Genetics of Acute Ethanol and Benzodiazepine Withdrawal Severity

by

Pamela Metten

A DISSERTATION

Presented to the Department of Behavioral Neuroscience
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

August 23, 1996

APPROVED:

(Professor in Charge of Thesis)

(Chairperson, Graduate Council)

TABLE OF CONTENTS

List of Tables and Figures

Tables

Figures

Acknowledgments

As for every large project, there are many people to thank for their assistance with and support of this research. In addition, the research and I were supported financially by grants AA06243, DA05228, AA10760, and DA07262. I have often thought that this section would turn out to have the most pages, since I have been fortunate enough to have the help of so many people! Unfortunately (!) for my Defense Committee, they have to actually *read* the rest of this document, and will be able to attest that the Acknowledgments didn't turn out to be longest after all...

I'd like to begin by saying that this project was, in part, a result of a conversation that Amanda Roberts and I had outside the Hyatt Hotel in La Jolla during my first attendance of a scientific meeting (Research Society on Alcoholism). We both were involved in ethanol withdrawal research, and I was then working on what would be my Master's thesis project. We wondered, aloud, and rather simultaneously (those who know Amanda know what I mean!), whether withdrawal convulsions occurred spontaneously (i.e., without precipitation) after single injections of benzodiazepines, as they do after ethanol and pentobarbital. As I recall, we came home from the meeting, got permission to order triazolam, and planned a couple of experiments. Amanda did most of the work, and I tried to help, learn, and stay out of her way! Eventually, we determined that triazolam might not produce withdrawal convulsions under the conditions we employed, although we could get precipitated withdrawal from it.

Neither of us had the time to pursue another drug at the time,

so we took our question to my mentor, Dr. John Crabbe. He deemed it worthy of pursuit, acquired other drugs, and assigned Catherine Merrill (technician extraordinaire!) to perform the experiments. I'd like to take this opportunity to thank Cathy for all her help. She did all of the Experiments (1 - 7) in WSP mice, as well as several pilot studies, while we tried to figure out dose ranges and time-courses. We had several exciting findings, but Amanda was pursuing her Ph.D. and I was defending my Master's, doing gene mapping, and taking qualifying exams, so the results went mostly unnoticed for a while.

When I caught my breath, I tested one of the drugs (zolpidem) using a panel of inbred strains that became available after another experiment. The results of that study occupied my thoughts for quite some time. Out of it was born the gene mapping portion of this dissertation. I owe many people a debt of gratitude for their help with that effort (Experiments 12 - 14) and the tests of genetic correlation (Experiments 8 - 11).

Dr. Tamara Phillips gave me space in her colony room for breeding my D1D2F2s, and went over simple main effects analyses with me (again!). I received the expert technical assistance of our (Tamara and John share) staff. I would like to give special thanks to Sue Burkhart-Kasch, Carrie McKinnon, Mary Huson, Emmett Young, Janet Dorow, and to fellow student Elaine Shen for their help with injections, weighing mice, and spleen dissections. Sue is the marvellous 8 mice-per-minute injector in Experiment 8!

Dr. John Belknap has been a "closet" co-mentor to me, and I owe to him a great deal of my education. He also involved me with his short-term selective breeding of ethanol withdrawal severity lines, and

allowed me considerable latitude in how I bred and used mice for testing correlated responses to selection. His technicians, Laurie O'Toole, Melinda Helms, and Susan Richards, gave me a lot of assistance with everything from mouse inventories to injections, and lessons in spectrophotometry, spleen DNA extractions, and PCR. I also thank them for allowing me to grace their doorstep (even when they said, "NOW WHAT?").

Dr. Kari Buck allowed me to use her lab and equipment to do the majority of the PCR (I owe her my UV-light-box tan). Tamara Lischka was an absolute brick during "crunch time." She gave me additional PCR instruction, lessons in gel photography, read gels, entered data, kept me supplied with reagents and materials, and took phone calls at home -- all with her soft-spoken patience and sense of humor. She also actually did the PCR on the final 6 markers in the D1D2F2s. Susan Richards and Melanie Ramsey also read gels.

Dr. Kristine Wiren generated and edited the computer-scanned image that became Figure 27. She has been wonderfully encouraging and has provided me with a very healthy different point of view on the way I think about behavioral data. She and Dr. Don Keith have made it a point to say frequently during the past year, "Stop worrying! You're ready!" at times when I needed it most.

Dr. Judy Grisel was marvellously supportive. She read drafts of this document, discussed statistical fine points with me, took me for a walk, and told me that feeling frustrated is part of the process. At times, I think she literally kept me sane! Dr. Steve Mitchell has also been great. He spent several hours with me bandying statistics back and forth (sometimes over e-mail) and provided me in early June with

an electronically-downloaded version of Mammalian Genome's Mouse Encyclopedia issue that saved me from re-writing portions of this document at the last minute.

I would like to express my thanks to the members of my Defense Committee: Drs. Susan Olson (Chair), John Belknap, Ed Gallaher, Kris Wiren, and especially to my mentor, John Crabbe. I have been extremely fortunate in my graduate career to have been able to work with John Crabbe. I consider him a brilliant scientist, and he has been absolutely wonderful to work with. He has been very generous with praise and opportunities for project involvement and publications. He has handed me a box of Kleenex on more than one occasion, and tolerated my tears and insecurities with equal aplomb. He has certainly been supportive and flexible regarding my hours during times when I've had family crises. I think that John is one of the rare individuals in the world who naturally treat everyone with respect. It's a quality I have tried to emulate. For his seemingly untiring endurance while I took seven years to graduate, I hereby christen him,

"St. John the Patient."

I would also like to thank Drs. Richard Askay and Susan Baillet, my undergraduate advisors at the University of Portland, who told me it was time to go to graduate school instead of stay another year to major in a third subject (biology). Although I wasn't sure at the time, I know in retrospect that they were right. They were so good for me that I was reluctant to leave!

I'd like to thank my various office mates, Lila Cormican, Char Wenger, Kelly Donan, Heather Hain, and newly, Steve Boehm, for putting up with me and my tensions during the past several months.

Fellow students, Shelly Dickinson, Sarah Coste, Gwen Schafer, and Sandra Angeli-Gade have been pillars of support for me, as I hope I have been for them.

Probably the person to have given me the most peace of mind throughout my undergraduate and graduate careers with the least recognition is Robin Cox. Robin has been a second mother to my children, providing them with her loving care and supervision. They have also been exposed to an organized and clean home at her house; something I have not been able to keep! She has rather selflessly accepted our inability to pick them up on time, and kept them in clean clothes, full bellies, and entertainment when we've worked really late unexpectedly. This dissertation literally would not have been possible without Robin's support, and I am forever grateful.

Finally, my husband and children have been the most wonderful sources of joy and serenity. I feel so fortunate! I have a job I love, and a family I adore. We experience Life together. I dedicate this dissertation

to
my husband and best friend,
Kelly Ray Mulholland

to
my daughter,
Kelsea Rae Mulholland

and to
my son,
Johnathan Ray Mulholland

I love you!

Abstract

Potentially life-threatening convulsions can occur following withdrawal from either ethanol or benzodiazepines. There is inconsistent evidence that these are partially determined by common genetic factors. Using genetic mouse models and a variety of benzodiazepine receptor ligands, the present studies tested the general hypothesis that ethanol and benzodiazepine withdrawal convulsions following a single drug injection share at least some genes underlying a common mechanism. Results confirm that withdrawal handling-induced convulsion severity following a single injection of diazepam is genetically mediated (controlling an estimated 62% of the variance) and demonstrate that it is genetically correlated with ethanol withdrawal severity in inbred strains. The proportion of variance accounted for by common genetic factors was estimated at 69%. These results contrast with previous data obtained in mice that were serially tested for withdrawal severity from ethanol, pentobarbital, and then diazepam (Metten & Crabbe, 1994a). Concurrent analysis of the previous and present diazepam withdrawal data showed that three of fourteen strains had significantly different diazepam scores in the two studies, arguing that serial testing of those strains significantly affected the previous results. Results in newly-derived HAW and LAW selectively bred lines provide independent confirmation of previous data obtained in WSP and WSR mice that ethanol and diazepam withdrawal convulsion severities are genetically correlated (Belknap et al., 1989; Crabbe et al., 1991a).

The occurrence of withdrawal convulsions following single injections of some benzodiazepine receptor ligands may depend both

on the drug and on whether withdrawal is precipitated. WSP mice are genetically susceptible to spontaneous withdrawal from a variety of central nervous system depressant drugs, and yet, they were not universally susceptible to all five drugs tested in these studies. However, the results imply that the genetic correlation with ethanol withdrawal may extend to several benzodiazepine receptor ligands using drug-appropriate (spontaneous or precipitated) paradigms.

Direct evidence that ethanol and zolpidem withdrawal are genetically correlated was seen in HAW and LAW mice using both spontaneous and precipitated withdrawal paradigms. Spontaneous withdrawal findings were extended in two independent experiments: the C57BL/6J, DBA/1J, and DBA/2J inbred strains, and in D1D2F2 mice. Results suggest both that a major gene influences zolpidem withdrawal severity and that DBA/2J alleles confer severe withdrawal.

The gene mapping study provides the first evidence suggesting the chromosomal location of any quantitative trait locus (QTL) influencing benzodiazepine withdrawal severity. Evidence was found for associations of three chromosomal regions with zolpidem withdrawal severity; each putatively controls only a small amount (<10%) of the genetic trait variance. Allele frequency at the microsatellite marker, *D17Mit66*, was associated at $p = 0.04$ with severity of zolpidem withdrawal in D1D2F2 mice. Pleiotropic influences of ethanol withdrawal QTLs on zolpidem withdrawal were suggested by nearly-significant association of markers on chromosomes 11 and 2. The putative QTLs on chromosomes 17 and 11 are negatively associated with zolpidem and ethanol withdrawal,

implying that DBA/2J alleles confer protection against withdrawal. Thus, neither QTL can be the major gene hypothesized to control a large proportion of the genetic variance in zolpidem withdrawal.

I. Introduction

I.A. Common Actions of Ethanol and Benzodiazepines

Dependence on alcohol (ethanol) or other central nervous system depressants, like benzodiazepines, produces signs and symptoms during withdrawal that are opposite in direction to those induced by intoxication (Victor & Adams, 1953; Kalant et al., 1971; Friedman, 1980; Jaffe, 1985); Swift, 1994 #644. Because the major effect of these drugs is depression of the central nervous system, withdrawal produces rebound neural hyperexcitability, which can manifest itself in several ways. Seizures are a potentially life-threatening consequence of alcohol withdrawal that is common to all species studied, including mice and humans (Kalant, 1977; Friedman, 1980).

Ethanol and benzodiazepines produce many common signs and symptoms upon withdrawal, suggesting that they share many mechanisms (Isbell et al., 1955; Jaffe & Ciraulo, 1985; Sellers, 1988; Edwards et al., 1990; Litten & Allen, 1991). Besides seizures or convulsions, common symptoms include anxiety, irritability, dizziness, tremor, excessive sweating, tachycardia, nausea, vomiting, insomnia, delirium, and hallucinations. Furthermore, cross-tolerance and -dependence is suggested by the fact that ethanol withdrawal is commonly treated in humans using benzodiazepines (Litten & Allen, 1991). In rodents, cross-tolerance and -dependence between ethanol and benzodiazepines has been shown in rats (Lê et al., 1986; Naruse & Asami, 1990; Mihic et al., 1992; Lytle et al., 1994) and mice (Chan et al., 1985; Chan et al., 1988; Chan et al., 1990; Buck et al., 1991). However, to date, only diazepam and chlordiazepoxide have been

studied. It appears that ethanol treatment may confer cross-tolerance to benzodiazepines but that only partial cross-tolerance is produced with the converse treatments. Further studies may sort out this issue. Certainly, it is clear that benzodiazepines can also reduce ethanol withdrawal convulsions in rodents (e.g., Crabbe, 1992; Goldstein, 1972a).

I.A.1. Ethanol and the GABA/Benzodiazepine Receptor Complex

Ethanol affects many neurotransmitter receptors, ion channels, and second-messenger systems. Many of these effects have been reviewed recently (Deitrich & Erwin, 1996). Relevant to the present studies is the evidence that ethanol acts on the γ-aminobutyric acid (GABA)/benzodiazepine receptor complex (GRC). This hetero-oligomeric complex is composed of several subunits (e.g., two α, two β, and one γ) and forms a chloride ionophore. Besides distinct sites for GABA and for benzodiazepines, the GRC has binding sites for many compounds, including neuroactive steroids, barbiturates, noncompetitive antagonists (e.g., picrotoxin), and zinc ions (Sieghart, 1992). Evidence suggests that the expression of a γ subunit of the GRC is required for benzodiazepine binding, while the subtype of α subunit affects benzodiazepine pharmacology (Pritchett et al., 1989; Olsen & Tobin, 1990; Burt & Kamatchi, 1991). Further evidence suggests that a splice variant of a γ subunit subtype (the γ_2 long) may be required for ethanol sensitivity of the GRC (Wafford et al., 1991; but see Grant, 1994). The apparent common requirement for the presence of a γ subunit by both benzodiazepines and ethanol provides an attractive rationale for an underlying functional similarity.

Ethanol and the benzodiazepines have been shown to increase GABA-stimulated chloride flux into the neural cell (see reviews by Grant, 1994; Mihic & Harris, 1996). Furthermore, GABA-stimulated chloride uptake into brain microsacs is altered when tissue is prepared from rats during withdrawal from chronic ethanol compared with non-withdrawn controls (Mihic & Harris, 1996). Chronic ethanol treatment has sometimes, but not always, been shown to decrease the number of $GABA_A$ agonist and benzodiazepine binding sites and increase inverse agonist binding sites (Buck & Harris, 1991). Thus, many researchers have shown *in vitro* an interaction of ethanol with the GRC, complementing and extending the common behavioral findings discussed above. Finally, chronic ethanol treatment *in vivo* has also been shown to alter GRC subunit mRNA levels (Buck, 1996).

I.B. Genetics of Ethanol and Benzodiazepine Withdrawal Severity

There is inconsistent evidence that genes affecting withdrawal from ethanol exert pleiotropic influences on withdrawal from other central nervous system depressant drugs. Studies in selectively bred mouse lines have supported this hypothesis for barbiturates and benzodiazepines, while tests in an inbred strain panel have found support for ethanol withdrawal gene pleiotropy for withdrawal from barbiturates but not benzodiazepines (Belknap et al., 1989; Crabbe et al., 1991a; Metten & Crabbe, 1994a; Metten & Crabbe, 1996). One purpose of this dissertation is to re-examine the general hypothesis that some of the same genes confer susceptibility to both ethanol and benzodiazepine withdrawal convulsions and attempt to resolve the apparently contradictory conclusions.

I.B.1. Methods of Studying Withdrawal Convulsions

Withdrawal from central nervous system depressant drugs has been measured in rodents by recording increased sensitivity to convulsant treatments, (e.g., pentylenetetrazol or electroconvulsive shock), or to convulsions induced by sensory stimulation (e.g., loud noise or handling). The present studies focus on the handling-induced convulsion, or HIC, developed by Goldstein and Pal (1971). The HIC method involves lifting the mouse by the tail, observing for convulsive signs, and if absent, gently spinning the mouse in a 180 - 360° arc, and again observing. Convulsions are rated on a scale from 0 (absent) to 7 (violent tonic-clonic convulsion resulting from cage disturbance). Goldstein established that the severity of the HIC during ethanol withdrawal is dose-dependent, heritable, and modifiable by a wide variety of drugs (Goldstein, 1972a; Goldstein, 1972b; Goldstein, 1973a; Goldstein, 1973b; Goldstein, 1974). Furthermore, HIC severity has been shown to increase during withdrawal from other central nervous system depressants, administered either chronically or acutely (Belknap et al., 1987; Belknap et al., 1988; Crabbe et al., 1991a; Metten & Crabbe, 1994a).

I.B.2. Methods of Studying Genetic Correlation

There are several ways to test the hypothesis that withdrawal severities following administration of ethanol and benzodiazepine receptor ligands (BZs) share a common genetic etiology.

I.B.2.a. Genetic Correlations in Selectively Bred Lines

One method of estimating genetic correlations is to test lines selectively bred bidirectionally for withdrawal severity from one drug

(e.g., ethanol) with the other drug (e.g., a benzodiazepine). Selectively bred lines are developed by testing animals from a heterogeneous stock on the trait of interest and then mating together extreme-scoring animals. Usually, bidirectionally selected lines (high- and low-response lines) plus control (nonselected) lines are established. Divergence of the high and low lines on the selection trait in opposite directions from the control lines over generations of selection is conclusive evidence that the trait is genetically influenced. During selection, genes influencing the trait become homozygously fixed, but remaining genes (i.e., those not influencing the trait) continue to segregate according to Mendelian law (Falconer, 1989). Establishment of independently selected replicate lines assists the researcher with the interpretation of results suggesting correlated responses to selection (i.e., differential sensitivity of the lines on a nonselected trait). Similar line differences in both replicates of the selected lines is strong evidence of pleiotropic influences of the genes fixed by selection (Crabbe et al., 1990).

Withdrawal Seizure Prone (WSP) mice were selectively bred in replicate from HS/Ibg stock for severe withdrawal HICs following chronic ethanol vapor inhalation (Crabbe et al., 1985). Selection pressure was applied for 26 generations. As of this writing, more than 20 generations of relaxed selection have taken place with no change in the magnitude of phenotypic expression (J. C. Crabbe, unpublished observations). These mice show severe withdrawal convulsions following acute (i.e., single administration) and/or chronic administration of several alcohols, barbiturates, and nitrous oxide (Belknap et al., 1987; Belknap et al., 1988; Crabbe et al., 1991a). WSR

mice were selectively bred in parallel for minimal ethanol withdrawal severity and are generally resistant to withdrawal convulsions from these drugs.

Differential susceptibility to withdrawal convulsions following depressant drugs does not imply that WSP mice are more susceptible than WSR mice to convulsions in general. These lines differ not at all or only slightly in susceptibility to convulsions induced by intravenous infusion of convulsant drugs, compared with their approximately ten-fold differences in ethanol withdrawal HICs (Kosobud & Crabbe, 1995). In fact, it has been shown that WSR mice are more sensitive than WSP mice to convulsions induced by infusion of *N*-methyl-D-aspartate (NMDA; (Kosobud & Crabbe, 1993). One replicate of the WSP lines was more sensitive to infusion of pentylenetetrazol-induced convulsions than its respective WSR line; however, in the other replicate, WSP mice were equally or less sensitive than WSR mice (Kosobud et al., 1992). Examination of these lines for other correlated responses to selection has been extensive. These data have been reviewed elsewhere (Phillips & Crabbe, 1991; Crabbe & Phillips, 1993; Kosobud & Crabbe, 1995; Metten & Crabbe, 1996).

In addition to the central nervous system depressant drugs mentioned above, WSP mice also show greater withdrawal than WSR mice following acute or chronic administration of diazepam when withdrawal convulsions are precipitated by the competitive benzodiazepine antagonist, flumazenil (Ro15-1788). Mice of selection generations 5 (S_5) and S_{13} were housed singly and fed diazepam in drug-adulterated food (1.5 mg/g diet) for seven days. On the morning of the eighth day, withdrawal was precipitated by injection of 20

mg/kg flumazenil (i.p.). HICs were measured at 2, 5, and 8 minutes later. Both replicates of the WSP line had significantly higher withdrawal scores than their WSR counterparts. In generation S_5, the magnitude of the difference between the lines was approximately two-fold, while in S_{13}, a ten-fold difference was seen. The magnitude of the differences between these lines in diazepam withdrawal closely paralleled that of ethanol withdrawal severity in both generations (Belknap et al., 1989).

The greater susceptibility of WSP mice compared with WSR mice to withdrawal from single injections of central nervous system depressants including ethanol and diazepam (i.e., acute withdrawal) has been demonstrated (Kosobud & Crabbe, 1986; Crabbe et al., 1991a). Separate groups of mice of each genotype were injected with vehicle or 20 mg/kg diazepam and scored for HIC 30 minutes later. Sixty minutes after injection, they were given 10 mg/kg flumazenil to precipitate withdrawal or vehicle. HICs were measured 1, 2, 3, 5, 8, and 20 minutes later. There was no elevation of HICs in any group of WSR mice. In contrast, WSP mice given diazepam followed by flumazenil had significantly increased HIC scores compared to WSP mice given vehicle followed by either flumazenil or vehicle (Crabbe et al., 1991a). Diazepam treated WSP mice given vehicle instead of flumazenil had no HICs. These studies strongly imply that the induction of convulsions by withdrawal from acute or chronic ethanol and diazepam occurs at least in part via a common genetic mechanism.

I.B.2.b. Genetic Correlations in Inbred Strains

Another way to assess genetic correlations among ethanol and

benzodiazepine withdrawal severities would be to test a number of inbred strains for withdrawal severity from each drug, and correlate strain means (Hegmann & Possidente, 1981). Inbred strains are developed by systematic inbreeding, commonly brother/sister matings, over 20 or more generations (Falconer, 1989). Therefore, members of any particular inbred strain are genetically identical except for gender (Crabbe, 1989; McClearn, 1991). Consequently, individual differences in responses within an inbred strain must be due to environmental influences. Furthermore, differences among several inbred stains can be attributed to genetic factors, given equivalent testing procedures. The utility of inbred strains and methodological considerations in pharmacogenetic research have been discussed in detail elsewhere (Belknap, 1980; Deitrich & Spuhler, 1984; Crabbe et al., 1990; McClearn, 1991). Use of relatively large panels of inbred strains ($\geq$ 12 strains) is recommended when attempting to ascertain genetically correlated responses because each strain represents a single genotype. This means that the genetic sample size equals the number of strains being tested. Furthermore, the genes of most inbred strains are fixed without respect to any particular phenotype, and therefore, any pair of strains is likely to have similarities and differences on several traits that are genetically unrelated. Thus, tests of too few strains would be more likely to identify spurious correlations than large panels.

Acute ethanol, pentobarbital, and precipitated diazepam withdrawal severities were previously assessed in 15 inbred strains using the same paradigm as described above for WSP and WSR mice. In apparent disagreement with those findings, no significant

correlation of ethanol and diazepam withdrawal severity strain means was found (Metten & Crabbe, 1994a). Consistent with the findings in WSP and WSR mice, a genetic correlation was demonstrated for ethanol and pentobarbital withdrawal severities. Pentobarbital and diazepam withdrawal strain means were also genetically correlated. The lack of demonstration of genetic correlation between ethanol and diazepam withdrawal severity means was surprising. However, the mice were serially tested for withdrawal from ethanol, pentobarbital, and then diazepam. Although mice were given a respite of a week between withdrawal episodes, an effect of repeated testing could not be ruled out.

I.B.3. Questions Generated by the Previous Studies*

I.B.3.a. Does Choice of Benzodiazepine in the Test Matter?

A possible difficulty with the hypothesis that ethanol and benzodiazepine withdrawal severity are mediated in part by some of the same genes is that all benzodiazepines are not alike. In fact, many drugs putatively acting at the benzodiazepine receptor (BZR) are not chemically classified as benzodiazepines at all. The BZR ligands used in the present studies are listed in Table 1 along with their functional and chemical classifications, and putative α subunit subtype affinity, if known.

BZR ligands have different potencies for producing sedative or hypnotic, anti-convulsant, and anxiolytic effects, and thus are differentially prescribed. For example, zolpidem and midazolam are frequently used as sedative/hypnotic agents, alprazolam is often

* Formal hypotheses are listed at the end of the Introduction.

Table 1

Benzodiazepine Receptor Ligands Used in the Present Studies

Ligand	**Functional * Classification**	**Chemical Classification**	**GRC α Subunit Affinity†**
Diazepam	full agonist	benzodiazepine	α2=α3>α1=α5
Alprazolam	full agonist	triazolo-benzodiazepine	α1
Triazolam	full agonist	triazolo-benzodiazepine	
Abecarnil	partial agonist/ full agonist	β-carboline	α1>α3>α5
Midazolam	full agonist	imidazo-benzodiazepine	α1
Zolpidem	full agonist	imidazopyridine	α1>α2=α3>>α5
Flumazenil	competitive antagonist	imidazo-benzodiazepine	α1=α2=α3=α5>α6

* Based on effect of ligand binding on magnitude of GABA agonist-stimulated chloride influx. Agonists enhance, inverse agonists reduce, and antagonists prevent agonists from enhancing chloride flux (Sieghart, 1992).

† If known. (Lüddens & Wisden, 1991; Doble & Martin, 1992; Wong et al., 1992; Knoflach et al., 1993)

prescribed for panic disorder, and diazepam and chlordiazepoxide are the drugs of choice for prevention of ethanol withdrawal seizures. It is tempting to attribute the differential behavioral effects of these ligands to their differential affinities for the GRC α subunit subtypes (Giusti et al., 1991). Support for such an hypothesis comes from a group of researchers who determined that subunit compositions of *in vivo* GRCs in many brain areas vary considerably (Laurie et al., 1992; Wisden et al., 1992). Binding of zolpidem in various brain regions has been found to correlate highly with ethanol enhancement of iontophoretically applied GABA (Criswell et al., 1993). A follow-up study found that neurons of the red nucleus and globus pallidus were either sensitive to both zolpidem and ethanol or insensitive to both. Furthermore, GRC subunit mRNA expression was assayed in brain regions sensitive to ethanol and zolpidem. Several subunits (α1 – α3, β2, β3, and γ2) were common to all brain regions in which ethanol and zolpidem enhanced GABA responses and several others were not (Criswell et al., 1995).

Clinical literature suggests that some benzodiazepines have more dependence liability than others, although there is dissension. Midazolam, triazolam, and lorazepam have been ranked as having "high" potential for withdrawal reactions in one review, while diazepam has been ranked as "moderate" (Tyrer, 1988). Abuse potential is partially responsible for the removal of triazolam from the market in the United Kingdom in 1991 (Robertson & Treasure, 1996). Another review examined both human and nonhuman studies comparing the abuse potential of alprazolam (not included in Tyrer's 1988 review) to other benzodiazepines, including diazepam, and

concluded that the literature did not support the belief that it had greater abuse potential (Rush et al., 1993). However, their review of actual dependence potential only included comparisons of alprazolam with diazepam (of the drugs in Table 1). Nevertheless, when looking for genetic commonalities in severity of withdrawal from ethanol and benzodiazepines, the choice of benzodiazepine receptor ligand may be important.

Although originally selected only for ethanol withdrawal severity, WSP mice are now believed to be a model of genetic vulnerability to central nervous system depressant drug withdrawal in general (Metten & Crabbe, 1996). One hypothesis that could be tested was whether the finding that WSP mice were sensitive to withdrawal from diazepam generalized to withdrawal sensitivity from other benzodiazepine receptor ligands. In this dissertation, the term "benzodiazepine withdrawal" will refer generally to withdrawal from ligands acting at the benzodiazepine receptor (BZR ligands) and not strictly to withdrawal from chemical benzodiazepines.

I.B.3.b. Does it Matter Whether Withdrawal Convulsions Were Precipitated?

Another possible difficulty with the previous tests of the hypothesis that ethanol and benzodiazepine withdrawal severity are genetically correlated is that they may have been comparing the proverbial apples and oranges. Withdrawal convulsions following single doses of some drugs occur in the mouse without antagonist precipitation (e.g., ethanol and pentobarbital, for which there are no known direct antagonists). However, in order to observe withdrawal

convulsions in mice reliably following a single injection of diazepam, precipitation of withdrawal by flumazenil may be required (Crabbe et al., 1991a; Crabbe, 1992).

Spontaneous withdrawal convulsions from some benzodiazepines are seen in humans (Martínez-Cano et al., 1995). Perhaps withdrawal convulsions following single injections of some BZR ligands will not require precipitation in the mouse. Swiss-Webster mice treated with alprazolam chronically admixed in their diet displayed handling-induced convulsions without precipitation (Gallaher et al., 1987b). Conversely, even after chronic treatment with abecarnil, convulsions were not observed in NMRI mice (Steppuhn et al., 1993; Rundfeldt et al., 1995). There is no reason *a priori* to suppose that genetic vulnerability common to both ethanol and benzodiazepine withdrawal convulsions will depend on whether those convulsions are precipitated. However, this has not been tested, and it is possible that precipitated and spontaneous benzodiazepine withdrawal convulsions may be differentiable on the genetic level. Thus, another testable hypothesis is whether spontaneous withdrawal convulsions are observed in WSP mice after a single injection of BZR ligand, or whether flumazenil-precipitation is required.

I.B.3.c. Are Ethanol and Diazepam Withdrawal *Really* Genetically Correlated in Inbred Strains?

A third hypothesis is that the serial testing of the inbred strains in Metten & Crabbe (1994a) interfered with the ability to detect the genetic correlation. Measuring diazepam withdrawal in naive animals of the same strains would permit comparison with the previous diazepam data as well as with the ethanol data.

Another way to address this issue is to test other selectively bred lines for BZR ligand withdrawal severity. High and Low Alcohol Withdrawal (HAW and LAW) mice were recently selectively bred from F2 intercross progeny from C57BL/6J (B6) and DBA/2J (D2) mice for differential withdrawal severity following a single, hypnotic dose of ethanol (4 g/kg, injected i.p.; Buck et al., submitted; Metten & Crabbe, 1996). The selection index was the residual from regression of the area under the handling-induced convulsion curve over time on baseline HIC severity. This index is a measure of withdrawal severity that is independent of baseline differences. These mice are in the very early generations of selection, which reduces the risk that trait-irrelevant genes have become fixed spuriously. Therefore, a test of these lines for diazepam withdrawal severity would provide independent evidence regarding the hypothesis that selective breeding for ethanol withdrawal severity produces lines that differ in benzodiazepine withdrawal severity. Furthermore, the HAW and LAW lines could be tested with other BZR ligands. No lines selectively bred for benzodiazepine withdrawal severity exist, making a test of the reciprocal hypothesis impossible at present.

I.B.3.d. Can a striking result of the above studies be exploited to map benzodiazepine withdrawal QTLs?

To anticipate the results of the studies addressing the above questions, the answers were generally "yes." Spontaneous and precipitated withdrawal in WSP mice were seen only with zolpidem and alprazolam. Diazepam withdrawal was correlated with ethanol withdrawal in inbred strains and in the HAW and LAW selected lines.

Furthermore, ethanol and zolpidem withdrawal were correlated in a subset of the inbred strains and the HAW and LAW lines.

Mice of fourteen inbred strains that were previously tested for hypothermic and activity effects of the dopaminergic drug, quinpirole, became available at about this time. They were allowed to rest for about a week before spontaneous zolpidem withdrawal severity assessment (unpublished data). Although all of the strains recovered basal levels of HIC sensitivity, only D2 mice had significant zolpidem withdrawal. This was an important study for two reasons. The first is that one of the other strains tested was DBA/1J (D1; see below); i.e., this closely related strain was apparently not susceptible to zolpidem withdrawal. These data suggested that zolpidem withdrawal genes could be mapped using an F2 intercross of DBA/1J X DBA/2J progenitors (i.e., D1D2F2s). The second reason was that these two DBA substrains also differ two-fold in acute ethanol withdrawal severity (Metten & Crabbe, 1994a). Furthermore, since ethanol and zolpidem withdrawal convulsion severities are genetically correlated (see Results), it was possible that the difference in ethanol withdrawal severity between these strains was mediated by a zolpidem withdrawal gene. Thus, the mapping strategy employed was to genotype D1D2F2 mice in the regions of ethanol withdrawal gene loci.

I.C. Mapping Genes Mediating Benzodiazepine Withdrawal

I.C.1. Ethanol Withdrawal Gene Mapping Efforts

Quantitative trait loci (QTLs) affecting acute ethanol withdrawal severity were recently mapped in intercross progeny of the B6 and D2 strains (Belknap et al., 1993c; Buck et al., submitted). A preliminary full-genome search was performed using BXD recombinant inbred (RI)

strains and then putative loci were confirmed using B6D2F2s and the HAW and LAW selectively bred lines. Significant linkage was found for ethanol withdrawal QTLs with markers on chromosomes 1, 4, and 11, with respective LOD (logarithm of the likelihood ratio for the presence of a QTL) scores of 5.8, 5.7, and 4.1, each controlling between 12 and 30% of the genetic variance (Buck et al., submitted). Suggestive linkage was found for another QTL on chromosome 2, using criteria for reporting linkage recommended by Lander and Kruglyak (1995).

There is presently no evidence to suggest the location of any putative QTLs for benzodiazepine withdrawal. Although mapping QTLs affecting benzodiazepine withdrawal could be undertaken in several ways, for example using B6 X D2 intercross progeny in an approach similar to the approach by Buck et al. (submitted), a single-step approach using D1D2F2s was employed.

I.C.2. Genetic Characterization of the DBA Substrains

DBA mice originated in 1909 in the lab of Clarence Cook Little (Morse, 1978). The acronym stands for homozygous recessivity of the strain's alleles at the dilute (D), brown (B), and agouti (A) coat color loci. Much of the breeding history is not clear. However, when the strain was about 45 generations inbred (1929), sublines (apparently from different colonies; distinct from "substrains" which are demonstrated to differ at least at one locus) were apparently intermated. Around 1932, the breeding stock was divided into three separate lines and inbreeding resumed: DBA/12 (now abbreviated DBA/1), DBA/212 (now DBA/2), and DBA/LiA. It has been estimated that there were still about 100 segregating genes prior to subline intermating (Bailey, 1978). The result of this geneology was that there

was more residual heterozygosity in the DBA strain prior to its separation into substrains than would have been predicted by continuous inbreeding from a single colony. Additionally, there may have been a period of random mating in the DBA/1 substrain after having been separated for about 25 generations. Bailey (1978) has estimated that the DBA substrains were about 98% genetically homologous at the time of the division of the substrains.

Festing has argued that the known genetic differences plus the accumulated differences implied by heterozygosity estimated at 2% of loci are substantial enough that DBA/1 and DBA/2 should be recognized as unique inbred strains rather than substrains (Festing, 1990; Festing, 1994). Recently, 18 inbred strains including D1 and D2 were examined for degree of relationship by examining of variation of minisatellite fragment lengths after restriction enzyme digestion with *Hae* III (Aker & Huang, 1996). These markers are highly polymorphic, centromeric repeat sequences of around 200 - 500 kilobases in length. D1 and D2 strains differed by 43% of fragments implying homology of only 57%. However, other very closely phylogenetically-related strains display even lower homology (e.g., B6 with C57L/J varied in 100% of these fragments; Aker & Huang, 1996). Thus this latter estimate of D1/D2 homology at functional genes is probably artificially low.

Genotypes of the D1 strain are known for only about 250 loci (GBASE; Festing, 1990; Festing, 1994). In marked contrast, the D2 strain has been genotyped for thousands of loci. Many of the loci genotyped in D2s are microsatellite markers, also called simple sequence length polymorphisms (SSLPs; Silver, 1992). Although

these microsatellites tend to be DNA sequences of variable-length repeated two-base motifs, they are surrounded by unique sequences. These highly polymorphic loci tend to be around 75 to 600 bases in length. Therefore, they are readily amplified with oligodeoxynucleotide primers by polymerase chain reaction (PCR; Dietrich et al., 1992; Sambrook et al., 1989). Unlike minisatellite markers, microsatellites are well-dispersed in the genome.

I.D. Hypotheses

I.D.1. Hypothesis 1

The first hypothesis is that some benzodiazepine receptor ligands (e.g., alprazolam, triazolam, midazolam, and zolpidem) can induce spontaneous withdrawal convulsions in mice after a single injection. It is predicted that abecarnil will not, since neither chronic treatment nor acute treatment induced convulsions previously (Crabbe, 1992; Steppuhn et al., 1993). After baseline HIC assessment, the benzodiazepine receptor ligands alprazolam, triazolam, abecarnil, midazolam, and zolpidem were injected into separate groups of WSP mice and HIC severity was monitored at several timepoints (time intervals and doses were determined by pilot studies with each benzodiazepine). These are Experiments 1 through 5, respectively.

I.D.2. Hypothesis 2

The second hypothesis is that each of the benzodiazepine receptor ligands tested in Hypothesis 1 will induce withdrawal convulsions when withdrawal is precipitated by flumazenil. Each of the five benzodiazepine receptor ligands was injected i.p. into WSP mice following baseline HIC assessments, and flumazenil was injected

20 to 60 minutes later to precipitate withdrawal. Control groups, when included, received an injection of the appropriate vehicle prior to injection of flumazenil. HICs were scored at 1, 3, 5, 8, and 12 minutes following flumazenil injection. Pilot experiments have determined that BZ withdrawal convulsion precipitation is essentially complete by 10 minutes after flumazenil injection. These are Experiments 6 and 7.

I.D.3. Hypothesis 3

The third hypothesis is that ethanol and precipitated benzodiazepine withdrawal severities are genetically correlated. The first test of this hypothesis was assessment of precipitated diazepam withdrawal severity in naive animals of the same 14 inbred strains previously tested by Metten & Crabbe (1994a). Withdrawal severity strain means were correlated with those collected previously for ethanol, pentobarbital, and precipitated diazepam withdrawal. Existing data led to conflicting predictions of the outcome of this experiment. Previously collected data on diazepam withdrawal severity in inbred strains predict no genetic correlation with ethanol withdrawal (Metten & Crabbe, 1994a). However, diazepam withdrawal experiments in WSP/WSR mice (Belknap et al., 1989; Crabbe et al., 1991a) predicted that inbred strain means of ethanol and diazepam withdrawal severities would be genetically correlated. This is Experiment 8. Due to the expense of standard inbred strains, no tests of other BZR agonists in the precipitated withdrawal paradigm were made.

