KINDLING BY REPEATED ALCOHOL DEPENDENCE AND WITHDRAWAL EXPERIENCES: EFFECTS ON HANDLING-INDUCED CONVULSIONS AND VOLUNTARY ALCOHOL DRINKING IN ETHANOL WITHDRAWAL SEIZURE –PRONE and –RESISTANT MOUSE SELECTED LINES

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CERTIFICATE OF APPROVAL

This is to certify that the Master's thesis of

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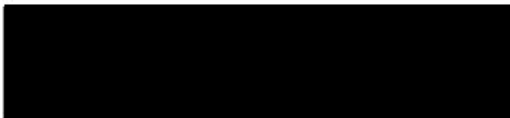

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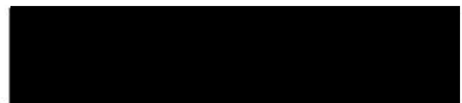

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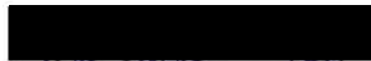

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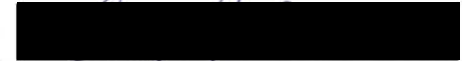

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ABSTRACT

The effects of multiple ethanol withdrawals on handling-induced convulsions (HICs; HIC singular) and voluntary ethanol ingestion were observed in the Withdrawal Seizure-Prone (WSP) and -Resistant (WSR) *Mus musculus* selected lines. Vapor inhalation chambers were used to administer single- or multiple-withdrawal treatments (SW or MW, respectively) to WSP and WSR mice, which differed in the number of exposures to 16-h of chronic ethanol vapor inhalation followed by 8-h of air inhalation (SW=one 16-h ethanol vapor treatment per phase, or two treatments total; MW=three 16-h ethanol vapor treatments per phase, or six treatments total). In this design, each single 16-h ethanol:8-h air exposure resulted in a short-term, chronic 16-h intoxication followed by an 8-h withdrawal experience, which resulted in distinct withdrawal histories for each treatment group.

Two separate experiments implemented this treatment schedule to explore the effects of a history of numerous alcohol intoxications and withdrawals on two different phenotypes. Experiment 1 examined the possible kindling-like effect of repeated ethanol withdrawals on handling-induced convulsions (HICs) in SW- and MW-treated mice of both WSP and WSR replicates; experiment 2 examined the effect of a withdrawal history on free-choice alcohol drinking in SW- and MW-treated mice in replicate 1 of WSP and WSR lines. For both experiments, blood ethanol concentrations were statistically equated prior to analyses. In experiment 1, main effects of treatment supported a kindling-like phenomenon for withdrawal-induced HICs in WSP and WSR lines, where MW groups had significantly greater HICs than SW groups within each line. Cumulative effects of withdrawal were observed in response to repeated treatment administration, though the effect was opposite in WSP and WSR lines: WSP mice showed a kindling-like potentiation of the HIC response during the second phase of the study, where WSR mice had more intense HIC responses during the first phase of experiment 1. Throughout the course of experiment 2, the WSR line voluntarily drank more ethanol than the WSP selected line. Changes in ethanol consumption were observed by line (WSP < WSR) and by treatment (SW > MW); significant within-line treatment effects were observed in the WSR line (SW groups drank

more than MW groups), but such effects were absent in the WSP line. Repeated measures analyses gave mixed results, depending on the index used to reference drinking (g/kg/day 10% ethanol consumption or preference ratio).

A kindling-like effect was observed for alcohol withdrawal-precipitated HICs, where multiple ethanol withdrawal experiences and repeated administration of treatment phases increased the severity of HICs. An inverse relationship between withdrawal experiences and free-choice alcohol drinking was observed in the WSR selected line, such that animals with multiple withdrawals were found to voluntarily drink less ethanol. These results suggest that the genes influencing HICs in the WSP and WSR selected lines might partially affect alcohol withdrawal-induced drinking.

INTRODUCTION

Alcohol addiction, abuse, or dependence occurs in approximately 10% of the United States' population (Crabbe, 1996), and the incidence of the disease exacts a large economic and social burden on communities that is disproportionately greater than the number of alcohol-dependent individuals in the population (Caetano and Cunradi, 2002). These costs stem from alcohol-related accidents, injuries, arrests, crime, legal problems, loss of productivity (due to injury, unemployment, or work-related issues), birth defects, medical complications, and affected interpersonal relationships (Caetano and Cunradi, 2002; Martin, 2001).

Thus, understanding the genetic factors influencing withdrawal and drinking would be an important augmentation to the existing knowledge of the disease, allowing the development of useful—possibly better—prevention and treatment strategies. Yet human genetic research has prominent ethical concerns and the findings are often controversial owing to the genetic heterogeneity within a natural population, poor environmental control, or poor subject compliance with treatment (Plomin *et al.*, 2001; Crabbe and Belknap, 1998). Because mice and humans are approximately 80-85% homologous and exhibit extensive synteny (Copeland *et al.*, 1993; Plomin *et al.*, 2001), the mouse genetic model has allowed the genetic underpinnings of many Mendelian traits to be elucidated in humans (Crabbe and Phillips, 2004). Mendelian phenotypes are "simple" phenotypes influenced by the inheritance of a single allele of large or dominant effect, rendering statistically predictable patterns of inheritance. By contrast, alcoholism is a quantitative—or "complex"—phenotype influenced by multiple genes, each of which contributes a small or modest effect to the overall display of the trait, in addition to environmental factors that foster or protect against the disease's emergence.

Several subcomponent behaviors underlie the development of alcohol abuse and dependence, including the choice to drink, the preference for an alcoholic drink, the pursuit of alcohol, the pattern of alcohol consumption, the subjective physiological/psychological experience of intoxication or withdrawal, and the long-term effects of repeated intoxication and withdrawal

cycles. Each of these behaviors is likely to be influenced by distinct, overlapping mechanisms, with each mechanism controlled by multiple genes influencing the overall phenotype. The multi-faceted nature of alcoholism is reflected by the additional criteria used to describe and categorize the disease: alcohol abuse and alcohol dependence (outlined by the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, revised; DSM-IV-R). The general characteristics describing a phenomenon of alcohol addiction would include tolerance to the subjective euphoric effects of alcohol resulting in increased volumes of alcohol ingestion over the course of the disease, the development of physical dependence such that "normal" functioning depends on the presence of alcohol, and compulsive behavior leading to alcohol obtainment and drinking (craving, drug seeking) (DSM-IV-R; Koob, 2004; Malcolm *et al.*, 2000; Flannery *et al.*, 2001). These attributes are also implicit in another prominent diagnostic standard, the International Classification of Diseases (ICD) assembled by the World Health Organization (Caetano and Cunradi, 2002).

Although the genetic effects on alcoholism, as indexed by the heritability, are estimated at 40% (Plomin *et al.*, 2001), the precise allelic contributions to the disease remain unknown. For this reason, it is advantageous that valid animal models of many particular subcomponent behaviors of alcoholism have been established to increase the knowledge of the human condition (Koob, 2004; Spanagel, 2001; Hitzemann, 2000; Crabbe, 1996; Tabakoff and Hoffman, 2000). Certain practical and methodological issues are reduced or resolved in animal models, such as the ability to better standardize age, condition of health, prior drug exposure, and prior experience of dependence, as well as increasing control of dose, duration, timing, mode of administration, and pattern of drug exposure variables (Becker, 2000). Anatomical manipulations (site-specific drug administration or lesioning, for example) and tissue-harvesting techniques also allow distinctions to be rendered regarding the *modus operandi* of the pharmacological agent.

In addition to being able to manipulate and restrict the testing environment, the use of controlled breeding strategies involving well-characterized inbred strains of the common laboratory mouse or animal populations derived from these strains (such as the ethanol Withdrawal Seizure-Prone,

WSP, and Withdrawal Seizure-Resistant, WSR, selected lines) improves the conditions for ascertaining specific alleles important in the development of alcohol dependence on multiple levels: behavioral, anatomically specific, cellular, subcellular, and molecular. To achieve this end, many inbred strains have been genotyped with thousands of molecular markers, allowing the refinement of genomic maps important for quantitative trait loci (QTL) discovery and rendering them powerful model populations for gene mapping (Phillips *et al.*, 2002; Hitzemann 2000).

The potential for research to lead to the discovery of genes and/or gene networks associated with the complex phenotype of alcohol abuse in humans is large. In support of using animal models to elucidate individual genes contributing to a quantitative trait, success has been demonstrated in the understanding of the *Mpdz* allele's contributions to seizure susceptibility during acute alcohol and barbiturate withdrawal in genetic mapping populations established from the C57BL/6J and DBA/2J inbred lines (BXD recombinant inbreds, B6D2 F2 intercross mice, and High- and Low-Alcohol Withdrawal selected lines; Buck *et al.*, 1997, 2002; Fehr *et al.*, 2004; Shirley *et al.*, 2004). The *Mpdz* allelic region codes for the multiple PSD95/DLG/ZO-1 domain protein. In addition to gene mapping, mouse models can be important for discovering pleiotropic or epistatic relationships worthy of additional study. Selected lines may be an early step in this endeavor, as they are a powerful medium for detecting genetic correlations (Crabbe *et al.*, 1990b; Crabbe, 1999a,b; WSP and WSR correlations are reviewed in Metten and Crabbe, 1996).

Alcohol effects: acute, chronic, and during withdrawal. The mechanisms of ethanol's effects are multiple, with several nonspecific actions affecting normal neuronal function. For example, alcohol's influences include neuronal membrane fluidity and composition, neuropeptide activity, growth and stress hormone levels, ion conductance, GABAergic and glutamatergic neurotransmitter contributions to neuroelectrical excitability, second messenger signaling mechanisms, and gene expression (Finn and Crabbe, 1997; Koob *et al.*, 1998a,b; Kreek and Koob, 1998; Ryabinin *et al.*, 2002; Littleton, 1998; Nevo and Hammon, 1995). The same neurotransmission systems are affected by acute or chronic ethanol exposure, but opposite effects are exerted depending on the dose and duration of alcohol intoxication (Kalant *et al.*,

1971). For example, initial, modest doses of ethanol have been reported to evoke subjective feelings of euphoria, with disinhibitive, stimulatory effects on behavior (Lovinger and Crabbe, 2005). These effects are mediated by the initial enhancement of dopaminergic, opioidergic, and glutamatergic systems, with an accompanying decrease in inhibitory GABAergic signaling (Koob and Le Moal, 1997; Finn and Crabbe, 1997; Metten and Crabbe, 1996). However, prolonged or high doses of alcohol generally act as a depressant on the central nervous system, with adaptations to prolonged alcohol exposure resulting in effects that are opposite to ethanol's initial action on the same neurotransmission systems once ethanol has been metabolized.

The emergence of the alcohol withdrawal syndrome (AWS) is in response to the neural hyperexcitability that results after circulating ethanol has been metabolized and the affected systems normally attenuating the near-constant presence of ethanol are left functioning unchecked before returning to "normal" function. Following a single large dosing or a chronic dose of long duration, ethanol has been documented to show neural hyperexcitability attributed to the decreased inhibitory chloride ion influx at the type A γ-aminobutyric acid (GABA) receptor ($GABA_A$) with a corresponding increase in excitatory Ca^{2+} ion influx at ligand-gated *N*-methyl-D-aspartic acid- (NMDA-) type glutamate receptors (Dietrich *et al.*, 1989; DeWitte, 2003; Metten and Crabbe, 1996). Chronic alcohol presence can also affect signaling by the upregulation of voltage-gated Ca^{2+} channels (Finn and Crabbe, 1997), change in receptor subunit composition, or increased activation of voltage-gated Ca^{2+} channels (responding to the ligand-gated changes in intracellular ionic levels that alter neural membrane potential) (Becker, 1998). As the experience of reduced sensitivity and increased tolerance attenuates the human subjective effects of intoxication, over time many alcohol users begin seeking greater volumes of alcohol to achieve the initial effects of euphoria and disinhibition, and the body adapts as an attempt to maintain the pre-alcohol levels of homeostasis within these chronically-treated systems (Koob and Le Moal, 1997). The combined adaptations of these systems influence broad physiological effects, such as increased tolerance and decreased sensitivity for a specific dose of alcohol, leading to a self-propagating cycle of abuse (*i.e.*, chemical dependence; DSM-IV-R).

Physical symptoms of AWS in humans include increased irritability, anxiety, headaches, nausea, insomnia, *delirium tremens* (general delirium with accompanying muscular tremulousness), hallucinations (auditory, tactile, or visual in nature), delusions, and/or *grand mal* seizures (*i.e.*, generalized tonic-clonic seizures) (DSM-IV-R). Additional autonomic nervous system effects of hyperventilation, vomiting caused by gastrointestinal malfunction, tachycardia, and hyperthermia (fever) contribute to the physical distress of AWS. Each symptom has a distinct period of onset and duration, with several symptoms overlapping at particular times, and some persisting for days after the abatement of others (Bayard *et al.*, 2004). The slow return to pre-dependence functioning and the persistence of such symptoms is believed to contribute to relapse.

This topic is of clinical interest, as experimental data have shown that withdrawal may stimulate a "self-medicating" behavior, where alcoholics have reported that drinking diminishes withdrawal symptoms or that the reward valence of alcohol may offset the negative affective and/or physiological symptoms experienced during the withdrawal that might lead to relapse (Koob *et al.*, 1998a). For example, rats taught to operantly respond to EtOH and then made alcohol-dependent by 2 weeks of chronic EtOH vapor exposure were demonstrated to self-administer alcohol in greater volumes during withdrawal than they did prior to a withdrawal experience (Roberts *et al.*, 1996). Additionally, voluntary responding was shown to persist and stabilize when animals experienced an additional alcohol detoxification. Protracted alcohol use in human subjects that have previously been hospitalized for withdrawal symptoms has shown that some alcoholics claim that their prolonged alcohol use serves to prevent future severe withdrawal symptoms. However, these clinical findings are contrasted by findings of the inverse genetic relationship between withdrawal severity and voluntary drinking in inbred mouse strains and selected lines (McClearn *et al.*, 1982; Metten *et al.*, 1998; Metten and Crabbe, 2005). This relationship is reviewed below.