The second test of this hypothesis assessed the genetic correlation in the HAW/LAW selectively bred lines. Separate groups of

animals of these lines were tested for precipitated benzodiazepine withdrawal from diazepam and zolpidem. It was predicted that HAW mice would show significantly greater precipitated BZ withdrawal than LAW mice. These are Experiments 10 and 11B.

I.D.4. Hypothesis 4

The fourth hypothesis is that ethanol and spontaneous BZ withdrawal severities are genetically correlated. In the first test of this hypothesis, HAW and LAW mice were tested for spontaneous zolpidem withdrawal severity (Experiment 11A). Zolpidem was chosen over the other drugs that were tested in Hypotheses 1 and 2 because it produced both spontaneous and precipitated withdrawal in WSP mice. It was predicted that if HAW mice were to show greater spontaneous zolpidem withdrawal than LAW mice, the combined results of Hypotheses 3 and 4 would be strong evidence in favor of genetic correlations among ethanol and spontaneous or precipitated BZR agonist withdrawal severities.

In a second test of this hypothesis, naive mice of the inbred strains, C57BL/6J, DBA/1J, and DBA/2J, were also tested for spontaneous zolpidem withdrawal severity (Experiment 9). If earlier findings were to be replicated (i.e., that the DBA substrains differ in withdrawal severity from this benzodiazepine agonist), then this would further support the correlation between ethanol and BZR agonist withdrawal severity. This result would also suggest an avenue to determine the location(s) of the gene or genes responsible for this difference.

I.D.5. Hypothesis 5

The fifth hypothesis is that DBA/1J and DBA/2J differ in

genotype at one or more ethanol withdrawal QTLs. Because DBA/1J mice have not previously been genotyped for many gene loci, it was necessary to genotype them and determine loci for which they are polymorphic with respect to DBA/2J mice. Mice from Experiment 9 were sacrificed for collection of genomic DNA after withdrawal testing. Polymerase chain reaction (PCR) was used to amplify and size DNA segments recognized by SSLP markers. For each marker, PCR-amplified DBA/2J genomic DNA samples were run on the same gel as those of DBA/1J in order to map polymorphisms between the DBA substrains (Experiment 12). C57BL/6J DNA samples were also run on each gel for control purposes and to determine whether the allele possessed by the DBA/1J substrain has the same base pair length as the C57 allele.

I.D.6. Hypothesis 6

The sixth hypothesis is that an F2 intercross between the DBA substrains could be used to map the gene(s) involved in the difference in the severity of zolpidem withdrawal. Naive DBA/1J X DBA/2J F2 generation intercross progeny (D1D2F2s) were tested first for spontaneous zolpidem withdrawal (Experiment 13), followed a week later by ethanol withdrawal (Experiment 14). Assessment of the phenotypic correlation between drug withdrawal severities was made in the same animals. The mice were sacrificed for spleen genomic DNA and the phenotypic extreme scorers for zolpidem withdrawal (top and bottom 18.75%) were genotyped for those markers shown to be polymorphic between DBA/1J and DBA/2J in Experiment 12. Differences in allele frequencies between the high and low scoring extremes would imply linkage of a QTL with the marker.

II. Methods

II.A. Materials and General Procedures

II.A.1. Animal Husbandry

All mice were *Mus musculus*-derived stock. Mice from the selectively bred lines and F2 intercross progeny of DBA/1J and DBA/2J inbred strains were bred at the Portland Department of Veterans Affairs Medical Center Veterinary Medical Unit (PVAMC VMU). The sexes were separated at weaning (21±1 days of age). Inbred strains were ordered from The Jackson Laboratory, Bar Harbor, Maine (unless otherwise noted), and were four to seven weeks of age at the time of arrival. They were allowed at least one week to acclimate to their new housing before testing. All mice were 50 - 105 days old at the time of testing.

Mice were housed by strain or selected line, 1 - 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±1.5 °C. During each experiment, food and water were available ad libitum and lights remained on.

II.A.2. Drugs

All drugs were freshly mixed the morning of each experiment. Diazepam and Ro15-1788 (flumazenil) were a gift of Dr. Edward J. Gallaher. Abecarnil was a gift of Dr. David Stephens. Midazolam was synthesized by Dr. Janice Stuart. Zolpidem was a gift of Synthelabo Recherché. Alprazolam and triazolam were purchased from Sigma. All BZR ligands were prepared in a vehicle containing 1 drop of Tween-

80 per 5 ml 0.9% physiological saline except as specified below. Midazolam was prepared in saline alone. For the inbred strain panel experiment (# 8), the vehicle for both diazepam and flumazenil contained 0.125 g/ml 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals Incorporated) in 0.9% physiological saline. Although no differences between vehicle effects have ever been detected in our hands, use of this vehicle allowed direct comparability of old and new diazepam withdrawal scores. Control animals for the precipitated benzodiazepine withdrawal experiments were injected with the appropriate vehicle. Benzodiazepine receptor ligands were injected in a 10 ml/kg volume. Doses are specified in the methods for each experiment, except for flumazenil, which was always 10 mg/kg.

Ethanol (200 proof; Pharmco Products, Inc.) was prepared as 20% ethanol v/v in 0.9% physiological saline, and injected intraperitoneally in a volume of 25.33 ml per kg body weight. The dose of ethanol used was 4 g/kg.

II.A.3. Handling-Induced Convulsion Scoring

The HIC scale used in the present studies has been published (Crabbe & Kosobud, 1990), and was modified from that of Goldstein (1972a). 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° arc and again observed. A score was assigned based on the specific convulsive sign and whether spinning was required to elicit a convulsion, as specified in Table 2.

Table 2

Rating of Handling-Induced Convulsions (HICs)

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

II.A.4. Experimental Procedures

Experiments commenced at 07:30. For all experiments, the animals were assessed first for baseline HIC and weighed. After approximately 20 minutes, a second baseline HIC was scored. Immediately thereafter, all animals were injected i.p. with drug (or vehicle) as rapidly as possible. For spontaneous withdrawal experiments, the help of 1 - 3 additional injectors was used, so that injections were complete in about the time it takes to score HICs on the required number of animals (typically 5 - 7 minutes). For precipitated withdrawal experiments, 2 cages of animals were injected by one person within one minute, and starting times were staggered for each pair of cages. Withdrawal HICs were scored at various intervals after injection (specified below). Withdrawal severity scores were calculated as the peak score (average of the maximum score plus the two adjacent scores) minus the average vehicle group score over the time of peak withdrawal, except where specified. All data analyses were performed using the SYSTAT® statistical package, version 5.1 for the Macintosh (Wilkinson, 1989).

II.A.4.a. Ethanol and spontaneous benzodiazepine withdrawal experiments

This paradigm involves baseline HIC assessment, injection of ethanol or BZR agonist, and assessment of HIC at intervals appropriate for each drug. Doses of BZR ligands and time-courses were determined by pilot studies and available information on duration of action. Control groups, when included, received identical treatment except that they were injected with the appropriate BZR agonist

vehicle in lieu of drug. Tables 3 and 4 list the drugs, doses, and time-courses of withdrawal tests using the spontaneous withdrawal paradigm. Table 3 lists the drug-screening tests performed in WSP mice; while Table 4 refers to the tests of ethanol/benzodiazepine withdrawal correlation using inbred strains, HAW and LAW mice, and D1D2F2s. Between HIC assessments, the animals were left undisturbed in the room.

II.A.4.b. Precipitated benzodiazepine withdrawal experiments

This paradigm involves baseline HIC assessment, injection of BZR agonist, assessment of agonist effects on HIC, injection of BZR antagonist (i.e., flumazenil), and subsequent measurement of precipitated withdrawal HIC severity. Control groups received identical treatment except that they were injected with the appropriate BZR agonist vehicle in lieu of drug, providing a measure of the weak anticonvulsant effect of flumazenil alone. Animals within each selected line or inbred strain were assigned pseudorandomly to the two drug treatment groups. For midazolam only, WSP mice were assigned pseudorandomly into four drug treatment groups (first/second injection): vehicle/vehicle, vehicle/flumazenil, midazolam/vehicle, and midazolam/flumazenil. This was necessary because midazolam was dissolved in a different vehicle (saline) from that used for the other BZR agonists.

Vehicle group performance by WSP mice in response to vehicle/BZR antagonist treatment has been quite reliable in our experience (unpublished observations). Therefore, in order to reduce the number of animals required for these studies, vehicle groups were

Table 3

Drugs, Doses, and Time-Courses of Spontaneous Withdrawal Tests in WSP Mice

Expt #	Drug	Doses (mg/kg)*	Times of HIC assessment (minutes post- injection)
1	Alprazolam	0.5, 0.75, 1.0	30, 60, 90, 120, 150, 180, 210, 240, 300, 420, 480
2	Triazolam	0.1, 0.25, 0.5	30, 60, 90, 120, 180, 240, 300, 360, 420
3	Abecarnil	1.0, 2.0, 3.0	30, 60, 120, 180, 240, 300, 360, 420
4	Midazolam	2.5, 5.0, 10.0	15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, 270, 330, 390
5	Zolpidem	0.3, 3.0, 30.0	10, 20, 30, 40, 50, 60, 120, 180, 240

Table 4

Drugs, Doses, and Time-Courses of Spontaneous Withdrawal Tests in Inbred Strains, HAW/LAW, and D1D2F2 Mice

Expt #	Drug/ Mice	Dose (mg/kg)	Times of HIC assessment (minutes post- injection)
9	Zolpidem/ Inbreds	20	15, 30, 45, 60, 75, 90, 105, 120, 150, 180
11A	Zolpidem/ HAW/LAW	20	15, 30, 45, 60, 75, 90, 105, 120, 150, 180
13	Zolpidem/ D1D2F2s	20	15, 30, 45, 60, 75, 90, 105, 120, 150, 180
14	Ethanol/ D1D2F2s	4 (g/kg)	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 (hours)

* 0 denotes the drug-appropriate vehicle (see Drugs section of Methods). All drugs were administered by single, intraperitoneal injection.

included in testing WSP mice for withdrawal from only three of the five BZR agonists (abecarnil, midazolam, and alprazolam). Vehicle group data were examined for differences, combined where appropriate, and used for withdrawal severity assessment from drugs with similar time-courses (see below). Specifically, vehicle group data from the alprazolam test and an unpublished study examining precipitated diazepam withdrawal (in WSP female mice of the same age and generation as those in the present studies) were combined for analysis of withdrawal severity from triazolam (and alprazolam). Similarly, vehicle group data from the abecarnil and midazolam tests were combined for analysis of withdrawal severity from zolpidem (as well as abecarnil and midazolam).

Two time-courses for precipitated withdrawal testing were suggested by the spontaneous withdrawal results. Tables 5 and 6 list the drugs, doses, and time-courses of precipitated withdrawal tests using WSP mice (Table 5) and inbred strains and HAW/LAW mice (Table 6). In both time-courses, HICs were assessed twice following injection of drug or vehicle to establish that HICs were depressed in the drug group. For abecarnil, midazolam, and zolpidem, HICs were assessed at 10 and 19 minutes following BZR agonist injection. For alprazolam, diazepam, and triazolam, HICs were assessed at 30 and 55 minutes following agonist injection. After the second time point, animals were injected with flumazenil (10 mg/kg) and scored for HICs 1, 3, 5, 8, and 12 minutes later.

Finally, the maximum number of animals that could be tested in one experimental day (pass) was ~60. Each pass of the inbred strain panel test (Experiment 8) included all fourteen inbred strains.

Table 5

Drugs, Doses, and Time-Courses of Precipitated Withdrawal Tests in WSP Mice

Expt #	Drug	Doses (mg/kg)*	Times of HIC assessment† (minutes post- injection)
6	Alprazolam	0, 0.5	30, 55, FMZ @ 60, 61, 63, 65, 68, 72
6	Triazolam	0.5	30, 55, FMZ @ 60, 61, 63, 65, 68, 72
7	Abecarnil	0, 2.0	10, 19, FMZ @ 20, 21, 23, 25, 28, 32
7	Midazolam	0, 2.5	10, 19, FMZ or VEH @ 20, 21, 23, 25, 28, 32
7	Zolpidem	30	10, 19, FMZ @ 20, 21, 23, 25, 28, 32

Table 6

Drugs, Doses, and Time-Courses of Precipitated Withdrawal Tests in Inbred Strains and HAW/LAW Mice

Expt #	Drug/ Mice	Dose (mg/kg)*	Times of HIC assessment† (minutes post- injection)
8	Diazepam/ Inbreds	0, 20.0	30, 55, FMZ @ 60, 61, 63, 65, 68, 72
10	Diazepam/ HAW/LAW	0, 20.0	30, 55, FMZ @ 60, 61, 63, 65, 68, 72
11B	Zolpidem/ HAW/LAW	0, 20.0	10, 19, FMZ @ 20, 21, 23, 25, 28, 32

* 0 denotes the drug-appropriate vehicle (see Drugs section of Methods). All drugs were administered by single, intraperitoneal injection.

† Times are minutes after BZR agonist (i.e., first) injection. FMZ (or VEH) @ 60 (or 20) means flumazenil (or vehicle) was injected 60 (or 20) minutes after BZR agonist injection. Thus, for all drugs, HICs after withdrawal precipitation occurred exactly at 1, 3, 5, 8, and 12 minutes following flumazenil.

Approximately 4 mice per strain were tested in each pass. Therefore, multiple passes were required to obtain sufficient numbers of animals per strain. All mice of each pass were tested within four 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 several times across the four-hour period.

II.A.4.c. PCR Genotyping

II.A.4.c.i. Spleen Genomic DNA Extraction

This procedure is a modification (Buck, unpublished) of Miller et al. (1988). D1D2F2, DBA/1J, DBA/2J, and C57BL/6J mice were killed by cervical dislocation within a few days after testing. For inbred strains, whole spleens were dissected and placed in a labelled tube containing 1 ml of ice-cold saline, immediately put in ice water, and frozen within 2 minutes on dry ice. For D1D2F2 mice, procedures were identical except that the spleens were cut in two, and each half was placed in its own labelled tube prior to freezing. All procedures were performed under sterile conditions. The tubes were then stored in a -80 °C freezer until processing.

On the morning of processing, the tubes were removed from the freezer and placed in a refrigerator for approximately one hour for tempered thawing. Saline was poured off and the spleen was added to a new tube containing 10 ml Hanks' Balanced Salt Solution [HBSS; Gibco BRL]. Each spleen was pressed through a sieve [a standard kitchen tea strainer]. The spleen extracts were spun at 1000 rpm at 25 °C for 10 minutes in a swinging bucket centrifuge. Supernatants were discarded, and each pellet was resuspended in 10 ml of room

temperature Lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2mM Na_2EDTA, pH 8.2). After addition of 40 µl of DNAse-free RNAse [Boehringer Mannheim] to each vial, the suspensions were incubated at 37 °C for one hour while gently shaking. Next, 670 µl of 10% sodium dodecylsulfate (SDS) solution and 785 µl of 8 mg/ml Proteinase K were added to each tube. Tubes were then incubated with gentle shaking at 50 - 55 °C overnight (approximately 16 hours).

The next day, samples were allowed to cool to room temperature (20 - 22 °C) before addition of 3.30 ml of saturated NaCl (~6M), and were shaken well for 15 seconds. Samples were then centrifuged at 3750 rpm at 4 °C for 20 minutes. Supernatants were transferred to a new tube, centrifuged as before, and retransferred. After equilibration to room temperature, two volumes of room temperature 100% ethanol was added to precipitate the DNA. DNA was collected on a hook made from a sterile Pasteur pipette, washed with 70% ethanol twice, and allowed to air dry for approximately four minutes. Each hook was then placed in a conical tube containing 10 ml of TE' buffer (10 mM Tris-HCl, 0.2 mM Na_2EDTA, pH 7.5). After 5 minutes, DNA was shaken off of the hook into the TE', and the sample was covered and kept at room temperature for 2 - 3 days to dissolve the DNA. Samples were assayed by spectrophotometry at 280/260 nm for DNA quantitation and purity and then refrigerated at 4 °C prior to PCR amplification.

II.A.4.c.ii. PCR Amplification and Genotyping

PCR amplification and genotyping of DBA/1J, DBA/2J, C57BL/6J, and D1D2F2 mice were performed according to a procedure slightly different from the modification of Serikawa et al.(1992) to the

technique of Dietrich et al.(1992). Forward and reverse sequence primers of simple sequence length polymorphisms (SSLPs) purchased from Research Genetics (Huntsville, Alabama) were used unlabelled to amplify DNA. Briefly, 150 - 200 ng genomic DNA was amplified in a final volume of 25 µl 1x PCR buffer containing the following: 100 nM of each primer, 200 µM each dinucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, 1 Unit of *Tfl* DNA polymerase [Epicenter Technologies] in a 96-well plate. The plate was placed in a thermal cycler 9600 [Perkin-Elmer Cetus], and thermocycled according to the following protocol: 3 minutes initial denaturation at 94 °C, followed by 40 cycles of: 94 °C for 1 minute, 56 °C for 2 minutes, and 72 °C for 3 minutes. A final elongation step of 72 °C for 7 minutes was followed by cooling to 4 °C. Five µl bromphenol blue/xylene cyanole dyes in 100% formamide [gel loading solution, Sigma] was then added to the PCR products before loading on a high resolution agarose gel [4% MetaPhor™ or NuSieve® GTG®, FMC BioProducts] in the presence of 1 µg/ml ethidium bromide. Electrophoresis was performed for 4 to 5 hours at ~185 V (~10 V/cm of gel). PCR product bands were visualized under UV light.

II.B. Experimental Designs

II.B.1. Screening Tests: Experiments 1 through 7

II.B.1.a. Animals

Subjects were adult mice of both replicates of the Withdrawal Seizure Prone (WSP1 and WSP2) selectively bred lines. Female mice were used because they were available in sufficient numbers for these studies. These mice were born at the PVAMC VMU and reared

according to our standard colony conditions. Naive mice of the 47th to 50th filial generations (26th selection generation; i.e., $S_{26}G_{47\text{-}50}$) were tested for spontaneous or precipitated benzodiazepine withdrawal from one of the following drugs: alprazolam, triazolam, abecarnil, midazolam, and zolpidem.

II.B.1.b. Designs and Data Analyses

Acute withdrawal scores were analyzed using previously published procedures (Crabbe et al., 1991a; Metten & Crabbe, 1994a). For these and all subsequent experiments, the two pre-drug baseline scores for each animal were averaged. Average baseline (AVB) scores were analyzed for group differences by analyses of variance (ANOVA). In addition, each experiment was analyzed for group differences using a Replicate X Drug group (or Dose) X Time repeated measures ANOVA. Results were taken to be significant at $p < 0.05$. Where no differences between replicates were found, data were collapsed on replicate line for further analysis (i.e., replicate was ignored).

The common hypothesis for these experiments was uni-directional in that only increases above vehicle scores were deemed important. Because it seemed likely that each dose of each drug could produce withdrawal at a different time, planned comparisons were made. For each drug dose in the spontaneous withdrawal studies, a uni-directional t-test was performed on peak scores of the dose group versus the average scores of the vehicle group at the time peak occurred. The family wise alpha level of significance was set at $\alpha_{FW} = 0.05$. Thus, for each comparison, the one-tailed $\alpha = (0.05/3) = 0.017$,

where 3 is the number of planned comparisons in the spontaneous withdrawal studies (one for each dose of drug). For the precipitated withdrawal experiments, only one dose of each drug was tested. The one-tailed alpha was set at 0.05 for these comparisons.

II.B.1.b.i. Spontaneous Withdrawal: Experiments 1 through 5

Pilot tests were performed to determine the most likely time-course and dose range for withdrawal from each drug. Factors influencing choices included duration and extent of observed decreases in HIC prior to recovery of basal levels of HIC severity and estimated drug half-lives in mouse. Doses and time-courses for each drug were given above in Table 3. Appropriate vehicle or saline groups were included in all experiments. Five to ten animals per genetic replicate per dose were tested. This yielded a minimum sample size of 12 per dose per drug group when replicate lines were combined.

II.B.1.b.ii. Precipitated Withdrawal: Experiments 6 and 7

A single dose of each drug was chosen from the spontaneous withdrawal experiments for use in precipitated withdrawal assessment. Doses and time-courses for each drug were given above in Table 5. These doses were effective in depressing HIC scores in Experiments 1 - 5 over the times required for the precipitated withdrawal time-courses (i.e., at 30 and 55 minutes for alprazolam and triazolam; and at 10 and 19 minutes for abecarnil, midazolam, and zolpidem). Appropriate vehicle or saline groups were included at the time of abecarnil, midazolam, and alprazolam testing. Five to fifteen animals per genetic replicate per drug group were tested, yielding a minimum sample size of fourteen per drug group when replicate line data were collapsed. ANOVA grouping factors were Replicate and

Drug. Withdrawal severity was calculated as for Experiments 1 through 5. When a drug-treated animal's first post-flumazenil HIC score was the maximum score, the average of the first three scores was used to assess the peak score.

II.B.2. Genetic Correlation Studies: Experiments 8 through 11

II.B.2.a. Experiments 8 and 9 - Inbred Strain Tests

II.B.2.a.i. Animals

Subjects in Experiment 8 were adult male mice from the following inbred strains: 129/J, A/HeJ, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6J, C57BR/cdJ, C57L/J, CBA/J, DBA/1J, DBA/2J, PL/J, SJL/J, and SWR/J. These particular inbred strains were chosen for their genetic diversity, availability, and because we have tested them for many other drug-related phenotypes (Belknap et al., 1993a; Belknap et al., 1993b; Crabbe, 1983; Crabbe et al., 1994b; Crabbe et al., 1980b; Crabbe et al., 1983; Crabbe et al., unpublished data; Kosobud & Crabbe, 1990; Metten & Crabbe, 1994a). In the present experiment, these strains were tested for precipitated withdrawal following 20 mg/kg diazepam. The withdrawal time-course for this experiment was specified in Table 6. The previous diazepam withdrawal data in these same inbred strains predicted that a minimum sample size of 4 to 6 per drug group would be adequate to produce reliable means (reliability: 0.93; Metten & Crabbe, 1994a). Therefore, sample sizes for each drug group were 5 to 6 animals.

Only C57BL/6J, DBA/1J, and DBA/2J mice were used in Experiment 9. C57BL/6J mice were included because they are one of the progenitors to the HAW and LAW selectively bred lines (the other progenitor is DBA/2J). Eight mice per strain were tested for

spontaneous withdrawal following 20 mg/kg zolpidem according to the time-course given in Table 4. Dose and time-course were modified from Experiment 5 (see Results). The dose was lowered to a dose intermediate between 3 and 30 mg/kg in order to preserve drug. The time-course was modified to decrease the number of HIC assessments during the first hour, and increase their number during the rest of the time-course. Both dose and time-course for Experiments 9, 11A, and 13 (see Table 4) were the same as used in the inbred panel test (Metten, unpublished). Spleens were harvested from two mice per strain for genotyping in Experiment 12.

II.B.2.a.ii. Designs and Data Analyses

Experiment 8 was designed as a replicate test of precipitated diazepam withdrawal using naive animals of the same strains, vehicle, and time-course as in Metten & Crabbe (1994a). Average baseline scores were analyzed statistically by two-way (Strain X Drug group) ANOVA. Peak withdrawal severity scores calculated as above were subjected to one-way (Strain) ANOVA. Withdrawal severity was also assessed in the manner described in Metten & Crabbe (1994a) for the purpose of assessing the genetic correlations with the previous diazepam, ethanol, and pentobarbital scores. Briefly, the strain-appropriate mean area under the withdrawal curve (AUC) of the vehicle groups were subtracted from the AUC of each animal in the drug group, and negative values were corrected to zero. Strain mean diazepam withdrawal scores were then calculated. These scores were subjected to ANOVA (Strain). The proportion of total phenotypic variance in peak 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 inbred strain mean acute withdrawal severities for diazepam in naive mice were genetically correlated with the previously collected scores (Metten & Crabbe, 1994a). A re-analysis of the genetic correlations among ethanol, pentobarbital, and diazepam withdrawal severities was also performed including means from the present diazepam data set. Statistical significance for the genetic correlations was based on a two-tailed test, with $\alpha = 0.05$. The percentage of the common phenotypic variance accounted for by genetic factors was estimated as the square of the correlation coefficient (Falconer, 1989).

Experiment 9 was designed to test spontaneous zolpidem withdrawal using naive animals from the C57BL/6J, DBA/1J, and DBA/2J inbred strains. Mice of these and 11 other strains had been tested for spontaneous zolpidem withdrawal assessment (unpublished data) after evaluating quinpirole effects on activity and hypothermia (various doses). In that study, only DBA/2J mice showed significant withdrawal although all strains appeared to recover to baseline levels of HIC severity.

Average baseline and withdrawal severity scores were analyzed statistically by one-way (Strain) analyses of variance. The proportion of total phenotypic variance in zolpidem withdrawal accounted for by genetic factors was estimated as above.

II.B.2.b. Experiments 10 and 11 - Tests of Selectively Bred Lines

II.B.2.b.i. Animals

Subjects were adult male and female mice of the High Alcohol Withdrawal (HAW) and Low Alcohol Withdrawal (LAW) selectively bred lines. These mice were born at the PVAMC VMU and reared according to our standard colony conditions. Mice of both sexes from the second selected generation were tested for precipitated withdrawal after 20 mg/kg diazepam (Experiment 10). Fourteen to fifteen mice per line were in the drug group, and five mice per line were in the vehicle group. Male mice of the third selected generation were tested first for spontaneous (Experiment 11A; n = 14 -17/line) and then a week later for precipitated (Experiment 11B; n = 7 - 9/drug/line) withdrawal after 20 mg/kg zolpidem. BZR agonist withdrawal scores were compared only with ethanol withdrawal scores from the same selection generation.

II.B.2.b.ii. Designs and Data Analyses

Experimental design and data analyses for Experiments 10 and 11B followed the methods outlined above for Experiment 8 (refer to Table 6 for time-courses). Similarly, Experiment 11A was designed and analyzed as above for Experiment 9 (refer to Table 4 for time-course). The grouping factor for analyses of variance was Line in lieu of Strain.

II.B.3. Gene Mapping: Experiments 12 through 14

II.B.3.a. Animals in Experiment 12

Subjects were two DBA/1J mice that were tested in Experiment 9. Mice were sacrificed, and spleens were collected and processed as described above for genotyping using SSLP markers. For control and

comparison purposes, genomic DNA from DBA/2J and C57BL/6J mice were also included on the same gels. As the term SSLP implies, the pair of oligonucleotide primers for each marker recognize a unique DNA segment whose length (number of base pairs of DNA) tends to vary. When two animals differ in the length of the amplified segment (i.e., have different genotypes), the PCR product band of the shorter DNA segment will run through a gel more quickly than the band of the longer segment. Thus, visualization of the difference in placement of PCR product bands on a gel is evidence of genetic polymorphism.

SSLP markers in the regions of putative ethanol withdrawal QTLs that were previously mapped using C57BL/6J X DBA/2J crosses (Belknap et al., 1993c; Buck et al., submitted) were chosen for genotyping DBA/1J mice (see Figure 1). All markers were from the MIT marker set, and were purchased from Research Genetics, Inc. Markers were chosen to be polymorphic between DBA/2J and C57BL/6J, with at least 4 base pairs difference in allele lengths. Detection of the D2/B6 polymorphism would thus confirm that the gel and PCR conditions were appropriate. Three to seven markers per ethanol withdrawal QTL region were attempted.

Another set of MIT markers was used to genotype DBA/1J mice: these amplify DNA from regions of the genome previously identified to be polymorphic with DBA/2J alleles by comparison of allelotypes of loci mapped by other methods, e.g., *Hc*, on chromosome 2 at 25 cM (see Figure 2). These regions seemed like good candidates for identification of SSLPs, and were reasonable candidates for benzodiazepine withdrawal gene mapping given the paucity of DBA/1J genotyping data and the lack of any previous benzodiazepine

Figure 1

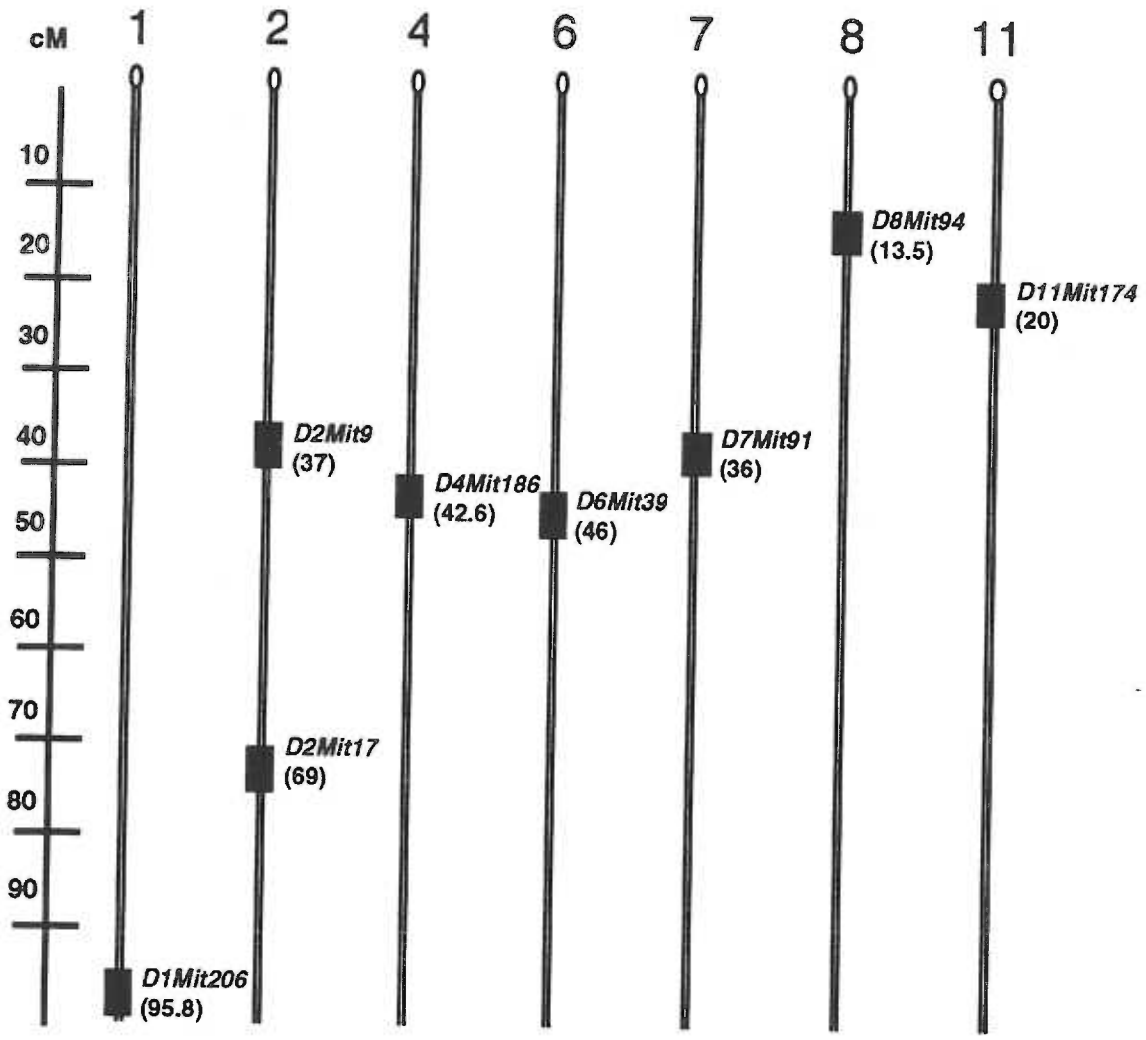

Figure 1: Locations of putative ethanol withdrawal QTLs (depicted by the black boxes) are shown on chromosome stick figures (numbered above the centromere). QTLs were mapped in B6 X D2 crosses (Belknap et al., 1993; Buck et al., submitted). SSLP marker names are shown to the right of each QTL and centiMorgan (cM) distances from the centromere are in parentheses (see scale at left). cM distances are from Silver et al. (1996). All chromosomes are depicted as 100 cM in total length, regardless of actual length.

Figure 2A

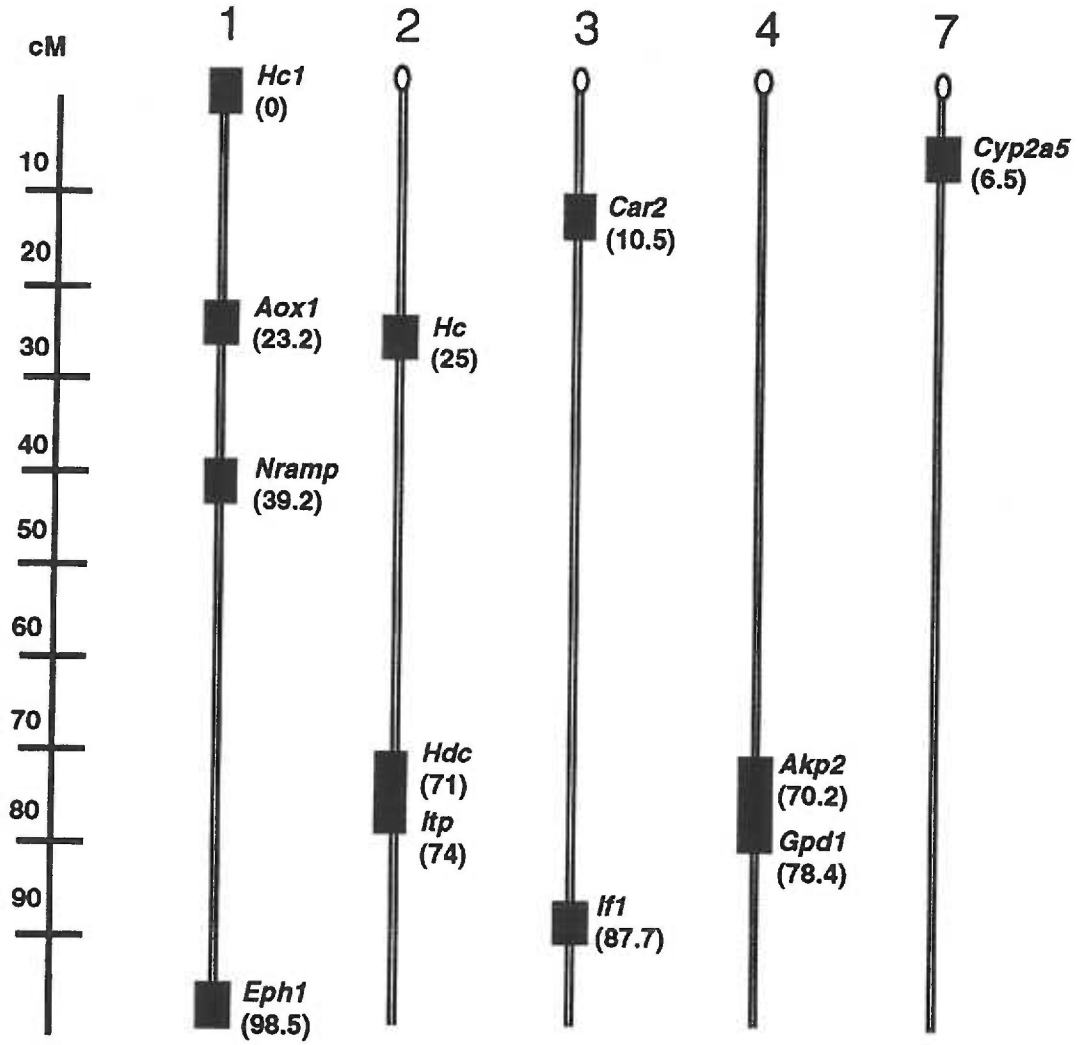

Figure 2: Regions of genetic polymorphism between DBA/1J and DBA/2J inbred strains are identified by a black box on chromosome stick figures (numbered above the centromere). Regions not marked by a box are not known to be polymorphic; isomorphism is assumed to be about 98% of the genome for these two strains. Locus names (shown to the right of each box) are those of actual polymorphic genes. CentiMorgan (cM) distances of each gene from the centromere are given in parentheses (see scale at left). Gene names and cM distances are from Silver et al. (1996). All chromosomes are depicted as 100 cM in total length, regardless of actual length.

Figure 2B

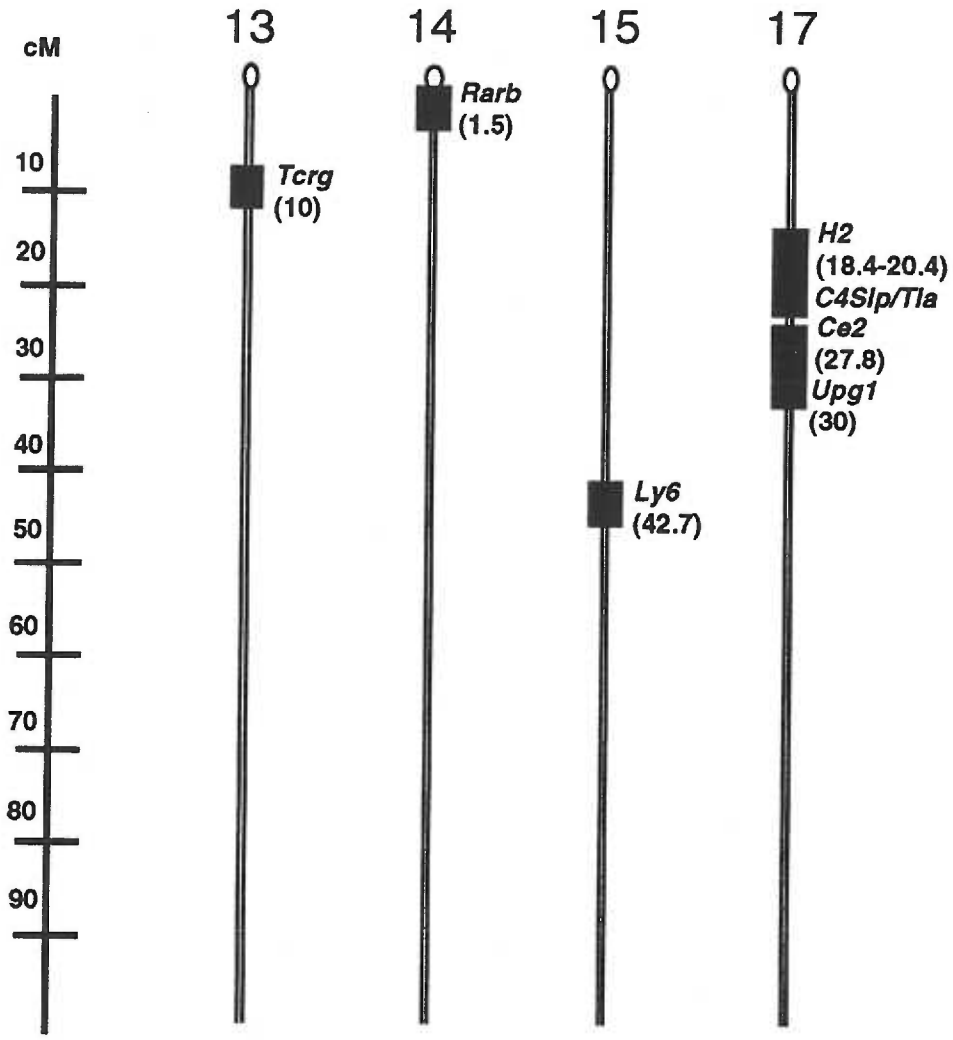

Figure 2B: Continued from previous page.

withdrawal QTL locations. Markers were chosen to be as close as possible to the known polymorphism. An attempt was made to obtain two to four markers as close to each polymorphism as possible (typically 0 to 5 cM proximal or distal), while still meeting the conditions described above. At the time of this experiment, no markers were available near *Hc1* (chromosome 1 at the centromere). PCR was attempted on the closest available marker, *D1Mit167* (chromosome 1 at 6.5 cM); however, neither the two-base pair polymorphism for D2/B6 nor a D1/D2 polymorphism were detected (data not shown).

II.B.3.b. Animals in Experiments 13 and 14

Subjects were adult male and female D1D2F2 mice (n = 160). The parental strains were also tested as available (D1: n = 10; D2: n = 19). These mice were born at the PVAMC VMU and reared according to our standard colony conditions. Mice were tested first for spontaneous zolpidem withdrawal (20 mg/kg), and then a week later for acute ethanol withdrawal (4 g/kg). Vehicle groups were not included in either study because the progenitors did not differ in baseline HICs or response to vehicle treatment in either Experiments 8 or 9, and because it would have increased the sample size required. After ethanol withdrawal testing, all D1D2F2 mice were sacrificed by cervical dislocation and spleens were harvested and frozen according to the methods described above.

II.B.3.c. Designs and Data Analyses

Estimation of the D1D2F2 sample size required for zolpidem withdrawal was made from the results of Experiment 9. First,

withdrawal severity data from the D1 and D2 strains only were subjected to a one-way ANOVA (Strain) and trait heritability (h^2) was estimated according to the expression,

$$h^2 = [\sigma^2_B/(\sigma^2_B + \sigma^2_W)],$$

where σ^2_W is estimated by the mean square within strains (MS_W) and σ^2_B is estimated by $(MS_B - MS_W)/n$, where MS_B is the mean square between strains and n is the average sample size per strain (Sokal & Rohlf, 1981). Hegmann and Possidente (1981) give the formula for estimation of expected heritability in an F2 intercross as,

$$h^2 = [\tfrac{1}{2}\sigma^2_B/(\tfrac{1}{2}\sigma^2_B + \sigma^2_W)],$$

where σ^2_B and σ^2_W are estimated from the overall ANOVA as above. Heritability is an estimate of the proportion of phenotypic variance attributable to genetic sources, and therefore, is an estimate of the genetic effect size (Belknap et al., 1996).

D1D2F2 sample size was then estimated according to the method described by Lander and Botstein (1989) as explicated by Belknap et al. (1996). As a conservative measure, it was assumed that two QTLs control the majority of the genetic variation in zolpidem withdrawal, and that a QTL would be detected with 90% probability (i.e., power) if it controlled at least 7% of the phenotypic variance. Criteria established by Lander and colleagues for statistical significance of a QTL at a single marker ($\alpha_S = 0.0001$) were created for whole genome searches (Lander & Schork, 1994; Lander & Kruglyak, 1995). This was inappropriately stringent for the present study. Predicted genetic homology between the DBA substrains (98%; Bailey, 1978)

precludes a whole genome search. The two-tailed α_S for the present study was set at 0.05. Therefore, according to the expression,

$$N_{(F2)} = (Z_\alpha + Z_\beta)^2(s^2_{RES}/s^2_{QTL})$$

the requisite sample size of D1D2F2s would be 139, where Z_α and Z_β are the normal variates of α_S (i.e., $Z_\alpha = 1.96$) and β (i.e, 1 - power; $Z_\beta = 1.28$), s^2_{QTL} is the proportion of the phenotypic variance controlled by a single QTL (i.e., 7%), and s^2_{RES} is the residual (remaining) variance. However, this number was increased to N = 160 due to the need for selective genotyping (Lander & Botstein, 1989). It was planned to genotype only the phenotypic extremes of the distribution, using approximately one third of the total sample. Selective genotyping doesn't result in significant loss of power with proper sample sizes (Lander & Botstein, 1989).

Withdrawal severity scores were calculated for each drug using both peak and area measures. Pearson's r was employed to assess the phenotypic correlation between zolpidem withdrawal and ethanol withdrawal in the same animals. Correlations were taken to be significant at $\alpha = 0.05$.

Markers identified to be polymorphic between DBA/1J and DBA/2J mice in Experiment 12 were chosen for genotyping via PCR of D1D2F2 mice from both phenotypic extremes. On each gel, DNA PCR products from DBA/1J, DBA/2J mice and a mixed lane (simulating D1D2F1) were run for comparison purposes with the D1D2F2s. Genotypes were interpreted by two people from the position of bands on the gel as described above. Gene dosages were assigned to the genotype for each marker according to the number of DBA/2J alleles

possessed by each F2 animal (i.e., D1D1 = 0, D1D2 = 1, and D2D2 = 2). The difference in allele frequency of the D2 allele between the phenotypic extreme groups (i.e., $q_H - q_L$), was analyzed at each marker by chi-square test. The conditions of the chi-square were set as a two-tailed test with one degree of freedom and $p < 0.05$, which is appropriate for a limited genome search. A significant chi-square would imply linkage of the trait with the marker, since allele frequencies at unlinked markers would be maintained at $p = q = 0.5$, according to Hardy-Weinberg equilibrium (Falconer, 1989). This strategy has been employed previously in selectively bred lines (Belknap et al., in press).

III. Results

III.A. BZR Ligand Screening Tests

III.A.1. Experiments 1 - 5 Spontaneous Withdrawal

Results of these experiments are shown in Figures 3 through 7. These studies demonstrate that WSP female mice are susceptible to withdrawal convulsions following treatment with some BZR ligands without precipitation. For all drugs except midazolam, baseline HIC severity scores did not differ among dose groups or replicates of the WSP line.

III.A.1.a. Experiment 1 Alprazolam

In this experiment, a significant interaction of replicate with dose and time was found in the repeated measures ANOVA ($F_{(33,572)} = 1.52$, $p = 0.03$). There were no other significant effects of replicate (all other $Fs < 2.35$, $ps > 0.08$). Closer examination of the interaction revealed that it was due primarily to lower scores among vehicle

treatment groups in the WSP2 replicate compared to WSP1 (data not shown). It also appeared that WSP2 mice treated with 0.75 mg/kg alprazolam had lower peak withdrawal scores relative to WSP1 mice (data not shown). Therefore, replicate-appropriate vehicle means were used when calculating withdrawal severity for drug-treated animals. Time-courses and withdrawal severity data (inset) are shown in Figure 3 collapsed on replicate. Repeated measures ANOVA also revealed significant main effects of dose and time and a significant dose by time interaction (all $Fs > 10.11$, $ps < 0.01$). All doses of alprazolam suppressed HIC at 30 minutes after injection. The modal time of peak HIC scores for 0.5 mg/kg alprazolam was 300 minutes post-injection. Modal peak for the other two doses was 360 minutes. The average vehicle group HIC scores used to calculate withdrawal severity for 0.5, 0.75, and 1.0 mg/kg dose groups were 2.10±0.26, 1.95±0.32, and 1.95±0.32 (WSP1) and 1.54±0.15, 1.67±0.14, and 1.67±0.14 (WSP2), respectively. Withdrawal severity scores were significantly elevated in the 0.5 and 1.0 mg/kg dose groups (see inset; $ts > 3.31$, $ps < 0.002$). Increases in withdrawal severity in the 0.75 mg/kg dose group fell just short of significance ($t = 2.10$, $p = 0.023$).

III.A.1.b. Experiment 2 Triazolam

There were no significant effects of replicate over the time-course of triazolam withdrawal testing (all $Fs < 1.65$, $ps > 0.11$). Therefore, time-courses and withdrawal severity data (inset) are shown in Figure 4 collapsed on replicate. Repeated measures ANOVA revealed that there were significant main effects of dose group and time, as well as a significant dose group X time interaction (all

Figure 3

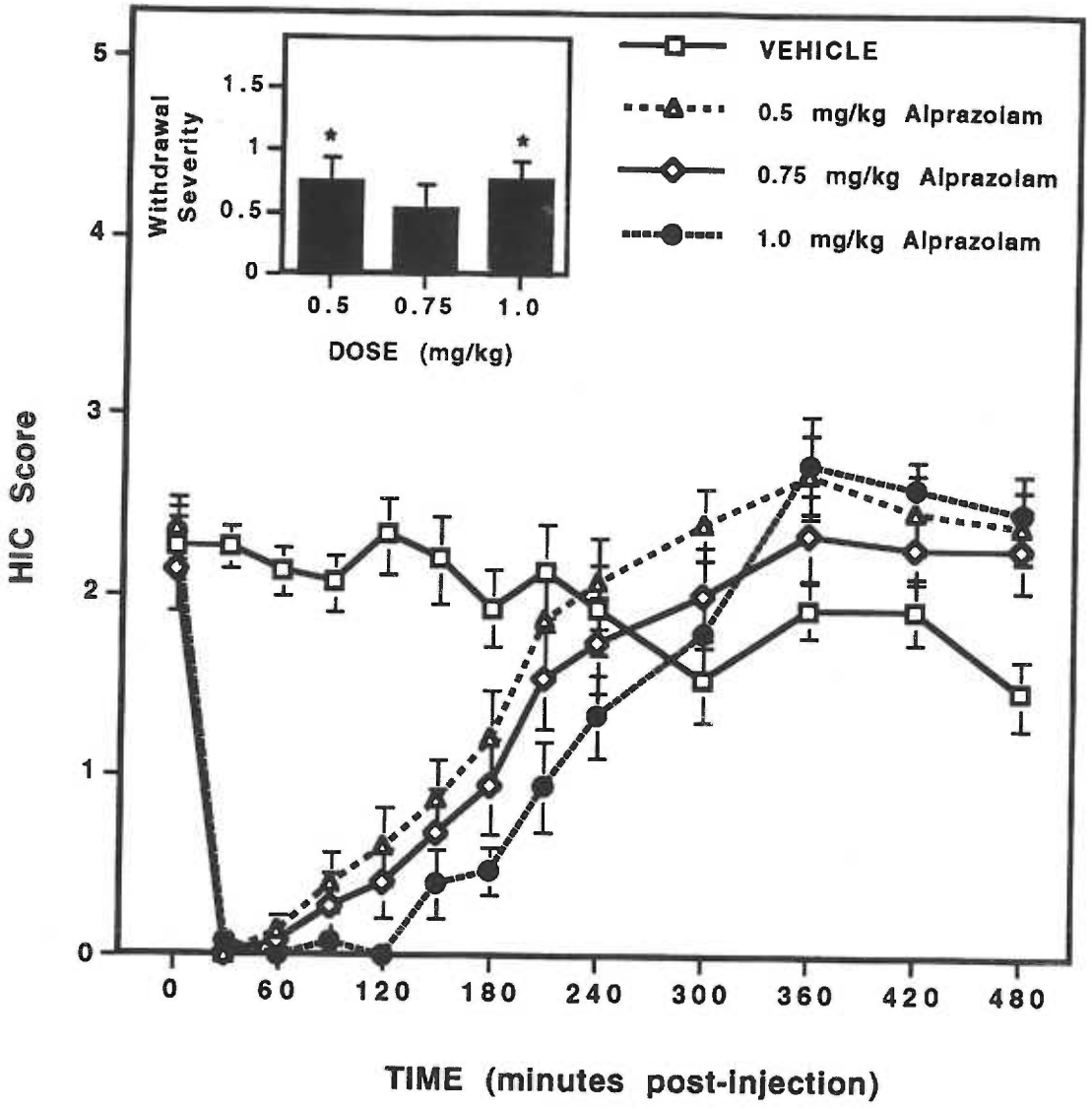

Figure 3: Time-course of spontaneous alprazolam withdrawal severity in WSP female mice. Alprazolam or vehicle was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following injection. **Inset:** Withdrawal severity scores among the three drug doses. Bars represent mean ± SE for each group. Withdrawal severity was calculated versus vehicle group as discussed in the text. Significant withdrawal was shown by animals in the 0.5 and 1.0 mg/kg dose groups (* $p < 0.002$). Withdrawal was not quite significantly elevated in the 0.75 mg/kg dose group ($p = 0.023$).

Figure 4

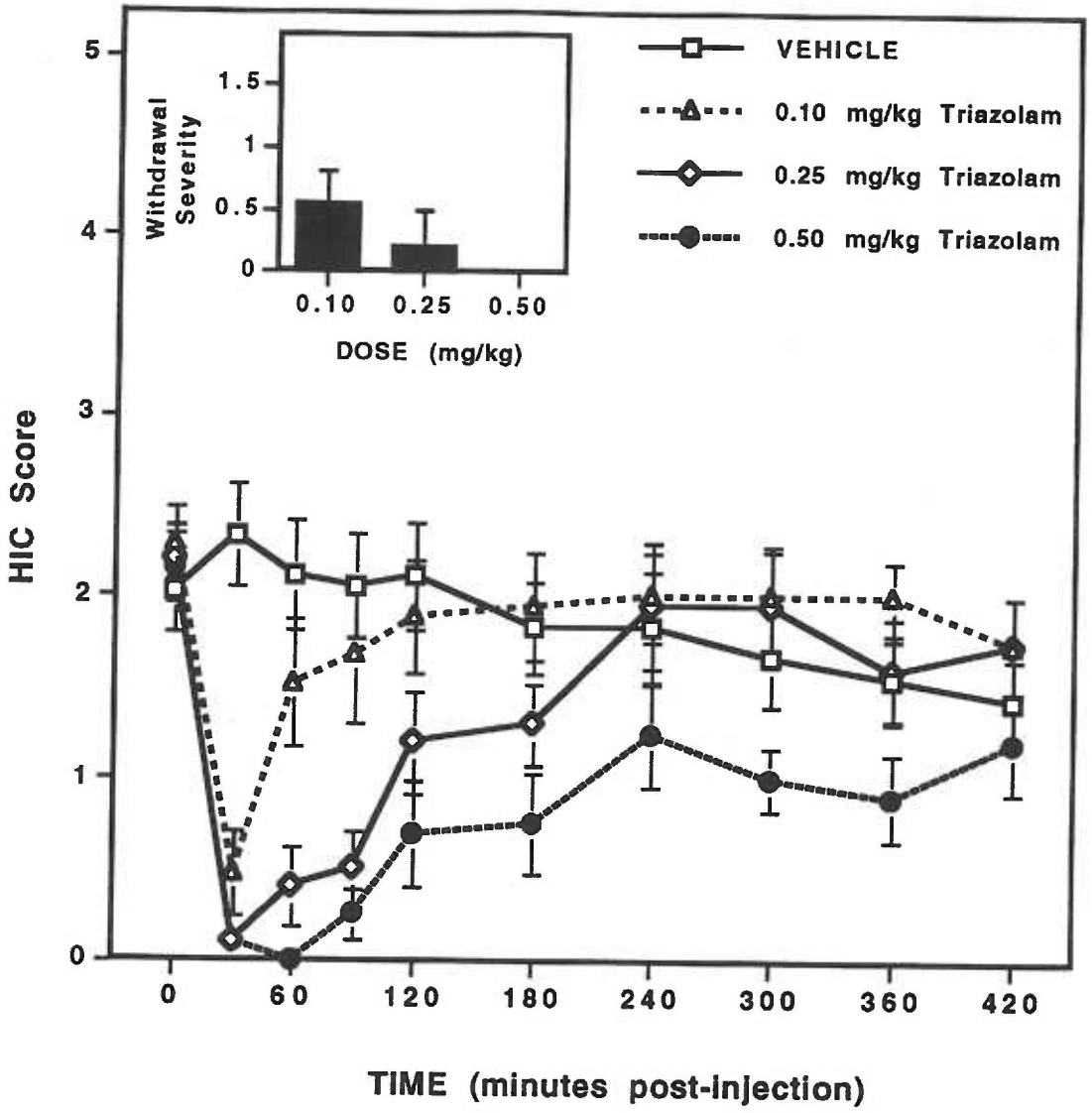

Figure 4: Time-course of spontaneous triazolam withdrawal severity in WSP female mice. Triazolam or vehicle was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following injection. **Inset:** Withdrawal severity scores among the three drug doses. Bars represent mean ± SE for each group. Withdrawal severity was calculated versus vehicle group as discussed in the text. There was no significant withdrawal in any of the dose groups.

Fs > 6.28, ps < 0.01). All doses of triazolam produced decreases in HIC at 30 minutes after injection, but complete depression was not observed at the lowest dose (0.1 mg/kg). The modal time of peak HIC scores was 240 minutes post-injection for all three doses. The average vehicle group HIC score used to calculate withdrawal severity was 1.78±0.25. Although there appeared to be modest increases in HIC above vehicle levels at later time-points with 0.1 and 0.25 mg/kg triazolam (see Figure 4), withdrawal severity was not significantly elevated at any dose (see inset; ts < |±1.68|; ps > 0.05, one-tailed).

III.A.1.c. Experiment 3 Abecarnil

No significant effects of replicate were found on baseline scores or the time-course of the abecarnil withdrawal test (all Fs < 1.84, ps > 0.18). Therefore, time-courses and withdrawal severity data (inset) are shown in Figure 5 collapsed on replicate. Repeated measures ANOVA revealed that there were significant main effects of dose group and time, as well as a significant dose group X time interaction (all Fs > 4.96, ps < 0.01). Although all doses of abecarnil produced decreases in HIC at 30 minutes after injection, the lowest dose (1 mg/kg) failed to completely suppress HIC. The modal times of peak HIC scores for 1, 2, and 3 mg/kg abecarnil were 180, 240, and 300 minutes post-injection, respectively. The average vehicle group HIC scores used to calculate withdrawal severity for each dose were 2.10±0.27, 2.05±0.23, and 2.02±0.24, respectively. Neither 2 nor 3 mg/kg abecarnil produced significant withdrawal in this paradigm (peak scores were not significantly greater than vehicle group scores (ts < |±0.47|).

Figure 5

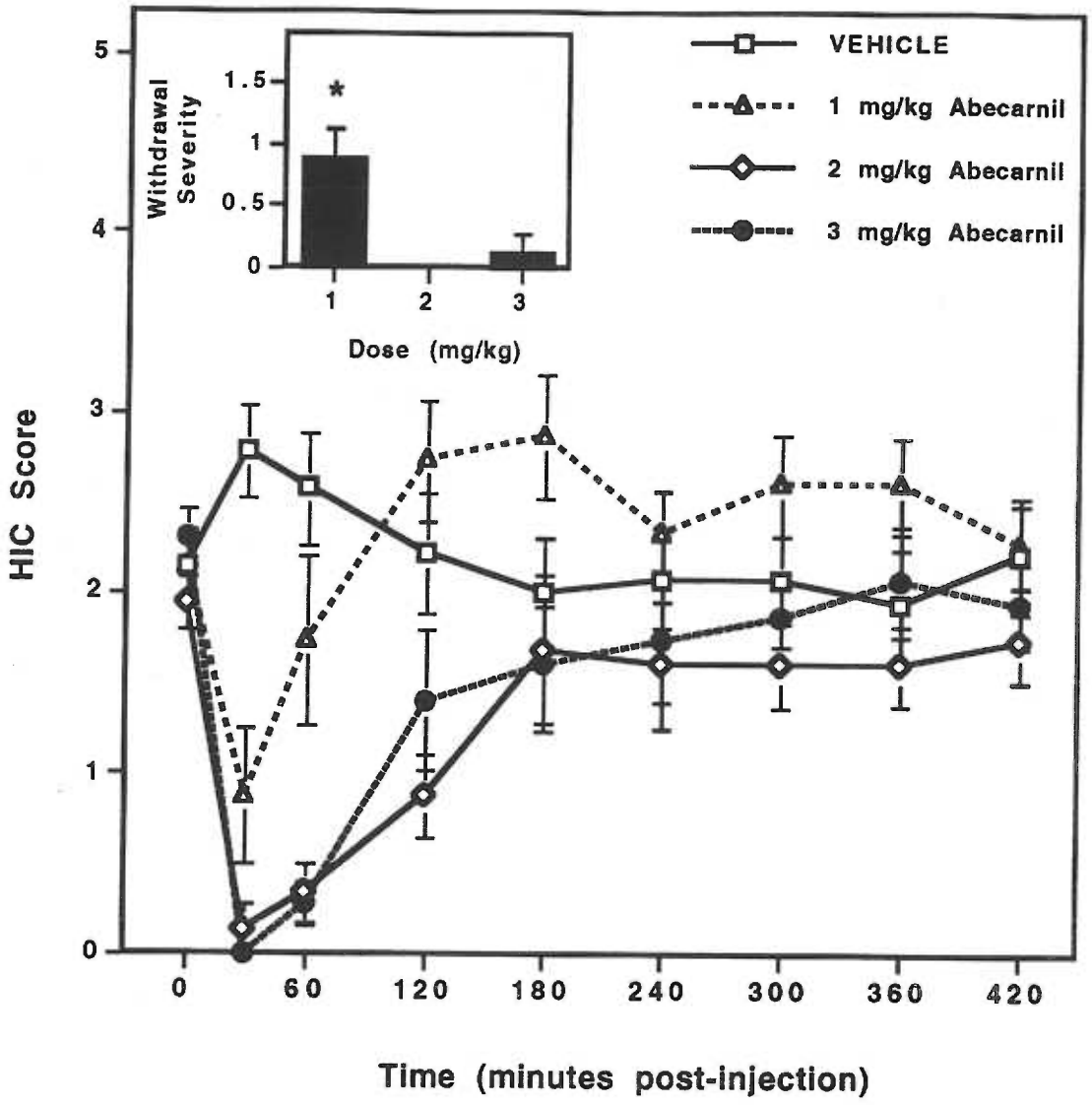

Figure 5: Time-course of spontaneous abecarnil withdrawal severity in WSP female mice. Abecarnil or vehicle was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following injection. **Inset:** Withdrawal severity scores among the three drug doses. Bars represent mean ± SE for each group. Withdrawal severity was calculated versus vehicle group as discussed in the text. Significant withdrawal was shown by animals in the 1 mg/kg abecarnil group only (* $p < 0.011$).

Withdrawal severity was significantly elevated in the 1 mg/kg group (see inset; $t = 2.44$, $p = 0.011$).

III.A.1.d. Experiment 4 Midazolam

A shortage of replicate 2 WSP females at the time of this experiment resulted in the use of over twice as many replicate 1 (WSP1, n = 10/dose) as replicate 2 (WSP2, n= 3 - 4/dose) mice. A significant effect of replicate was found for baseline HIC (WSP2: 2.37±0.16 > WSP1: 1.83±0.11; $F_{(1,47)} = 6.18$, $p < 0.02$). There also was a significant main effect of replicate in the Replicate X Dose X Time repeated measures ANOVA ($F_{(1,47)} = 9.29$, $p < 0.01$), but no interactions were significant (all Fs < 1.28, ps > 0.21). Therefore, replicate-appropriate vehicle means were used when calculating withdrawal severity scores for drug-treated animals. Time-course and withdrawal severity data (inset) are shown in Figure 6 collapsed on replicate. Repeated measures ANOVA revealed that there were significant main effects of dose group and time, as well as a significant dose group X time interaction (all Fs > 5.69, ps < 0.01). All doses of midazolam suppressed HIC scores as soon as 15 minutes after injection. The mean peak score of animals treated with the highest dose of midazolam was clearly lower than the vehicle group mean; thus, this dose failed to produce acute withdrawal (see Figure 6). HIC scores returned to control levels at about 90 - 120 minutes after injection in animals receiving the lowest dose of midazolam, 2.5 mg/kg. Modal peak for this dose was a four-way tie (among 120, 150, 270, and 330 minutes) with two animals scoring maximum at each point. As a tie-breaker, the time of mean peak for the entire dose

Figure 6

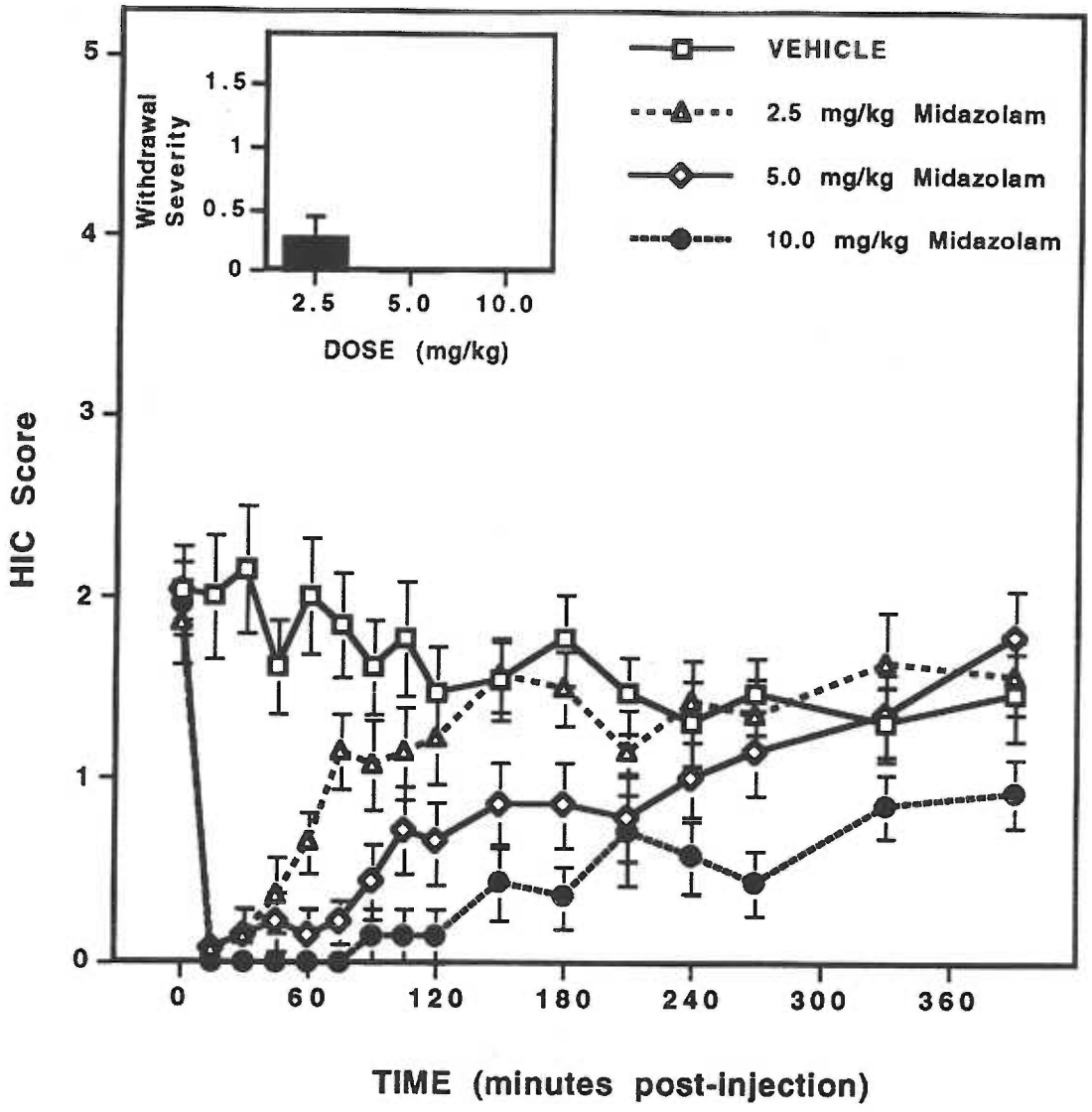

Figure 6: Time-course of spontaneous midazolam withdrawal severity in WSP female mice. Midazolam or vehicle was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following injection. **Inset:** Withdrawal severity scores among the three drug doses. Bars represent mean ± SE for each group. Withdrawal severity was calculated versus vehicle group as discussed in the text. There was no significant withdrawal in any of the dose groups.

group (i.e., 330 minutes) was used to calculate the vehicle group mean. The average vehicle group scores were 1.23±0.24 (WSP1) and 2.00±0.00 (WSP2). For the 5 mg/kg dose, the modal peak occurred at 390 minutes, the last time-point. The average vehicle group scores were the same as for the 2.5 mg/kg dose, since the three scores used to compute peak for both doses were those occurring at 270, 330, and 390 minutes. No withdrawal was shown for either of the two lower doses (Figure 6 and inset; both ts < $|\pm 1.29|$; ps > 0.11, one-tailed).

III.A.1.e. Experiment 5 Zolpidem

The lowest dose of zolpidem, 0.3 mg/kg, was totally ineffective in suppressing HIC at any time point measured (data not shown). Therefore, this dose was dropped from the analyses. No significant effects of replicate were found on the time-course of zolpidem withdrawal, and time-courses and withdrawal severity data (inset) are shown in Figure 7 collapsed on replicate. Repeated measures ANOVA revealed that there was a significant main effect of time, as well as a significant dose group X time interaction (both Fs > 12.77, ps < 0.01); however, a main effect of dose was not apparent ($F_{(2,30)} = 0.93$, p = 0.41). Both remaining doses of zolpidem decreased HIC at ten minutes after injection, but with 3 mg/kg suppression was incomplete (see Figure 7). The modal times of peak HIC scores were 30 (3 mg/kg dose; mean vehicle score: 1.89±0.24) and 120 (30 mg/kg dose; 1.61±0.29) minutes post-injection. Withdrawal severity scores were significantly elevated in the 30 mg/kg dose group (see inset; t = 3.44, p = 0.001). Withdrawal severity just missed being significantly

Figure 7

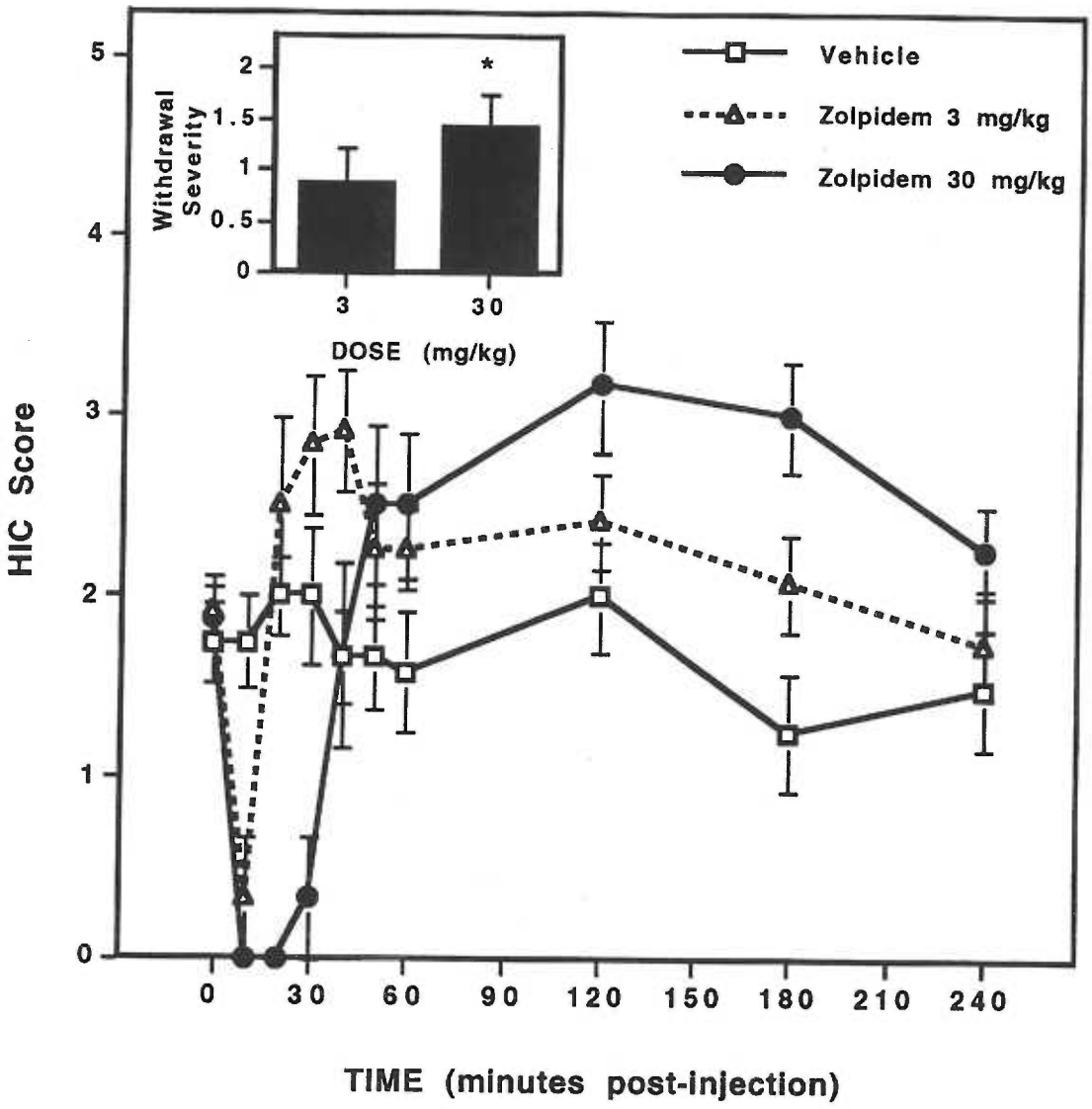

Figure 7: Time-course of spontaneous zolpidem withdrawal severity in WSP female mice. Zolpidem or vehicle was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following injection. **Inset:** Withdrawal severity scores of the two drug doses. Bars represent mean ± SE for each group. Withdrawal severity was calculated versus vehicle group as discussed in the text. Significant withdrawal was shown by animals given 30 mg/kg, but not 0.3 mg/kg (data not shown; * $p < 0.001$). Withdrawal severity just missed significance in the 3 mg/kg dose group ($p = 0.020$).

elevated in the 3 mg/kg dose group ($t = 2.19$, $p = 0.020$) using the alpha level of 0.017, derived from $\alpha_{FW} = 0.05$, as discussed previously.

III.A.1.f. Summary of Spontaneous Withdrawal Experiment Results

The results of Experiments 1 through 5 show clearly that WSP female mice are sensitive to withdrawal from some BZR ligands without precipitation. Alprazolam, abecarnil, and zolpidem were effective in inducing spontaneous withdrawal convulsions (Figures 3, 5, and 7). Neither triazolam nor midazolam produced withdrawal in this paradigm over the time-courses employed (Figures 4 and 6).