Kindling: electrogenic, chemogenic, withdrawal-induced. Goddard *et al.*, introduced the term "kindling" in 1969 to describe the sensitization process that occurred with daily limbic electrical stimulation in rats, such that the application of an originally subthreshold electroconvulsive shock eventually precipitated full motor convulsions with recurrent administration, and additional applications of the same stimulus continued to exacerbate subsequent seizures. A chemogenic kindling phenomenon has also been observed through the repeated application of convulsant drugs working by way of distinct mechanisms (Gilbert, 1992; Post *et al.*, 1983). In either case, eventual seizure manifestation was attributed to heightened brain excitability following each stimulus presentation, so that the electrical excitability levels were reset to a "primed" state following each stimulus presentation, thus resulting in a seizure threshold reduction. As such, the repetition of stimulus application is a required characteristic of kindling (Becker, 1998).

In the presentation of AWS, "kindling" is hypothesized to be the sensitization to the repeated neural hyperexcitability of withdrawal, and repeated stimulus applications will evoke progressively potentiated withdrawal symptoms, including seizures and motor convulsions. Ballenger and Post (1978) were the first to ascribe the progressive intensification of alcohol-withdrawal symptoms following repeated alcohol detoxifications to a persistent, kindling-like neural excitability based on clinical observations of alcoholics hospitalized for withdrawal. Regardless of the patient's age, withdrawal symptom severity correlated with the duration of "heavy daily alcohol abuse." Multiple detoxification periods following heavy alcohol use enhanced susceptibility for seizures and sensitized other adverse withdrawal symptoms unrelated to seizures. These AWS symptoms were found to increase with continued chronic alcohol consumption and multiple alcohol withdrawals. Ballenger and Post postulated that the apparent sensitization to alcohol withdrawal episodes was attributable to increased central nervous system excitability.

In 1988, a retrospective case-controlled study conducted by Brown *et al.*, revealed an association between histories of repeated withdrawal and withdrawal-precipitated seizures in alcoholics hospitalized during ethanol withdrawal. Patients with a history of multiple alcohol detoxifications

were predisposed to have withdrawal-induced seizures prior to hospitalization (48% of the patients examined had $\geq$ 5 prior alcohol withdrawals) compared with the patients that did not experience withdrawal (12% of the group had experienced $\geq$ 5 prior alcohol withdrawals). This was interpreted to mean that each withdrawal episode served as a kindling stimulus that predisposed patients for more severe symptoms during future withdrawals. Similarly, Booth and Blow (1993) conducted a database review of patients undergoing alcohol detoxification and found that the patients presenting with seizures had a history of multiple alcohol withdrawals. Additionally, the clinical presentation of seizures supported a predictive relationship: seizing patients were more likely to be readmitted with an alcoholism-related diagnosis and to experience seizures during future withdrawals than were the hospitalized withdrawal patients that did not experience withdrawal-related seizures.

Patients with histories of multiple detoxifications from heavy, prolonged ethanol consumption patterns are more likely to experience AWS symptoms of central and autonomic nervous system hyperactivity. Experimental findings in animals that have experienced multiple intoxication and withdrawal cycles demonstrated region specific responses to electrical brain stimulation, where stimulation of the inferior colliculus (a region involved in auditory processes) generated seizures faster in withdrawal-experienced animals than in control animals (McCown and Breese, 1990). However, seizure threshold was increased in the amygdala (a region involved in the processing of emotions and in the consolidation of memories). Because many withdrawal symptoms involve negative affect, this finding supports the notion that repeated cycles of withdrawal cause long-term changes in limbic processing (McCown and Breese, 1990; Stephens *et al.*, 2001). On this note, kindling has been shown to mediate intense alcohol craving and obsessive-compulsive thoughts in detoxified patients (Malcolm *et al.*, 2000), increasing the risk of relapse (Becker, 1998) and stabilizing alcohol consumption patterns with repeated withdrawal exposure (Roberts *et al.*, 1996).

Real-time electrophysiological alterations in brain activity have been measured following withdrawal in experimental and clinical studies, supporting the notion of hyperexcitability with localized measurements of electrical aberrances in repeatedly withdrawn subjects. An electroencephalograph (EEG) is the collective measurement of spontaneous neural firing as it occurs in the subject. When EEG electrodes are positioned on the scalp, EEG measures cortical activity. However, electrodes can also be subcranially implanted to measure of the activity of distinct brain regions. EEG measurements of resting frontal cortex beta brain wave activity was found to be more random in relapsing alcoholics than in non-relapsers (Begleiter and Porjesz, 2003). This measurement suggests an imbalance of neuronal networks, a predictive feature of future relapse and a predisposing variable to the development of alcohol dependence (Begleiter and Porjesz, 1999). *In vitro* studies of repeated alcohol exposure and withdrawal (washouts) in rat hippocampal preparations showed that multiple withdrawal treatments exacted more NMDA-induced neural excitotoxicity than treatments that received an equal dose and duration of alcohol exposure without intermittent withdrawal washouts (Becker and Littleton, 1996). Veatch and Gonzalez (1996) independently varied the duration of ethanol exposure and the number of withdrawals to examine cortical and subcortical EEG brain waves in rats. Both variables were important contributors to a greater incidence of aberrant spike and sharp wave (SSW) signaling activity, with regional and treatment-specific sensitivity observed within the hippocampus: CA1 regions responded most to a longer duration of ethanol exposure, while more SSWs were recorded in the CA3 region following multiple withdrawals. In 1997, Veatch and Gonzalez demonstrated that the EEG changes caused by both of these alcohol treatment variables were long lasting, as they persisted for at least 2 weeks after treatment ended. Interestingly, alcohol-treated groups were found to be seizure protected when electrical stimulation was applied long after the cessation of treatment—a finding that did not correlate with acute withdrawal signs or the relative amount of ethanol exposure. This finding might be related to the persistent excitability of NMDA-influenced systems resulting in excitotoxicity (Becker and Littleton, 1996), thus interfering with electrogenic seizure propagation. However, it did not interfere with the potentiation of withdrawal-induced convulsions evidenced soon after the termination of alcohol exposure (Veatch

and Gonzalez, 1997; Gonzalez, 1998). Results might also be influenced by the location of the electrical stimulation, since the generation of withdrawal-induced seizures or convulsions might have a different locus of origination.

Selective breeding. Though quantitative phenotypic traits are determined in part by genetics and in part by environment, a controlled laboratory setting minimizes environmental effects, allowing the manifestation of behaviors of interest to reflect primarily genetic influences. Selective breeding utilizes the realized heritability of a desired, heritable, quantifiable, additive, and well-defined phenotype to alter the frequency of alleles underlying the trait *en masse* (Crabbe, 1999a). Starting with a founding population, phenotypes are quantified and individual mice registering extremely high or low phenotypic performance for the desired trait are mated to produce the subsequent generation, such that individuals with high ranking or performance are bred to each other to create a high-ranking line, and individuals with low ranking or performance are bred to each other to create a low-ranking line. Such is the case with WSP mice, which have been selected for their high handling-induced convulsion (HIC; HICs plural) severity during withdrawal from chronic ethanol exposure, and WSR mice, which were concurrently selected for their low manifestation of HICs during chronic ethanol withdrawal (Kosobud and Crabbe, 1986a,b). In subsequent generations, high-ranking mice for the HIC phenotype were bred to other high-ranking mice within the WSP line, thus increasing the fixation of alleles at genes underlying the manifestation of the trait of interest (ethanol withdrawal-precipitated HICs). Similarly, mice with low HIC responsiveness were bred with other low-ranking mice within the WSR line, resulting in the fixation of gene alleles predisposing toward low HIC scores after identical alcohol exposures in the offspring of low-responding parents. The result was that after multiple generations of artificial selection, the WSP mice have become enriched for alleles at genes responsible for the tendency to display extreme HICs during ethanol withdrawal, while WSR mice have relatively few of the same alleles by comparison. In measures unrelated to the selection trait, the high- and low- selected lines are similar in every regard excepting traits that are pleiotropically influenced by the genes underlying the primary trait of interest (HIC

susceptibility/intensity during ethanol withdrawal) and sex chromosome alleles (Falconer and Mackay, 1996). This makes the selected lines valuable for determining the influence of the fixed genes underlying the selected trait on other phenotypic measures, and a statistical correlation of such traits is suggestive of a common genetic architecture. If such genetic correlations are significant, they imply that the selected trait and the correlated trait share common genes.

Origin of WSP and WSR selection lines. WSP and WSR mice originated from genetically heterogeneous mouse stock created from 8 inbred strains (HS/Ibg stock developed by GE McClearn at the Institute for Behavioral Genetics, University of Colorado, Boulder, CO, USA) and were selectively bred for their response to HIC elicitation during ethanol withdrawal following an induced ethanol dependence precipitated by three days of ethanol vapor inhalation (Crabbe *et al.*, 1985). Described briefly, two independent selection line replicates were mated by standard within-family selection, and HIC intensity was observed to peak in the first 8 hours following removal from the ethanol vapor inhalation chamber. By the fifth selection generation (S5), WSP mice showed 2.71 (replicate line 1) times or 4.56 (replicate line 2) times greater HIC susceptibility than WSR mice (Crabbe *et al.*, 1985). The HIC phenotype increased in response to additional selection pressure over time, with WSP lines showing 15 times greater HIC susceptibility than WSR mice at S26 (Kosobud and Crabbe, 1993). Evidence for allelic fixation was evident in the predictably stable phenotype following the termination of selection pressure at S26, with WSP and WSR mice typically showing a 10-fold differential response despite 76 generations of relaxed selection where no selective breeding was practiced (the lines are now at generation 101; Pamela Metten, personal communication).

Replicate lines. A strength of the WSP and WSR selected lines is that two replicates were generated and maintained in parallel, each originating from the same foundation stock. After the initial round of choosing high HIC responders and low HIC responders to parent the first selection generation, progeny from these responders were parsed into replicates and only mated to other high or low responders within their respective replicate line. The presence of an additional replicate line bred for the same trait allows the fixation of genetic underpinnings of the trait to be

attributed to the influence of alleles affecting the trait of interest, *versus* the event of random genetic fixation, where genetic influences irrelevant to the trait are inherited (or not inherited, as in the case of the WSR line) along with the desired genes (Falconer and Mackay, 1996). The chance phenomenon of systematically inheriting (and/or enriching) for genes that are not of interest to the selection phenotype can occur for various reasons, such as close chromosomal proximity of the genes (thus decreasing the probability of recombination during meiotic events). More importantly, random fixation could occur by chance. The presence of an independently generated replicate during the artificial selection process allows comparisons to be made on the phenotypic traits observed. If the trait observed is qualitatively and metrically similar in both replicates, it can be assumed that the same genes are influencing the generation of that phenotype. In such cases, if the two replicate lines are not statistically different for the measure of interest, they can be combined to increase statistical power. An existing replicate allows observation of errors in the selection process if, for example, phenotypic differences on measures of the selected trait are observed in the multiple replicates when experimental treatment is the same. The "checkpoint" of multiple replicates is helpful in interpreting correlated traits as genetically related as well.

Though selected for HICs precipitated by EtOH withdrawal, WSP and WSR selected lines have been well characterized on many pharmacological indices involving seizure susceptibility (Crabbe *et al.*, 1983a,c; Crabbe and Kosobud, 1986; McSwigan *et al.*, 1984). It has been determined that the HICs observed are specific to the withdrawal experience and not attributed to basal central nervous system excitability or hyperactivity in WSP mice (McSwigan *et al.*, 1984), or strain-specific differences in ethanol dosing or metabolism (Crabbe *et al.*, 1985). Both lines have been differentially responsive to chemical precipitation of HICs by pentylenetetrazol (a $GABA_A$ receptor antagonist) (Kosobud *et al.*, 1992) and by NMDA (agonist of NMDA-type glutamate receptors) (Kosobud and Crabbe, 1993). Mice have shown a tendency to kindle for HIC severity precipitated by acute ethanol withdrawal (Kosobud *et al.*, 1988; "acute" refers to the administration of a single injection of EtOH). However, kindling to chronic ethanol withdrawal has

not yet been tested in these mice. In addition, this is the first study to assess the effect of multiple withdrawal experiences on voluntary ethanol drinking preference in WSP and WSR selected lines.

Voluntary alcohol ingestion. Assessment of the willingness of mice to drink alcohol has been reliably tested using the two-bottle choice model for nearly 50 years (McClearn and Rodgers, 1959, 1961). This model has also allowed C57BL/6J and DBA/2J inbred mouse strains to be repeatedly established as near extremes for free choice alcohol drinking among inbred mouse strains, where high-preferring C57BL/6J mice typically show undeniable avidity for 10% ethanol solutions in preference to tap water (C57BL/6J 10% ethanol preference ratio=0.55 – 0.75; alcohol consumption=6.5 g/kg/day) and low-preferring DBA/2J mice typically exhibit strong ethanol avoidance (DBA/2J 10% ethanol preference ratio≤0.07; alcohol consumption=0.5 g/kg/day) (preference ratio values taken from Belknap *et al.*, 1993 and Phillips *et al.*, 1994; consumption values taken from Phillips *et al.*, 1994; see also McClearn and Rodgers, 1959; Belknap *et al.*, 1997). Within the WSP and WSR selected lines, Kosobud *et al.* (1988) tested the voluntary consumption of alcohol-containing solutions and found that WSP and WSR selected lines consume approximately 2 – 8 g/kg daily. As such, the WSP and WSR lines are "mild-to-moderate" drinkers when compared to the extremes of ethanol consumption observed in high-preferring C57BL/6J (>10 g/kg/day) and low-preferring DBA/2J (<1 g/kg/day) inbred mice (Belknap *et al.*, 1993; DA Finn and P Metten, personal communications).

Inverse genetic relationship between alcohol withdrawal severity and alcohol consumption. There have been almost a dozen reports in the literature of an inverse relationship between withdrawal severity and voluntary alcohol consumption (10% EtOH *vs.* tap water), which have noted that mice genetically predisposed to display higher withdrawal severity tend to avoid alcohol consumption more than mice that are genetically predisposed to experience less intense withdrawal symptoms (McClearn *et al.*, 1982; Metten *et al.*, 1998; Metten and Crabbe, 2005). This includes studies among inbred strains, recombinant inbred strains, and lines of mice selected for either withdrawal severity or consumption (Metten *et al.*, 1998). This inverse

relationship was reported for the WSP and WSR selected lines, where WSP mice voluntarily drank less alcohol than WSR lines (Kosobud *et al.*, 1988). Interestingly, it has also held true in two independent rat lines selectively bred to differ for alcohol consumption: Preferring (P) and High Alcohol Drinking (HAD) selected lines experienced milder EtOH withdrawal than the Nonpreferring (NP) and Low Alcohol Drinking (LAD) lines (Chester *et al.*, 2002, 2003).

Effects of multiple ethanol withdrawal cycles in mice. HC Becker and colleagues were the first to test the EtOH withdrawal kindling model in mice. They tested the effects of one or more 16-h EtOH intoxication:8-h withdrawal cycles on subsequent withdrawal severity and also on preference drinking using a design similar to that used in experiments 1 and 2 of the present work with some differences, especially for experiment 2 as noted below. They were able to show that repeated cycles of withdrawal increased subsequent withdrawal severity and also increased preference drinking in a manner consistent with the kindling model (Becker, 1994; Becker and Lopez, 2004). None of this work focused on genotype (strain) comparisons. For example, this repeated withdrawal procedure evoked a kindling effect, or sensitization of brain neurohyperexcitability, manifested as potentiated HIC severity and increased frequency of anomalous EEG activity in C3H/He inbred mice (Becker and Hale, 1993; Becker, 1994; Becker *et al.*, 1997a,b; Veatch and Becker, 2002). In C57BL/6J inbred mice, this testing paradigm was observed to increase and stabilize voluntary ethanol drinking during 2-h access sessions after mice had been trained to self-administer 15% EtOH using a sucrose fading technique (Becker and Lopez, 2004; Lopez and Becker, 2005). In both cases, the behavior observed following repeated withdrawal experiences was increased in the multiple withdrawal groups compared to single withdrawal groups. However, a crossover application of this kindling model for each inbred strain was not reported. Thus, it is unknown whether voluntary alcohol drinking in C3H/He mice is influenced by multiple withdrawals, or whether C57BL/6J mice kindle for the HIC response following multiple withdrawals.

Specific aims. Alcohol addiction is a phasic and progressive disease that is composed of psychological and physiological aspects of dependence. To address the phasic aspect of alcoholism (binge periods followed by abstinence), alcohol dependence and withdrawal were induced by chronic, intermittent vapor inhalation. To address the progressive aspect of the disease, effects of repeated withdrawal on HICS and drinking preference were examined. Although addiction cannot be measured in animal models *per se*, longitudinal behavioral assessments such as HICs allow the physiological aspects of alcohol dependence to be studied. As we are unable to know the psychological experience of alcohol intoxication in mice directly, it is likely that indexing motivated behavior, such as voluntary consumption of ethanol solutions in a two-bottle choice paradigm will reveal *preference* drinking, thus modeling an aspect of the psychological experience.

The present study asked whether the alleles influencing ethanol withdrawal-induced HICs in the WSP and WSR selected lines would also influence the alcohol ingestion phenotype when using an experimental design similar to that used by Becker and colleagues (described above). The WSP and WSR lines have proved useful for suggesting pleiotropic influences of genes associated with ethanol withdrawal severity (quantified by HICs) on several genetically correlated responses to ethanol (Crabbe and Belknap, 1992; Crabbe and Phillips, 2004). The specific goals of this study were to test the influence of genetics and a multiple withdrawal environment in terms of the following general hypotheses.

Multiple ethanol dependence and withdrawal cycles will "kindle" ethanol withdrawal severity (indexed by HICs) in WSP and WSR selected lines, such that animals with a history of more withdrawals will show a sensitization to HIC severity. This hypothesis is supported by the kindling effect that repeated EtOH withdrawals induced in the C3H/He inbred strain (Becker and Hale, 1993; Becker, 1994).

"Prone" genes influencing the WSP line's high HIC response will contribute to greater kindling in the WSP mice compared to the WSR mice on the HIC measure. This hypothesis was influenced by Kosobud *et al.*'s 1988 study that suggested that kindling a potentiation in the HIC phenotype could occur following multiple acute alcohol withdrawal treatments delivered by i.p. injection (the model of acute withdrawal).

Baseline voluntary ethanol ingestion will be greater in the WSR line than in the WSP line. At selection generation 17, WSR-2 mice drank more ethanol than the WSR-1 line, and the combined WSR drinking preference results were substantially greater than those of the WSP lines (WSP-1 and WSP-2 were not significantly different from each other) (Kosobud *et al.*, 1988). However, this contrasts with results obtained in a recent, unpublished study in our laboratory, where WSP and WSR lines did not differ in their willingness to consume alcohol (Barkley-Levenson *et al.,* 2005). Given these conflicting findings, we sought to reassess preference drinking in the WSP and WSR selection lines prior to any withdrawal experiences as part of this project.

Repeated ethanol withdrawal experiences will decrease voluntary ethanol drinking in the WSP and WSR selected lines as compared to baseline alcohol consumption observed prior to the intoxication:withdrawal treatment(s). As was reviewed above, the preclinical genetic literature has reported an inverse genetic relationship between preference drinking and withdrawal severity among genotypes known to differ markedly for at least one of these two behavioral domains (Metten *et al.*, 1998; Chester *et al.*, 2002, 2003). Therefore, we predict that any treatment that increases withdrawal severity, such as multiple withdrawal treatment, will also reduce drinking more than single withdrawal treatment.

METHODS

Animals. Female mice from the WSP and WSR lines were tested at the 99th or 100th filial generation, following relaxed artificial selection pressure for HIC response beginning with selection generation 26 (WSP-1, S26.G100; WSR-1, S26.G100; WSP-2, S26.G99; WSR-2, S26.G100). These selected lines were developed and are maintained at the Portland Alcohol Research Center, Department of Veterans Affairs Medical Center, in Portland, OR, USA by JC Crabbe. Uniform housing conditions included a 12-h lights on:12-h lights off schedule that began at 6:00 A.M., group-housing with 1-4 littermates in clear 28 x 13 x 18-cm polypropylene "shoebox" cages, corn cob bedding, two weekly cage changes, and *ad libitum* access to food and tap water for the duration of the study. During SW or MW treatment in the inhalation chambers, mice were housed in cages made of ¼-inch-wide stainless steel wire mesh, with water and chow pellets still freely available. All experimental procedures were approved by the vivarium's Institutional Animal Care and Use Committee and were compliant with the National Institutes of Health's guidelines for animal experimentation and maintenance.

At 50-75 days-of-age, siblings were divided near equally and randomly assigned into single withdrawal (SW) or multiple withdrawal (MW) treatment groups. Treatment groups were designed to examine the effects of one or more cycles of alcohol (*i.e.*, ethanol, or EtOH) dependence and withdrawal on the kindling of HICs (experiment 1; see Figure 1 for timeline) and on voluntary ingestion of 10% EtOH (experiment 2; see Figure 2 for timeline).

Outline of experimental design. To address both questions, administration of EtOH vapors using an inhalation chamber apparatus was implemented on experimentally naïve SW- and MW-treatment groups in each experiment. MW and SW groups were tested in parallel and received comparable handling (weighings, pyrazole HCl injections, inter-chamber transfers, and tail blood samplings), but differed in the number of 16-h EtOH:8-h air cycles experienced over the course of 72 hours. According to a previously established HIC-kindling model (Becker and Hale, 1993; Becker, 1994; Becker *et al.*, 1997a,b), MW groups experienced three consecutive 16-h EtOH:8-h

Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6

Phase 1: SW TREATMENT			
24-h Air only	24-h Air only	16-h EtOH:8-h Air	25-h EtOH Withdrawal with HIC testing
			D3BEC
Phase 1: MW TREATMENT			
16-h EtOH:8-h Air	16-h EtOH:8-h Air	16-h EtOH:8-h Air	25-h EtOH Withdrawal with HIC testing
	D1BEC	D2BEC	D3BEC

Day 7	Day 8	Day 9	Day 10	Day 11

Phase 2: SW TREATMENT			
24-h Air only	24-h Air only	16-h EtOH:8-h Air	25-h EtOH Withdrawal with HIC testing
			D10BEC
Phase 2: MW TREATMENT			
16-h EtOH:8-h Air	16-h EtOH:8-h Air	16-h EtOH:8-h Air	25-h EtOH Withdrawal with HIC testing
	D8BEC	D9BEC	D10BEC

Figure 1: Experiment 1 timeline: Kindling-like potentiation of the HIC withdrawal response. WSP and WSR mice from both replicates were included in each treatment group and were run in parallel. Single withdrawal (SW) mice received comparable handling during their 24-h air inhalation periods, and received sham blood sampling on days 1, 2, 8, and 9. Animals used during Phase 1 received the same treatments during Phase 2, such that the SW group received a total of 2 withdrawal periods and the multiple withdrawal (MW) group received a total of 6 withdrawal periods during the 11 days of experimentation. Animals were euthanized after the 25-hour HIC test on day 11. Review text for details of design. Abbreviations: h=hour; D=day; EtOH=ethanol; BEC=blood ethanol concentration.

Phase 0: D-6 Individually House 2-bottle choice setup:H2O(L/R)	Phase 0: D-5 Color Code, Ear Punch H2O(L/R)	Phase 0: D-4 Weigh H2O(L/R)	Phase 0: D-3 2-bottle choice setup: 5% (L)	Phase 0: D-2 Read 5% (L) Start 5% (R)	Phase 0: D-1 Read 5% (R) Start 5% (L)	Phase 0/1: D0 Read 5% (L) Start 10% (R)
Phase 1: D1 Read 10% (R) Refill 10% (R)	Phase 1: D2 Weigh Read 10% (R) Start 10% (L)	Phase 1: D3 Read 10% (L) Refill 10% (L)	Phase 1: D4 Read 10% (L) Start 10% (R)	Phase 1: D5 Read 10% (R) Refill 10% (R)	Phase 1: D6 Read 10% (R) Start 10% (L)	Phase 1: D7 Read 10% (L) Refill 10% (L)
Phase 1: D8 Read 10% (L) Start 10% (R)	Phase 1: D9 Read 10% (R) Refill 10% (R)	Phase 1/2: D10 Weigh Read 10% (R) Start 16-h Tx	Phase 2: D11 Weigh D11BEC 8-hAir:16-h Tx	Phase 2: D12 Weigh D12BEC 8-hAir:16-hEtOH	Phase 2: D13 Weigh D13BEC Give H20(L/R) Air WD	Phase 3: D14 Weigh Start 10% (L)
Phase 3: D15 Read 10% (L) Refill 10% (L)	Phase 3: D16 Read 10% (L) Start 10% (R)	Phase 3: D17 Read 10% (R) Refill 10% (R)	Phase 3: D18 Weigh Read 10% (R) Start 10% (L)	Phase 3: D19 Read 10% (L) Refill 10% (L)	Phase 3: D20 Read 10% (L) Start 10% (R)	Phase 3: D21 Read 10% (R) Start 10% (L)
Phase 3&4: D22 Weigh Read 10% (L) Start 16-h Tx	Phase 4: D23 Weigh D23BEC 8-hAir:16-h Tx	Phase 4: D24 Weigh D24BEC 8-hAir:16-hEtOH	Phase 4: D25 Weigh D25BEC Give H20(L/R) Air WD	Phase 4/5: D26 Weigh Start 10% (L)	Phase 5: D27 Read 10% (L) Refill 10% (L)	Phase 5: D28 Read 10% (L) Start 10% (R)
Phase 5: D29 Read 10% (R) Refill 10% (R)	Phase 5: D30 Read 10% (R) Start 10% (L)	Phase 5: D31 Read 10% (L) Refill 10% (L)	Phase 5: D32 Weigh Read 10% (L) Start 10% (R)	Phase 5: D33 Read 10% (R) Refill 10% (R)	Phase 5: D34 Read 10% (R) Start 10% (L)	Phase 5: D35 Read 10% (L) Refill 10% (L)
Phase 5: D36 Read 10% (L) Start 10% (R)	Phase 5: D37 Read 10% (R) Refill 10% (R)	Phase 5: D38 Read 10% (R) Start 10% (L)	Phase 5: D39 Read 10% (L) Refill 10% (L)	Phase 5: D40 Weigh Read 10% (L) Start 10% (R)		

Figure 2: Experiment 2 timeline: Effects of repeated withdrawal history on voluntary 10% ethanol ingestion. WSP and WSR mice from replicate 1 were run in parallel for multiple and single withdrawal treatments. Animals had 24-h access to tubes containing ethanol and water. Bottle positions were alternated every 2 days. Fluids were refilled as needed and fresh water or 10% ethanol was presented regularly. Review text for details of design. Abbreviations: D=day; (L)=left tube placement; (R)=right tube placement; %=volume:volume EtOH dilution factor; BEC=blood ethanol concentration; h=hour; Tx=Treatment (Air for Single Withdrawal, EtOH for Multiple Withdrawal); WD=withdrawal.

air cycles in one 72-h period (48 total hours of intermittent EtOH vapor inhalation) while SW groups experienced only one 16-h EtOH:8-h air cycle on the third day of the 72-h period (16 total hours of EtOH vapor inhalation). The third day of each chamber treatment phase marked the third chronic EtOH vapor inhalation session for MW groups and the first for SW groups. Herein, each 16-h EtOH:8-h air period is referred to as one dependence and withdrawal cycle or period. Each 72-h MW- or SW-treatment constitutes one "phase."