III.A.2. Experiments 6 and 7 Precipitated Withdrawal

Based on the results of the spontaneous withdrawal experiments, a dose of each drug was chosen for testing in the precipitated withdrawal paradigm. Each dose was chosen based on its demonstration of complete HIC suppression for the time points prior to that for injection of flumazenil (the antagonist). Results of these experiments are shown in Figures 8 through 12. Briefly, these studies indicate that WSP female mice are sensitive to precipitated withdrawal convulsions following a variety of BZR ligands. Analyses of baseline HIC scores for each drug test indicated that these did not differ between replicates of the WSP line (all $Fs < 3.62$, $ps > 0.06$). Comparison of time-course-appropriate vehicle groups indicated that they differed neither for replicate nor test day ($Fs < 2.41$, $ps > 0.13$ for the 32 minute time-course; $Fs < 2.16$, $ps > 0.15$ for the 72 minute time-course). Therefore, all data were collapsed on replicate for analyses and presentation. Composite vehicle groups were formed for further analyses. For zolpidem-treated animals only, baseline HICs were

higher than those in the composite vehicle group ($F_{(1,56)} = 9.22$, $p < 0.01$). Therefore, as a more conservative approach, zolpidem withdrawal severity was assessed both as a difference from vehicle (between groups) and as a difference from baseline (within animal).

III.A.2.a. Experiment 6 Long Time-Course

III.A.2.a.i. Alprazolam

Repeated measures ANOVA of the precipitated alprazolam withdrawal test revealed that there were significant main effects of drug group and time, as well as a significant drug group X time interaction (all Fs > 10.70, ps < 0.01). Consistent with Experiment 1, 0.5 mg/kg alprazolam depressed HIC scores at 30 and 55 minutes post-injection (Figure 8). Maximal withdrawal scores were obtained at the first time-point (i.e., 1 minute) after flumazenil injection. Therefore, peak was calculated as the average of the scores at the first three time-points (61, 63, and 65) and compared to the average vehicle score (1.57±0.10) over the same time period. Precipitated withdrawal severity was significantly elevated in the drug group ($t = 8.79$, $p < 0.01$; Figure 8, inset).

III.A.2.a.ii. Triazolam

There were significant main effects of drug group and time, as well as a significant drug group X time interaction in the repeated measures analysis of variance of the precipitated triazolam withdrawal test (all Fs > 32.83, ps < 0.01). Consistent with its effect in the spontaneous withdrawal paradigm (Experiment 2), 0.5 mg/kg triazolam depressed HIC scores at 30 and 55 minutes after injection (Figure 9). Maximal withdrawal HIC scores were obtained at 63

Figure 8

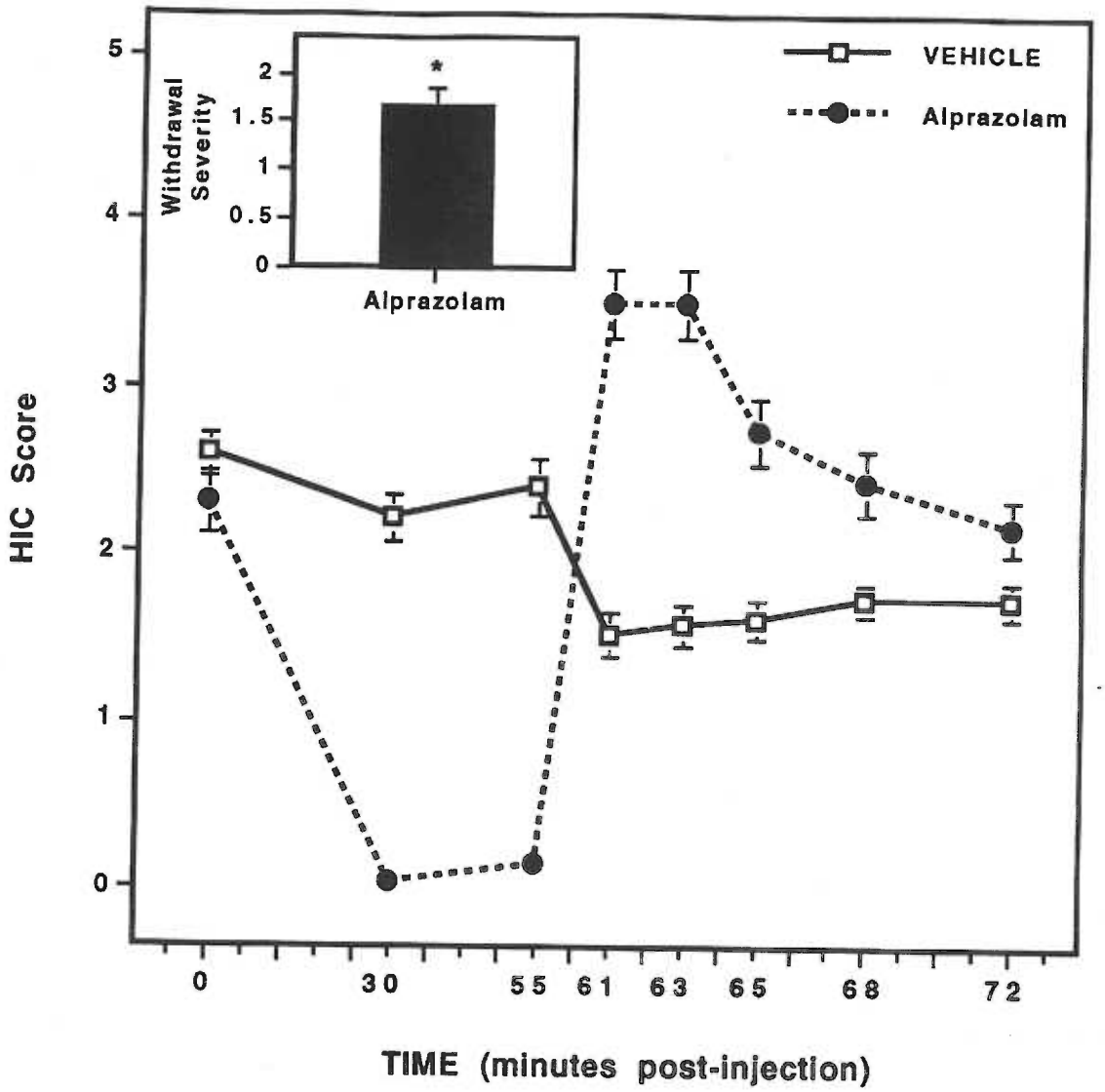

Figure 8: Time-course of alprazolam precipitated withdrawal severity in WSP female mice. Alprazolam or vehicle was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). HICs were scored at 30 and 55 minutes after injection. At 60 minutes, the BZR antagonist, flumazenil, was injected, and HICs were scored 1, 3, 5, 8, and 12 minutes later. Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following BZR agonist or vehicle injection. **Inset:** Alprazolam withdrawal severity score. Bar represents mean ± SE. Withdrawal severity was calculated as discussed in the text. Withdrawal was significantly elevated in the alprazolam-treated group compared to vehicle (* $p < 0.05$).

Figure 9

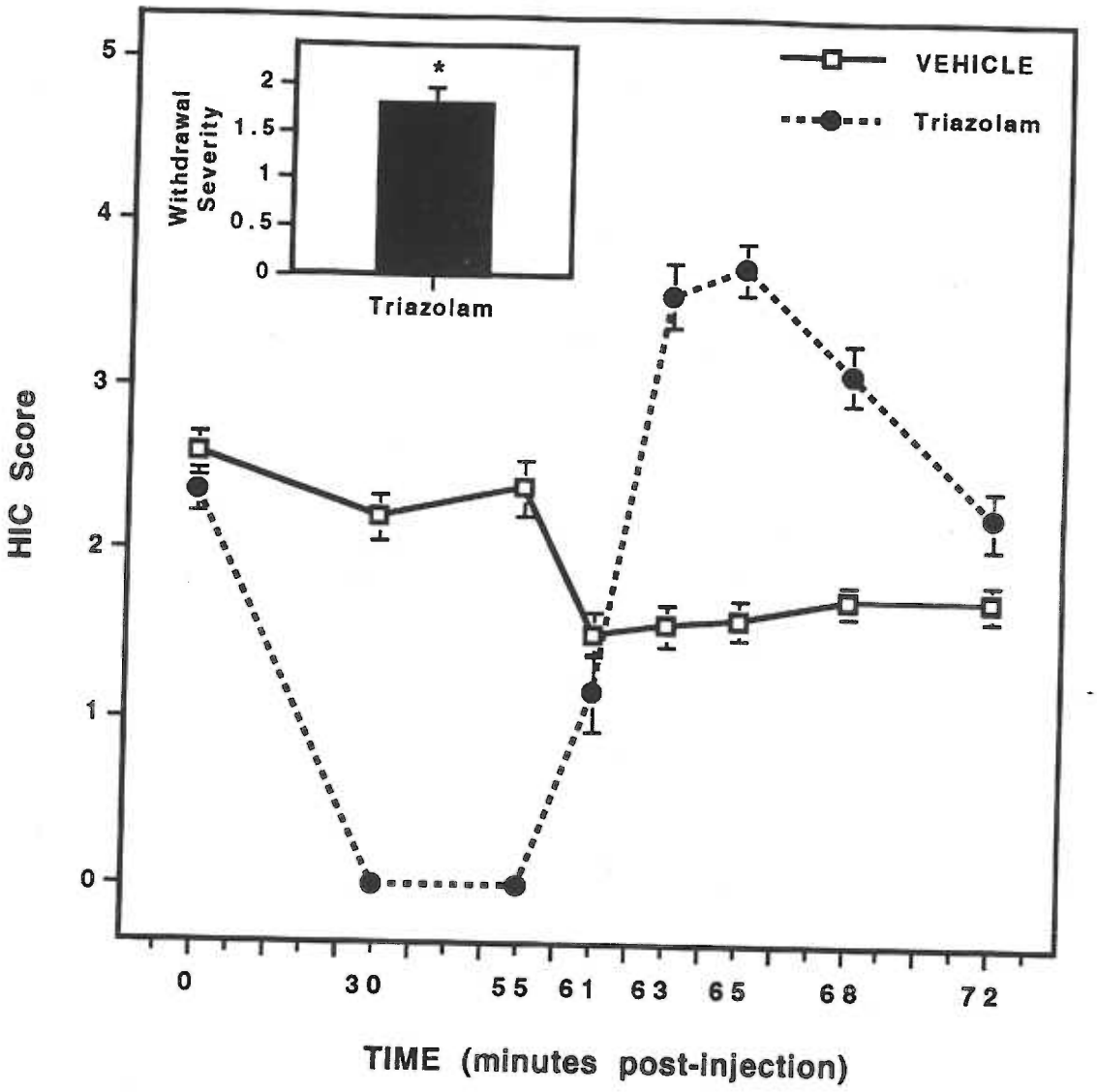

Figure 9: Time-course of triazolam precipitated withdrawal severity in WSP female mice. Triazolam or vehicle was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). HICs were scored at 30 and 55 minutes after injection. At 60 minutes, the BZR antagonist, flumazenil, was injected, and HICs were scored 1, 3, 5, 8, and 12 minutes later. Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following BZR agonist or vehicle injection. **Inset:** Triazolam withdrawal severity score. Bar represents mean ± SE. Withdrawal severity was calculated as discussed in the text. Withdrawal was significantly elevated in the triazolam-treated group compared to vehicle (* $p < 0.05$).

minutes (i.e., 3 minutes after flumazenil injection), so peak withdrawal in the drug group and average vehicle scores were calculated as the average of the first three time-points. The average vehicle group score was 1.64±0.09. In contrast to the response seen in the spontaneous withdrawal paradigm, precipitated withdrawal was significantly elevated in the triazolam-treated group compared to control ($t = 10.53$, $p < 0.01$).

III.A.2.b. Experiment 7 Short Time-Course

III.A.2.b.i. Abecarnil

Repeated measures ANOVA indicated that there were significant main effects of drug and time, as well as a significant drug X time interaction (all $Fs > 7.05$, $ps < 0.01$). In agreement with Experiment 3, 2.0 mg/kg abecarnil depressed HIC scores at 10 and 19 minutes post-injection (Figure 10). Interestingly, injection of flumazenil 20 minutes later appeared to have no effect on HIC expression in abecarnil-treated animals. In contrast to the findings in Experiment 3 in which abecarnil (1.0 mg/kg) produced spontaneous withdrawal convulsions (see Figure 5), HIC scores only gradually returned to vehicle levels by 32 minutes after abecarnil injection. No rebound was seen; thus, abecarnil did not produce withdrawal in the precipitated paradigm.

III.A.2.b.ii. Midazolam

A shortage of replicate 2 WSP mice at the time of this experiment resulted in the use of twice as many WSP1 as WSP2 mice. When the data were examined for effects of replicate, a significant main effect of replicate was found in the repeated measures ANOVA ($F_{(1,41)} = 10.08$, $p < 0.01$). Since there were no interactions (all

Figure 10

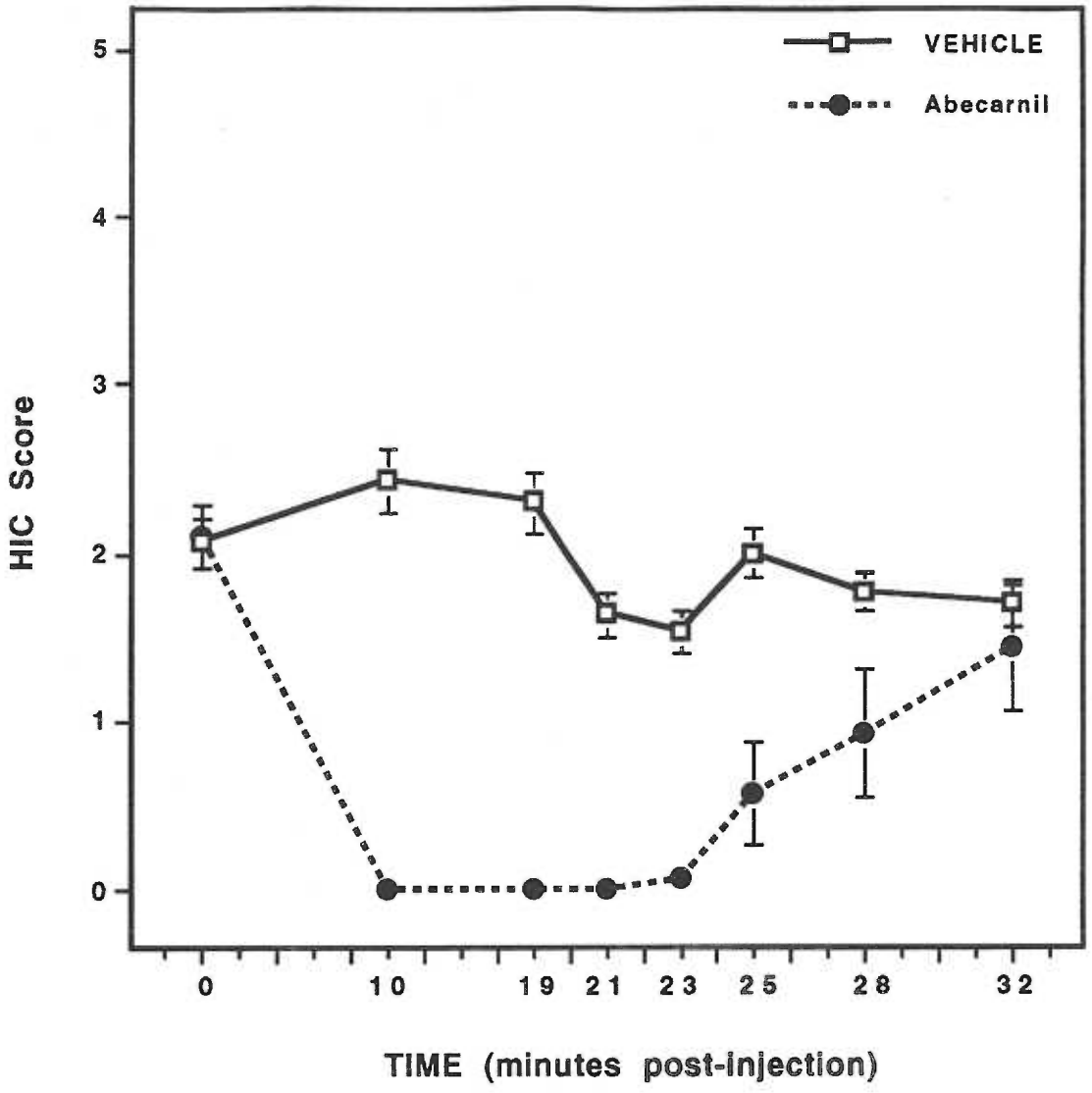

Figure 10: Time-course of precipitated abecarnil withdrawal severity in WSP female mice. Abecarnil or vehicle was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). HICs were scored at 10 and 19 minutes after injection. At 20 minutes, the BZR antagonist, flumazenil, was injected, and HICs were scored 1, 3, 5, 8, and 12 minutes later. Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following BZR agonist or vehicle injection. Abecarnil did not produce withdrawal in this paradigm.

Fs < 2.67, ps > 0.11), replicate-appropriate vehicle means were used when calculating withdrawal severity scores for drug-treated animals. All four drug treatment groups responded in exactly the manner predicted by previous experience (Crabbe et al., 1991a). In other words, the responses of the vehicle/vehicle and vehicle/flumazenil groups were almost identical, and the midazolam/vehicle group's scores were zero throughout the time-course. Therefore, the midazolam withdrawal time-course for only the midazolam/flumazenil versus vehicle/flumazenil groups and withdrawal severity data (inset) are shown in Figure 11 (collapsed on replicate). There were also significant main effects of drug group and time, and a significant drug group X time interaction (all Fs > 7.03, ps < 0.01). Consistent with its effect in Experiment 4, 2.5 mg/kg midazolam depressed HIC scores at 10 and 19 minutes post-injection (Figure 11). Maximal withdrawal HIC scores were obtained at the first time-point after flumazenil injection. Peak withdrawal in the drug group and average vehicle scores (1.72±0.11) were calculated as the average of the first three time-points. Unlike the response seen in the spontaneous withdrawal paradigm, flumazenil-precipitated withdrawal from 2.5 mg/kg midazolam was significantly elevated compared to vehicle treatment ($t = 6.82$, $p < 0.01$; Figure 11, inset).

III.A.2.b.iii. Zolpidem

Repeated measures ANOVA of precipitated zolpidem withdrawal data revealed that there were significant main effects of drug group and time, as well as a significant drug group X time interaction (all Fs > 25.15, ps < 0.01). In agreement with the results of Experiment 5, 30 mg/kg zolpidem depressed HIC scores at 10 and 19 minutes post-

Figure 11

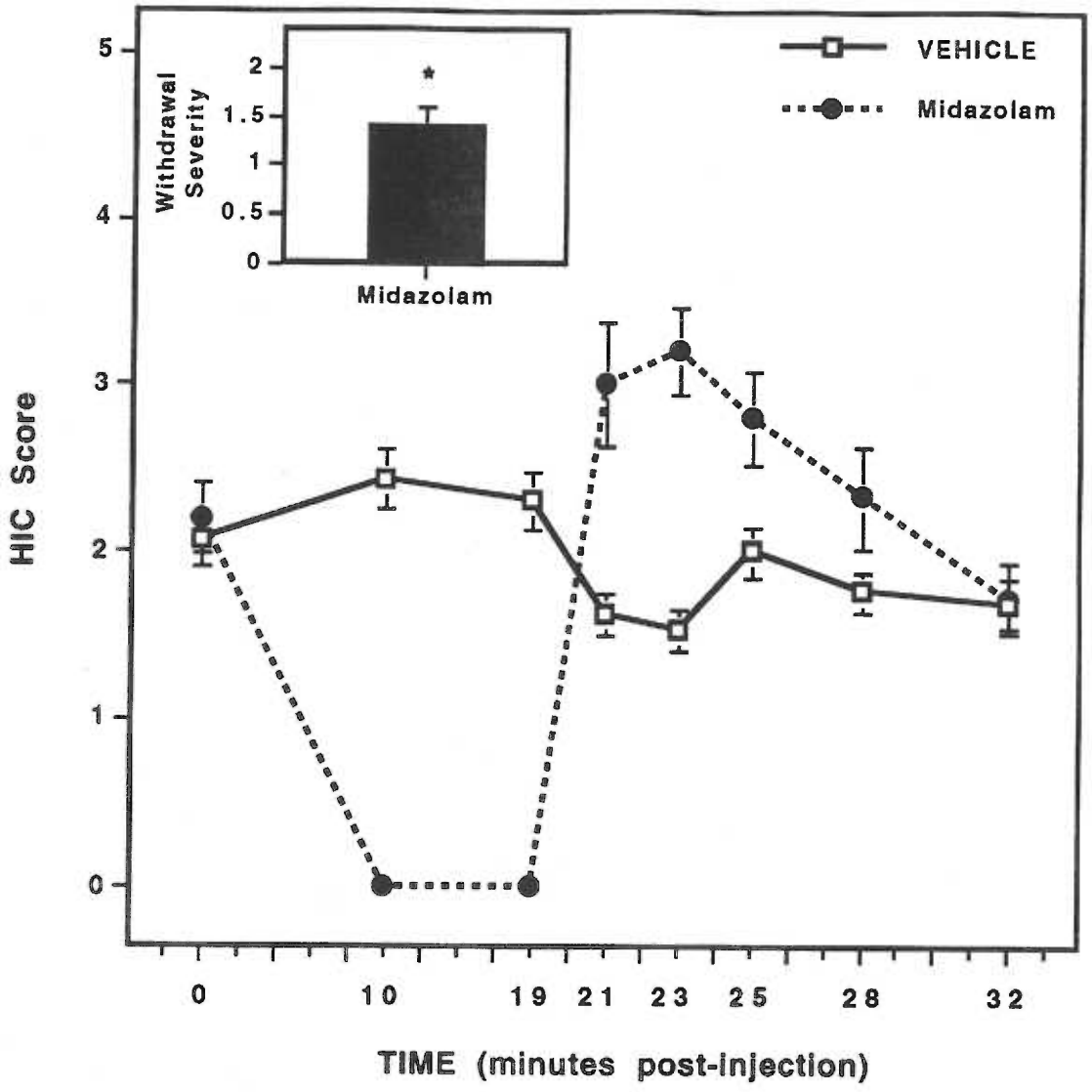

Figure 11: Time-course of precipitated midazolam withdrawal severity in WSP female mice. Midazolam or vehicle was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). HICs were scored at 10 and 19 minutes after injection. At 20 minutes, the BZR antagonist, flumazenil, was injected, and HICs were scored 1, 3, 5, 8, and 12 minutes later. Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following BZR agonist or vehicle injection. **Inset:** Midazolam withdrawal severity score. Bar represents mean ± SE. Withdrawal severity was calculated as discussed in the text. Withdrawal was significantly elevated in the midazolam-treated group compared to vehicle (* $p < 0.05$).

injection (Figure 12). Maximal withdrawal scores were obtained at 21 minutes (i.e., 1 minute after flumazenil injection), and so peak withdrawal in the drug group and average vehicle scores were calculated as the average of the first three time-points. The average vehicle group score was 1.72±0.11. Calculated as the difference between the scores of the drug treated animals and the average vehicle group, precipitated withdrawal was significantly elevated in the drug group ($t = 12.66$, $p < 0.01$; Figure 12, inset). Using the more conservative approach of estimating withdrawal severity as the difference between post-flumazenil peak and pre-zolpidem average baseline scores (i.e., within animal), precipitated withdrawal severity was still significantly elevated in the drug group ($t = 8.87$, $p < 0.01$).

III.A.2.c. Summary of Precipitated Withdrawal Experiment Results

The results of Experiments 6 and 7 show clearly that WSP female mice are sensitive to precipitation of withdrawal from some benzodiazepine receptor ligands. Flumazenil precipitated withdrawal from all of the BZR agonists except abecarnil (cf. Figure 10 with 8, 9, 11, 12).

III.A.3. Summary of BZR Agonist Withdrawal Screening Tests

Overall, these studies found that alprazolam and zolpidem were capable of producing significant withdrawal in WSP female mice regardless of whether withdrawal convulsions were precipitated. In contrast, significant withdrawal from triazolam and midazolam was only produced when convulsions were precipitated. Abecarnil was unusual in that spontaneous, but not precipitated, withdrawal was shown by WSP mice.

Figure 12

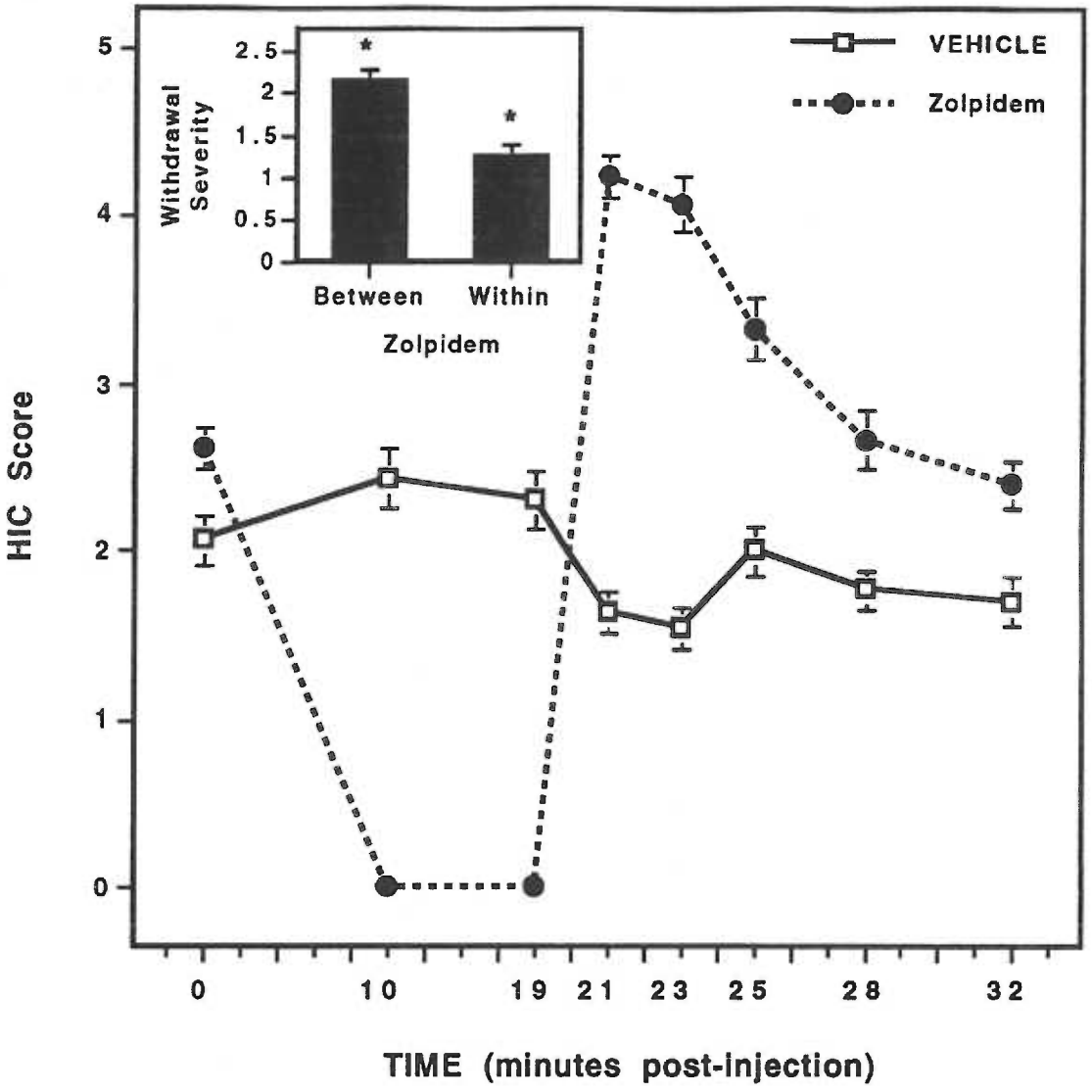

Figure 12: Time-course of precipitated zolpidem withdrawal severity in WSP female mice. Zolpidem or vehicle was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). HICs were scored at 10 and 19 minutes after injection. At 20 minutes, the BZR antagonist, flumazenil, was injected, and HICs were scored 1, 3, 5, 8, and 12 minutes later. Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following BZR agonist or vehicle injection. **Inset:** Zolpidem withdrawal severity score. Bars represent mean ± SE. Withdrawal severity was calculated as discussed in the text (Between - between groups correction; Within - within animal correction). Withdrawal was significantly elevated in the zolpidem treatment group compared to either vehicle or baseline (* $p < 0.05$).

III.B. Tests of Genetic Correlation

III.B.1. Experiment 8: Diazepam Withdrawal in Inbred Strains

Results of this experiment are shown in Figures 13 through 15. As we had seen previously, the inbred strains differed significantly in withdrawal convulsion severity following precipitation of withdrawal after 20 mg/kg diazepam (Metten & Crabbe, 1994a). The time-courses of precipitated diazepam withdrawal for the fourteen strains are depicted in Figures 13 and 14. The inbred strains are known to differ considerably in basal HIC severity (Crabbe et al., 1980a; Metten & Crabbe, 1994a). Consistent with these data, a significant main effect of strain on baseline HIC was detected ($F_{(13,140)} = 31.05$, $p < 0.01$; range: 0 - 4). As expected, groups within a strain did not differ with respect to baseline HICs. 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. The modal peak time for all strains was either 61 or 63 minutes; therefore, the average of the first three time-points was used as the index of peak HIC severity.

Analysis of the vehicle group data revealed significant differences among strains for the sum of the post-flumazenil HIC scores ($F_{(13,69)}$= 24.17, $p < 0.01$). This finding indicated the need to control for basal differences in responding to flumazenil for the precipitated diazepam withdrawal groups. Therefore, for each vehicle-treated animal, the sum of the first three post-flumazenil HIC scores was determined. To index diazepam withdrawal, the appropriate vehicle group strain mean was subtracted from the peak HIC score for each individual animal in

Figure 13

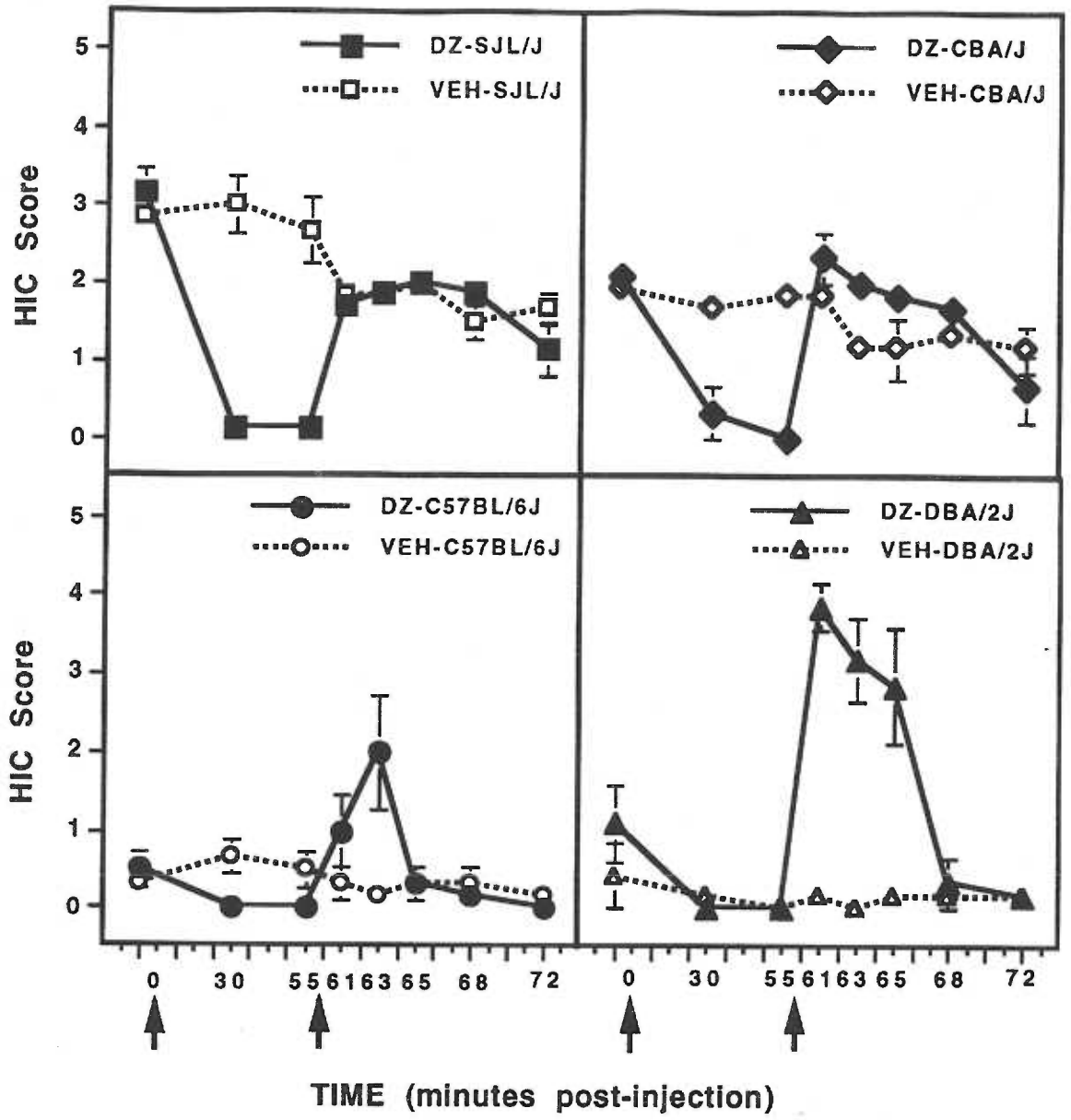

Figure 13: Time-courses of precipitated diazepam withdrawal in four of fourteen inbred strains. Symbols represent mean±SEM for each strain. Standard error bars not shown are smaller than the symbol. Y-axes: handling-induced convulsion (HIC) score. X-axes: time, in minutes, following diazepam or vehicle injection. Axis breaks were omitted for clarity. Diazepam or vehicle injection occurred at the first (left) arrows, immediately following pre-drug baseline HIC assessments. Flumazenil was injected at the second (right) arrows (at 60 minutes). Closed symbols represent the diazepam-treated animals. Withdrawal severity scores are shown in Figure 15.

Figure 14

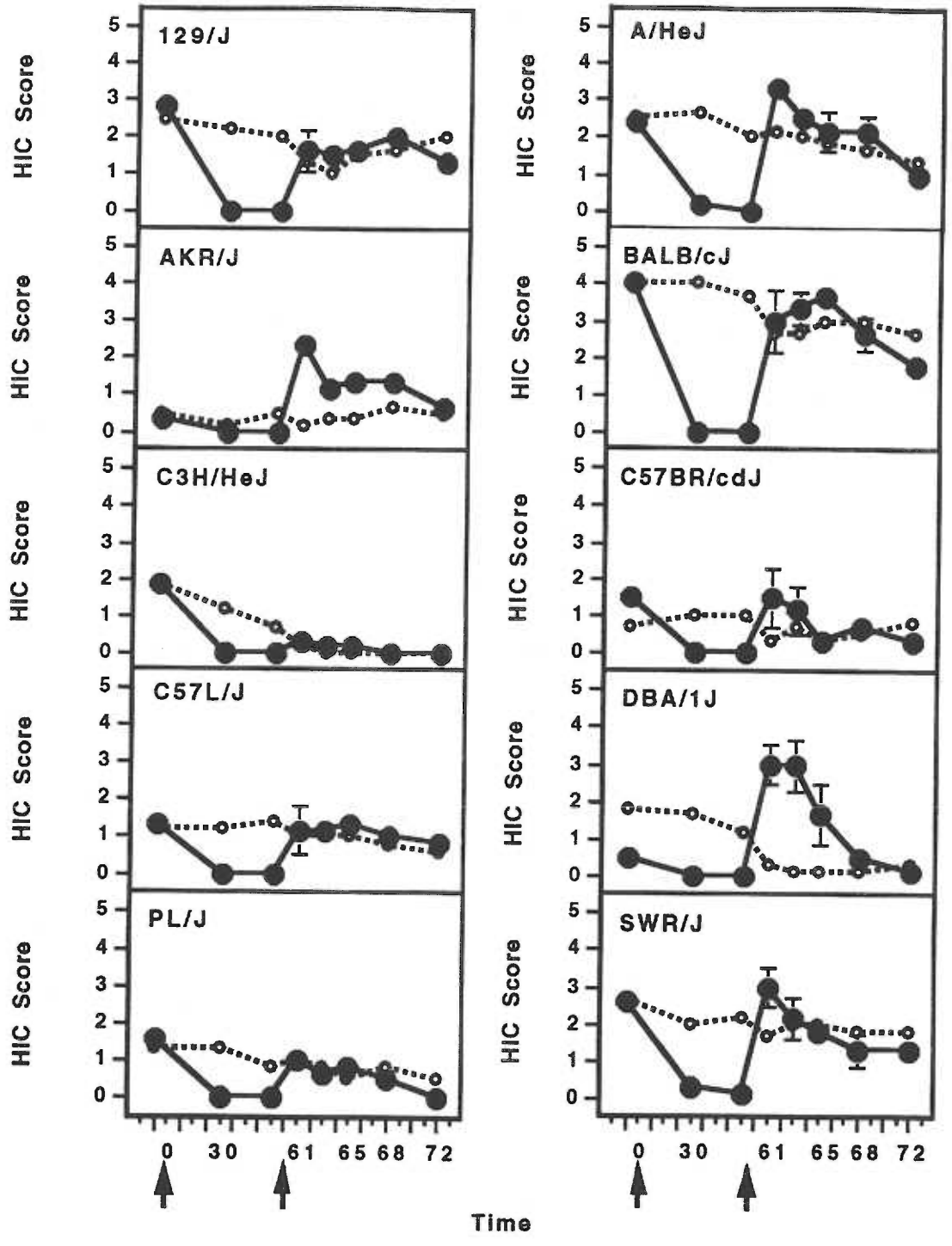

Figure 14: Time-courses of precipitated diazepam withdrawal in ten inbred strains. See Figure 13 legend for details. Standard error bars for diazepam groups (solid symbols) are shown where larger than the symbol; those for vehicle groups were omitted for clarity.