Pyrazole. At the beginning of each day during a 72-h phase, all mice were weighed and administered an intraperitoneal (i.p.) 1-mmol pyrazole HCl (68.1 mg) injection per kg body weight. Pyrazole, a competitive inhibitor of alcohol dehydrogenase, allows stable, physiologically relevant blood ethanol concentrations (BECs; BEC singular) to be achieved at the onset of placement in the EtOH inhalation chambers and maintained for the duration of the 16-h intoxication while minimizing individual variations in EtOH metabolism. This is important for comparative purposes when assessing treatment effects between and within groups, helping to eliminate a potential confound of relative dose. Early literature claimed that pyrazole did not exert effects on the EtOH withdrawal experience (Goldstein, 1972a); however, Kosobud and Crabbe (1986a) showed that pyrazole injection resulted in a small increase in WSP non-withdrawal HIC scores at selection generation 9 (see also Crabbe *et al.*, 1981). However, the relative influence of pyrazole on withdrawal HICs was considered nominal compared to the large, order-of-magnitude differences bred into the two selected lines following equivalent or near equivalent exposure to EtOH. Because equal pyrazole dosing was part of the treatment process used in the selective breeding program to develop the WSP and WSR selected lines, equal pyrazole dosing was routinely administered to all genotypes (WSP and WSR) and treatment (MW and SW) groups throughout both experiments in this study. Pyrazole HCl was dissolved in one of two solutions for delivery: (a) in 0.9% NaCl (SW days 1-2), or (b) in a 1.6 g/kg (8% weight/volume) priming dose of EtOH (20% v/v EtOH in 0.9% NaCl) (SW day 3; MW days 1-3). The pyrazole/EtOH injection additionally served as an EtOH priming dose prior to 16-h placement in the EtOH vapor inhalation chamber. MW groups received the pyrazole/EtOH injection for all three days of each intoxication

phase of the study. SW mice received the pyrazole injection dissolved in saline prior to 16-h placement in an air inhalation chamber on the first two days of the 72-h SW treatment, and received a pyrazole/EtOH injection prior to placement in the EtOH vapor chambers for 16-h on day 3 of each 72-h SW treatment.

Equating BEC among lines or treatment groups. With regard to ethanol dosing, chronic intoxication/dependence was induced by exposing mice to 16-h of continuous EtOH vapor in an inhalation chamber at an air:EtOH flow rate that was adjusted to obtain a physiologically relevant, target BEC of 1.5 mg/ml during all 16-h intoxication periods. Individual dosing was quantified by BECs when mice completed each 16-h EtOH vapor exposure. Upon removal from the inhalation chamber, 20 μl of blood was sampled from the distal 1 mm of the tail with capillary tubes in MW groups to quantify the BEC obtained at the end of each 16-h intoxication period, while SW groups were tail nicked (without collecting tail blood samples) to equate handling between the groups. The BEC assessments of the MW mice on the first two days of each 72-h phase were used to adjust the flow rate ratio of vaporized ethanol to air in order to optimize the EtOH concentration within the inhalation chambers and to produce equivalent BECs as much as possible, as BECs were used as an indirect assessment of individual alcohol exposure.

Two EtOH dependence:withdrawal treatment phases in both experiments. Each 16-h EtOH vapor exposure period was followed by 8-h of regular air exposure in a clean, adjacent inhalation chamber to mimic a natural detoxification/withdrawal period (Goldstein, 1972b). The third day of the first 72-h chamber inhalation treatment phase marked the third EtOH inhalation session for MW groups and the first for SW groups, allowing direct comparisons between the two treatment groups because all of the mice were withdrawing at the same time. This took place at the end of Phase 1. Each treatment group was scheduled to receive two phases of 72-h inhalation chamber exposures for their respective MW or SW treatment conditions during the course of each experiment. In other words, the MW or SW treatment regimen for the first 72-h dependence:withdrawal phase was repeated again during the course of each experiment (repeated measures), resulting in two dependence:withdrawal phases for each experiment.

Experiment 1: Examination of multiple, intermittently repeated, ethanol withdrawals on kindling for HICs. Mice from both replicates of the WSP and WSR selected lines (WSP-1, WSR-1, WSP-2, and WSR-2) were exposed to MW or SW conditions, resulting in 8 treatment groups. The age range of mice tested was 50 to 75 days, with the mean age on day 0 being 60 days. MW and SW treatment groups were tested in tandem to reduce environmental effects on treatment. Physical dependence on alcohol results in withdrawal symptoms that include convulsions during detoxification, and has been shown to occur rapidly in animal models (Essig and Lam, 1968; Ellis and Pick, 1969; Freund, 1969). Upon removal from the EtOH chamber on day 3 of Phase 1, MW and SW groups were immediately scored for the 0-hour (or, T=0) HIC score; blood was sampled, and the mice re-housed with their original cage mates. Rather than commencing 8 hours of air inhalation in a vapor chamber, mice were relocated to an alternative procedure room to conduct hourly HIC scoring during the withdrawal period.

HIC scoring. Scoring for HICs was done according to previously published scales (Kosobud and Crabbe, 1990; Becker, 1994). This procedure involves lifting the animal by the tail, gently spinning it 180-360° if necessary, and observing convulsions. HIC scores ranging from 1-3 require a gentle spin following a vertical lift to elicit a tonic or clonic convulsion, whereas convulsions elicited by merely lifting the mouse by the tail are ranked from 4-6, depending on relative severity. The HIC index provides a quantitative measure of the magnitude of physical dependence (as assessed indirectly by the withdrawal symptom severity) according to the following ordinal rank: 0=no response following spin, 1=facial grimace or tension following spin, 2=tonic convulsion following spin, 3=tonic/clonic convulsion following spin, 4=tonic convulsion without spin (elicited by tail lift stimuli), 5=tonic/clonic convulsion without spin, 6=severe tonic/clonic convulsion of long duration without spin, and 7=severe tonic/clonic convulsion induced in the absence of handling. Mice were graded for HICs hourly for 10 consecutive hours immediately following removal from the ethanol chambers on day 3 of Phase 1 (day 10 of Phase 2), allowed to rest during their standard dark cycle, and again scored for HICs at hours 24 and 25 on day 4 of Phase 1 (day 11 of Phase 2) (see Figure 1).

A 3-day rest period commenced following the final (day 3) intoxication period of Phase 1 (mice rested on days 4 to 7). This scheduled rest preceded the initiation of a second round of intermittent, chronic MW or SW treatment with HIC testing during Phase 2 (72-h of inhalation chamber treatment on days 7-10; HIC assessment on days 10 and 11). On the last day of the study (day 11), mice were euthanized by CO_2 asphyxiation following the 25th hour HIC assessment. See Figure 1 for the experimental design.

Area under the curve assessment. To assess withdrawal HIC severity, the area under the curve for the first 25 hours of ethanol withdrawal (AUC25) was figured according to Metten and Crabbe (2005), where AUC was calculated for the first 10 hours of HIC scoring followed by the calculation of AUC25:

- AUC10 = (Hour 0/2) + (Hour 1 + Hour 2 + Hour 3 + Hour 4 + Hour 5 + Hour 6 + Hour 7 + Hour 8 + Hour 9) + (Hour 10/2)
- AUC25 = (AUC10) + (Hour 10/2) + [13 x ((Hour 10 + Hour 24)/2)] + Hour 24 + Hour 25/2)

HIC AUC25 values for Phase 1 and Phase 2 withdrawal periods are the main quantitative measure used to determine differences in withdrawal experienced by line (WSP *vs.* WSR), replicate (1 *vs.* 2), and/or by treatment (MW *vs.* SW).

Experiment 2: Effects of repeated withdrawals on voluntary 10% ethanol ingestion.

Experiment 2 aimed to investigate whether kindling resulting from one or more cycles of intoxication and withdrawal would influence a different alcohol-related phenotype–voluntary ethanol drinking. Experimentally naïve WSP-1 and WSR-1 mice used in this experiment ranged in age from 67 to 74 days, with the mean for each treatment group falling between 70 and 71 days. One experimenter handled mice and recorded fluid volumes during the two-bottle choice drinking preference study, and fluid measurements were taken at the same time every day to reduce environmental variables that might influence this behavior (Crabbe, 1997; Kopp, 2001). Five phases of data collection were used to test the hypothesis that free-choice alcohol ingestion would decrease with multiple experiences of intoxication and withdrawal: two 72-h ethanol vapor dependence:withdrawal phases (Phases 2 and 4) interspersed between three EtOH ingestion phases (Phases 1, 3, and 5 offered 24-hour access to 10% EtOH *vs.* tap water). As in experiment 1, experimentally naïve mice of each genotype (WSP *vs.* WSR) were randomly assigned to two treatment groups (MW *vs.* SW) and tested in tandem to reduce environmental effects. However, unlike experiment 1, HIC scoring did *not* take place, and only replicate 1 of the selected lines was tested (see Figure 2).

Two-bottle choice preference drinking. A preparatory phase was initiated to acclimate the animals to the preference testing regimen, but no data were collected. The continuous two-bottle choice delivery presents tap water in one stoppered, inverted 25-ml volumetric cylinder beside another, identical cylinder that delivers a volume:volume dilution of 200-proof ethanol in tap water. The design used in this paper is most like that of Belknap *et al.* (1997), in that 10% ethanol testing was sustained for at least 8 days during each voluntary drinking phase. On Day –6 (Phase 0, or the preparatory phase), mice were individually housed in clear polypropylene boxes accommodated with two drinking tubes constructed from inverted 25-ml graduated cylinders capped with rubber stoppers and metal sipping spouts and suspended at a 45° angle from a slanted wire cage top to allow easy fluid intake. From Day –6 through Day –3, mice were presented with only tap water in a two-bottle choice setup to acclimate the animals to the choice

paradigm. Beginning on Day –3, 5% EtOH (volume:volume dilution of 200-proof EtOH in tap water) was given in one tube to gradually introduce EtOH to the animals. The positions of the ethanol- and water-containing tubes were alternated every other day to diminish possible confounds of placement preference on the relative consumption of each fluid, thus allowing more stable ingestion patterns to be recorded (Bachmanov *et al.*, 2002; Crawley, 2000).

During Phase 1 (days 0 to 10), baseline voluntary 10% EtOH ingestion was measured by continuing the two bottle choice procedure. This baseline served as the benchmark to assess the effect of withdrawal kindling on voluntary ethanol drinking to be tested later in the experiment. Phase 2 (days 10 to 13) began with the introduction to the ethanol vapor (or air) inhalation chamber, as mice were parsed into MW or SW treatment groups. BECs were assessed as in experiment 1. After all mice exited the alcohol chambers on Day 13, they were singly housed and presented with tap water in both of the cylinders for the first 24 hours of withdrawal. This recovery period was to allow the mice time to fully metabolize remaining ethanol and pyrazole and to eliminate any potential confounds of alcohol-influenced ataxia or, more likely, other physical symptoms of withdrawal (AE Ryabinin, personal communication) on drinking measures.

Phase 3 (days 14 to 22) reinstated the two-bottle choice of 10% EtOH *vs.* water and daily data collection commenced to measure the effects of intermittent vapor exposure on the voluntary alcohol drinking. In Phase 4 (days 22 to 25), a second 72-h chamber immersion cycle began according to the same guidelines outlined for Phase 2. MW groups again experienced 3 additional dependence:withdrawal cycles, and SW groups experienced one additional dependence:withdrawal cycle. Mice were again given 24 hours before introducing 10% EtOH in one tube during Phase 5 (days 26 to 40), the final post-withdrawal voluntary 10% EtOH ingestion period. Following the final reading of fluid volumes on Day 40, all MW and SW mice were weighed and then euthanized by CO_2 asphyxiation.

The repeated ethanol dependence and withdrawal cycles used in Phase 2 and Phase 4 are identical to the multiple withdrawal procedure used in experiment 1, except that HICs were not elicited. Also, the voluntary two-bottle choice drinking setup is the same during Phase 1, 3, and 5, excepting the duration of each phase. Fresh tap water and ethanol dilutions were presented every 2-4 days. Uninhabited control cages were also used to account for fluid evaporation or leakage, and the ethanol drinking measures were adjusted accordingly. See Figure 2 for the experimental outline.

Alcohol consumption and preference ratio indices. Voluntary 10% EtOH ingestion was measured according to two indices, the ethanol preference ratio and ethanol consumption, each calculated from the daily, volumetric 10% EtOH and tap water measurements taken on the second day of each tubes' positioning. The daily ethanol preference ratio was calculated as the volume of 10% EtOH consumed divided by the total volume of all fluids consumed (10% EtOH plus tap water). Daily ethanol consumption was measured as the grams of EtOH consumed per animal adjusted for the individual body weight of that animal in kilograms, thereby correcting the ingestion measure for relative body size (g/kg/day). As such, the focus of repeated withdrawal on ethanol ingestion was indexed primarily by the consumption (g/kg) measure, since it takes into account the individual growth of the animal over the forty-day course of experiment 2 as well as individual differences in body weight. Note that BECs were not measured during the continuous access voluntary drinking periods (Phase 1, Phase 3, or Phase 5).