Figure 15

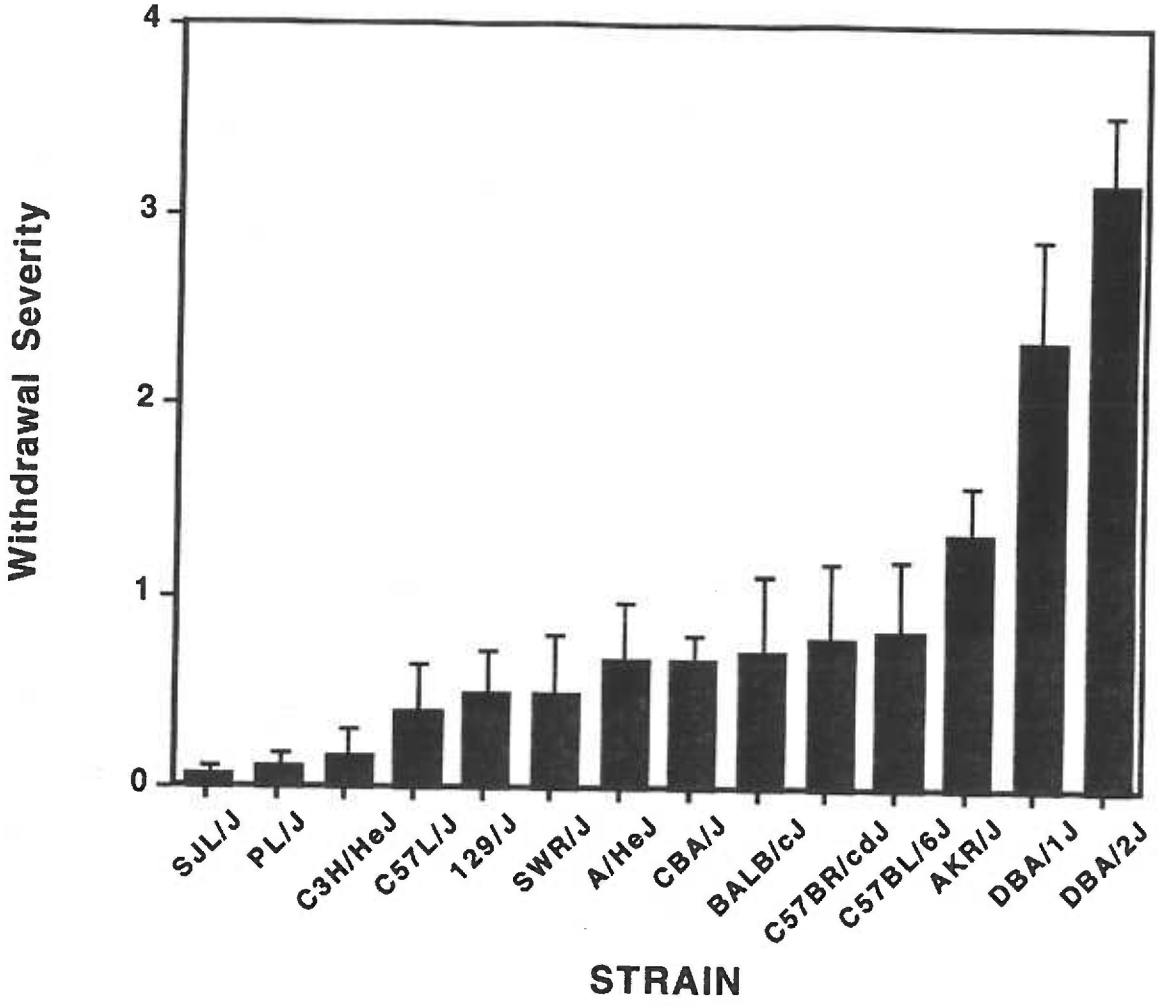

Figure 15: Rank ordered diazepam withdrawal severity in fourteen inbred strains. X-axis: Inbred strains, rank-ordered by withdrawal severity. Y-axis: Strain mean peak withdrawal severity, calculated as discussed in the text. Error bars represent SEM. Strains differed significantly in withdrawal severity ($F_{(13,71)}= 8.91$, $p < 0.01$).

the diazepam group. Strains differed significantly ($F_{(13,71)}$= 8.91, $p < 0.01$) in diazepam withdrawal severity (Figure 15). Some strains (e.g., SJL/J, solid squares and CBA/J, solid diamonds) had insignificant or slight withdrawal from diazepam, as shown in Figures 13 and 15. Other strains (e.g., C57BL/6J, solid circles and DBA/2J, solid triangles) had severe withdrawal from diazepam. Time-courses for the other ten strains are shown in Figure 14. The proportion of variance accounted for by genetic factors was 0.62 in this experiment.

III.B.1.a. Comparison of Present with Previous Data

As discussed above, the previous diazepam withdrawal data were collected in another group of mice of these same inbred strains (plus one additional strain, CE/J, which was not available for testing at this time). Those animals were tested serially for withdrawal from ethanol, pentobarbital, and diazepam (Metten & Crabbe, 1994a). The ethanol and pentobarbital withdrawal tests were both performed with the spontaneous withdrawal paradigm (no receptor-competitive antagonists are available). Also, withdrawal time-courses from both drugs lasted twelve hours (not minutes as with precipitated diazepam withdrawal) from the time of injection. In that study, diazepam withdrawal was calculated as the area under the curve of the diazepam-treated animals minus the strain mean area of the vehicle treated animals (i.e., between groups). Diazepam withdrawal was also calculated the same way as for ethanol and pentobarbital withdrawal (i.e., within subjects). These two measures of diazepam withdrawal were significantly genetically correlated ($r = 0.79$; Metten & Crabbe, 1994a).

The present data were examined for comparability with the previous data set by first recalculating the present withdrawal severity means using the between groups area measure. Strain mean withdrawal severities using both the peak and area measures are presented in Figure 16. Peak and area withdrawal severities were significantly genetically correlated (r_{12} = 0.99, $p < 0.01$) and strains differed significantly in withdrawal severity using the area measure ($F_{(13,71)}$= 8.76, $p < 0.01$). Scatterplots and least-squares regression lines representing the genetic correlation between the present and previous data sets are shown in Figures 17A and B. The apparent lack of correlation between the two data sets seemed to be due to an outlier strain, DBA/2J (Figure 17A: r_{12} = 0.37, $p = 0.20$). Therefore, the correlation was also performed without the DBA/2J strain (B: r_{11} = 0.74, $p < 0.01$). To address the issue of whether the withdrawal severity scores of any strains were significantly different between the two passes, the data were subjected to an Experiment X Strain ANOVA. As expected, there was a significant main effect of Strain ($F_{(13,146)}$ = 7.66, $p < 0.01$), and a significant Experiment X Strain interaction ($F_{(13,146)}$ = 3.98, $p < 0.01$). The main effect of Experiment was not significant ($F_{(1,146)}$ = 0.08, $p = 0.78$). The significant interaction was pursued by simple main effects analyses on six strains which had mean ± SEM scores between the two experiments that did not overlap (data not shown). Results of these analyses were that two strains, C57BR/cdJ and CBA/J, had significantly lower scores in the present experiment than in Metten and Crabbe (1994a; both $Fs_{(1,146)} >$ 7.66, $ps < 0.01$). DBA/2J had significantly higher scores in the present experiment $F_{(1,146)}$ = 39.42, $p < 0.01$), while no differences in

Figure 16

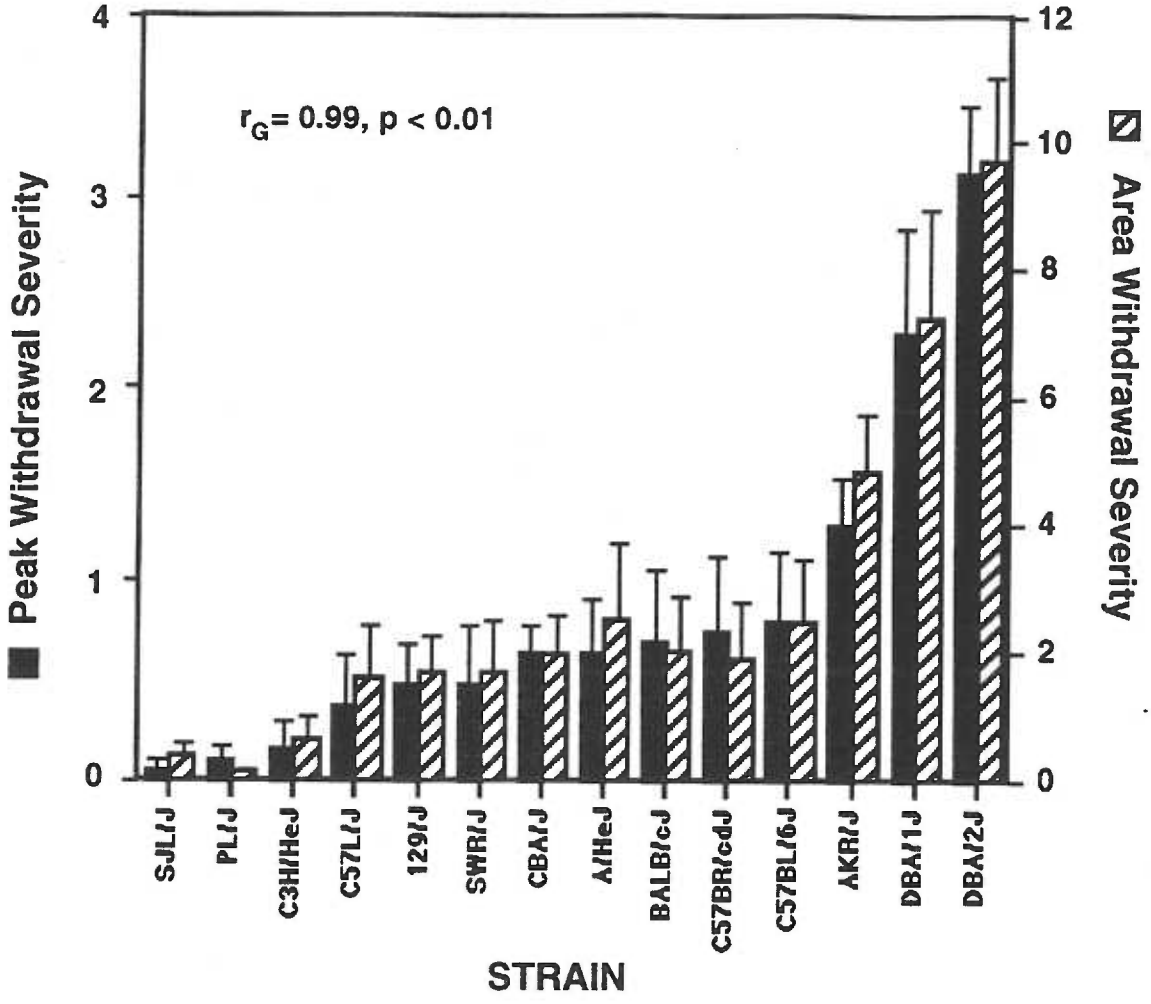

Figure 16: Withdrawal severity in fourteen inbred strains. Peak and area withdrawal severity strain means are genetically correlated (r_G is shown in the upper left-hand corner). X-axis: Inbred strains, rank-ordered by peak withdrawal severity. Left Y-axis: Strain mean peak withdrawal severity (black bars), calculated as discussed in the text. Right Y-axis: Strain mean area withdrawal severity (hatched bars), calculated as discussed in the text. Error bars represent SEM.

Figure 17A

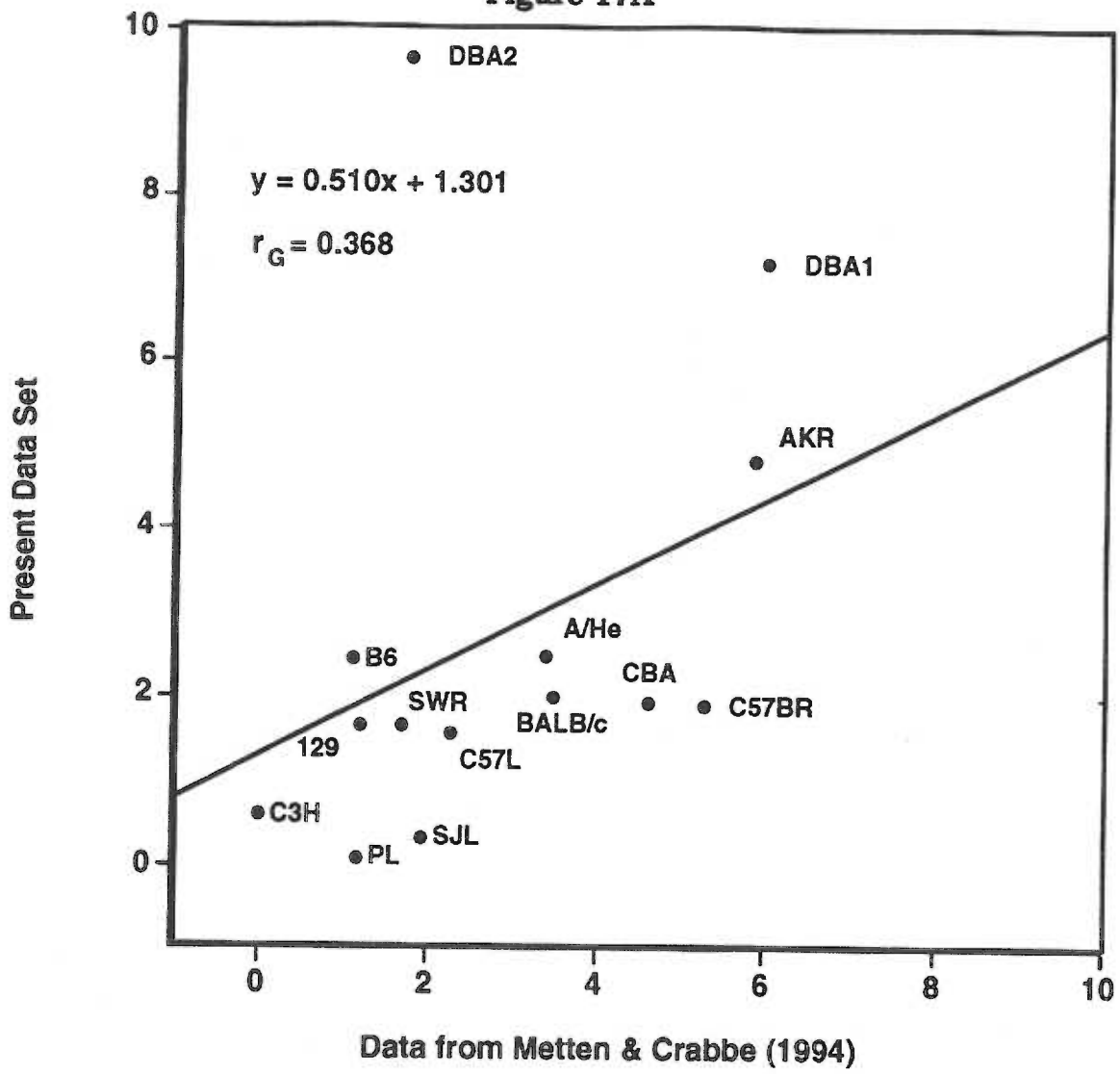

Figure 17A: Scatterplot and line of least-squares regression of diazepam withdrawal severity strain means from Metten & Crabbe (1994a; X-axis) and the present data set (Experiment 8; Y-axis). Withdrawal severities in both studies were calculated as the between groups area measure, as discussed in the text. Labelled symbols represent inbred strain means. Equation is that of the regression line. r_G is the Pearson's correlation coefficient. Withdrawal severities between the two studies were not significantly genetically correlated. Note the position of the DBA/2J strain relative to the regression line.

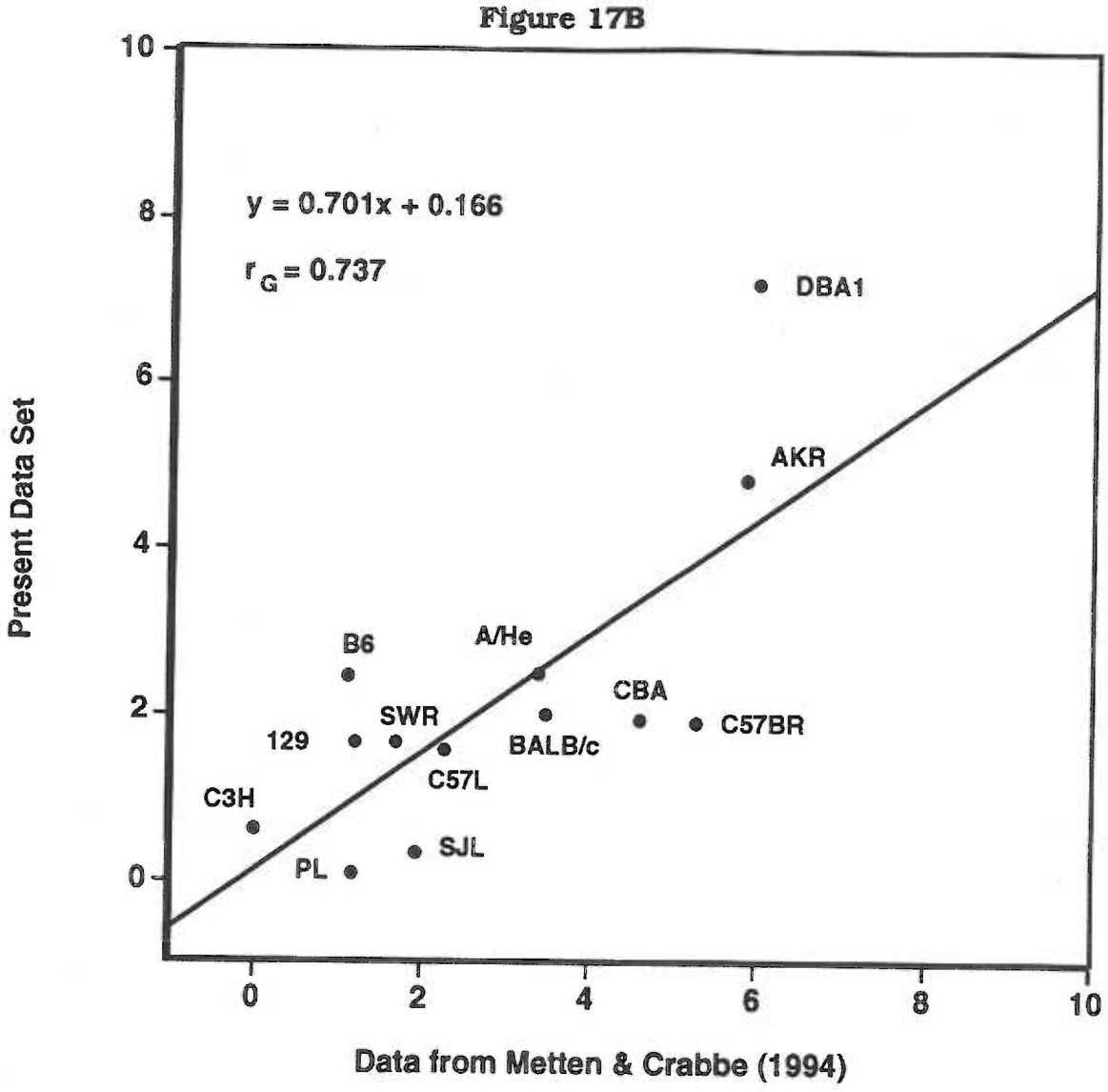

Figure 17B: The same data as in Figure 17A, except that the data for the DBA/2J strain were omitted, as discussed in the text. Scatterplot and line of least-squares regression of diazepam withdrawal severity strain means from Metten & Crabbe (1994a; X-axis) and the present data set (Experiment 8; Y-axis). Withdrawal severities in both studies were calculated as the between groups area measure, as discussed in the text. Labelled symbols represent inbred strain means. Equation is that of the regression line. r_G is the Pearson's correlation coefficient. Withdrawal severity strain means were found to be significantly genetically correlated when the DBA/2J strain data were removed from the analysis ($p < 0.01$).

scores were detected in C3H/HeJ, PL/J, or SJL/J (all Fs < 1.69, ps > 10).

III.B.1.b. Genetic Correlations Among Ethanol, Pentobarbital, and Diazepam

Figures 18 and 19 show the scatterplots and lines of least-squares regression of the genetic correlations of the present diazepam withdrawal severity means with those of ethanol and pentobarbital, respectively (Metten & Crabbe, 1994a). In contrast to previous findings, ethanol and diazepam withdrawal severity scores were significantly genetically correlated, indicating that there is substantial overlap in genes influencing acute withdrawal from these two drugs (r_{12} = 0.83, $p < 0.01$; cf. Metten & Crabbe, 1994a). Additionally, diazepam withdrawal severity scores correlated significantly with corresponding strain mean pentobarbital withdrawal severities, in agreement with previous findings (r_{12} = 0.75, $p < 0.01$). The proportions of phenotypic variance accounted for by common genetic factors were 0.69 and 0.56, respectively.

In addition, correlations were also calculated after excluding the DBA/2J strain since it had an extremely high score for ethanol withdrawal (Metten & Crabbe, 1994a) and since its exclusion restored the correlation between naive and non-naive diazepam withdrawal strain means (compare Figures 17A and B). Removal of the DBA/2J strain from the current experiment did not affect the conclusions regarding the genetic correlations of diazepam with ethanol and pentobarbital withdrawal severities. Ethanol and diazepam withdrawal severities remained significantly genetically correlated (r_{11} = 0.58,

Figure 18

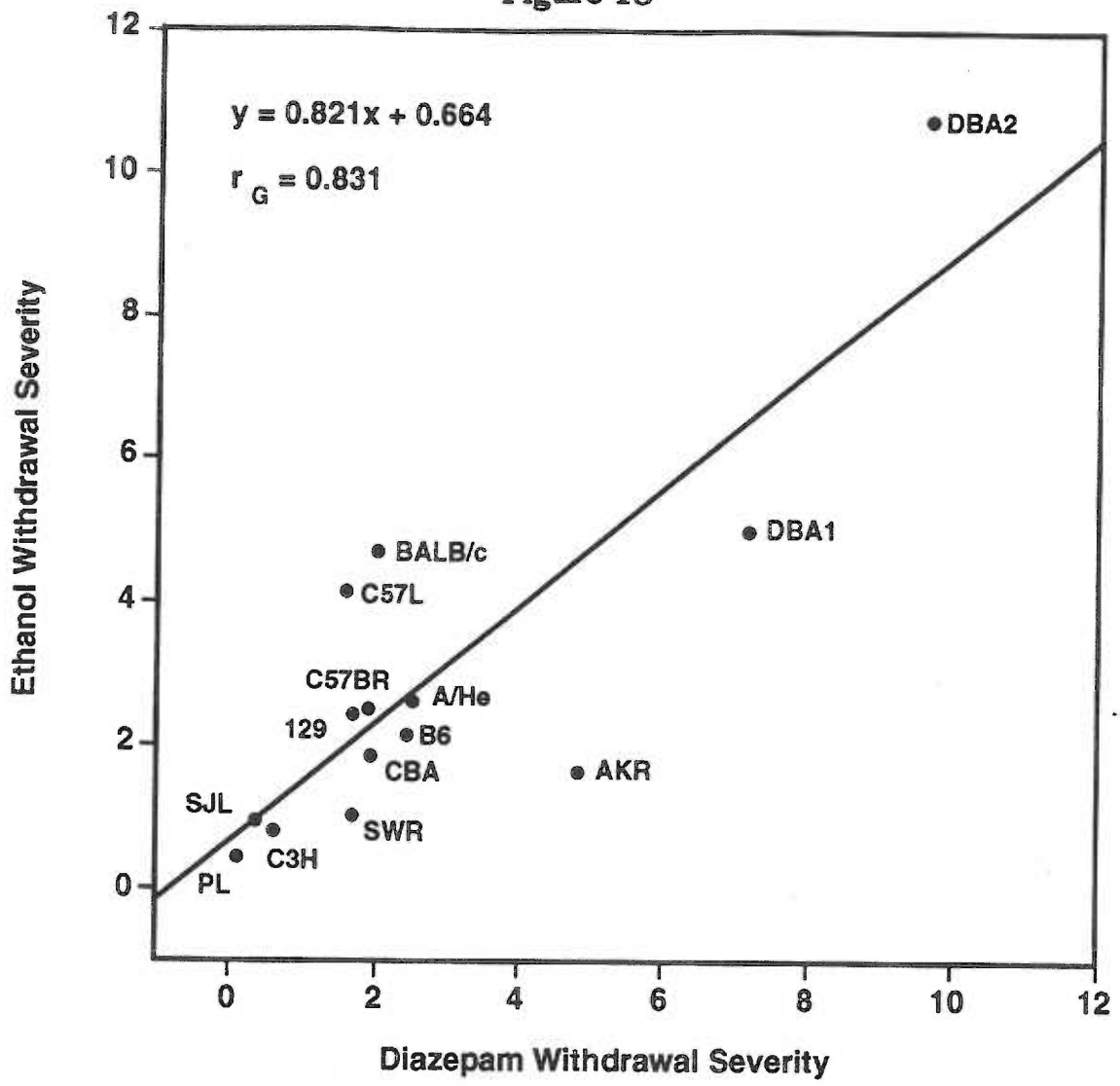

Figure 18: Scatterplot and line of least squares regression of strain means showing genetic correlation of withdrawal severity scores of ethanol (Metten & Crabbe, 1994a; Y-axis) and diazepam (present data; X-axis). Labelled symbols represent inbred strain means. Equation is that of the regression line. r_G is the Pearson's correlation coefficient. Ethanol and diazepam withdrawal severities were significantly genetically correlated using the present data set ($p < 0.01$).

Figure 19

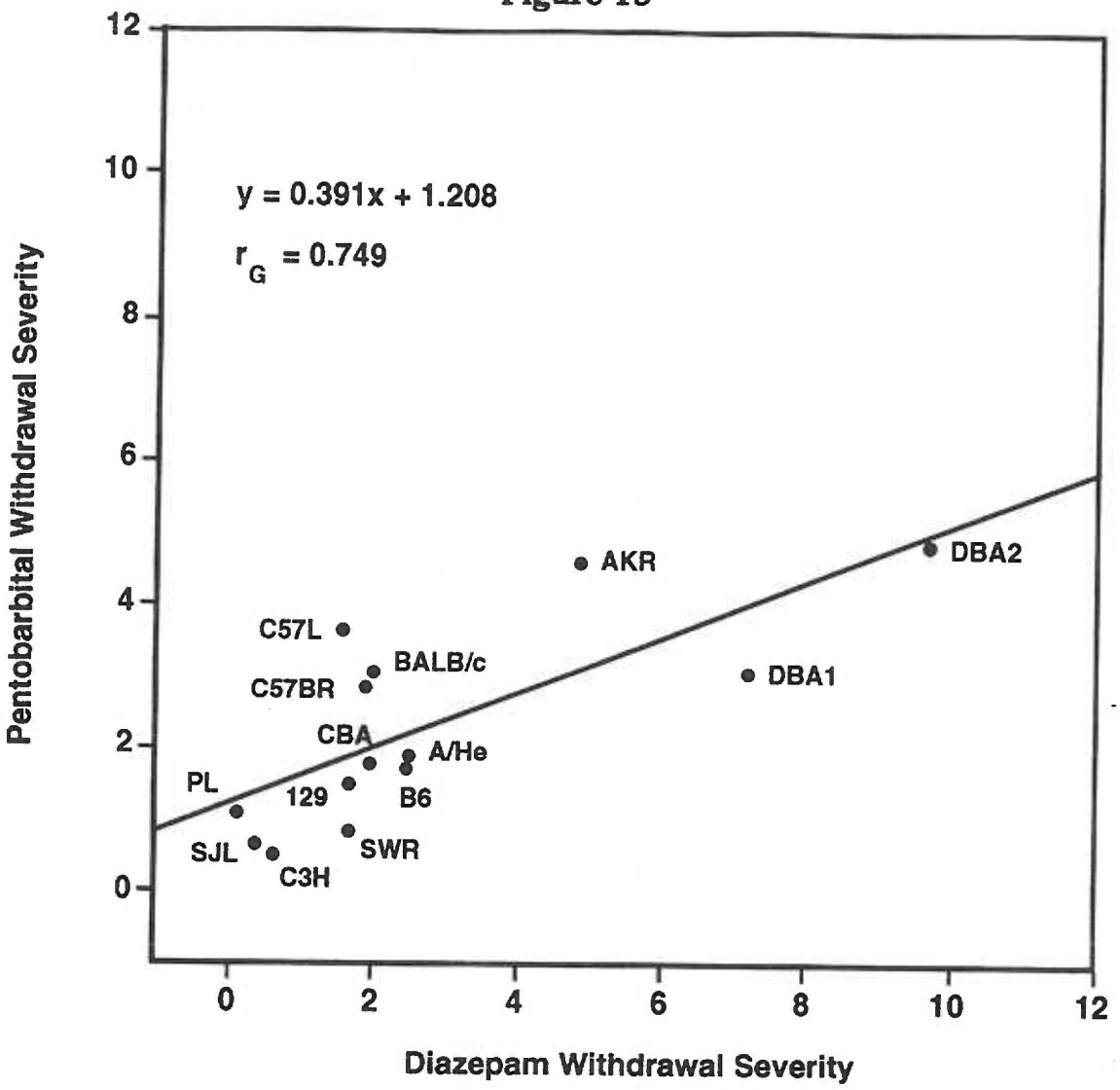

Figure 19: Scatterplot and line of least squares regression of strain means showing genetic correlation of withdrawal severity scores of pentobarbital (Metten & Crabbe, 1994a; Y-axis) and diazepam (present data; X-axis). Labelled symbols represent inbred strain means. Equation is that of the regression line. r_G is the Pearson's correlation coefficient. Pentobarbital and diazepam withdrawal severities were significantly genetically correlated using the present data set ($p < 0.01$).

$p < 0.05$), as did pentobarbital and diazepam withdrawal severity scores ($r_{11} = 0.64$, $p < 0.05$). Without DBA/2J, the proportions of variance accounted for by common genetic factors were 0.33 and 0.40, respectively.

III.B.1.c. Data Reliability Assessment

The current diazepam withdrawal severity scores, calculated as the peak corrected for vehicle treatment, were examined for split-half reliability. Animals were pseudorandomly assigned to one of two groups, A or B, for data re-analysis. This yielded sample sizes of three animals per strain per drug group. Strain means within each half were calculated for the average vehicle score and split-half correlations were performed using Pearson's r (i.e., the strain means for half A were correlated with the strain means for half B). The Spearman-Brown correction [2r/(1+r)] was employed to assess reliability of the correlation (McNemar, 1966; p. 150). Since vehicle group strain means were reliable ($r = 0.96$, reliability - 0.98, $p < 0.01$), withdrawal scores were examined without recalculation. Diazepam withdrawal severity strain means were significantly reliable ($r = 0.88$, reliability - 0.93, $p < 0.01$). The "true" genetic correlations of diazepam with ethanol and pentobarbital withdrawal severities were estimated according to McNemar (1966; p. 153). This measure estimates the true genetic correlation from the observed one by dividing by the product of the square roots of the split half reliability scores for the two traits. The measure is termed the correction for attenuation (i.e., unreliability of measurement). Using this correction and split half reliability scores for ethanol and pentobarbital from my Master's thesis

(Metten, 1993), the ethanol/diazepam correlation was significant ($r = 0.91$, $p < 0.01$); likewise, the pentobarbital/diazepam correlation was significant ($r = 0.88$, $p < 0.01$).

III.B.1.d. Summary

The results of Experiment 8 show clearly that there is considerable genetic variability among strains in precipitated diazepam withdrawal severity, confirming the findings of our earlier study (Metten & Crabbe, 1994a). However, unlike our previous findings, the present results support a genetic correlation between ethanol and diazepam withdrawal. Comparison of the two data sets suggests that most of the strains would have scored the same if they had been naive when tested (Figure 17B). However, some strains, most notably, DBA/2J, had significantly different withdrawal scores in the present study from those reported previously (Figure 17A).

III.B.2. Experiment 9: Zolpidem Withdrawal in Inbred Strains

Results of this experiment are shown in Figure 20. This study confirms our earlier finding that there is genetic variability in severity of withdrawal following a single zolpidem injection (unpublished data). Average baseline HIC severities ranged from 0 to 1 and did not differ among strains in this experiment ($F_{(2,21)} = 1.15$, $p = 0.34$). Peak withdrawal scores were corrected within-animal for baseline differences prior to analysis of the data by one-way ANOVA (Strain). Zolpidem (20 mg/kg) decreased HICs at 15 and 30 minutes after injection in all animals having non-zero baselines, regardless of strain (see Figure 20). Withdrawal severity differed significantly among strains ($F_{(2,21)} = 66.69$, $p < 0.01$). HIC scores were elevated in the D2

Figure 20

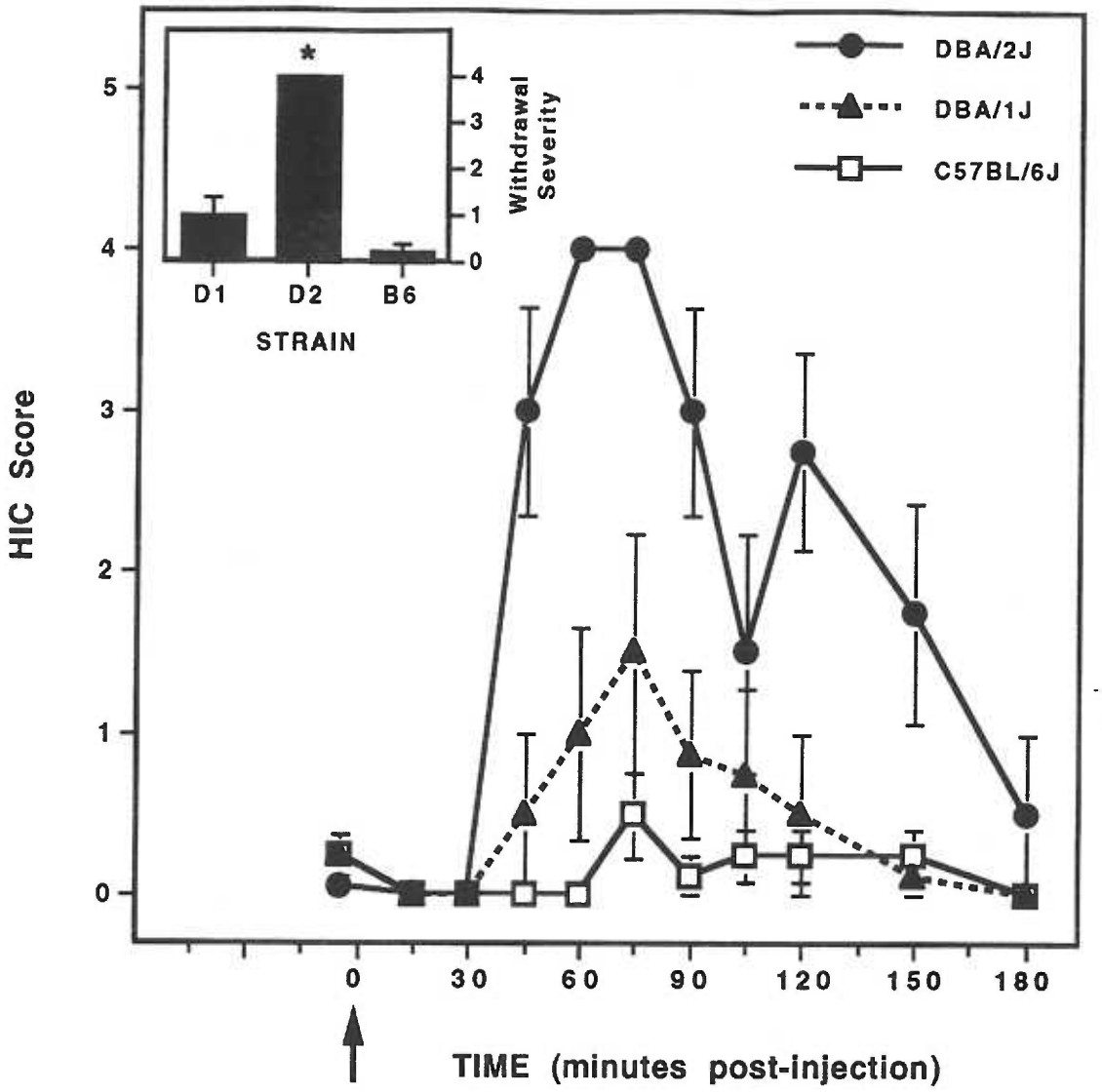

Figure 20: Time-course of spontaneous zolpidem withdrawal in DBA/1J (D1), DBA/2J (D2), and C57BL/6J (B6) mice. Eight male mice of each strain were tested. Zolpidem (20 mg/kg) was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). Symbols represent mean ± SE for each group. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following injection. **Inset:** Withdrawal severity scores (peak) of the three inbred strains. Bars represent mean ± SE for each group. There was no variation in peak score for the D2 strain. Withdrawal severity was calculated as discussed in the text. Significant withdrawal was shown by D2 mice only.

strain by the 45 minute time-point. In fact, the peak withdrawal severity displayed by D2 mice was significantly greater than that of either D1 or B6 mice (Tukey's HSD, both ps < 0.01). DBA/1J (D1) mice appeared to have modest withdrawal scores, peaking at the 75 minute time-point (see Figure 20), while C57BL/6J (B6) mice did not show significant withdrawal from zolpidem. Despite a tendency for greater withdrawal in D1 than B6 mice, peak withdrawal severity did not differ between these two strains (Tukey's HSD, p = 0.10). The proportion of variance accounted for by genetic factors was 0.86 in this experiment.

A visual inspection of these data suggested that D2 mice not only had higher peak withdrawal than either of the other two strains, but also suggested that their HIC scores were elevated for a longer period of time (see Figure 20). This hypothesis was tested by analysis of withdrawal severity calculated as the area measure (see Experiment 8). D2 mice also had significantly greater withdrawal than D1 and B6 mice using this measure ($F_{(2,21)} = 36.51$, $p < 0.01$; Tukey's HSD: ps < 0.01). D1 and B6 mice also did not differ in withdrawal severity when calculated this way (Tukey's HSD: p = 0.42). Further *post-hoc* analyses revealed that withdrawal for the DBA/2J strain began at about the 45 minute time-point and ended about 2 1/2 hours after zolpidem administration.

Finally, the data were analyzed by Kruskal-Wallis ANOVA for ordinal data (Siegel, 1956). By far, the two most common HIC scores from the 45 minute time-point onward were 0 (no convulsion; 65% of scores) and 4 (tonic convulsion with only tail lift; 26% of scores). D2 animals had 82% of scores of 4, while having only 16% of scores of 0.

In contrast, D1 mice had only 18% of the scores of 4, and B6 mice had none. Regardless of whether peak or area withdrawal severity was examined, the analysis confirmed a significant main effect of strain (Kruskal-Wallis test statistics > 15.36, ps < 0.01, 2 df).

III.B.2.a. Summary

The results of Experiment 9 show clearly that D2 mice have significantly greater spontaneous withdrawal from zolpidem compared to either D1 or B6 mice. The difference in BZR agonist withdrawal severity between the two DBA substrains seen after zolpidem was not apparent when they were tested for precipitated diazepam withdrawal (see Figure 15).

III.B.3. Experiments 10 and 11- BZR Withdrawal in Selectively Bred Lines

III.B.3.a. Diazepam Withdrawal in HAW and LAW Mice

Results of this experiment are shown in Figure 21. HAW mice from S_2 displayed significantly greater diazepam withdrawal than LAW mice. The lines differed significantly in baseline HIC severity (HAW > LAW; range: 0 to 2; $F_{(1,35)} = 9.82$, $p < 0.01$), but no drug effects were significant. Modal peak scores occurred at the 61 minute time-point for both lines. After correction for the line-appropriate average vehicle score, the lines differed significantly in peak withdrawal severity ($F_{(1,27)} = 13.51$, $p < 0.01$). Figure 21 shows the magnitude of the diazepam withdrawal difference between the lines, as well as the line difference in ethanol withdrawal severity in mice of the same selection generation.

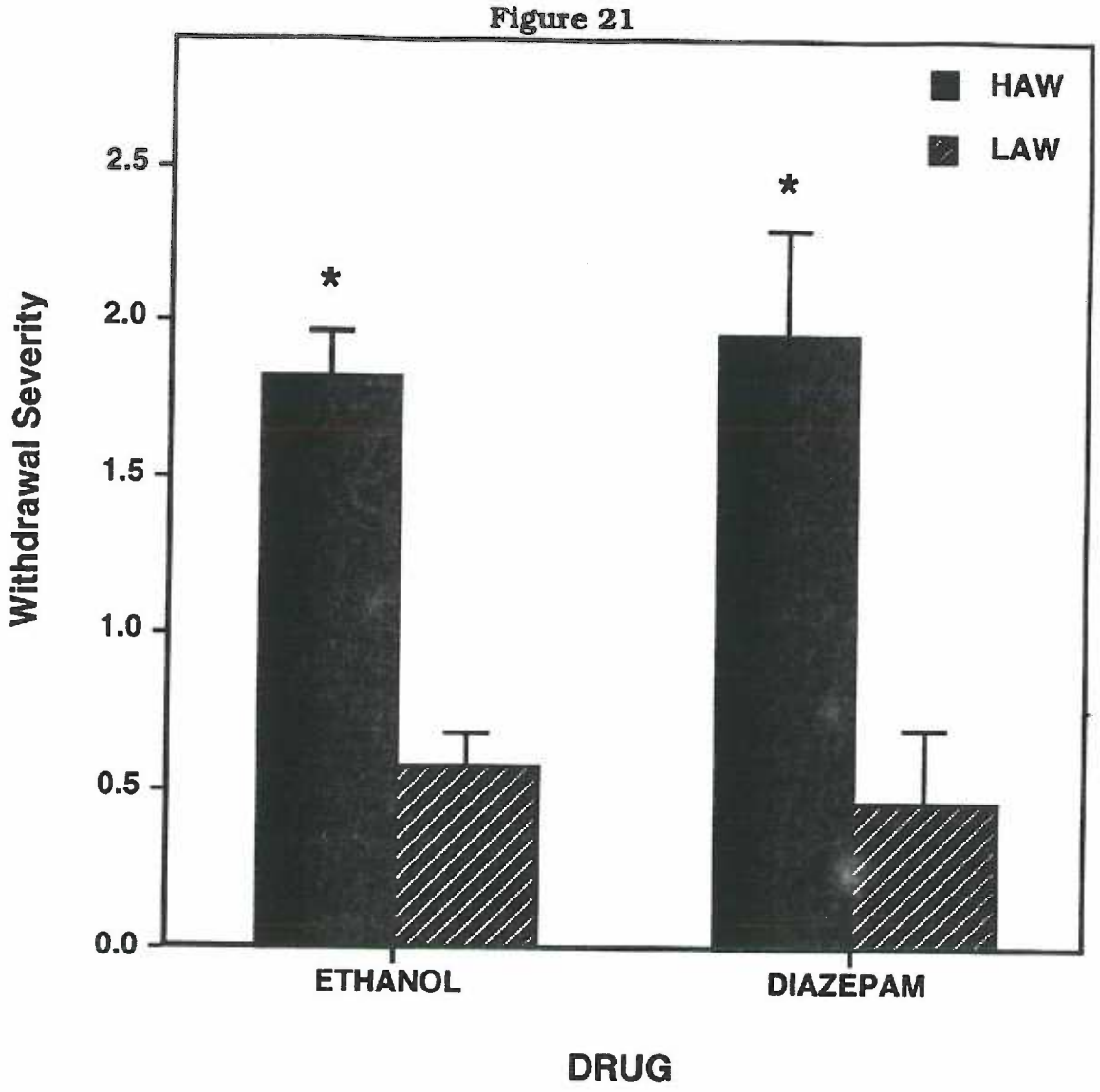

Figure 21: Ethanol and diazepam withdrawal severities in HAW and LAW mice selectively bred from an F2 intercross between C57BL/6J (B6) and DBA/2J (D2) mice. Male and female mice of the second selection generation were tested for withdrawal severity from either ethanol (n = 55 - 65/line) or diazepam (n = 14 - 15/line). Bars represent mean ± SE for each group. Y-axis: Peak withdrawal severity. X-axis: Drug withdrawal test: ethanol (left) or diazepam (right). Withdrawal severity was calculated as discussed in the text. HAW mice (solid bars) displayed significantly greater withdrawal from both drugs compared to LAW mice (hatched bars; * $p < 0.05$).

III.B.3.b. Summary

The results of Experiment 10 show clearly that HAW mice displayed significantly greater diazepam withdrawal than LAW mice. Thus, selection for ethanol withdrawal severity differences has produced lines of mice which differ early in diazepam withdrawal severity, providing strong evidence in favor of a genetic correlation between these two characters rather than random drift. This finding that lines of mice selectively bred for ethanol withdrawal severity differences also differ in diazepam withdrawal severity is consistent with previous findings in WSP and WSR mice (Crabbe et al., 1991a).

III.B.3.c. Zolpidem Withdrawal in HAW and LAW Mice

Results of these experiments are shown in Figure 22. These studies demonstrated that HAW mice of S_3 had significantly greater spontaneous and precipitated zolpidem withdrawal than LAW mice. Precipitated zolpidem withdrawal test results are discussed below. Before the spontaneous withdrawal test, the lines differed significantly in baseline HIC severity (HAW > LAW; range: 0 to 2.5; $F_{(1,28)} = 7.56$, $p = 0.01$). Zolpidem decreased HIC scores at fifteen minutes post-injection in animals having non-zero baselines. Peak withdrawal scores were corrected for pre-drug scores within animal. The lines differed significantly in spontaneous zolpidem withdrawal severity (HAW > LAW; $F_{(1,28)} = 25.24$, $p < 0.01$). The center of Figure 22 shows the magnitude of the spontaneous zolpidem withdrawal difference between the lines, as well as the line difference in ethanol withdrawal severity of mice of the same selection generation (left).

Figure 22

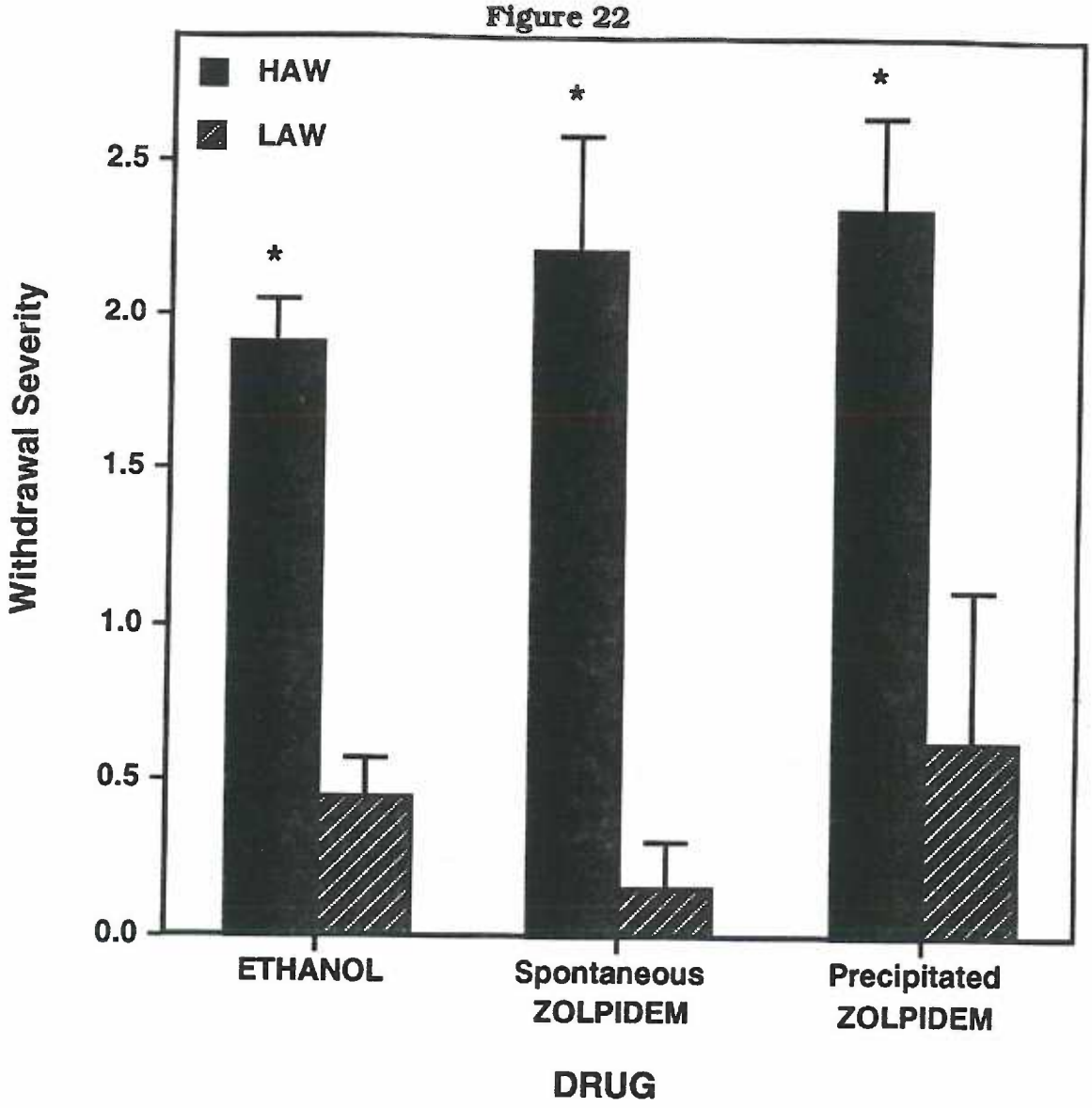

Figure 22: Ethanol and zolpidem withdrawal severities in HAW and LAW mice selectively bred from an F2 intercross between C57BL/6J (B6) and DBA/2J (D2) mice. Mice of the third selection generation were tested for withdrawal severity from ethanol (n = 47 - 52/line; both sexes) or zolpidem (spontaneous test: n = 14 - 16/line; precipitated test: n = 8 - 9/line). Bars represent mean ± SE for each group. Y-axis: Peak withdrawal severity. X-axis: Drug withdrawal test: ethanol (left), spontaneous zolpidem (center), or precipitated zolpidem (right). Withdrawal severity was calculated as discussed in the text. HAW mice (solid bars) displayed significantly greater withdrawal from both drugs regardless of test paradigm compared to LAW mice (hatched bars; * $p < 0.05$).

The lines also differed significantly in baseline HIC severity before the precipitated withdrawal test (HAW > LAW; $F_{(1,26)} = 11.97$, $p < 0.01$). Again, no effects of drug group were found (both Fs < 0.45, ps > 0.50). Modal peak scores occurred at the 21 minute time-point for both lines, so the average vehicle score for each line was calculated as previously. HAW mice had significantly greater precipitated zolpidem withdrawal than LAW mice ($F_{(1,15)} = 9.86$, $p < 0.01$) using the peak withdrawal measure. The right side of Figure 22 shows the magnitude of the precipitated zolpidem withdrawal difference between the lines.

III.B.3.d. Summary

The results of Experiment 11 show clearly that mice of the HAW line display significantly greater zolpidem withdrawal than their LAW counterparts, regardless of whether withdrawal is spontaneous or precipitated. The lines also differed significantly in the same direction in severity of diazepam withdrawal (Experiment 10). These results combined are consistent with the hypothesis that ethanol and benzodiazepine withdrawal severities are genetically correlated. In other words, these data suggest that at least one of the ethanol withdrawal modulating genes selected for in the HAW/LAW selection also modulates benzodiazepine withdrawal.

III.C. Gene Mapping

III.C.1. Experiment 12: Genotyping DBA/1J Mice

In all, DBA/1J mice were genotyped for 61 SSLP markers from the MIT marker set (Dietrich et al., 1992). Thirty-one of these were in regions of ethanol withdrawal QTLs. The other thirty markers were

used to genotype DBA/1J mice in twelve regions already known to be polymorphic with DBA/2J (Festing, 1990; Festing, 1994; GBASE, December, 1994).

III.C.1.a. Ethanol Withdrawal QTL Markers

Table 7 lists the markers associated with ethanol withdrawal in the B6 X D2 crosses, the markers genotyped in DBA/1J mice in each QTL region, and the result of the test for polymorphism with DBA/2J for each marker. The table also lists the base pair length of the DBA/2J allele (from Research Genetics, Inc., Murine Map Pairs, April, 1995 release, version 8/29/95) and the estimated base pair length of the DBA/1J allele. Gel conditions were sufficient to reliably detect differences in base pair number as small as four. Therefore, the conclusion for markers listed as not polymorphic should be taken as tentative, as differences smaller than four would possibly not be detectable. Eight markers in four regions for which polymorphism was definitely established are in bold print. These markers were used to genotype the D1D2F2s following Experiment 14.

III.C.1.b. Markers in Known Polymorphic Regions

Table 8 lists the names of the genes for which the two DBA substrains have different allele forms, the SSLP markers genotyped in DBA/1J mice in each region, and the result of the test for polymorphism with DBA/2J for each marker. Base pair lengths of the alleles are given or estimated as above. Seven markers in six regions for which polymorphism was established are listed in bold print. These markers were also used to genotype D1D2F2 mice following Experiment 14.

Table 7

DBA/1J Genotypes Using Ethanol Withdrawal QTL Markers*

Ethanol Withdrawal QTL Marker	Chr./ cM	Genotyped Markers	cM	Estimated DBA/1J allele (bp)	DBA/2J allele (bp)	Poly-morphic Status
D1Mit206	1/ 95.8	*D1Mit33*	81.6	122	122	No
		D1Mit206	95.8	114	114	No
		D1Mit221	102.5	125	125	No
D2Mit9	2/ 37.0	*D2Mit61*	34.0	156	156	No
		D2Mit9	37.0	195	195	No
		D2Mit91	37.0	194	194	No
		D2Mit379	37.0	92	92	No
		D2Mit472	37.5	88	88	No
D2Mit17	2/ 69.0	*D2Mit17*	69.0	220	220	No
		D2Mit134	69.0	196	196	No
		D2Mit77	**74.0**	**~178**	**174**	**Yes**
D4Mit186	4/ 42.6-45.5	*D4Mit142*	36-38	139	139	No
		D4Mit185	**32-43**	**~118**	**122**	**Yes**
		D4Mit186	42-46	149	149	No
D6Mit149	6/ 47.0	*D6Mit39*	46.0	118	118	No
		D6Mit104	46.0	154	154	No
		D6Mit149	**47.0**	**~209**	**199**	**Yes**
		D6Mit54	49.0	178	178	No
D7Mit91	7/ 36.0	*D7Mit91*	36.0	132	132	No
		D7Mit30	37.0	242	242	No
		D7Mit122	37.0	120	120	No
		D7Mit147	37.0	126	126	No
D8Mit94	8/ 13.5	*D8Mit281*	12.0	117	117	No
		D8Mit94	13.5	130	130	No
D11Mit174	11/ 20.0	*D11Mit163*	16.0	155	155	No
		D11Mit108	**18.0**	**~162**	**159**	**Yes**
		D11Mit217	**19.0**	**~136**	**118**	**Yes**
		D11Mit20	**20.0**	**~146**	**140**	**Yes**
		D11Mit174	**20.0**	**~147**	**165**	**Yes**
		D11Mit296	**20.0**	**~100**	**126**	**Yes**
		D11Mit238	23.0	174	174	No

* From Belknap et al., 1993 and Buck et al., submitted 6/96. Confirmed QTLs are on Chr. 1, 4, & 11. Abbreviations: Chr. - chromosome, cM - centiMorgan, bp - base pair number. Polymorphic status of each marker is given with respect to the comparison of DBA/1J and DBA/2J alleles. For reasons discussed in the text, only "Yes" status should be taken as confirmed. cM locations are from Silver et al. (1996), except for those of *D2Mit379*, *D2Mit472*, and *D7Mit122* which were estimated from the Research Genetics, Inc., Mouse Map Pairs, April, 1995 release, version 8/29/95.

Table 8

DBA/1J Genotypes Using SSLP Markers in Polymorphic Regions†

Known Polymorphism	Chr./ cM	SSLP Marker	cM	Estimated DBA/1J allele (bp)	DBA/2J allele (bp)	Poly-morphic Status
Aox1	1/ 23.2	*D1Mit211*	15.0	146	146	No
		D1Mit70	17.8	190	190	No
Nramp	1/ 39.2	***D1Mit128***	**36.9**	**~147**	**141**	**Yes**
Hc	2/ 25.0	*D2Mit7*	28.0	142	142	No
		D2Mit238	**28.0**	**~135**	**153**	**Yes**
		D2Mit369	28.5	110	110	No
		D2Mit370	28.5	104	104	No
Car2	3/ 10.5	*D3Mit118*	13.8	153	153	No
If1	3/ 87.7	***D3Mit116***	**84.9**	**~263**	**275**	**Yes**
		D3Mit89	86.1	216	216	No
		D3Mit19	87.6	176	176	No
Akp2 and *Gpd1*	4/ 70.2 & 78.4	***D4Mit13***	**71.0**	**~107**	**97**	**Yes**
		D4Mit33	**79.0**	**~128**	**144**	**Yes**
Cyp2a5	7/ 6.5	*D7Mit57*	4.0	136	136	No
		D7Mit179	4.0	145	145	No
		D7Mit115	8.0	195	195	No
Tcrg	13/ 10.0	***D13Mit57***	7.0	**~148**	**156**	**Yes**
		D13Mit217	7.0	110	110	No
		D13Mit219	7.0	272	272	No
		D13Mit115	11.0	141	141	No
Rarb	14/ 1.5	*D14Mit98*	3.0	146	146	No
		D14Mit99	3.0	106	106	No

(continued on the next page)

† Names of loci known to be polymorphic between DBA/1J and DBA/2J strains were compiled from several sources (Festing, 1990, 1994; GBASE, 1994). *Nramp* (Chr. 1, 39.2 cM) replaces the genes named *Ity* and *Lsh* (Silver et al., 1996). Not included in the table are two Chr. 2 polymorphisms, *Hdc* (71 cM) and *Itp* (74 cM) that map to roughly the same region as a putative ethanol withdrawal QTL (see Table 7). Abbreviations are the same as for Table 7. cM locations for *D2Mit238*, *D2Mit369*, and *D2Mit370* were estimated from the Research Genetics, Inc., Mouse Map Pairs, April, 1995 release, version 8/29/95.

Table 8, continued

DBA/1J Genotypes Using SSLP Markers in Polymorphic Regions†

Known Polymorphism	Chr./ cM	SSLP Marker	cM	Estimated DBA/1J allele (bp)	DBA/2J allele (bp)	Poly-morphic Status
Ly6	15/ 42.7	*D15Mit71*	40.9	132	132	No
		D15Mit158	40.9	170	170	No
		D15Mit29	42.8	186	186	No
C4Slp, H2-D,	17/ 18.83	*D17Mit102*	18.5	123	123	No
		D17Mit64	20.6	136	136	No
H2T18, H2-T3	19.8-20.0	*D17Mit10*	24.5	148	148	No
		D17Mit66	**24.5**	**~115**	**132**	**Yes**
Ce2, Upg	17/ 27.8-30	*D17Mit139*	30.2	164	164	No

† Names of loci known to be polymorphic between DBA/1J and DBA/2J strains were compiled from several sources (Festing, 1990, 1994; GBASE, 1994). *Nramp* (Chr. 1, 39.2 cM) replaces the genes named *Ity* and *Lsh* (Silver et al., 1996). Not included in the table are two Chr. 2 polymorphisms, *Hdc* (71 cM) and *Itp* (74 cM) that map to roughly the same region as a putative ethanol withdrawal QTL (see Table 7). Abbreviations are the same as for Table 7. cM locations for *D2Mit238, D2Mit369,* and *D2Mit370* were estimated from the Research Genetics, Inc., Mouse Map Pairs, April, 1995 release, version 8/29/95.

III.C.1.c. Summary of DBA/1J Genotyping Data

Fifteen of 61 SSLP markers were shown to be polymorphic between the DBA substrains. The majority of these polymorphic markers seemed to be isolated cases among the markers tried. One notable exception was found. There appears to be a cluster of polymorphic loci between approximately 18 - 20 cM on chromosome 11 (see Table 7). This region has been confirmed as containing an ethanol withdrawal severity-modulating gene in B6 X D2 crosses (Buck et al., submitted).

Genotyping of DBA/1J mice was attempted with eight other SSLP markers, but was unsuccessful using the standardized PCR conditions employed here. After at least two attempts, no bands were visible on the gels for markers *D1Mit7*, *D8Mit289*, and *D14Mit171*, while smeared multiple bands were seen for markers *D1Mit273* and *D7Mit114*. It was not possible to detect the B6/D2 polymorphism for markers *D3Mit130*, *D1Mit167*, *D4Mit14*. Repetition of these markers under modified PCR conditions may resolve these results.

III.C.2. Experiments 13 & 14: Zolpidem & Ethanol Withdrawal in D1D2F2s

III.C.2.a. Estimation of D1D2F2 Sample Size

Data from Experiment 9 for the two DBA parental strains were used to calculate estimates of zolpidem withdrawal heritability according to the methods described above. Heritabilities (Sokal & Rohlf, 1981) were estimated as 0.88 and 0.80 for peak and area withdrawal severity, respectively. The estimates of expected trait heritability in the D1D2F2s (Hegmann & Possidente, 1981) were 0.78

and 0.67, respectively. These data thus predict that a large proportion of the phenotypic variance in zolpidem withdrawal severity is accounted for by genetic factors. This can be readily appreciated by looking at the frequency histogram (see Figure 23) for these strains. All of the D2 mice had peak withdrawal scores of exactly 4, while 6 of 8 D1 mice had peak scores of less than 2 (all had scores ≤ 3), suggesting that zolpidem withdrawal is a bimodal function. The high degree of genetic homology between D1 and D2 mice (Bailey, 1978) coupled with these findings implies that a major gene controls most of the genetic trait variance. Thus, the assumptions delineated in the methods section regarding statistical power of detection of a QTL controlling a minimum of 7% of the genetic trait variance yielded a conservative estimate of the required D1D2F2 sample size.

III.C.2.b. Phenotypic Results

III.C.2.b.i. Zolpidem Withdrawal Frequency Distribution

Zolpidem withdrawal data from the parental strains in Experiment 13 were compiled with those from Experiment 9 and analyzed for experiment-wise differences. Data from one D2 mouse in Experiment 13 were eliminated for apparent injection failure. Its peak score was zero, more than 4 SDs less than the mean of the D2 strain. DBA substrain withdrawal scores did not differ across the two experiments (all $Fs < 1.75$, $ps > 0.10$).

Average baseline scores prior to zolpidem withdrawal testing of the D1D2F2s ranged from 0 to 4 (mean ± SEM: 0.73 ± 0.09; only 37 animals had scores higher than 1). Zolpidem peak and area scores were phenotypically correlated 0.83. Baseline scores were weakly

Figure 23

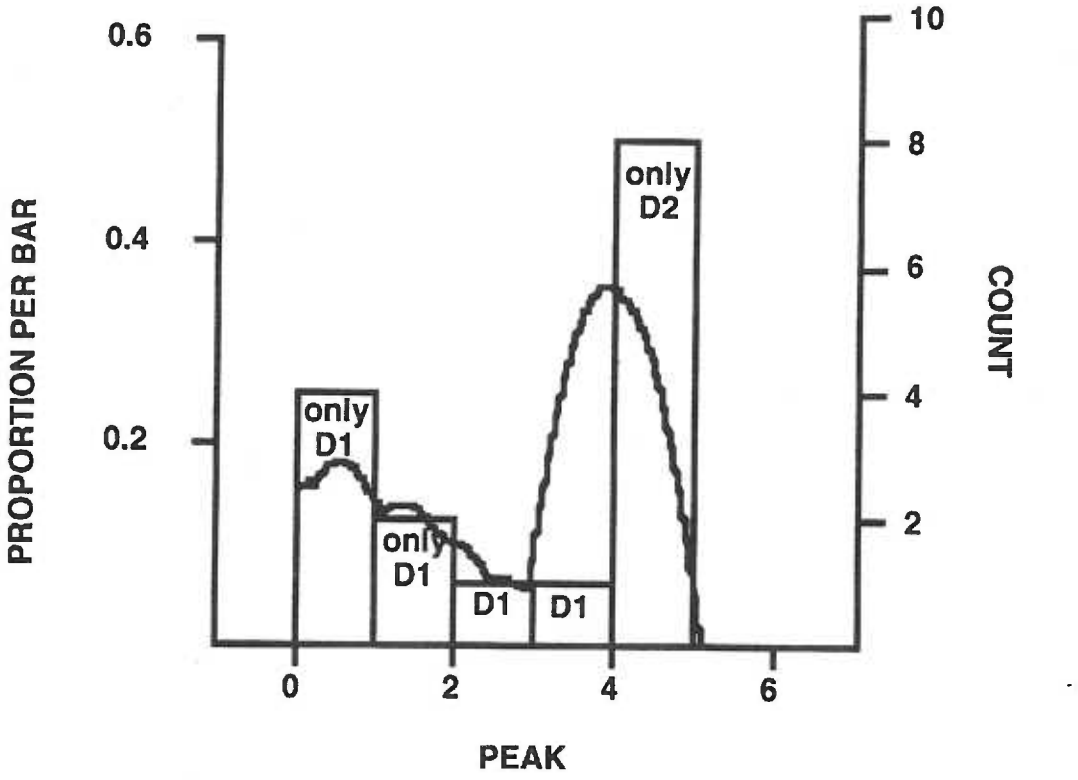

Figure 23: Peak zolpidem withdrawal frequency histogram and Epanechnikov kernel density function for D1 and D2 mice from Experiment 9 (Silverman, 1986; tension = 0.287). Left axis: proportion of animals per bar. Right axis: actual n per bar. The dissociation of the D2 animals from the D1 animals strongly suggests a bimodal distribution.

correlated with peak and area scores (0.29 and 0.39, respectively), so regression residuals were calculated for both peak and area on baseline. When regression residuals were correlated with their respective peak and area scores, they were correlated 0.95 and 0.92, arguing that either raw scores or their appropriate regression residuals could be used to rank order the F2s and select extremes for genotyping. Since there were many ties for raw scores at both extremes of the distribution, residual scores were used to break ties. Ultimately, animals were rank-ordered by peak and then residual peak and the top and bottom 22 (13.75%, plus ties) animals were chosen for genotyping (ultimately 59 total). To be certain of obtaining the animals whose withdrawal was most severe, all animals were re-ranked by area and then residual area. Those animals that were among the top and bottom 22 animals that were not already chosen were added to the genotyping groups. Thus, four additional animals were added to the low scoring group, and five to the high group. The choice of animals in this manner had the effect of emphasizing animals that scored 0 for baseline. Most animals at the low scoring extreme had scores of 0 for baseline, peak, and area (21/29); while most high scoring animals scored 0 for baseline, 4 for peak, and between 22 and 41 for area (16/30).

The peak score frequency distribution for the D1D2F2s and parental strains is shown in Figure 24. These data were subjected to analysis for bimodality according to the method described by Belknap et al. (1992). First, the data were sorted into two clusters using K-means cluster analysis (Wilkinson, 1989). All of the D1 strain (white hatched bars) and all but two of the low scoring F2 extreme group

Figure 24

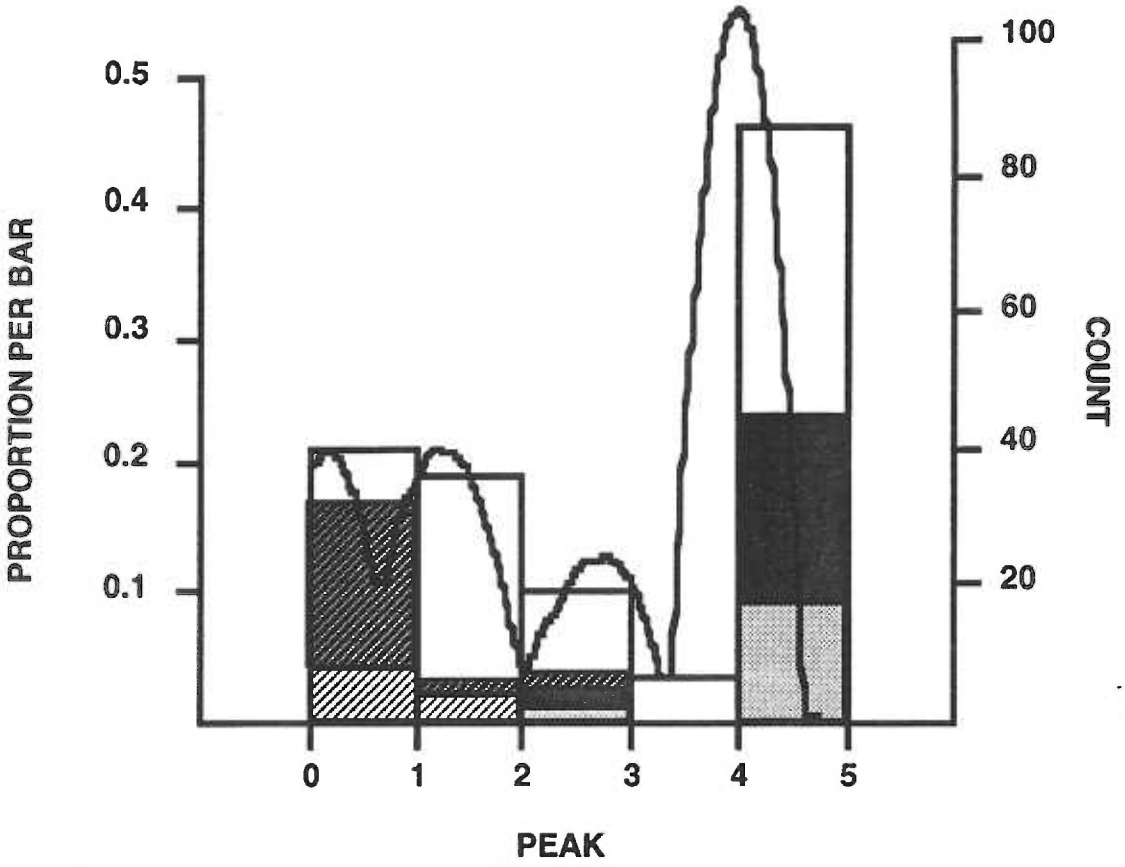

Figure 24: Peak zolpidem withdrawal frequency histogram and Epanechnikov kernel density function for D1D2F2, D1, and D2 mice from Experiment 13 (Silverman, 1986; tension = 0.175). Left axis: proportion of animals per bar. Right axis: actual n per bar. Portions of bars are coded to show the number of mice of each genotype (D1, D2, or D1D2F2) and their peak withdrawal score. D1 inbred mice (white hatched bars) and all but two genotyped low-scoring F2s (black hatched bars) had peak scores less than 2. D2 inbreds (white stippled bars) and genotyped high-scoring F2s (black stippled bars) had peak scores greater than 2. White portions of bars depict the peak scores of F2s that were ungenotyped. The data are consistent with a bimodal distribution (see text).

(black hatched bars) were sorted by SYSTAT into one cluster; while all of the D2 strain (white stippled bars) and all of the high scoring F2 group (black stippled bars) were sorted into the other. Both of the two low scoring F2s that were sorted into the high cluster were animals that would not have been chosen for genotyping by peak alone -- they were added based on residual area. The mean peak scores of the two clusters were 0.74 (± 0.63, SD) and 3.74 (± 0.53), respectively, and differed significantly, ($F_{(1,186)} = 1234.2$, $p < 0.001$). Next, the actual data within each cluster were regressed on their expected values if the data were unimodally normally distributed, and the y-intercepts of the two regression lines were examined by t-test and found to differ significantly ($t = 22.24$, $df = 184$, $p < 0.001$). Therefore, this test suggests that the data fit a bimodal distribution better than a unimodal one.

III.C.2.b.ii. Zolpidem and Ethanol Withdrawal Severity

The time-course of zolpidem withdrawal in D1D2F2 mice is shown in Figure 25. Also shown are the time-courses of the progenitor strains and the mean time-courses of the two sets of genotyped F2 extreme scorers. The data clearly show that the high scoring F2s are D2-like, while the low scoring F2s are D1-like.