Statistics. Systat 11.0 was used to analyze primary comparisons within replicate lines and to test the hypothesis that both WSP and WSR groups would kindle for HICs (experiment 1) or alter their voluntary 10% alcohol ingestion (experiment 2) following one or more ethanol dependence and withdrawal cycles. Data were primarily analyzed by factorial and repeated measures analyses of variance (ANOVAs; ANOVA singular) and analyses of covariance (ANCOVAs; ANCOVA singular) for consistency in generating results. Two-tailed tests were used except where the hypothesis being tested was directional, in which case, a one-tailed test was used. A p-value less than or equal to 0.05 was considered significant. When relevant to the hypothesis

under test, the magnitude of a line, treatment, or phase effect was quantified using the proportion of the variance accounted for by each effect (R^2).

RESULTS

The findings of experiments 1 and 2 are organized and presented according to the hypothesis-driven statistical analyses used in each experiment. *F* and *p* values demonstrating statistical significance or important trends are included within the text below. The interested reader will find the *F* and *p* values of all statistical analyses in Appendix Table 1 (experiment 1) and Appendix Table 4 (experiment 2). R^2 indices of effects size are presented within the text when relevant to the hypothesis under test. Descriptive statistics and adjusted least squares means derived for the hypothesis tests (ANOVA and/or ANCOVA) for experiment 1 can be found in Table 1 and Appendix Tables 2 and 3; descriptive statistics for experiment 2 can be found in Table 2. Figures 3 and 4 detail BEC and HIC AUC25 differences within and between experiment 1 groups. Figures 5, 6, and 7 address the BEC, voluntary ethanol consumption, and 10% ethanol preference differences within and between experiment 2 groups.

Experiment 1.

Differences in BEC among lines, treatment groups, or replicates. Before proceeding to HIC AUC25 comparisons, preliminary analyses focused on determining whether significant BEC differences existed between the groups to be compared. Day 3 BEC of Phase 1 and day 10 BEC of Phase 2 (D3BEC; D10BEC) were examined in order to equate ethanol exposure received within and between groups during each phase of treatment. To this end, main effects of line (WSP *vs.* WSR), treatment (MW *vs.* SW), and replicate (1 *vs.* 2) were tested using a one-way ANOVA by each factor for D3BEC and D10BEC. Following this, two-way ANOVAs were run on D3BEC and D10BEC values to determine if interactions between line and treatment, line and replicate, or treatment and replicate existed. These days were selected because, as shown in Figure 1, day 3 was when the MW treatment groups had completed their third 16-h ethanol dependence period and SW treatment groups had completed their first during Phase 1 of testing. By day 10 (end of Phase 2), MW groups had completed their sixth dependence period and SW groups had completed their second.

When significant BEC differences were observed, BEC was used as a covariate in a subsequent ANCOVA (analysis of covariance) to assess AUC25 differences among these same groups as the dependent variable. This served to statistically correct for any observed BEC differences by insuring that the influence of BEC on HIC AUC25 was exactly zero. In other words, the regression of AUC25 on BEC becomes zero as a result of the ANCOVA, eliminating the potential confound of BEC on the HIC AUC25 withdrawal score. On the other hand, if no significant BEC differences were seen, a standard ANOVA without a covariate was used. More detailed comparisons were made within-line to examine control (SW) and kindled (MW) treatment effects. In cases where both replicates of WSP and both replicates of WSR followed the same behavioral trend, replicates were collapsed to increase statistical power for data analyses. Repeated measures ANOVAs or ANCOVAs assessed the possible effect of a second 72-h testing period (Phase 2) on HIC AUC25 scores. Refer to Table 1 and Appendix Tables 2 and 3 for descriptive statistics of experiment 1.

Experiment 1, hypothesis 1: WSP mice will show greater HIC AUC25 scores than WSR mice following a single 16-h EtOH inhalation period. WSP and WSR mice were developed by artificially selecting for their HIC scores during withdrawal from 72 hours of chronic ethanol vapor inhalation. The hypothesis that the withdrawal response of the selected lines would be just as divergent following an abbreviated, 16-h ethanol vapor inhalation period was supported by previous observations of a large, characteristic difference in WSP and WSR withdrawal HICs following a 24-h chronic ethanol inhalation treatment (Finn and Crabbe, 1999). To test this for the 16-h treatment, the WSP and WSR SW treatment groups (which received only one 16-h ethanol vapor inhalation during Phase 1) were first tested for BEC differences using a one-way ANOVA by line to examine the line differences on D3BEC scores. WSP and WSR lines did not significantly differ on D3BECs (see Figure 3 and Appendix Table 1), so Phase 1 HIC AUC25 scores for the SW treatment groups were analyzed with ANOVA by line (see Figure 4). These results revealed significant differences between WSP and WSR SW groups, with WSP mice

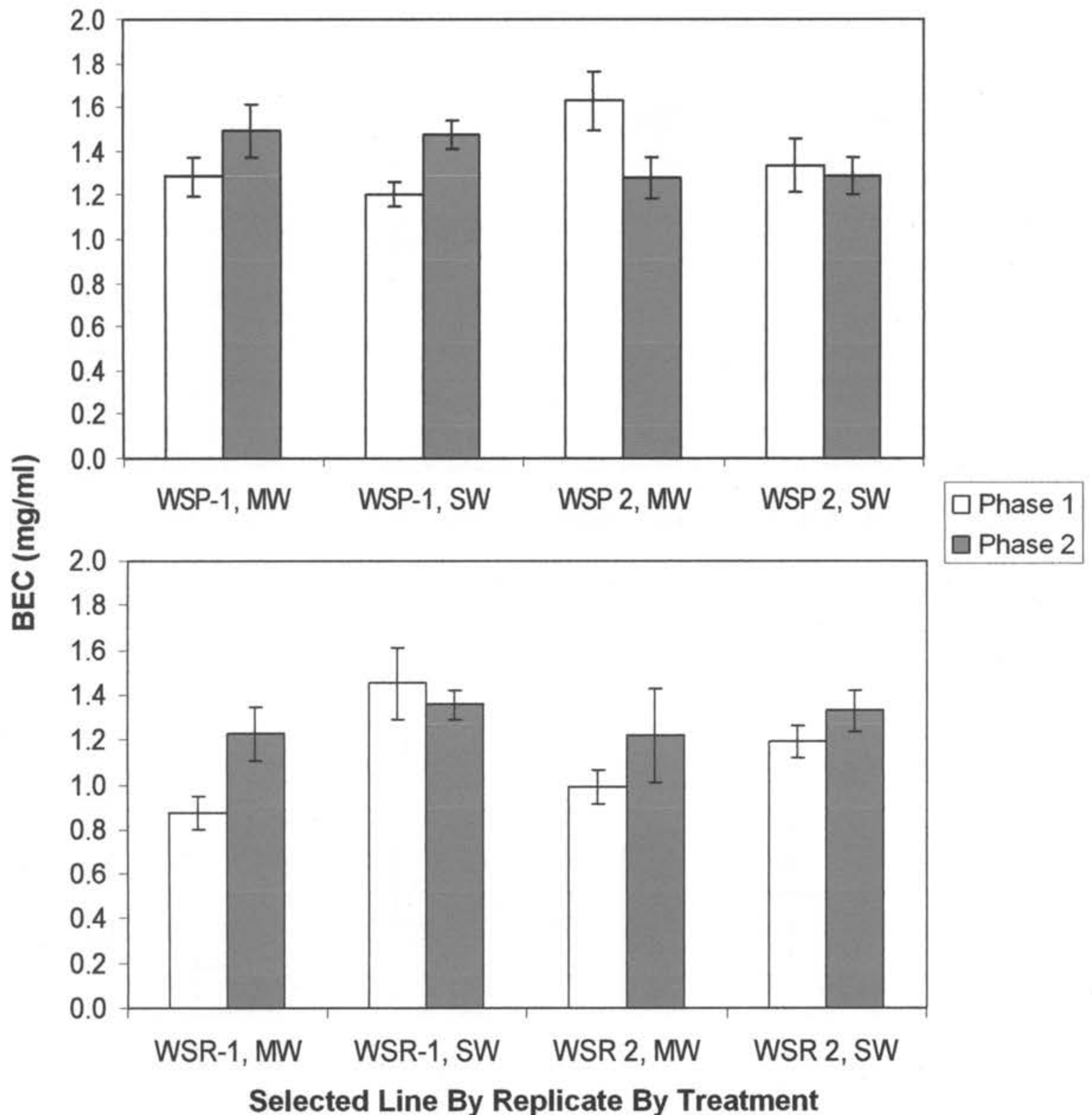

Figure 3: Experiment 1 assessment of blood ethanol concentrations (BECs; BEC singular) following removal from ethanol vapor inhalation chambers. D3BEC and D10BEC correspond to the third day of 72-h MW and SW treatments for Phase 1 and Phase 2, respectively. See text for designations of significance for hypothesis-driven inquiries of effects. Values indicate the group mean plus the standard error of the mean (SEM). Abbreviations: WSP=Withdrawal Seizure-Prone selected line; WSR=Withdrawal Seizure-Resistant selected line; MW=multiple withdrawal treatment; SW=single withdrawal treatment.

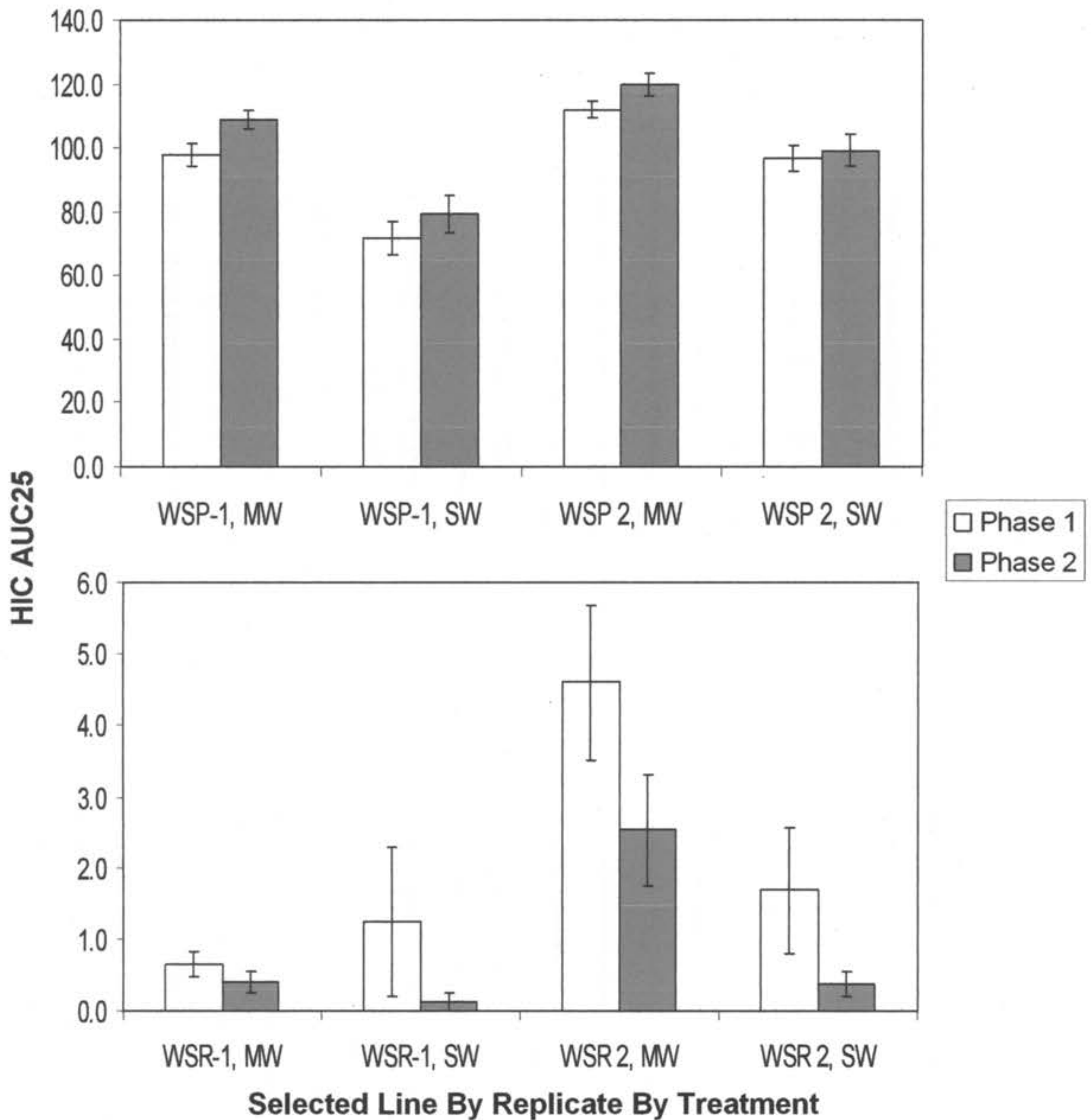

Figure 4: Experiment 1 assessment of handling-induced convulsion (HIC) area under the curve (AUC) over 25 hours of ethanol withdrawal by selected line, replicate, and treatment. See text for designations of significance for hypothesis-driven inquiries of effects. Values indicate the group mean plus the standard error of the mean (SEM). Abbreviations: WSP=Withdrawal Seizure-Prone selected line; WSR=Withdrawal Seizure-Resistant selected line; MW=multiple withdrawal treatment; SW=single withdrawal treatment.

Phase 1 HIC AUC25 Scores

Selected Line	Replicate	Treatment	Statistic	Desc Stats	H1- ANOVA	H2a- ANOVA	H2a- ANCOVA	H2b- ANOVA	H2b- ANCOVA	H3- ANOVA	H3- ANCOVA	H4- ANOVA	H4- ANCOVA
			Mean	**94.74**						94.74	94.34		
WSP			SEM	**2.45**						1.85	1.96		
			Mean	**2.30**						2.11	1.96		
WSR			SEM	**0.50**						2.83	2.98		
			Mean	**57.77**								57.52	58.95
	1		SEM	**5.35**								5.74	5.61
			Mean	**72.07**								72.22	69.31
	2		SEM	**5.75**								5.53	5.55
			Mean	**66.25**						53.94	53.77	65.94	64.35
		MW	SEM	**5.81**						2.12	2.21	5.36	5.31
			Mean	**63.95**						42.91	42.52	63.80	63.91
		SW	SEM	**5.29**						2.63	2.76	5.90	5.79
			Mean	**105.13**		105.13	104.77	105.13	104.98	105.13	104.67		
WSP		MW	SEM	**2.34**		3.14	3.39	3.14	3.16	2.61	2.86		
			Mean	**84.36**	84.36	84.36	83.99	84.36	84.51	84.36	84.01		
WSP		SW	SEM	**3.77**	3.30	3.14	3.28	3.14	3.16	2.61	2.69		
			Mean	**2.75**		2.75	3.23	2.75	3.03	2.75	2.88		
WSR		MW	SEM	**0.68**		0.62	0.60	0.62	0.62	3.34	3.59		
			Mean	**1.47**	1.47	1.47	0.10	1.47	0.94	1.47	1.04		
WSR		SW	SEM	**0.66**	5.77	0.85	0.89	0.85	0.87	4.57	4.81		
			Mean	**60.95**								60.95	63.79
	1	MW	SEM	**8.13**								7.82	7.74
			Mean	**54.09**								54.09	54.10
	1	SW	SEM	**6.76**								8.41	8.12
			Mean	**70.93**								70.93	64.90
	2	MW	SEM	**8.29**								7.34	7.40
			Mean	**73.52**								73.52	73.72
	2	SW	SEM	**7.84**								8.28	8.25
			Mean	**71.71**	71.71								
WSP	1	SW	SEM	**5.30**	4.79								
			Mean	**96.50**	96.50								
WSP	2	SW	SEM	**4.17**	4.70								
			Mean	**1.25**	1.25								
WSR	1	SW	SEM	**1.04**	0.97								
			Mean	**1.69**	1.69								
WSR	2	SW	SEM	**0.88**	0.97								

Phase 2 HIC AUC25 Scores

Selected Line	Replicate	Treatment	Statistic	Desc Stats	H2a- ANOVA	H2a- ANCOVA	H2b- ANOVA	H2b- ANCOVA	H3- ANOVA	H3- ANCOVA	H4- ANOVA	H4- ANCOVA
			Mean	**101.95**					101.95	101.91		
WSP			SEM	**2.64**					1.93	1.96		
			Mean	**1.09**					0.89	0.89		
WSR			SEM	**0.32**					2.96	3.01		
			Mean	**63.91**							63.61	61.91
	1		SEM	**5.89**							6.28	6.31
			Mean	**75.09**							75.10	76.85
	2		SEM	**6.27**							6.05	6.07
			Mean	**71.61**					58.02	58.01	71.38	71.86
		MW	SEM	**6.38**					2.21	2.27	5.87	5.90
			Mean	**67.45**					44.82	44.79	67.33	66.90
		SW	SEM	**5.67**					2.75	2.78	6.46	6.41
			Mean	**114.51**	114.51	114.55	114.51	114.89	114.51	114.49		
WSP		MW	SEM	**2.34**	3.29	3.36	3.29	3.30	2.73	2.78		
			Mean	**89.39**	89.39	89.39	89.39	89.01	89.39	89.32		
WSP		SW	SEM	**4.02**	3.29	3.32	3.29	3.30	2.73	2.76		
			Mean	**1.53**	1.53	1.41	1.53	1.82	1.53	1.53		
WSR		MW	SEM	**0.46**	0.38	0.30	0.38	0.35	3.49	3.60		
			Mean	**0.25**	0.25	0.11	0.25	-0.30	0.25	0.25		
WSR		SW	SEM	**0.11**	0.52	0.40	0.52	0.48	4.77	4.82		
			Mean	**67.74**							67.74	65.81
	1	MW	SEM	**8.93**							8.56	8.62
			Mean	**59.47**							59.47	58.01
	1	SW	SEM	**7.46**							9.20	9.18
			Mean	**75.01**							75.01	77.91
	2	MW	SEM	**9.12**							8.03	8.11
			Mean	**75.18**							75.18	75.79
	2	SW	SEM	**8.40**							9.06	9.00
			Mean	**79.25**	79.25	79.26						
WSP	1	SW	SEM	**5.65**	4.48	4.58						
			Mean	**99.12**	99.12	99.05						
WSP	2	SW	SEM	**5.08**	4.32	4.28						
			Mean	**0.13**	0.13	0.15						
WSR	1	SW	SEM	**0.13**	0.18	0.18						
			Mean	**0.38**	0.38	0.19						
WSR	2	SW	SEM	**0.18**	0.92	0.62						

Table 1: Experiment 1 descriptive statistics and ANOVA/ANCOVA adjusted least squares means values. Area under the curve (AUC) scores reflect the composite HIC scoring for 25 hours of ethanol withdrawal. "Adjusted values" refer to the least squares mean and corresponding standard error calculated for ANOVA or ANCOVA analyses. Mean and SEM from descriptive statistics are used to generate Figures 3 and 4. See text for additional information. Additional abbreviations: H=hypothesis; WSP=ethanol Withdrawal Seizure-Prone selected line; WSR=ethanol Withdrawal Seizure-Resistant selected line; MW=multiple withdrawal treatment; SW=single withdrawal treatment.

having a greater than fifty-fold difference in AUC25 scores than WSR mice following a single16-h EtOH inhalation treatment ($F_{(1,63)}$=155.62, p<0.001, R^2=0.71; see Table 1 for descriptive statistics, and Appendix Table 1 for ANOVA results). These results support the hypothesis that WSP and WSR lines retained characteristic withdrawal differences in response to an abbreviated 16-h ethanol treatment.

In the above tests, replicate 1 and replicate 2 were pooled to focus on line differences. However, within-line comparisons of replicate withdrawal differences in response to the SW ethanol vapor inhalation treatment were also addressed. That is, WSP-1 *vs.* WSP-2 and WSR-1 *vs.* WSR-2 were each analyzed for SW treatment effects on the D3BEC and HIC AUC25 variables. Because within-line WSP and WSR replicate differences on the D3BEC variable were insignificant (see Figure 5), potential within-line replicate 1 *vs.* replicate 2 differences were tested using standard one-way ANOVA by replicate for Phase 1 HIC AUC25 scores. The WSP-1 SW group had significantly lower Phase 1 AUC25 scores than the WSP-2 SW group ($F_{(1,47)}$=13.65, p<0.001, R^2=0.23; refer to Appendix Tables 2 and 3 for BEC values; see also Appendix Table 1), but such differences were not significant in the WSR line. Together, these results show that replicate 2 of the WSP selected line had greater withdrawal severity than replicate 1 (as indexed by HIC AUC25 scores) following one 16-h alcohol intoxication period, but replicate differences were not observed within the WSR line (see Figure 6).

Experiment 1, hypothesis 2: HIC withdrawal symptoms will increase in severity with repeated withdrawal experiences, such that: (a) MW treatment groups will demonstrate higher HIC AUC25 scores than SW treatment groups during Phase 1 and Phase 2, providing evidence for withdrawal-induced HIC kindling, and (b) Phase 2's MW and SW treatment administrations will generate larger HIC AUC25 scores than Phase 1's MW and SW treatment administrations due to the increased HIC severity caused by additional withdrawal experience(s). Within-line differences in D3BEC and D10BEC values were tested for each line using a one-way ANOVA by treatment. WSP mice of MW and SW treatments had comparable D3BEC levels, and as such, the WSP line's Phase 1 AUC25 treatment effects were

analyzed with a one-way ANOVA by treatment. ANOVA results revealed that the WSP MW group had significantly higher HIC scores than the WSP SW group ($F_{(1,96)}$=21.92, p<0.001, R^2=0.18; see Table 1 and Appendix Table 1). In contrast, WSR MW mice showed significantly lower D3BEC levels than the WSR SW mice ($F_{(1,43)}$=14.94, p<0.001), resulting in a one-way ANCOVA by treatment (D3BEC as a covariate) to determine differences in Phase 1 AUC25 scores. ANCOVA results showed significantly higher HIC AUC25 scores in the WSR MW *versus* the WSR SW group ($F_{(1,42)}$=7.53, p=0.009, R^2=0.15). In summary, Phase 1 MW treatments evoked higher HIC AUC25 scores than the SW treatments within the WSP and WSR selected lines (see Figure 3). Therefore, hypothesis 2a was supported.

The same analyses of the MW kindling hypothesis were extended to Phase 2. D10BEC values did not differ by treatment for WSP or WSR lines (see Figure 3), which allowed Phase 2 AUC25 scores to be assessed with a one-way ANOVA by treatment for WSP and WSR mice (see Figure 4). The WSP MW group demonstrated significantly higher Phase 2 AUC25 scores than the WSP SW group, while the WSR MW group showed a strong trend of larger Phase 2 AUC25 scores than WSR SW group (for WSP, $F_{(1,96)}$=29.19, p<0.001, R^2=0.23; for WSR, $F_{(1,44)}$=4.00, p=0.052, R^2=0.08; see Table1 and Appendix Tables 2 and 3). This further supported hypothesis 2a.

The cumulative effect of Phase 1 and Phase 2 treatment experiences on HIC AUC25 scores was assessed using a repeated measures ANCOVA by treatment to test whether within-line MW *vs.* SW effects were greater in Phase 2 than in Phase 1, as predicted by hypothesis 2b. To account for the total influence of BEC over the course of experiment 1, a new variable was calculated as the mean of D3BEC and D10BEC (referred to as D3D10AVGBEC). The choice to covary BEC was made after consideration of the fact that the WSR selected line had a significant main effect of treatment on D3BEC (but not D10BEC; WSP did not have significant effects for D3BEC or D10BEC, though there was a strong trend of a treatment effect for D3BEC). Within the WSP line, a significant main effect of treatment showed that MW-treated mice had larger AUC25 scores than SW-treated mice during Phase 1 and Phase 2 ($F_{(1,95)}$=27.40, p<0.001, R^2=0.22). A within-WSP subjects effect of repeated withdrawal showed that HIC AUC25 scores were more severe

during Phase 2 than during Phase 1 ($F_{(1,95)}$=21.04, p<0.001, R^2=0.18). Thus, the WSP line's ANCOVA analyses of AUC25 HIC scores supported hypotheses 2a (treatment effect; WSP MW > WSP SW) and 2b with a cumulative treatment, or repeated measures, effect on HIC AUC25 scores (WSP Phase 2 > WSP Phase 1). In the WSR selected line, between subjects main effects of treatment and D3D10AVGBEC were found for AUC25 scores (for treatment, $F_{(1,43)}$=8.38, p=0.006; for D3D10AVGBEC, $F_{(1,43)}$=8.82, p=0.005, R^2=0.17), which supported hypothesis 2a with the confirmation that MW and SW treatments significantly affected HIC severity (WSR MS > WSR SW). However, hypothesis 2b was not supported by the within subject WSR data, which showed a *decrease* in Phase 2 HIC severity compared with Phase 1 (though not statistically significant).

This fundamental difference in neurohyperexcitability (indexed by HIC AUC25) contrasted with the significant effect of treatment observed (listed above; also indexes withdrawal history). This comment is backed up by the interaction of repeated treatment experiences with selected line observed in a between line ANCOVA (D3D10AVGBEC covariate; $F_{(1,141)}$=26.96, p<0.001, R^2=0.16). The between selected line analyses also showed main effects of treatment and repeated withdrawal (for treatment, $F_{(1,141)}$=653.45, p<0.001, R^2=0.82; for repeated withdrawal $F_{(1,141)}$=13.38, p<0.001, R^2=0.09; see also Appendix Table 1).

Experiment 1, hypothesis 3: The WSP selected line will kindle more than the WSR selected line. This was predicted because WSP mice display more severe ethanol withdrawal experiences than WSR mice (as assessed by HICs; see Hypothesis 1 above). Thus, WSP mice should experience a greater stimulus for kindling development if withdrawal symptom severity is related to withdrawal-induced kindling.