The time-course of ethanol withdrawal in D1D2F2 mice is shown in Figure 26. The figure also shows the progenitor strain time-courses and those of the genotyped D1D2F2 animals. Note that D1 mice have significant ethanol withdrawal compared to their lack of zolpidem withdrawal, and that D2 mice have even higher ethanol withdrawal than D1 mice. These results are consistent with earlier findings (Crabbe et al., 1983; Metten & Crabbe, 1994a). While the different

Figure 25

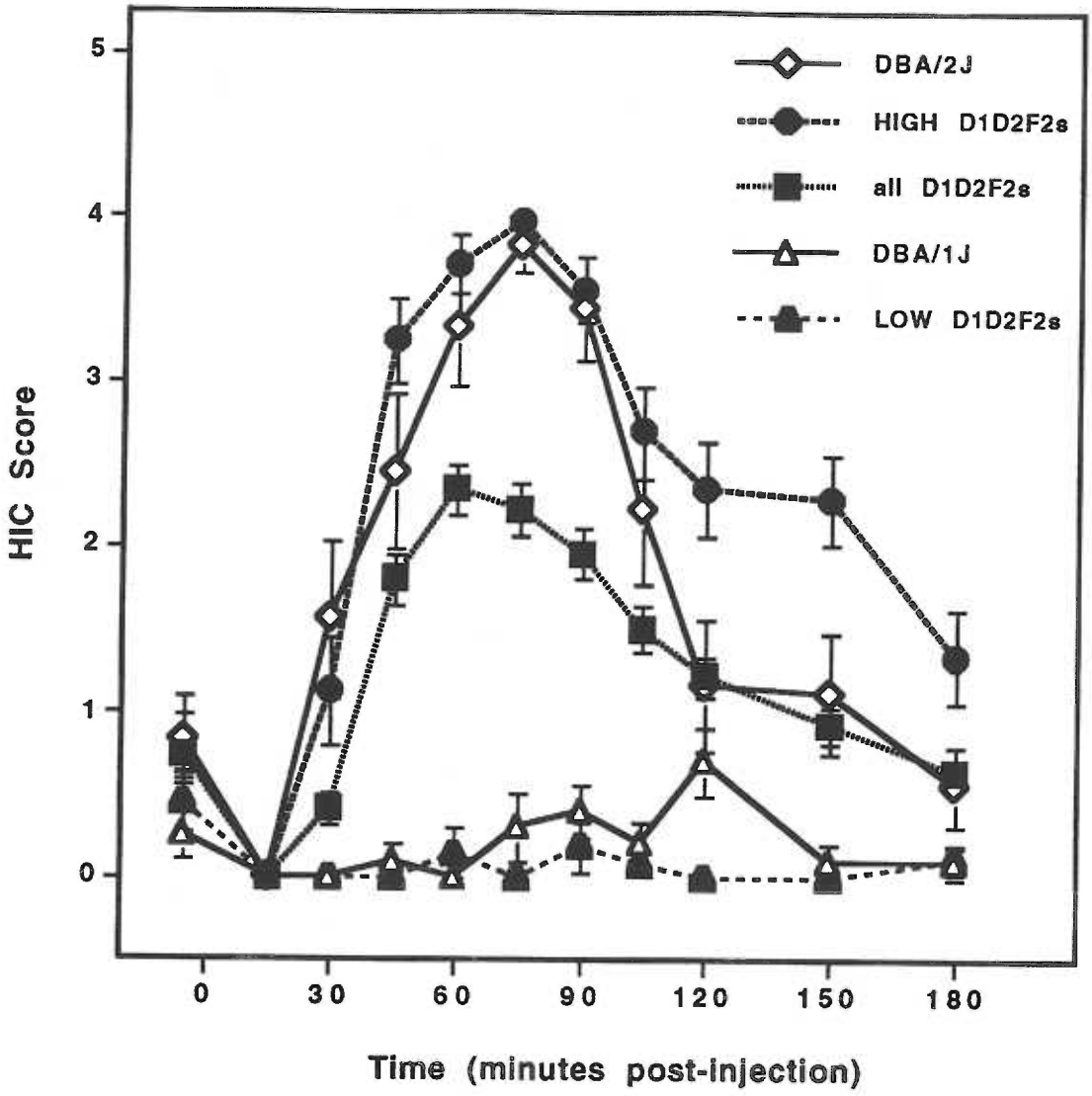

Figure 25: Time-course of spontaneous zolpidem withdrawal in D1D2F2, D1, and D2 mice. Zolpidem (20 mg/kg) was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). Symbols represent mean ± SE for each group. The entire group of D1D2F2 mice is shown, as well as only those in the two (high and low scoring) genotyped groups. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in minutes, following injection.

Figure 26

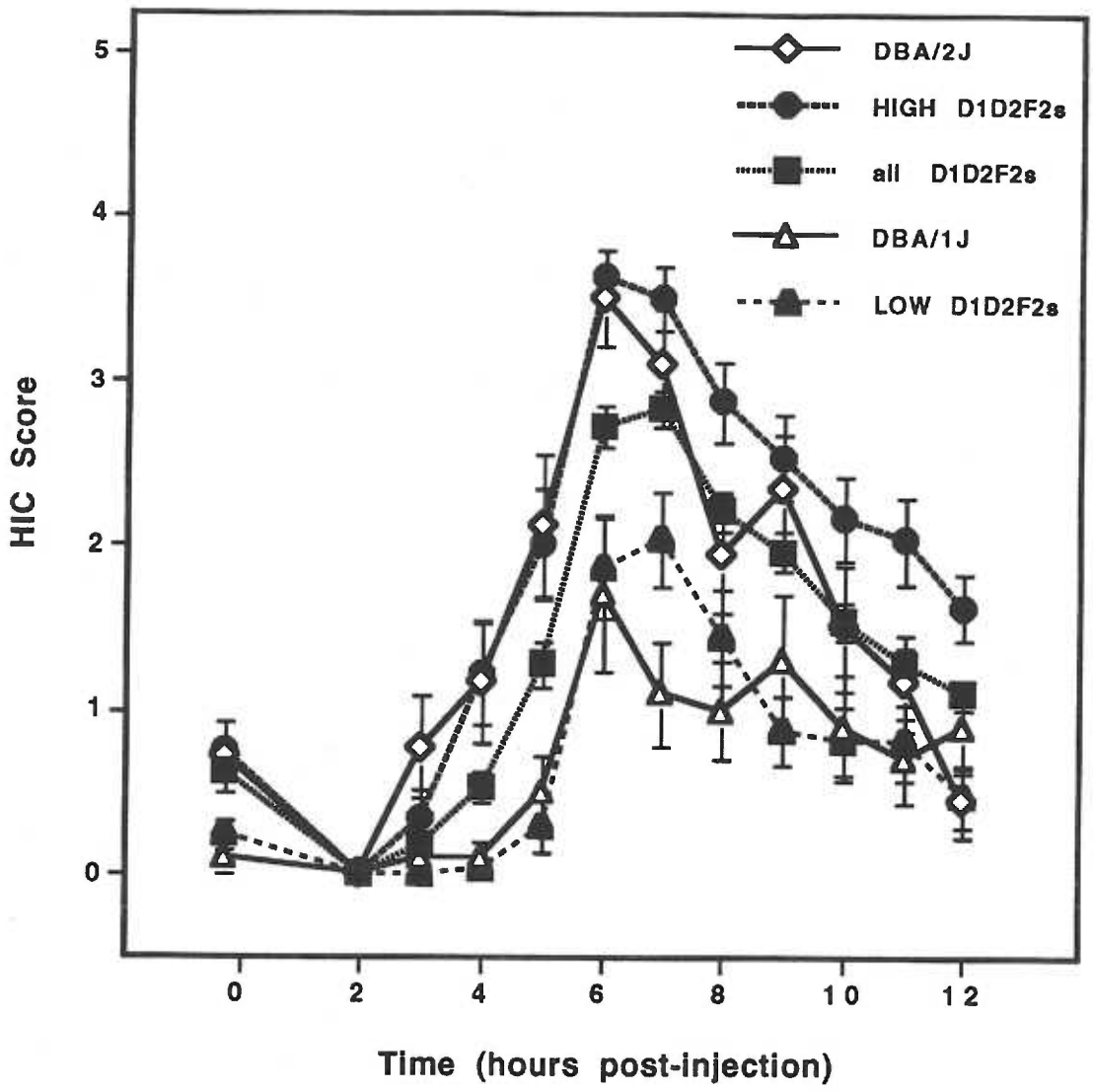

Figure 26: Time-course of ethanol withdrawal in D1D2F2, D1, and D2 mice. Ethanol (4 g/kg) was injected i.p. into separate groups of mice at time = 0, immediately following second baseline HIC assessment (see text). Symbols represent mean ± SE for each group. The entire group of D1D2F2 mice is shown, as well as only those in the two (high and low scoring) genotyped groups. Y-axis: Handling-induced convulsion (HIC) score (see Table 2). X-axis: Time, in hours, following injection. Ethanol and zolpidem withdrawal area scores in D1D2F2s were significantly phenotypically correlated (see text).

D1D2F2 groups are somewhat less distinct than in the zolpidem withdrawal time-course (cf. Figure 25), there is a clear trend in the same direction. When the zolpidem and ethanol withdrawal area scores of the D1D2F2 mice alone were correlated, a significant positive phenotypic correlation was shown, indicating that mice that had high scores for one trait also had high scores for the other (r_{158} = 0.74, $p < 0.01$). The genetic correlation of zolpidem and ethanol withdrawal severities in this population was estimated from the phenotypic correlation according to Falconer (1989; r_G = 0.84). This calculation requires estimates of heritability and the proportion of the phenotypic correlation attributable to environmental factors. Heritability was estimated as above, and the environmental correlation was estimated as the greater (D1) of the within strain correlations for D1 and D2 (r_E = 0.627). Thus, these data further support the hypothesis that ethanol and zolpidem withdrawal severities are mediated, in part, by the same gene(s).

III.C.2.c. Genotypic Results and Summary

DNA samples from 59 D1D2F2 mice (29 animals from the low extreme and 30 from the high extreme; ~37% of the total phenotypic distribution) were ultimately genotyped for the polymorphic markers listed in Tables 7 and 8. Allele frequencies of the phenotypic extremes for each marker were analyzed for differences by chi-square (χ^2) test (2-tailed test, 1 df, α = 0.05). CentiMorgan locations, chi-square values, number of mice successfully genotyped, and the respective allele frequencies (expressed as the proportion of D2 alleles, *q*) in each phenotypic extreme group are given for each

marker in Table 9. Discrepancy in genotype interpretation resulted in the elimination of data for one F2 animal for *D4Mit33*. Other deviations of the number of animals genotyped from 59 were due to inexplicable missing bands from the gels. It can be seen that only one of the allele frequencies (*D17Mit66*) differed significantly at the $p < 0.05$ level. Figure 27 is a computer-scanned representation of the photographs of the gels resolving genotypes of D1, D2, and D1D2F2 mice at *D17Mit66*. Table 9 also shows that allele frequency differences for *D2Mit238* and four markers on chromosome 11 approached significance (ps <0.10). Interestingly, for all five markers on chromosome 11 and the chromosome 17 marker, the number of D2 alleles was greater in the low scoring group than in the high scoring group, indicating that there may be QTLs controlling minor proportions of the genetic variance that are negatively correlated with zolpidem withdrawal in these two regions. Thus, it is likely that the major gene influencing zolpidem withdrawal severity differences between the DBA substrains is not near any of the markers genotyped in the present study, since D2 alleles at such a QTL would have to confer high withdrawal scores.

IV. Discussion

IV.A. Current Status of the Genetic Correlation Hypothesis

The results of these studies provide strong evidence that there are common genetic determinants of ethanol and benzodiazepine withdrawal convulsions. Thus, evidence is now consistent that genes affecting ethanol withdrawal convulsions exert pleiotropic influences on withdrawal from a variety of central nervous system depressant drugs, including benzodiazepines. The inbred strain mean correlation

Table 9

Chi-square (χ^2) values for Allele Frequency Differences (q_H - q_L) between Phenotypic Extremes in D1D2F2 Mice†

Marker	cM	n	χ^2	q_L	q_H
D1Mit128	36.9	57	0.91	0.50	0.59
D2Mit238	28	48	2.70*	0.44	0.61
D2Mit77	74	59	0.19	0.34	0.38
D3Mit116	84.9	59	0.12	0.53	0.57
D4Mit185	32-43	57	1.79	0.41	0.53
D4Mit13	71	59	0.02	0.40	0.38
D4Mit33	79	51	1.59	0.50	0.38
D6Mit149	47	53	2.64	0.64	0.48
D11Mit108	18	58	3.49*	0.66	0.48
D11Mit217	19	59	3.52*	0.66	0.48
D11Mit20	20	59	2.84*	0.64	0.48
D11Mit174	20	59	2.84*	0.64	0.48
D11Mit296	20	59	2.23	0.62	0.48
D13Mit57	7	59	0.04	0.45	0.47
D17Mit66	24.5	59	4.05¥	0.57	0.38

† Markers are ordered by chromosome and centimorgan (cM) location. χ^2 values were calculated using Sokal and Rohlf's G test for selective genotyping in a 2 X 2 contingency table. G is a likelihood ratio chi square test. Williams Correction was employed to give an adjusted G. With $\alpha = 0.05$, the critical value of $\chi^2 = 3.84$. Allele frequencies (q_L, q_H) are expressed as the proportion of D2 alleles in each of the phenotypic extremes (L - low extreme; H - high extreme). Also given is the number (n) of mice successfully genotyped for each marker.

* $p \leq 0.10$.

¥ $p < 0.05$.

Figure 27

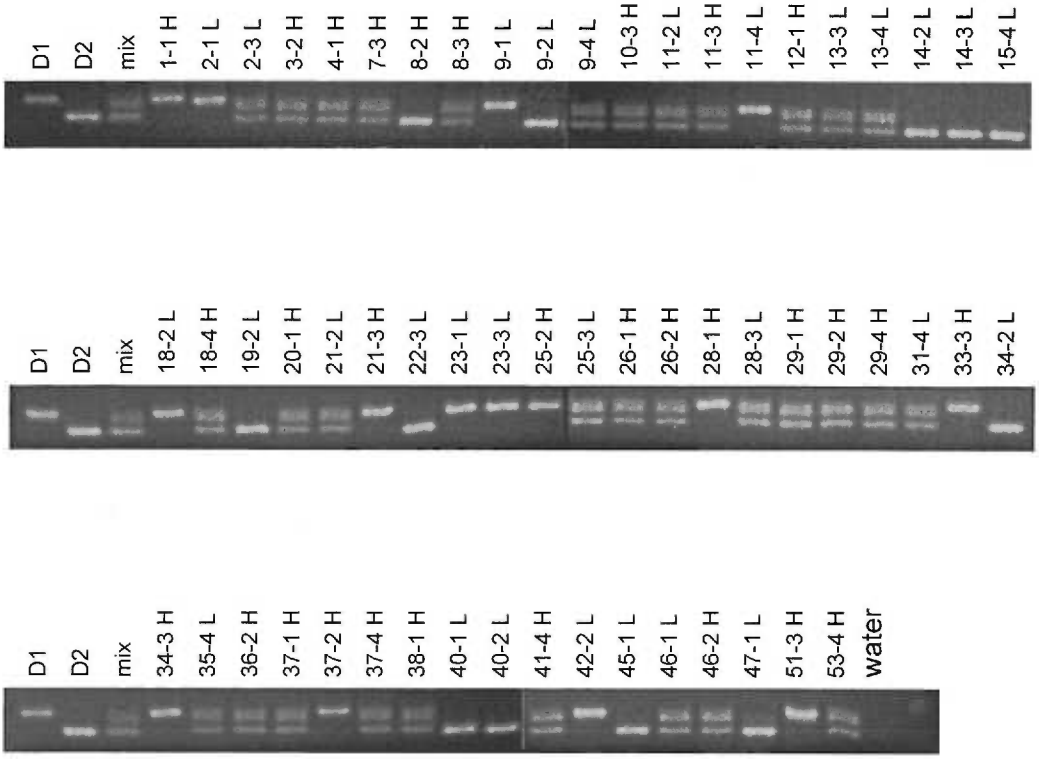

Figure 27: Computer-scanned images of photographed agarose gels resolving genotypes of D1, D2, and D1D2F2 mice at *D17Mit66*, a microsatellite marker. Each lane is labelled according to the identity of the mouse contributing DNA. D1 and D2 DNA were loaded in the first and second lanes of each gel, and D1 and D2 DNA were combined in the lanes labelled "mix," simulating a heterozygote animal. Each genotyped D1D2F2 mouse is identified by a unique number (e.g., 34-2) and phenotype (H - high scorer or L - low scorer), labelled at the top of the appropriate lane. Interpretations of F2 genotypes were made by two persons who were not aware of the phenotypic scores of the animals.

of ethanol and diazepam withdrawal (Experiment 8) is now concordant with evidence in WSP and WSR mice (Belknap et al., 1989; Crabbe et al., 1991a), and the finding generalized to the HAW and LAW lines (Experiment 10). Two other sets of selectively bred lines have also recently been used to test this hypothesis (unpublished data). The High Withdrawal (HW) and Low Withdrawal (LW) lines are being selectively bred for susceptibility and resistance, respectively, to elevated HICs following chronic ethanol vapor inhalation in an almost exact replication of the WSP/WSR selection protocol (V. G. Erwin, unpublished; Metten & Crabbe, 1996). Female mice of S_4 from one replicate of these lines were tested for precipitated withdrawal from diazepam using the same protocol as in the present studies. HW mice had significantly greater diazepam withdrawal HIC scores than LW mice (HW: 2.47 ± 0.22 [mean ± SEM]; LW: 0.43 ± 0.29; $t_{17} = 5.66$, $p < 0.01$; unpublished data). The second replicate is three generations behind the first, and has not yet been tested as of this writing.

The other selection project is one in which mice were selectively bred for sensitivity and resistance to the ataxic effects of diazepam, measured as latency to recover ability to stay on a fixed-speed rotarod (Gallaher et al., 1987a). Diazepam Resistant (DR) mice had significantly greater ethanol and diazepam withdrawal than Diazepam Sensitive (DS) mice (Metten & Crabbe, 1994b). These lines were tested late in selection (S_{26}), when accumulated inbreeding and the extent of chance fixation were high. The results concerning correlations among central nervous system depressant drug withdrawal and diazepam-induced ataxia should be interpreted cautiously. However, the fact that both ethanol and diazepam

withdrawal were greater in one line over the other is suggestive. Given the strength of the other evidence, the implication of this finding seems sound.

Withdrawal convulsion severity following zolpidem also was found to correlate genetically with ethanol withdrawal in several experiments, supporting the corollary hypothesis that benzodiazepine withdrawal convulsions in general are mediated by some of the same genes as ethanol withdrawal. Furthermore, the genetic correlation does not appear to require use of a strictly identical withdrawal paradigm, since HAW mice had significantly greater spontaneous and precipitated benzodiazepine withdrawal than LAW mice. It might be argued that this is a limited finding. HAW and LAW mice were derived without replicate lines from a genetically segregating B6D2F2 founder population, and interpretation of genetic correlations in unreplicated lines must be considered as tentative without corroborative evidence (Crabbe et al., 1990). However, the main reason for creating replicate lines is to protect against effects of random genetic drift, which is less likely to be a serious problem with short-term selection. Also, replicate lines can be re-derived readily in just a few generations, using the short-term selection protocol (Metten & Crabbe, 1996; Belknap et al., in press). The replicate lines could then be tested for spontaneous and precipitated benzodiazepine withdrawal, including diazepam and zolpidem, in order to replicate and extend these results.

It might also be argued that the generalizability of the HAW/LAW results are potentially limited to B6 and D2 inbred strains and their intercross progeny. The present studies do not provide sufficient evidence to conclude that precipitated zolpidem withdrawal would be

genetically correlated with ethanol withdrawal in other test crosses. However, spontaneous zolpidem and ethanol withdrawal were shown to be correlated in D1D2F2s, extending the finding to at least one more population. Furthermore, the HW and LW lines discussed above could be tested with several BZR ligands in both withdrawal paradigms. Like the WSP and WSR lines, these lines were derived from HS/Ibg stock, produced from crosses among eight inbred strains (A, AKR, BALB/c, C3H, C57BL, Is/Bi, RIII, and DBA/2; McClearn & Kakihana, 1981).

Several groups of researchers have failed to show dependence on zolpidem in mice (VonVoigtlander & Lewis, 1991; Perrault et al., 1992; Schoch et al., 1993). In one study, zolpidem or vehicle was administered to male CD-1 mice by gastric intubation (30 mg/kg, twice daily) for ten days. Dependence was measured in separate groups of mice at 3, 6, 14, 24, 42, and 67 hours after the last treatment as the decrease in latency to isoniazid-induced convulsions. An increase in convulsion latencies (i.e., protection) was seen at 3 hours in zolpidem-treated mice compared to controls, but no differences were detected at other times (Perrault et al., 1992). Another group of zolpidem-treated mice was injected with flumazenil (5 mg/kg, i.p.) ten minutes after isoniazid. There were no differences in convulsion latencies compared with chronic vehicle-treated mice, suggesting that flumazenil antagonized the protective effect of zolpidem (Perrault et al., 1992).

Although the study of Perrault et al. is not easily comparable to the present studies, several possiblities can be suggested for the discrepancy in conclusions. First, Perrault et al. may have looked too

infrequently or too late: their first time-point was 3 hours, a time when mice in the present studies are beginning to recover basal levels of HIC severity (see Figures 7, 20, 25). Second, it may be that zolpidem withdrawal after repeated injection is less intense than after a single injection; however, repeated ethanol exposures given to heterogeneous mice have been shown to induce successively greater withdrawal episodes (Goldstein, 1974). Third, convulsant drug treatment is rather harsh compared to the handling-induced convulsion, which is the exacerbation of a natural reflex (Chance, 1953; Goldstein, 1972b). It may be that the intensity of the convulsive response to isoniazid masked the zolpidem withdrawal-induced hyperexcitability. Finally, the outbred CD-1 mice may not possess the full range of genes conferring zolpidem withdrawal severity. The results of the present studies and our preliminary study in 14 inbred strains (unpublished) suggest that genetic susceptibility to zolpidem withdrawal convulsions may be rare.

IV.B. D2, D1, and B6 Mice and Withdrawal Severity

Closer examination of the data from the inbred strain diazepam and zolpidem withdrawal tests (Experiments 8 and 9) and ethanol withdrawal test (Metten & Crabbe, 1994a) revealed an interesting pattern of responses among the B6, D1, and D2 strains. The DBA substrains had greater withdrawal from both ethanol and diazepam compared to the B6 strain (or any other strain). The D2 strain's ethanol withdrawal mean was twice that of the D1 strain; however, there was no difference in their diazepam withdrawal means (see Figure 15; $p = 0.77$, Tukey's HSD). The DBA substrains also differed in the same direction for zolpidem withdrawal; therefore, there exists at

least one gene common to ethanol and zolpidem withdrawal severity that is not among the genes affecting diazepam withdrawal. Furthermore, it appears from the results of Experiments 13 and 14 that this gene(s) may control a relatively large percentage of the genetic trait variance in zolpidem and ethanol withdrawal in crosses with a D2 progenitor. We also tested D1D2F1 mice for zolpidem withdrawal. Sixteen of 18 F1s had peak withdrawal scores of 4; the remaining two animals had scores of zero throughout the time-course (unpublished observations). These data support the major gene hypothesis and further suggest that there may be dominance for the effect of the D2 allele since F1 mice are isogenic.

The D2 and WSP zolpidem withdrawal results (Figures 7, 12, and 20) suggest that genetic susceptibility to zolpidem withdrawal may be an indicator of susceptibility to extreme ethanol withdrawal severity. It is possible that D2 and WSP mice share common allelotype(s) at pleiotropic withdrawal QTL(s). The progenitor stock of WSP mice was HS/Ibg (see above). Thus, all DBA alleles extant in the WSP line are DBA/2 in origin if unique to one DBA substrain. As a test of this hypothesis, WSP and WSR mice are presently being genotyped for ethanol withdrawal QTL markers (K. J. Buck, personal communication).

Among the 23 loci known to be polymorphic between D1 and D2 mice prior to the present studies, genotypes for the B6 strain are known for 19. Of these, the D1 substrain has the same allele as the B6 strain for 13 loci; i.e., there is an estimated 68% genetic homology between these two strains (GBASE, 1994; Festing, 1990; Festing, 1994). The present studies found that the D1 strain showed zolpidem

withdrawal severity that was not significantly different from that of B6 (Figure 20). This suggests that the D1 strain may have the same allele(s) as B6 at the QTL(s) affecting zolpidem withdrawal. This is plausible given the D1/B6 homology estimate given above. The percentage of minisatellite fragments (estimated by digestion with the restriction enzyme *Hae* III) that vary between D1 and B6 is 68%, implying homology of centromeric DNA of no more than 32% (Aker & Huang, 1996). Another estimator of the proportion of D1/B6 homology is available from the present genotyping results. D1 alleles appeared to isomorphic with B6 alleles for only 5 of fifteen D1/D2 polymorphic markers (i.e., 33%). Like that from minisatellite markers, this estimate should be interpreted cautiously, since gel resolution is approximately 4 base pairs. Furthermore, alleles appearing to be identical in base pair number may still have different base pair identities. Finally, eleven other strains did not exhibit significant zolpidem withdrawal (unpublished observations). It is equally plausible that the D1 strain shares allelotype at the zolpidem QTL with one of these strains.

IV.B.1. Genotyping and QTLs

The present studies provide the first evidence suggesting the chromosomal location of any QTL affecting benzodiazepine withdrawal. At the time of this writing, a mapping effort for QTLs affecting precipitated withdrawal from acute diazepam is underway in BXD recombinant inbred (RI) strains (E. J. Gallaher, personal communication). It will be interesting to compare the ethanol/ diazepam genetic correlation in the RI strains with that of the inbred strains. The chromosomal locations of QTLs affecting ethanol and the

two BZR ligands can also be compared (Crabbe et al., 1994a). The results of the present studies predict that there will be some overlap in the QTLs observed from ethanol and diazepam, and that at least one QTL will be common to ethanol and zolpidem, but not diazepam. It can be predicted that the unique ethanol/zolpidem QTL will not be in the chromosome 11 region (18 - 20 cM) or near *D17Mit66* (24.5 cM) because of the negative association of these markers with zolpidem and ethanol withdrawal (Table 9).

The D1D2F2 genotyping data from polymorphic markers on chromosome 11 were analyzed using MAPMAKER/EXP and MAPMAKER/QTL (Lincoln et al., 1993a; Lincoln et al., 1993b) despite the lack of statistical significance. MAPMAKER/EXP 3.0 constructed the primary linkage map for this region, giving the order of the markers (centromere to distal end) as: *D11Mit217*, *D11Mit108*, *D11Mit174*, *D11Mit20*, *D11Mit296*. The order of markers is consistent with the Enclopedia of the Mouse Genome V (Silver et al., 1996), except for the first two markers which currently are assigned to 19 and 18 cM, respectively. The other three markers are assigned to 20 cM; however, the present data suggest that *D11Mit296* may be slightly further distal than *D11Mit20* and *D11Mit174*.

MAPMAKER/QTL 1.1 was employed to determine whether D1D2F2 acute ethanol withdrawal and spontaneous zolpidem withdrawal data were independently associated with any of these markers. This program uses linear regression of phenotype on gene dosage, and therefore, the p values obtained are the same as for correlation of phenotype with gene dosage (Lincoln et al., 1993b; Belknap et al., 1995). However, MAPMAKER/QTL 1.1 also includes

interval analysis using maximum likelihood estimation and a built-in genotyping error check, and assesses both additive and dominance effects of a QTL. MAPMAKER/QTL 1.1 calculated a LOD score for the presence of a zolpidem withdrawal QTL in this region as 1.384 ($p = 0.04$), best fitting either a D1-dominant or additive genetic model. This putative QTL localized to the interval between *D11Mit217* and *D11Mit108*, and was calculated to control about 6.7% of the phenotypic variance in zolpidem withdrawal. These results do not meet the suggestive linkage standard set up for reporting linkage (LOD thresholds 1.9 - 2.0 for intercrosses with 1 d.f.) by Lander and Kruglyak (1995). However, several caveats should be mentioned before disregarding this region as a potential QTL site. First, MAPMAKER/ QTL 1.1 assumes that the phenotypic data are normally distributed. That is clearly not the case with these data. In fact, the probable degree (98%) of genetic homology between D1 and D2 strains and the bimodality of the distribution (Figure 24) argues that a nonparametric linkage program would be more appropriate for analysis of these data. Such a program is in development (Kruglyak & Lander, 1995). Second, the numbers of animals tested (160) and genotyped (59) in this study generally were calculated to detect a major gene affecting the trait, not QTLs affecting less than 7% of the trait variance.

Third, the chromosome 11 markers were also found to be associated with ethanol withdrawal in the D1D2F2s (LOD 1.711, best fitting an additive model, $p = 0.02$, explaining 10.2% of the trait variance). The chromosome 11 markers were negatively associated with both zolpidem and ethanol withdrawal. This is the same

direction as found in B6 X D2 crosses (Belknap et al., 1993c; Buck et al., submitted), lending further support to the results. Markers on chromosome 11 (16 - 23 cM) were chosen for genotyping D1 mice not only because a putative ethanol withdrawal QTL maps there, but because the α1, α6, and γ2 subunits of the GABA receptor complex are co-localized to that region (Silver et al., 1996). The human homolog of the GRC β2 subunit is located in the same gene cluster as the α1, α6, and γ2 subunits (Russek & Farb, 1994), suggesting that the β2 mouse homolog will map to murine chromosome 11.

There was also a negative association of *D17Mit66* at 24.5 cM with zolpidem withdrawal ($p < 0.05$). This area of chromosome 17 is one of the historically-identified regions containing polymorphisms between the DBA substrains (Hoffman, 1978). No other chromosome 17 markers were identified as D1/D2 polymorphic in Experiment 12, precluding analysis with MAPMAKER. Marginal support was found for a positively associated marker on chromosome 2, *D2Mit238* (28 cM; $p = 0.10$). This marker is in the general region of another putative ethanol withdrawal QTL (~37 cM) located by mapping in B6 X D2 intercrosses (Belknap et al., 1993c; Buck et al., submitted).

IV.B.2. Future Directions

It seems unlikely that any of the marginally associated markers just discussed can account for the bimodality in the D1D2F2 data. Therefore, the present study did not successfully map the putative major gene affecting zolpidem withdrawal severity to any of the regions tested in the present studies. One possibility that must be considered is that the polymorphism resulted from mutation, rather than residual heterozygosity at the time of substrain separation.

Assuming that the mutation were linked to a region of polymorphism preserved from residual heterozygosity, it should be possible to detect it by performing a full-genome scan of D1D2F2 mice with microsatellite markers. Spacing the markers about 10 cM apart would permit interval mapping, such as that employed by MAPMAKER, including the nonparametric version (Kruglyak & Lander, 1995). This approach will also work if the polymorphism did not result from mutation. In either case, it should be unnecessary to breed and test additional D1D2F2s since only a miniscule portion of DNA was used in the present studies.

However, a much more extensive effort in genotyping the D1 progenitor than the present studies would probably be necessary in order to identify *de novo* polymorphic loci. The approach used here was to use markers that were known to be B6/D2 polymorphic. There is certainly no requirement for this. Other markers known to be polymorphic between at least two commonly used inbred strains should be equally useful. For example, the polymorphism detected with *D4Mit13* revealed that the D1 strain has neither the D2 (97 bp) nor the B6 (92 bp) allelotype. The D1 allele appeared to be around 105 - 115 bp, which suggests that it may share allelotype with C3H/HeJ (108 bp) or AKR/J (111 bp).

To the best of my knowledge, no other group has any interest in genotyping DBA/1J mice. However, D1 mice are commonly used in immunologic research as a genetic model susceptible to arthritis (e.g., collagen- or pristane-induced) and D2 mice are resistant (Chapedelaine et al., 1991). Differences in a two-way active-avoidance shuttle box task and hippocampal CA3 pyramidal cell spontaneous and

evoked bursting between the DBA/1Halle and DBA/2Gat strains have also been identified (Yanovsky et al., 1995). Thus, it seems likely that the genotyping of D1 mice would prove useful for other fields of science as well as that of drug withdrawal.

IV.C. Implications of Screening Test Results for the Correlation Hypothesis

Although the fact that WSP mice were selectively bred for susceptibility to severe ethanol withdrawal convulsions and both replicate lines are susceptible to benzodiazepine withdrawal is strongly suggestive of common genetic determinants of withdrawal among these drugs, alternative explanations are possible. Two possibilities are (1) that random inbreeding produced homozygous fixation of genes affecting benzodiazepine withdrawal and (2) that genes having a minor effect on the exacerbation of chronic ethanol withdrawal convulsions but a major effect on diazepam withdrawal became fixed later in selection. Several lines of evidence argue against these possibilities. First, despite the fact that WSR mice were not tested in the present studies, selective breeding pressure against susceptibility for ethanol withdrawal convulsions following chronic vapor inhalation has rendered both WSR replicates almost universally resistant to withdrawal from central nervous system depressants, including diazepam (Belknap et al., 1987; Belknap et al., 1988; Belknap et al., 1989; Crabbe et al., 1991a). Furthermore, this differential sensitivity of the lines is specific to depressants, since WSR mice may be more sensitive than WSP mice to naloxone-precipitated morphine withdrawal (Belknap, unpublished).

Second, the differential sensitivity of the WSP and WSR lines to diazepam withdrawal was initially demonstrated in both replicates early in selection (S_5). Differential fixation of trait-irrelevant genes this early in selection in both replicates is highly unlikely under the breeding conditions employed (Crabbe et al., 1985; Crabbe et al., 1990).

Third, the ethanol/diazepam correlation has been confirmed early in selection in two other sets of lines selectively bred for differential ethanol withdrawal (HAW/LAW: Experiment 10; HW/LW: discussed above) and extended to zolpidem (Experiment 11A & B). Therefore, the results in WSP mice imply that withdrawal severity following BZR ligands other than diazepam and zolpidem are also genetically correlated with ethanol withdrawal severity.

It might also be argued that WSP mice are simply sensitive to convulsions in general, and HICs in particular. After 26 generations of selective breeding, there is evidence that WSP mice are slightly more sensitive than WSR mice to convulsions induced by infusion of chemical convulsants (Kosobud & Crabbe, 1995). The observation that WSR mice are more sensitive to NMDA-induced convulsions after intraveneous infusion indicates that this finding is not universal (Kosobud & Crabbe, 1993). WSP mice are also more sensitive than WSR mice to HICs induced by intraperitoneal injection of a variety of convulsant drugs; however, it is clear that WSR mice do display HICs in response to some convulsant drugs (Crabbe et al., 1991b).

It may be that these differences between the lines developed late in selection. After three generations of selection, HAW and LAW mice did not differ in sensitivity to NMDA-induced convulsions after

infusion; in animals from S_5, no differences between the lines were found for either kainic acid- or pentylenetetrazol- induced convulsions after infusion (Metten et al., unpublished observations). In contrast to WSR mice, LAW mice still display baseline and mild withdrawal-elevated HICs indicating that selection pressure has not yet neutralized the capability for these responses (Figures 21 and 22; baseline data not shown). The HAW and LAW lines have not yet been examined for differential sensitivity to elevation of HICs following injection of convulsants or following withdrawal from the wide variety of drugs examined in WSP and WSR lines, so that the extent of the specificity remains unclear. However, HAW mice display greater withdrawal than LAW mice following pentobarbital and nitrous oxide, in addition to the BZR ligands examined in the present studies (Metten et al., unpublished observations). Thus, the implications of these findings support the conclusion that selection for severe ethanol withdrawal convulsions confers sensitivity to withdrawal to central nervous system depressant drugs in general, but not necessarily to convulsions induced by other methods.

IV.C.1. Spontaneous versus Precipitated Withdrawal Convulsions

In the present studies, WSP mice displayed spontaneously produced withdrawal convulsions following a single dose of zolpidem or alprazolam and withdrawal from each could also be precipitated by flumazenil, but precipitation of convulsions was required for both midazolam and triazolam. In marked contrast, HIC scores following abecarnil injection were only elevated above basal levels when the spontaneous withdrawal paradigm was employed. Thus, only

alprazolam and zolpidem produced withdrawal convulsions using both the spontaneous and precipitated withdrawal paradigms.

The results of the screening tests suggest that expression of withdrawal convulsions after single injections of different BZR ligands may depend on the withdrawal paradigm (i.e., spontaneous versus precipitated). For example, precipitated triazolam withdrawal was seen, but none of three doses produced spontaneous withdrawal convulsions. An inverse dose-response relationship might be hypothesized from the results of the spontaneous triazolam withdrawal test (Experiment 2; see Figure 4 inset). This suggests that withdrawal from higher doses might have been seen later after injection. However, in a separate study, we monitored withdrawal convulsion severity at approximately 3 hour intervals for 3 days following administration of 0.5, 1, 5, 10, or 20 mg/kg triazolam to WSP mice; no dose produced elevation above vehicle levels (unpublished observations).

Another group has compared the proportions of DBA/2J mice displaying benzodiazepine withdrawal convulsions following precipitation with Ro15-3505, a partial inverse agonist at the BZR (Moreau et al., 1990). Young (~35 day old) D2 mice were implanted for 7 days with minipumps containing triazolam, alprazolam, diazepam, or vehicle. Five hours after removal of the minipumps, they were injected through the tail vein with Ro15-3505. Significantly greater proportions of benzodiazepine-treated mice displayed clonic convulsions during the next 30 minutes than vehicle-treated mice (Moreau et al., 1990). No subjects were administered vehicle in lieu of Ro15-3505, prohibiting examination of the possibility of spontaneous

convulsions following this treatment paradigm. However, like the WSP results, this study implies that ethanol and benzodiazepine withdrawal convulsions are genetically correlated because of the use of D2 mice.

Chronic administration of midazolam has previously been shown to induce dependence in mice and rats (Boisse et al., 1990; Perrault et al., 1992). CD-1 mice 14 or more hours withdrawn from midazolam displayed decreased latency to isoniazid-induced convulsions compared to mice administered vehicle, suggesting that midazolam withdrawal produced a hyperexcitable state. Flumazenil treatment antagonized the protective effect of midazolam seen at 3 and 6 hours after cessation of midazolam treatment, but did not further decrease convulsion latencies compared to the midazolam/no flumazenil group at later times (Perrault et al., 1992). Thus, this study suggests that withdrawal with or without precipitation occurs after chronic treatment with midazolam.

The data presented in Figure 6 suggest that a lower dose of midazolam than 2.5 mg/kg might have produced spontaneous withdrawal convulsions and that recovery was just beginning in the 5 mg/kg dose group when the time-course was stopped. Another possibility for the lack of observance of withdrawal exists. It has repeatedly been noted that HIC scores diminish over time in repeatedly-tested vehicle-treated animals. Withdrawal HICs were assessed every 15 minutes for two hours following midazolam treatment in the present study. The data suggest that about 30 to 60 minutes between assessments would have been adequate (Figure 6). It is conceivable that following a more loosely-spaced and longer time-

course might have allowed expression of spontaneous midazolam withdrawal convulsions.

Finally, the finding that abecarnil produced spontaneous withdrawal was unexpected. The spontaneous withdrawal results are not consistent with those seen previously (Crabbe, 1992), using the same dose, mouse line and sex, HIC rater, and almost identical time-course. The lack of concordance with the literature (Crabbe, 1992; Steppuhn et al., 1993; Rundfeldt et al., 1995), and the very small withdrawal scores, suggests that the present result should be taken as tentative until replicated. The lack of precipitated withdrawal (Figure 10) was inconsistent with the prediction that abecarnil acts as a full or partial agonist at the BZR; however it has been suggested that abecarnil's agonist properties may depend on the trait being assessed (Lytle et al., 1995). It is possible that another antagonist (e.g., Ro15-3505, a partial inverse-agonist; Moreau et al., 1990) would produce a different result.

IV.C.2. Pharmacology of BZR Ligands vis á vis Withdrawal Expression

It is possible that differences among the BZR ligands with regard to mechanism of action (e.g., GRC binding specificity and affinity, functional agonism) or pharmacology (e.g., brain regional binding, pharmacokinetics) may underlie their respective abilities to induce spontaneous withdrawal convulsions.

IV.C.2.a. Pharmacokinetic Factors

Three of the BZR ligands in these studies are classified as ultra-short acting. Midazolam has an elimination half-life ($t_{1/2}$) of about 1.5 to 2.5 hours in healthy human subjects. A pharmacologically active

metabolite, α-hydroxymidazolam, has a $t_{1/2}$ of about 1 hour (Garzone & Kroboth, 1989). Zolpidem's $t_{1/2}$ is also about 1.5 to 2.5 hours. Three major metabolites have been identified, but they appear to lack pharmacological activity (Langtry & Benfield, 1990). Midazolam and zolpidem both have similar times to maximum plasma concentration (30 - 45 minutes). Triazolam's $t_{1/2}$ is about 2.5 to 4 hours and its metabolites are very rapidly glucuronidated and excreted in urine (Garzone & Kroboth, 1989). Abecarnil is described as a short acting partial-to-full agonist. Its $t_{1/2}$ is about 3.5 to 8 hours. Four metabolites of abecarnil have been identified. They have longer half-lives (~22 hours) and very low binding affinity (~40-fold less) compared to abecarnil and may not cross the blood-brain barrier (Spencer & Benfield, 1995). Alprazolam is classified as intermediate in duration of action, with a $t_{1/2}$ of about 10 to 15 hours. Twenty-nine metabolites of alprazolam have been identified in urine; however, the major metabolites appear to be rapidly glucuronidated and excreted (Garzone & Kroboth, 1989). These latter three drugs have similar times to maximum plasma concentration (about 1.25 to 1.75 hours). Finally, these drugs all have similar volumes of distribution (0.5 - 1 L/kg), although there is large intersubject variability (0.4 - 2 L/kg) in this measure with midazolam (Garzone & Kroboth, 1989).

The half-lives of the BZR ligands used in the present studies do not explain their relative abilities to induce spontaneous withdrawal convulsions. A common assumption is that withdrawal is more severe in humans following cessation of treatment with short-acting benzodiazepines than long-acting ones. In fact, a common first step of treatment for people undergoing withdrawal from a rapidly eliminated

benzodiazepine is to switch them to a drug with a long $t_{1/2}$, commonly diazepam (Sellers, 1988), although this is not always effective in preventing seizures (Schneider et al., 1987). Yet, if a rapid elimination half-life were either necessary or sufficient to determine withdrawal severity, then triazolam, zolpidem, and midazolam should all have produced spontaneous convulsions upon withdrawal.

Elimination half-life should not be confused with duration of action. Several other pharmacokinetic properties are important to action duration, including absorption half-time, rate of brain uptake and clearance, and total volume of distribution (Amrein et al., 1983; Arnold, 1991). The lipid solubility of a drug is related to both rates of brain uptake and clearance and has been advocated as a preferable way to categorize BZR ligands by effective duration over half-life (Arnold, 1991). Another way of ranking these drugs is by the residual fraction, which is the plasma concentration at 12 hours after drug intake divided by the maximal plasma concentration (Amrein et al., 1983). With this method, a drug with rapid clearance would be termed short-acting compared to one which clears more slowly, although equivalent maximum concentrations were administered. In fact, time-to-peak withdrawal score for each drug would predict that the following rank order of duration of action would be observed: zolpidem, abecarnil, triazolam, midazolam, alprazolam. This measure might be supposed to be correlated with time to maximum plasma concentration and duration of action. The rank order observed indicates that this estimate may also not be a good predictor of withdrawal severity.

Although it has been demonstrated that withdrawal HIC severity after chronic exposure to ethanol is dose- and duration-dependent

(Goldstein, 1972b), we have shown previously using Swiss-Webster mice that the magnitude of ethanol and diazepam withdrawal severity did not differ across a range of doses in the acute paradigm (Metten & Crabbe, 1994b). Furthermore, inbred strain mean brain diazepam concentrations 30 minutes after a similar dose (16 mg/kg) (Crabbe et al., manuscript in preparation) were not genetically correlated with withdrawal severity ($r = -0.33$, $p = 0.25$). Withdrawal severity was weakly genetically correlated with blood ethanol concentrations at 30 minutes after 4 g/kg ethanol ($r = 0.53$, $p < 0.04$), but not at 90, 150, or 210 minutes or when the four concentrations were pooled (Metten & Crabbe, 1994b). Taken together, these data suggest that pharmacokinetic factors are not crucially important in determining drug withdrawal severity.

IV.C.2.b. Pharmacodynamic Factors

The potencies of the drugs in the present studies at producing withdrawal (regardless of paradigm) are in general accord with their recognized potency ranges at producing pharmacological effects in humans (Garzone & Kroboth, 1989; Langtry & Benfield, 1990; Spencer & Benfield, 1995). GRC subunit binding specificity, brain regional binding, and fractional receptor occupancy have been forwarded as potential explanations of the potency (affinity/efficacy) of BZR ligands at producing behavioral effects (e.g., Giusti et al., 1991; Jones et al., 1994). No data that I am aware of address these hypotheses with regard to withdrawal convulsion severity *per se*. At the present time, the neural circuitry involved in the HIC is unknown, so that a regionally-directed search for brain areas involved in

withdrawal convulsions has been primarily focussed on obvious areas, such as hippocampus. Whole brain messenger RNA for α3, α6, and β2 subunits of the GRC are lower in ethanol naive WSP compared to WSR mice (see review by Buck, 1996), indicating that selective breeding for ethanol withdrawal HIC severity has differentially fixed regulators of GRC subunit expression. Therefore, further investigation into the pharmacodynamic properties of withdrawal from ethanol and BZR ligands may prove fruitful.

IV.D. Summary

The results of the present studies indicate that estimation of withdrawal severity following single injections of different BZR agonists depends on the drug, the withdrawal paradigm, and/or the mice being tested. However, the data from multiple studies support the hypothesis that there is a genetic correlation between ethanol and benzodiazepine withdrawal severities when mice are tested using the drug-appropriate paradigm.

Evidence suggests that zolpidem withdrawal susceptibility may be a behavioral marker of extreme ethanol withdrawal susceptibility. It is therefore advantageous to determine the genetic basis of this susceptibility. D1D2F2 mice tested for zolpidem withdrawal were genotyped for 15 SSLP markers using PCR-based genotyping techniques. Three chromosomal regions were weakly associated with zolpidem withdrawal severity: ~24 cM distal from the centromere on chromosome 17, ~20 cM on chromosome 11, and ~28 cM on chromosome 2. Evidence was found for a major gene effect on zolpidem withdrawal, but the chromosomal location of this gene remains unknown.

V. References

Aker, M., & Huang, H. V. (1996). Extreme heterogeneity of minor satellite repeat arrays in inbred strains of mice. Mammalian Genome, 7, 62-64.

Amrein, R., Eckert, M., Haefeli, H., & Leishman, B. (1983). Pharmacokinetic and clinical considerations in the choice of a hypnotic. British Journal of Clinical Pharmacology, 16, 5S-10S.

Arnold, J. (1991). Determinants of pharmacologic effects and toxicity of benzodiazepine hypnotics: Role of lipophilicity and plasma elimination rates. Journal of Clinical Psychiatry, 52(9 (supplement)), 11-14.

Bailey, D. W. (1978). Sources of subline divergence and their relative importance for sublines of six major inbred strains of mice. In H. C. Morse III (Ed.), Origins of Inbred Mice, (pp. 197 - 215). New York: Academic Press.

Belknap, J. K. (1980). Genetics factors in the effects of alcohol: neurosensitivity, functional tolerance and physical dependence. In H. Rigter & J. C. Crabbe (Eds.), Alcohol Tolerance and Dependence, (pp. 157-180). Amsterdam: Elsevier/North-Holland Biomedical Press.

Belknap, J. K., Crabbe, J. C., & Laursen, S. E. (1989). Ethanol and diazepam withdrawal convulsions are extensively codetermined in WSP and WSR mice. Life Sciences, 44(26), 2075-80.

Belknap, J. K., Crabbe, J. C., Plomin, R., McClearn, G. E., Sampson, K. E., O'Toole, L. A., & Gora-Maslak, G. (1992). Single-locus control of saccharin intake in BXD/Ty recombinant inbred (RI) mice: some methodological implications for RI strain analysis. Behavior Genetics, 22(1), 81-100.

Belknap, J. K., Crabbe, J. C., Riggan, J., & O'Toole, L. A. (1993a). Voluntary consumption of morphine in 15 inbred mouse strains. Psychopharmacology, 112(2-3), 352-8.

Belknap, J. K., Crabbe, J. C., & Young, E. R. (1993b). Voluntary consumption of ethanol in 15 inbred mouse strains. Psychopharmacology, 112(4), 503-10.

Belknap, J. K., Danielson, P. W., Lame, M., & Crabbe, J. C. (1988). Ethanol and barbiturate withdrawal convulsions are extensively codetermined in mice. Alcohol, 5(2), 167-71.

Belknap, J. K., Dubay, C., Crabbe, J. C., & Buck, K. J. Mapping Quantitative Trait Loci for Behavioral Traits in the Mouse. In K. Blum & E. P. Noble (Eds.), Handbook of Psychiatric Genetics. Boca Raton, Florida: CRC Press, in press.

Belknap, J. K., Laursen, S. E., & Crabbe, J. C. (1987). Ethanol and nitrous oxide produce withdrawal-induced convulsions by similar mechanisms in mice. Life Sciences, 41(17), 2033-40.

Belknap, J. K., Metten, P., Helms, M. L., O'Toole, L. A., Angeli-Gade, S., Crabbe, J. C., & Phillips, T. J. (1993c). Quantitative trait loci (QTL) applications to substances of abuse: physical dependence studies with nitrous oxide and ethanol in BXD mice. Behavior Genetics, 23(2), 213-22.

Belknap, J. K., Mogil, J. S., Helms, M. L., Richards, S. P., O'Toole, L. A., Bergeson, S. E., & Buck, K. J. (1995). Localization to chromosome 10 of a locus influencing morphine analgesia in crosses derived from C57BL/6J and DBA/2J strains. Life Sciences, 57, PL 117-124.

Belknap, J. K., Richards, S. P., O'Toole, L. A., Helms, M. L., & Phillips, T. J. Short term selective breeding as a tool for QTL mapping: Alcohol preference drinking in mice. Behavior Genetics, in press.

Boisse, N. R., Quaglietta, N., Samoriski, G. M., & Guarino, J. J. (1990). Tolerance and physical dependence to a short-acting benzodiazepine, midazolam. Journal of Pharmacology and Experimental Therapeutics, 252, 1125-1133.

Buck, K. J. (1996). Molecular genetic analysis of the role of GABAergic systems in the behavioral and cellular actions of alcohol. Behavior Genetics, 26(3), 313-323.

Buck, K. J., & Harris, R. A. (1991). Neuroadaptive responses to chronic ethanol. Alcoholism: Clinical and Experimental Research, 15(3), 460-470.

Buck, K. J., Heim, H., & Harris, R. A. (1991). Reversal of alcohol dependence and tolerance by a single administration of flumazenil. Journal of Pharmacology and Experimental Therapeutics, 257(3), 984-989.

Buck, K. J., Metten, P., Belknap, J. K., & Crabbe, J. C. Loci influencing genetic predisposition to acute alcohol dependence and withdrawal map to murine chromosomes 1, 4 and 11. Submitted.

Burt, D. R., & Kamatchi, G. L. (1991). GABA A receptor subtypes: from pharmacology to molecular biology. The FASEB Journal, 5(November), 2916-2923.

Chan, A. W. K., Langan, M. C., Leong, F. W., Penetrante, M. L., & Schanley, D. L. (1990). Partial cross-dependence on ethanol in mice dependent on chlordiazepoxide. Pharmacology, Biochemistry & Behavior, 35(2), 379-384.

Chan, A. W. K., Langan, M. C., Leong, F. W., Schanley, D. L., & Penetrante, M. L. (1988). Does chronic ethanol intake confer full cross-tolerance to chlordiazepoxide? Pharmacology, Biochemistry & Behavior, 30, 385-389.

Chan, A. W. K., Schanley, D. L., Aleo, M. D., & Leong, F. W. (1985). Cross-tolerance between ethanol and chlordiazepoxide. Alcohol, 2, 209-213.

Chance, M. R. A. (1953). The posture of a falling mouse. British Journal of Animal Behavior, 1, 118-119.

Chapedelaine, J. M., Whalen, J. D., & Wooley, P. H. (1991). Pristane induced arthritis. II. Genetic regulation in F1 hybrid mice and cellular immune abnormalities following pristane injection. Autoimmunity, 8(3), 215-220.

Crabbe, J., & Kosobud, A. (1990). Alcohol withdrawal seizures: Genetic animal models. In R. J. Porter, R. H. Mattson, J. A. Cramer, & I. Diamond (Eds.), Alcohol and Seizures, (pp. 126-139). Philadelphia: F.A. Davis Company.

Crabbe, J. C. (1983). Sensitivity to ethanol in inbred mice: genotypic correlations among several behavioral responses. Behavioral Neuroscience, 97(2), 280-9.

Crabbe, J. C. (1989). Genetic animal models in the study of alcoholism. Alcoholism, Clinical & Experimental Research, 13(1), 120-7.

Crabbe, J. C. (1992). Antagonism of ethanol withdrawal convulsions in Withdrawal Seizure Prone mice by diazepam and abecarnil. European Journal of Pharmacology, 221(1), 85-90.

Crabbe, J. C., Gallaher, E. S., Phillips, T. J., & Belknap, J. K. (1994). Genetic determinants of sensitivity to ethanol in inbred mice. Behavioral Neuroscience, 108(1), 186-95.

Crabbe, J. C., Janowsky, J. S., Young, E. R., & Rigter, H. (1980a). Handling induced convulsions in twenty inbred strains of mice. Substance & Alcohol Actions/Misuse, 1(2), 159-63.

Crabbe, J. C., Janowsky, J. S., Young, E. R., & Rigter, H. (1980b). Neurosensitivity to ethanol in inbred mouse strains: genetic correlations. Proceedings of the Western Pharmacology Society, 23, 225-7.

Crabbe, J. C., Kosobud, A., Young, E. R., Tam, B. R., & McSwigan, J. D. (1985). Bidirectional selection for susceptibility to ethanol withdrawal seizures in *Mus musculus*. Behavioral Genetics, 15, 521-536.

Crabbe, J. C., Merrill, C., & Belknap, J. K. (1991a). Acute dependence on depressant drugs is determined by common genes in mice. Journal of Pharmacology & Experimental Therapeutics, 257(2), 663-7.

Crabbe, J. C., Merrill, C. D., & Belknap, J. K. (1991b). Effects of convulsants on handling-induced convulsions in mice selected for ethanol withdrawal severity. Brain Research, 550(1), 1-6.

Crabbe, J. C., & Phillips, T. J. (1993). Selective breeding for alcohol withdrawal severity. Behavior Genetics, 23(2), 171-7.

Crabbe, J. C., Phillips, T. J., Kosobud, A., & Belknap, J. K. (1990). Estimation of genetic correlation: interpretation of experiments using selectively bred and inbred animals. Alcoholism, Clinical & Experimental Research, 14(2), 141-51.

Crabbe, J. C., Jr., Young, E. R., & Kosobud, A. (1983). Genetic correlations with ethanol withdrawal severity. Pharmacology, Biochemistry, & Behavior, 18(Suppl. 1), 541-547.

Criswell, H. E., Simson, P. E., Duncan, G. E., McCown, T. J., Herbert, J. S., Morrow, A. L., & Breese, G. R. (1993). Molecular basis for regionally specific action of ethanol on gamma-aminobutyric acidA receptors: generalization to other ligand-gated ion channels. Journal of Pharmacology & Experimental Therapeutics, 267(1), 522-37.

Criswell, H. E., Simson, P. E., Knapp, D. J., Devaud, L. L., McCown, T. J., Duncan, G. E., Morrow, A. L., & Breese, G. R. (1995). Effect of zolpidem on gamma-aminobutyric acid (GABA)-induced inhibition predicts the interaction of ethanol with GABA on individual neurons in several rat brain regions. Journal of Pharmacology & Experimental Therapeutics, 273(1), 526-36.

Deitrich, R. A., & Erwin, V. G. (Eds.). (1996). Pharmacological Effects of Ethanol on the Nervous System. Boca Raton, FL: CRC Press.

Deitrich, R. A., & Spuhler, K. (1984). Genetics of alcoholism and alcohol actions. In R. G. Smart, H. D. Cappell, F. B. Glazer, Y. Israel, H. Kalant, R. Popham, W. Schmidt, & E. M. Sellers (Eds.), Research Advances in Alcohol and Drug Problems, (Vol. 8, pp. 47-98). New York: Plenum Press.

Dietrich, W., Katz, H., Lincoln, S. E., Shin, H.-S., Friedman, J., Dracopoli, N. C., & Lander, E. S. (1992). A genetic map of the mouse suitable for typing intraspecific crosses. Genetics, 131, 423-447.

Doble, A., & Martin, I. L. (1992). Multiple benzodiazepine receptors: no reason for anxiety. Trends in Pharmacological Sciences, 13, 76-81.

Edwards, J. G., Cantopher, T., & Olivieri, S. (1990). Benzodiazepine dependence and the problems of withdrawal. Postgraduate Medical Journal, 66(Suppl. 2), S27-S35.

Falconer, D. S. (1989). Introduction to Quantitative Genetics. (Third ed.). New York: Longman Scientific & Technical.

Festing, M. F. W. (1990). Inbred Strains of Mice, 12th. listing, and distribution of some polymorphisms among inbred strains. Mouse Genome, 88(October), 19-112.

Festing, M. F. W. (1994). Inbred Strains of Mice. Mouse Genome, 92(3), 420-426.

Friedman, H. J. (1980). Assessment of physical dependence on and withdrawal from ethanol in animals. In H. Rigter & J. C. Crabbe Jr. (Eds.), Alcohol Tolerance and Dependence, (pp. 93-121). Amsterdam: Elsevier/North-Holland Biomedical Press.

Gallaher, E. J., Hollister, L. E., Gionet, S. E., & Crabbe, J. C. (1987a). Mouse lines selected for genetic differences in diazepam sensitivity. Psychopharmacology, 93, 25-30.

Gallaher, E. J., Jacques, C. J., & Hollister, L. E. (1987b). Alprazolam dependence in mice. Alcohol and Drug Research, 7, 503-510.

Garzone, P. D., & Kroboth, P. D. (1989). Pharmacokinetics of the newer benzodiazepines. Clinical Pharmacokinetics, 16, 337-364.

Giusti, P., Guidetti, G., Costa, E., & Guidotti, A. (1991). The preferential antagonism of pentylenetetrazole proconflict responses differentiates a class of anxiolytic benzodiazepines with potential antipanic action. Journal of Pharmacology & Experimental Therapeutics, 257(3), 1062-8.

Goldstein, D. B. (1972a). An animal model for testing effects of drugs on alcohol withdrawal reactions. Journal of Pharmacology and Experimental Therapeutics, 183, 14-22.

Goldstein, D. B. (1972b). Relationship of alcohol dose to intensity of withdrawal signs in mice. Journal of Pharmacology and Experimental Therapeutics, 180, 203-215.

Goldstein, D. B. (1973a). Alcohol withdrawal reactions in mice: Effects of drugs that modify neurotransmission. Journal of Pharmacology and Experimental Therapeutics, 186(1), 1-9.

Goldstein, D. B. (1973b). Inherited differences in intensity of alcohol withdrawal reactions in mice. Nature, 245(September 21), 154-156.

Goldstein, D. B. (1974). Rates of onset and decay of alcohol physical dependence in mice. Journal of Pharmacology and Experimental Therapeutics, 190, 377-383.

Goldstein, D. B., & Pal, N. (1971). Alcohol dependence produced in mice by inhalation of ethanol: Grading the withdrawal reaction. Science, 172, 288-290.

Grant, K. A. (1994). Emerging neurochemical concepts in the actions of ethanol at ligand-gated ion channels. Behavioural Pharmacology, 5, 383-404.

Hegmann, J., & Possidente, B. (1981). Estimating genetic correlations from inbred strains. Behavior Genetics, 11, 103-114.

Hoffman, H. A. (1978). Genetic quality control of the laboratory mouse (Mus musculus). In H. C. Morse III (Ed.), Origins of Inbred Mice, (pp. 217-234). New York: Academic Press.

Isbell, H., Fraser, H. F., Wikler, A., Belleville, R. E., & Eisenman, A. J. (1955). An experimental study of the etiology of "rum fits" and delirium tremens. Quarterly Journal of Studies on Alcohol, 16, 1-33.

Jaffe, J. H. (1985). Drug addiction and drug abuse. In A. G. Gilman, L. S. Goodman, T. W. Rall, & F. Murad (Eds.), Goodman and Gilman's The Pharmacological Basis of Therapeutics, (Seventh ed., pp. 532-581). New York: Macmillan Publishing Company.

Jaffe, J. H., & Ciraulo, D. A. (1985). Drugs used in the treatment of alcoholism. In J. H. Mendelson & N. K. Mello (Eds.), The Diagnosis and Treatment of Alcoholism, (Second ed., pp. 355-389). New York: McGraw-Hill Book Company.

Jones, G. H., Schneider, C., Schneider, H. H., Seidler, J., Cole, B. J., & Stephens, D. N. (1994). Comparison of several benzodiazepine receptor ligands in two models of anxiolytic activity in the mouse: an analysis based on fractional receptor occupancies. Psychopharmacology, 114, 191-199.

Kalant, H. (1977). Alcohol withdrawal syndromes in the human: Comparison with animal models. In M. Gross (Ed.), Alcohol Intoxication and Withdrawal-IIIb, (Vol. IIIb, pp. 57-64). New York: Plenum Press.

Kalant, H., LeBlanc, A. E., & Gibbins, R. J. (1971). Tolerance to, and dependence on, some non-opiate psychotropic drugs. Pharmacological Reviews, 23(3), 135-191.

Keppel, G. (1991). Design and Analysis: A Researcher's Handbook. (Third ed.). Englewood Cliffs, New Jersey: Prentice Hall, Inc.

Knoflach, F., Drescher, U., Scheurer, L., Malherbe, P., & Mohler, H. (1993). Full and partial agonism displayed by benzodiazepine receptor ligands at recombinant gamma-aminobutyric acidA receptor subtypes. Journal of Pharmacology & Experimental Therapeutics, 266(1), 385-391.

Kosobud, A., & Crabbe, J. C. (1986). Ethanol withdrawal in mice bred to be genetically prone or resistant to ethanol withdrawal seizures. Journal of Pharmacology and Experimental Therapeutics, 238(1), 170-177.

Kosobud, A., & Crabbe, J. C. (1995). Genetic influences on the development of alcohol dependence and withdrawal. In H. Begleiter & B. Kissin (Eds.), The Genetics of Alcoholism, (pp. 221-256). Oxford, United Kingdom: Oxford University Press.

Kosobud, A. E., & Crabbe, J. C. (1990). Genetic correlations among inbred strain sensitivities to convulsions induced by 9 convulsant drugs. Brain Research, 526(1), 8-16.

Kosobud, A. E., & Crabbe, J. C. (1993). Sensitivity to *N*-methyl-D-aspartic acid-induced convulsions is genetically associated with resistance to ethanol withdrawal seizures. Brain Research, 610, 176-179.

Kosobud, A. E., Cross, S. J., & Crabbe, J. C. (1992). Neural sensitivity to pentylenetetrazol convulsions in inbred and selectively bred mice. Brain Research, 592(1-2), 122-8.

Kruglyak, L., & Lander, E. S. (1995). A nonparametric approach for mapping quantitative trait loci. Genetics, 139(March), 1421-1428.

Lander, E., & Kruglyak, L. (1995). Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nature Genetics, 11, 241-247.

Lander, E. S., & Botstein, D. (1989). Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics, 121(January), 185-199.

Lander, E. S., & Schork, N. J. (1994). Genetic dissection of complex traits. Science, 265(5181), 2037-48. [Published erratum appears in Science 1994 Oct 21;266(5184):353].

Langtry, H. D., & Benfield, P. (1990). Zolpidem. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential. Drugs, 40(2), 291-313.

Laurie, D. J., Seeburg, P. H., & Wisden, W. (1992). The distribution of 13 GABA A receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. The Journal of Neuroscience, 12(3), 1063-1076.

Lê, A. D., Khanna, J. M., Kalant, H., & Grossi, F. (1986). Tolerance to and cross-tolerance among ethanol, pentobarbital and chlordiazepoxide. Pharmacology, Biochemistry & Behavior, 24, 93-98.

Lincoln, S. E., Daly, M. J., & Lander, E. S. (1993a). Constructing genetic linkage maps with MAPMAKER/EXP Version 3.0: A tutorial and reference manual (Version 3.0): Whitehead Insitute for Biomedical Research.

Lincoln, S. E., Daly, M. J., & Lander, E. S. (1993b). Mapping genes controlling quantitative traits with MAPMAKER/QTL 1.1: A tutorial and reference manual (Version 1.1): Whitehead Institute for Biomedical Research.

Litten, R. Z., & Allen, J. P. (1991). Pharmacotherapies for alcoholism: promising agents and clinical issues. Alcoholism: Clinical and Experimental Research, 15(4), 620-633.

Lüddens, & Wisden. (1991). Function and pharmacology of multiple GABAA receptor subunits. Trends in Pharmacological Sciences, 12(2), 49-51.

Lytle, D. A., Egilmez, Y., Rocha, B. A., & Emmett-Oglesby, M. W. (1994). Discrimination of ethanol and of diazepam: differential cross-tolerance. Behavioural Pharmacology, 5, 451-460.

Lytle, D. A., Emmett-Oglesby, M. W., & Stephens, D. N. (1995). Discriminative stimulus effects of midazolam and abecarnil in rats treated chronically with diazepam or abecarnil. Psychopharmacology, 121, 339-346.

Martínez-Cano, H., Vela-Bueno, A., de Iceta, M., Pomalima, R., & Martínez-Gras, I. (1995). Benzodiazepine withdrawal syndrome seizures. Pharmacopsychiatry, 28, 257-262.

McClearn, G. E. (1991). The tools of pharmacogenetics. In J. C. Crabbe & R. A. Harris (Eds.), The Genetic Basis of Alcohol and Drug Actions, (pp. 1-23). New York: Plenum Press.

McClearn, G. E., & Kakihana, R. (1981). Selective breeding for ethanol sensitivity: Short-Sleep and Long-Sleep mice. In G. E. McClearn, R. A. Deitrich, & V. G. Erwin (Eds.), Development of Animal Models as Pharmacogenetic Tools, (Research Monograph No. 6, pp. 147-159). Washington, D. C.: U.S.D.H.H.S.-N.I.A.A.A.

McNemar, Q. (1966). Psychological Statistics. (Third ed.). New York: John Wiley and Sons, Inc.

Metten, P. (1993). Common genetic determinants of severity of acute withdrawal from ethanol, pentobarbital, and diazepam in inbred mice. Unpublished Thesis, Master of Science, Oregon Health Sciences University.

Metten, P., & Crabbe, J. C. (1994a). Common genetic determinants of severity of acute withdrawal from ethanol, pentobarbital and diazepam in inbred mice. Behavioural Pharmacology, 5, 533-547.

Metten, P., & Crabbe, J. C. (1994b). Genetic correlations among CNS depressant drug withdrawal and diazepam-induced ataxia. Alcoholism: Clinical and Experimental Research, 18(2), 486. (Abst.)

Metten, P., & Crabbe, J. C. (1996). Dependence and Withdrawal. In R. A. Deitrich & V. G. Erwin (Eds.), Pharmacological Effects of Ethanol on the Nervous System, (pp. 269-290). Boca Raton: CRC Press.

Mihic, S. J., & Harris, R. A. (1996). Alcohol actions at the GABA A receptor/chloride channel complex. In R. A. Deitrich & V. G. Erwin (Eds.), Pharmacological Effects of Ethanol on the Nervous System, (pp. 51-72). Boca Raton, FL: CRC Press.

Mihic, S. J., Kalant, H., Liu, J.-F., & Wu, P. H. (1992). Role of the γ-aminobutyric acid receptor/chloride channel complex in tolerance to ethanol and cross-tolerance to diazepam and pentobarbital. The Journal of Pharmacology and Experimental Therapeutics, 261(1), 108-113.

Miller, S. A., Dykes, D. D., & Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research, 16(3), 1215.

Moreau, J.-L., Jenck, F., Pieri, L., Schoch, P., Martin, J. R., & Haefely, W. E. (1990). Physical dependence induced in DBA/2J mice by benzodiazepine receptor full agonists, but not by the partial agonist Ro16-6028. European Journal of Pharmacology, 190, 269-273.

Morse III, H. C. (1978). Introduction to Historical Perspective on the Development of Inbred Mice. In H. C. Morse III (Ed.), Origins of Inbred Mice, (pp. 3-22). New York: Academic Press, Inc.

Naruse, T., & Asami, T. (1990). Cross-dependence on ethanol and pentobarbital in rats reinforced on diazepam. Archives Internationales de Pharmacodynamie et de Therapie, 304(Mar-Apr), 147-162.

Olsen, R. W., & Tobin, A. J. (1990). Molecular biology of GABA A receptors. The FASEB Journal, 4(March), 1469-1480.

Perrault, G., Morel, E., Sanger, D. J., & Zivkovic, B. (1992). Lack of tolerance and physical dependence upon repeated treatment with the novel hypnotic zolpidem. Journal of Pharmacology & Experimental Therapeutics, 263(1), 298-303.

Phillips, T. J., & Crabbe, J. C. (1991). Behavioral Studies of Genetic Differences in Alcohol Action. In J. C. Crabbe & R. A. Harris (Eds.), The Genetic Basis of Alcohol and Drug Actions, (pp. 25-104). New York: Plenum Press.

Pritchett, D. B., Sontheimer, H., Shivers, B. D., Ymer, S., Kettenmann, H., Schofield, P. R., & Seeburg, P. H. (1989). Importance of a novel GABA A receptor subunit for benzodiazepine pharmacology. Nature, 338, 582-585.

Robertson, J. R., & Treasure, W. (1996). Benzodiazepine abuse: Nature and extent of the problem. CNS Drugs, 5(2), 137-146.

Rundfeldt, C., Wla´z, P., Hönack, D., & Löscher, W. (1995). Anticonvulsant tolerance and withdrawal characteristics of benzodiazepine receptor ligands in different seizure models in mice. Comparison of diazepam, bretazenil and abecarnil. The Journal of Pharmacology and Experimental Therapeutics, 275(2), 693-702.

Rush, C. R., Higgins, S. T., Bickel, W. K., & Hughes, J. R. (1993). Abuse liability of alprazolam relative to other commonly used benzodiazepines: A review. Neuroscience and Biobehavioral Reviews, 17, 277-285.

Russek, S. J., & Farb, D. H. (1994). Mapping of the beta 2 subunit gene (*GABRB2*) to microdissected chromosome 5q34-q35 defines a gene cluster for the most abundant GABAA isoform. Genomics, 23, 528-533.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Second ed.). (Vol. 2): Cold Springs Harbor Press.

Schneider, L. S., Syapin, P. J., & Pawluczyk, S. (1987). Seizures following triazolam withdrawal despite benzodiazepine treatment. Journal of Clinical Psychiatry, 48(10), 418-419.

Schoch, P., Moreau, J. L., Martin, J. R., & Haefely, W. E. (1993). Aspects of benzodiazepine receptor structure and function with relevance to drug tolerance and dependence. Biochemical Society Symposia, 59, 121-134.

Sellers, E. M. (1988). Alcohol, barbiturate and benzodiazepine withdrawal syndromes: Clinical management. Canadian Medical Association Journal, 139, 113-120.

Serikawa, T., Kuramoto, T., Hilbert, P., Mori, M., Yamada, J., Dubay, C. J., Lindpainter, K., Ganten, D., Guénet, J.-L., Lathrop, G. M., & Beckmann, J. S. (1992). Rat gene mapping using PCR-analyzed microsatellites. Genetics, 131, 701-721.

Siegel. (1956). Nonparametric Statistics for the Behavioral Sciences. New York: McGraw-Hill Book Company.

Sieghart, W. (1992). $GABA_A$ receptors: ligand-gated Cl^- ion channels modulated by multiple drug-binding sites. Trends in Pharmacological Sciences, 13(December), 446-450.

Silver, L. M. (1992). Bouncing off microsatellites. Nature, 2(September), 8-9.

Silver, L. M., Nadeau, J. H., & Goodfellow, P. N. (1996). Encyclopedia of the Mouse Genome V, December 1995. Mammalian Genome, 6(Special Issue), S1-S352.

Silverman, B. W. (1986). Density Estimation for Statistics and Data Analysis. (Vol. 26). London: Chapman and Hall.

Sokal, R. R., & Rohlf, F. J. (1981). Biometry. San Francisco: Freeman.

Spencer, C. M., & Benfield, P. (1995). Abecarnil in generalised anxiety disorder: An initial appraisal of its clinical potential. CNS Drugs, 3(1), 69-82.

Steppuhn, K. G., Schneider, H. H., Turski, L., & Stephens, D. N. (1993). Long-term treatment with abecarnil does not induce diazepam-like dependence in mice. The Journal of Pharmacology and Experimental Therapeutics, 264(3), 1395-1400.

Tyrer, P. (1988). Dependence as a limiting factor in the clinical use of minor tranquilizers. Pharmacology & Therapeutics, 36, 173-188.

Victor, M., & Adams, R. D. (1953). The effect of alcohol on the nervous system. Association of Research on Nervous and Mental Disorders, 32, 526-573.

VonVoigtlander, P. F., & Lewis, R. A. (1991). A rapid screening method for the assessment of benzodiazepine receptor-related physical dependence in mice. Evaluation of benzodiazepine-related agonists and partial agonists. Journal of Pharmacological Methods, 26(1), 1-5.

Wafford, K. A., Burnett, D. M., Leidenheimer, N. J., Burt, D. R., Wang, J. B., Kofuji, P., Dunwiddie, T. V., Harris, R. A., & Sikela, J. M. (1991). Ethanol sensitivity of the GABA A receptor expressed in xenopus oocytes requires 8 amino acids contained in the γ2L subunit. Neuron, 7, 27-33.

Wilkinson, L. (1989). SYSTAT: The System for Statistics (Version 5.1). Evanston, IL: SYSTAT, Inc.

Wisden, W., Laurie, D. J., Monyer, H., & Seeburg, P. H. (1992). The distribution of 13 GABA A receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. The Journal of Neuroscience, 12(3), 1040-1062.

Wong, G., Sei, Y., & Skolnick, P. (1992). Stable expression of type 1 gamma-aminobutyric acidA/benzodiazepine receptors in a transfected cell line. Molecular Pharmacology, 42(6), 996-1003.

Yanovsky, Y., Brankack, J., & Haas, H. L. (1995). Differences of CA3 bursting in DBA/1 and DBA/2 inbred mouse strains with divergent shuttle box performance. Neuroscience, 64(2), 319-325.