To test this hypothesis, a two-way ANOVA by line and treatment was used on the Phase 1 and Phase 2 AUC25 variables. A significant line-by-treatment interaction would indicate that the two genotypes (WSP and WSR) responded differently to treatment, as predicted by hypothesis 3. Preliminary differences in D3BEC values (main effect of line at $F_{(1,134)}$=7.16, p=0.008; interaction

of line and treatment at $F_{(1,134)}$=11.42, p=0.001) led to a two-way ANCOVA for Phase 1 HIC AUC25 analyses (D3BEC as the covariate). Phase 1 AUC25 ANCOVA results showed significant main effects of line and treatment, with a significant line-by-treatment interaction (line effect supported WSP > WSR, $F_{(1,133)}$=653.29, p<0.001, R^2=0.85; treatment effects supported MW > SW, $F_{(1,133)}$=10.10, p=0.002, R^2=0.85; see Table 1 and Appendix Tables 1 and 2). The significant line-by treatment interaction supports hypothesis 3, because the WSR line did not respond to treatment as much as the WSP line during Phase 1 (interaction $F_{(1,133)}$=6.58, p=0.011, R^2=0.85). For Phase 2, precursory analyses of D10BEC did not highlight any significant differences, allowing Phase 2 HIC AUC25 scores to be assessed with an ANCOVA. Phase 2 AUC25 ANCOVA results showed the same points of significance and the same direction of effect (by line WSP > WSR, $F_{(1,137)}$=788.55, p<0.001, R^2=0.87; by treatment MW > SW, $F_{(1,137)}$=13.58, p<0.001; for line-by-treatment interaction, $F_{(1,137)}$=11.07, p=.011, R^2=0.87; see Table 1 and Appendix Tables 1 and 3). The presence of a line-by-treatment interaction, and the reiteration of the pattern of main effects and interactions, suggest that the lines responded differently to the MW and SW treatments during Phase 1 as well as during Phase 2. More specifically, although the MW treatment increased withdrawal severity more than the SW treatment in both lines, the WSP selected line showed a greater increase than the WSR selected line. This conclusion was also supported by evidence for hypothesis 2b, where within-line analyses showed greater treatment effects (R^2) in WSP mice than in WSR mice.

Experiment 1, hypothesis 4: Within-line analyses will support greater kindling in replicate 2 than in replicate 1 (WSP-2 > WSP-1; WSR-2 > WSR-1), in keeping with the replicate difference results observed in hypothesis 1. This prediction was supported by the outcome for hypothesis 1, where WSP-2 had greater HIC AUC25 responses than WSP-1 following a single 16-h EtOH dependence (the SW treatment) in the WSP line. Hence, it was expected that the experience of greater withdrawal severity would contribute to larger increases in withdrawal severity due to repeated withdrawal periods. This was tested separately in each selected line, using a two-way ANCOVA by replicate and treatment to test Phase 1 and Phase 2 AUC25

scores. For Phase 1, D3BEC was the covariate, and D10BEC was the Phase 2 covariate. Again, a significant replicate-by-treatment interaction would indicate that the two replicates responded differently to treatment, as predicted by hypothesis 4.

In the WSP line, Phase 1 and 2 HIC AUC25 scores showed significant main effects of replicate and treatment, but no replicate-by-treatment interaction. Phase 1 results showed that replicate 1 had smaller AUC25 scores than replicate 2, and that MW had greater AUC25 scores than the SW treatment (by replicate, $F_{(1,88)}$=22.19, p<0.001; by treatment, $F_{(1,88)}$=25.25, p<0.001). Phase 2 results showed the same main effects and patterns of significance (replicate 2 greater than replicate 1 at $F_{(1,92)}$=13.19, p<0.001; MW greater than SW treatment at $F_{(1,92)}$=31.29, p<0.001). The WSP line did not display replicate-by-treatment interactions during Phase 1 or Phase 2, which suggested that both replicates responded similarly to treatment. These findings do not support hypothesis 4 for the WSP mice.

The WSR line also showed significant main effects of replicate and treatment on HIC AUC25 scores during Phases 1 and 2. Replicate 1 had lower AUC25 scores than replicate 2 during both phases (for Phase 1, $F_{(1,40)}$=5.02, p=0.031; for Phase 2, $F_{(1,40)}$=5.54, p=0.024), and the MW groups showed greater HIC AUC25 scores than the SW treatment groups (for Phase 1, $F_{(1,40)}$=7.10, p=0.011; for Phase 2, $F_{(1,40)}$=7.88, p=0.008). WSR mice did not support hypothesis 4, as no significant replicate-by-treatment interactions were seen during Phase 1 or Phase 2, indicating that the two replicates responded similarly to treatment. Thus, hypothesis 4 was not supported by the WSP or WSR selected lines.

Experiment 2.

Experimental design. Experiment 2 used a design much like that of experiment 1, where effects of MW *versus* SW treatments are examined. The primary difference between experiments 1 and 2 is that experiment 1 tested a withdrawal-induced kindling-like potentiation of the HIC withdrawal symptom, whereas experiment 2 tested whether repeated withdrawals would affect voluntary ethanol consumption. Additional distinctions between experiments included the presence of baseline measurements of voluntary 10% ethanol ingestion in experiment 2 (no pre-withdrawal HICs were measured in experiment 1), the absence of HIC testing during experiment 2 alcohol withdrawal(s), and a difference in the number of "phases" during each experiment. Whereas experiment 1 consisted of two testing phases (Phase 1 was the first round of ethanol vapor treatments followed by HIC assessment; Phase 2 was the second round of treatments and HIC testing), experiment 2 consisted of 5 testing phases. Phase 1 measured baseline 10% alcohol ingestion; Phase 2 introduced the first round of ethanol vapor inhalation treatments; Phase 3 measured withdrawal-influenced voluntary 10% alcohol ingestion; Phase 4 was the second round of ethanol vapor treatments; Phase 5 was the final period of withdrawal-influenced voluntary 10% alcohol ingestion measurements. (See Table 2 for descriptive statistics.)

Analysis of BECs following vapor inhalation focused on the final day of each treatment in order to minimize the possible confounds of disproportionate ethanol BEC values on subsequent drinking analyses (day 13 BEC for Phase 2, or D13BEC; day 25 BEC for Phase 4, or D25BEC). The final day of exposure was selected because it was when both MW and SW groups received 16-h EtOH vapor inhalation. Where significant effects of BEC existed, ANCOVAs were run with BEC as the corrective covariate. Refer to Table 2 for the descriptive statistics of experiment 2.

Experiment 2, hypothesis 1: The WSR line will voluntarily consume more 10% ethanol than the WSP line during baseline ingestion measurements. Baseline free-choice 10% ethanol drinking was recorded during Phase 1 before the commencement of MW and SW ethanol vapor inhalation treatments that began during Phase 2 (see Figure 2). This eliminated the confounding influence of treatment conditions on baseline voluntary ingestion. The directional hypothesis that WSR would freely drink more alcohol than the WSP selected line was tested using a one-tailed test. Tests of voluntary consumption data supported hypothesis 1 at p=0.043 (R^2=0.03; mean WSR=4.2 g/kg/day; mean WSP=2.8 g/kg/day). However, a WSP *vs.* WSR line difference for baseline voluntary alcohol preference ratio was not observed. (Refer to Figures 6 and 7.)

One-way ANOVAs by treatment did not reveal within- or between-line differences in body weight during Phase 1 (see Appendix Table 4). In other words, WSP *vs.* WSR, WSP MW *vs.* WSP SW, and WSR MW *vs.* WSR SW tests of body weight differences were not statistically significant.

Experiment 2, hypothesis 2: MW and SW treatment groups will both show a reduction in free-choice alcohol drinking compared to baseline (Phase 1) values. This will be observed by (a) a substantially reduced voluntary 10% ethanol ingestion in MW groups compared to SW groups, and (b) a general reduction in free-choice drinking for both MW- and SW-treatment groups when assessed with repeated measures tests of Phase 2 and Phase 4 treatment effects. For hypothesis 2a, comparisons of the treatment effects on alcohol consumption were assessed between and within the WSP and WSR selected lines for drinking during phases 3 and 5 (each phase examined separately). First, the possibility of a BEC confound on Phase 3 free-choice drinking was assessed within each line using a one-way ANOVA by treatment on the Phase 2 D13BEC variable (see Figure 5). WSP MW and WSP SW groups were not shown to differ from one another on the BEC measure. Because Phase 3 voluntary alcohol drinking was expected to decrease following the withdrawal experience, this variable was analyzed with a one-tailed test by treatment. Significant Phase 3 treatment effects were not observed in the WSP line for the ethanol consumption index or preference ratio, even

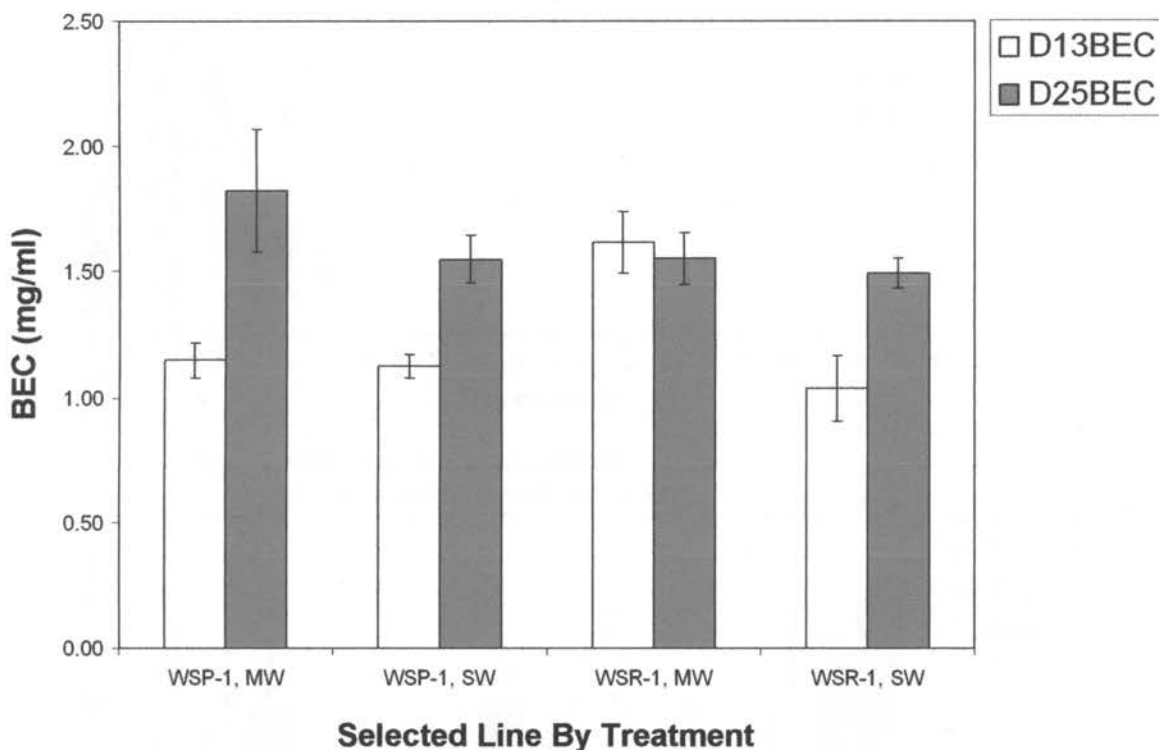

Figure 5: Experiment 2 assessment of blood ethanol concentrations (BECs; BEC singular) by selected line and treatment following removal from ethanol vapor inhalation chambers. D13BEC and D25BEC correspond to the third day of 72-h MW and SW treatments for Phase 2 and Phase 4, respectively. Only replicate 1 mice are represented in this experiment. See text for designations of significance for hypothesis-driven inquiries of effects. Values indicate the group mean plus the standard error of the mean (SEM). Abbreviations: WSP=Withdrawal Seizure-Prone selected line; WSR=Withdrawal Seizure-Resistant selected line; MW=multiple withdrawal treatment; SW=single withdrawal treatment.

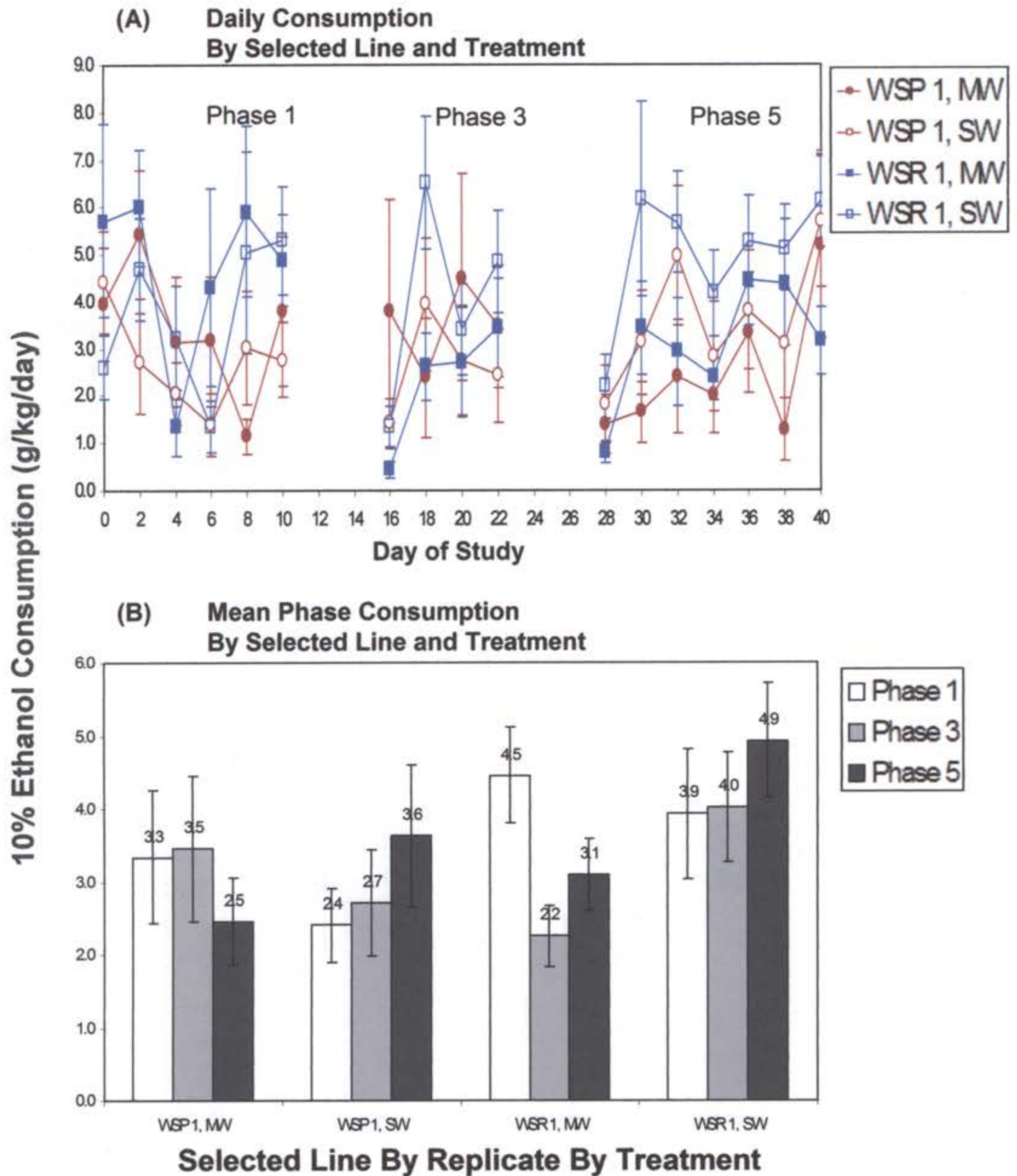

Figure 6: Experiment 2 assessment of repeated withdrawal experiences on the ethanol withdrawal-induced potentiation of voluntary ethanol consumption (g/kg). See text for designations of significance for hypothesis-driven inquiries of effects. Only replicate 1 is represented in this experiment. Values indicate the group mean plus the standard error of the mean (SEM). Abbreviations: WSP=Withdrawal Seizure-Prone selected line; WSR=Withdrawal Seizure-Resistant selected line; MW=multiple withdrawal treatment; SW=single withdrawal treatment.

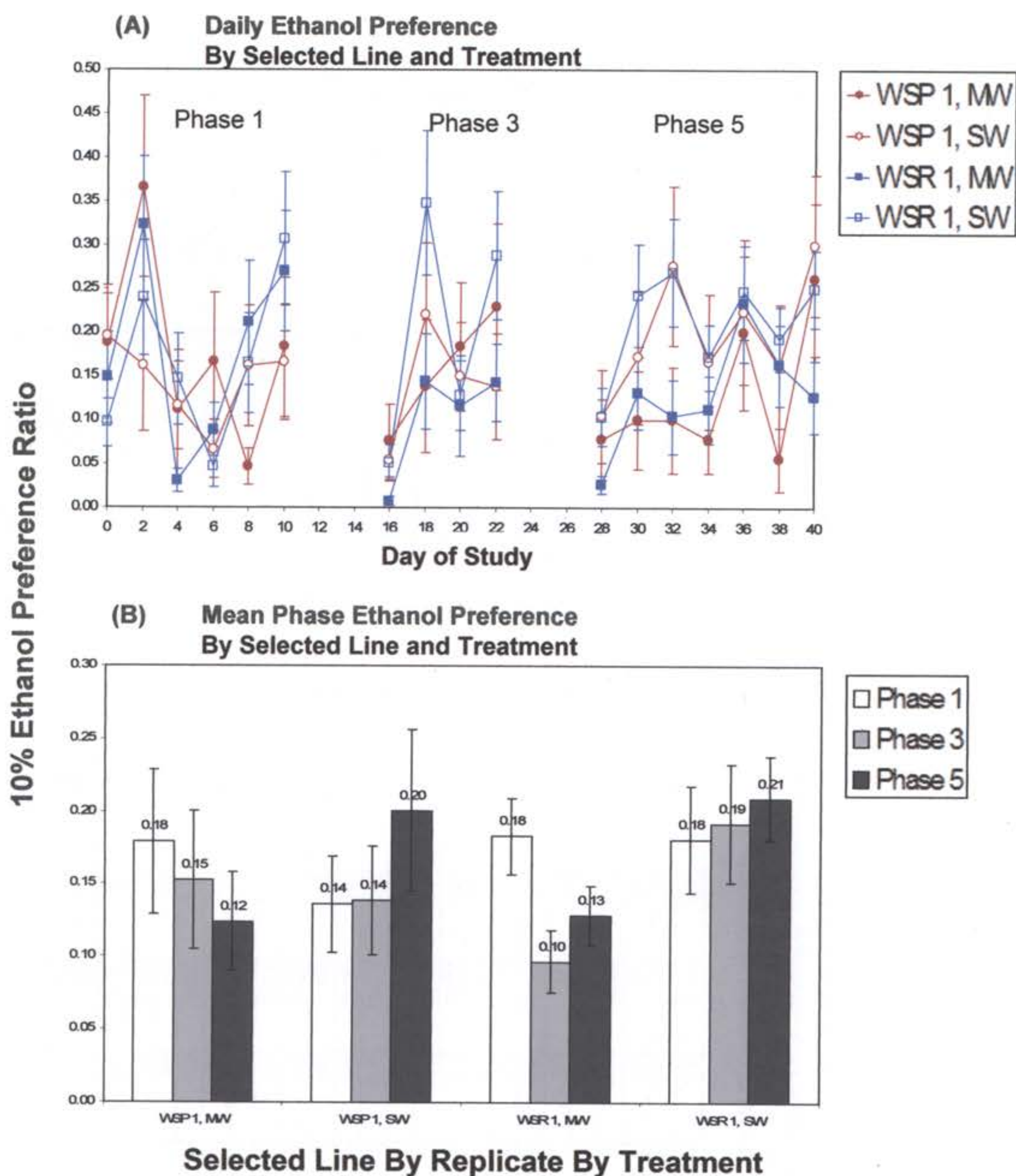

Figure 7: Experiment 2 assessment of repeated withdrawal experiences on the ethanol withdrawal-induced potentiation of voluntary 10% ethanol preference (relative to tap water). See text for designations of significance for hypothesis-driven inquiries of effects. Only replicate 1 is represented in this experiment. Values indicate the group mean plus the standard error of the mean (SEM). Abbreviations: WSP=Withdrawal Seizure-Prone selected line; WSR=Withdrawal Seizure-Resistant selected line; MW=multiple withdrawal treatment; SW=single withdrawal treatment.

Selected Line	Replicate	Treatment	Statistic	D0 Age	Phase 1 g/kg	Phase 1 PR	Phase 1 Vol 10%	Phase 1 Vol H2O	Phase 2 D13BEC	Phase 3 g/kg	Phase 3 PR	Phase 3 Vol 10%	Phase 3 Vol H2O	Phase 4 D25BEC	Phase 5 g/kg	Phase 5 PR	Phase 5 Vol 10%	Phase 5 Vol H2O
All	All	All	n	94	94	94	94	94	93	94	94	94	94	93	94	94	94	94
All	All	All	Mean	71.6	3.6	0.17	1.0	6.1	1.25	3.1	0.15	0.9	6.2	1.59	3.6	0.17	1.1	6.1
All	All	All	SEM	0.2	0.4	0.02	0.1	0.2	0.06	0.4	0.02	0.1	0.2	0.06	0.4	0.02	0.1	0.2
WSP	1		n	39	39	39	39	39	38	39	39	39	39	39	39	39	39	39
WSP	1		Mean	71.8	2.8	0.16	0.8	5.8	1.14	3.1	0.15	0.9	5.7	1.68	3.1	0.17	0.9	5.7
WSP	1		SEM	0.3	0.5	0.03	0.1	0.3	0.04	0.6	0.03	0.2	0.3	0.12	0.6	0.03	0.2	0.3
WSR	1		n	55	55	55	55	55	55	55	55	55	55	54	55	55	55	55
WSR	1		Mean	71.4	4.2	0.18	1.2	6.4	1.32	3.1	0.15	0.9	6.5	1.52	4.0	0.17	1.2	6.4
WSR	1		SEM	0.2	0.6	0.02	0.2	0.3	0.10	0.4	0.02	0.1	0.3	0.06	0.5	0.02	0.1	0.2
	1	MW	n	45	45	45	45	45	44	45	45	45	45	44	45	45	45	45
	1	MW	Mean	71.5	4.0	0.18	1.1	6.1	1.44	2.7	0.12	0.8	6.6	1.66	2.8	0.13	0.9	6.7
	1	MW	SEM	0.3	0.5	0.02	0.2	0.3	0.09	0.5	0.02	0.1	0.3	0.12	0.4	0.02	0.1	0.2
	1	SW	n	49	49	49	49	49	49	49	49	49	49	49	49	49	49	49
	1	SW	Mean	71.6	3.3	0.16	0.9	6.1	1.07	3.5	0.17	1.0	5.8	1.52	4.4	0.21	1.3	5.5
	1	SW	SEM	0.2	0.6	0.03	0.2	0.3	0.08	0.5	0.03	0.2	0.3	0.05	0.6	0.03	0.2	0.3
WSP	1	MW	n	18	18	18	18	18	17	18	18	18	18	18	18	18	18	18
WSP	1	MW	Mean	71.7	3.3	0.18	0.9	5.6	1.15	3.5	0.15	1.0	5.7	1.82	2.5	0.12	0.7	6.1
WSP	1	MW	SEM	0.4	0.9	0.05	0.3	0.5	0.07	1.0	0.05	0.3	0.4	0.24	0.6	0.03	0.2	0.3
WSP	1	SW	n	21	21	21	21	21	21	21	21	21	21	21	21	21	21	21
WSP	1	SW	Mean	71.9	2.4	0.14	0.7	5.9	1.12	2.7	0.14	0.8	5.7	1.55	3.6	0.20	1.1	5.3
WSP	1	SW	SEM	0.4	0.5	0.03	0.1	0.3	0.05	0.7	0.04	0.2	0.4	0.09	1.0	0.06	0.3	0.5
WSR	1	MW	n	27	27	27	27	27	27	27	27	27	27	26	27	27	27	27
WSR	1	MW	Mean	71.4	4.5	0.18	1.3	6.4	1.62	2.2	0.10	0.7	7.1	1.55	3.1	0.13	0.9	7.2
WSR	1	MW	SEM	0.3	0.7	0.03	0.2	0.4	0.12	0.4	0.02	0.1	0.3	0.10	0.5	0.02	0.1	0.3
WSR	1	SW	n	28	28	28	28	28	28	28	28	28	28	28	28	28	28	28
WSR	1	SW	Mean	71.4	3.9	0.18	1.1	6.3	1.04	4.0	0.19	1.2	5.9	1.49	4.9	0.21	1.5	5.6
WSR	1	SW	SEM	0.3	0.9	0.04	0.3	0.4	0.13	0.8	0.04	0.2	0.4	0.06	0.8	0.03	0.2	0.3

Table 2: Descriptive statistics for experiment 2 (effects of repeated withdrawals on voluntary 10% ethanol ingestion). D13BEC and D25BEC represent the blood ethanol concentrations (BECs; BEC singular) following the third day of ethanol vapor inhalation treatment. See text for additional information. Additional abbreviations: D=day; WSP=Withdrawal Seizure Prone; WSR=Withdrawal Seizure Resistant; MW=multiple withdrawal; SW=single withdrawal; g/kg=gram per kilogram, a measure of absolute ethanol consumption; PR=10% ethanol preference ratio; Vol 10% EtOH=Volume of 10% ethanol ingested; Vol H2O=Volume of tap water ingested from the water-only drinking tube.

when analyzed with one-tailed tests. Next, a one-way ANOVA by treatment revealed that the WSR MW group obtained substantially greater BECs than the WSR SW group ($F_{(1,53)}$=10.35, p=0.002, R^2=0.16; also see Appendix Table 4). Although multiple treatments were hypothesized to decrease drinking measures within the WSR line (in other words, a directional effect), the significant within-line difference in the WSR mice's D13BEC dictated that a one-way ANCOVA by treatment be used to examine each alcohol ingestion variable in addition to the one-tailed test by treatment, with D13BEC used as the covariate. ANCOVA results suggested a strong trend for the WSR MW group to have lower alcohol consumption and preference ratio measurements than the WSR SW group (for consumption, $F_{(1,52)}$=4.01, p=0.050, R^2=0.07; for preference ratio, $F_{(1,52)}$=2.94, p=0.092, R^2=0.08), and one-tailed analyses showed a significant treatment effects on voluntary ethanol consumption (p=0.025; mean MW=2.2 g/kg/day; mean SW=4.0 g/kg/day) and for the 10% ethanol preference ratio (p=0.046; mean MW=0.1; mean SW=0.2) (see also Table 2 and Appendix Table 4). Thus, hypothesis 2a was supported by the WSR data, but not the WSP data, for Phase 3.

The within-line analyses for Phase 4 BEC values (D25BEC) and Phase 5 drinking variables were examined in a like fashion. D25BEC was not found to show significant within-line treatment effects (tested with a one-way ANOVA by treatment for each line). The withdrawal experience was hypothesized to decrease voluntary alcohol ingestion, so drinking indices were tested with a one-tailed test in WSP and WSR lines. Again, no differences in voluntary alcohol ingestion or ethanol drinking preference were observed within the WSP line. In the WSR line, no treatment group differences for D25BEC were observed, but significant treatment effects were seen for consumption (p=0.028, R^2=0.09; mean MW=3.1 g/kg/day; mean SW=4.9 g/kg/day) and ethanol preference (p=0.013, R^2=0.09; mean MW=0.1; mean SW=0.2), such that WSR MW groups consumed less alcohol and had a significantly lower alcohol preference ratio than the WSR SW groups. Thus, hypothesis 2 was supported by the WSR data, but not the WSP data for Phase 5.

For hypothesis 2b, repeated measures ANOVAs were used to test whether kindling effects on alcohol consumption (g/kg/day) or preference ratio values occurred after Phase 2 (with animals drinking more during Phase 3 than during Phase 1) or Phase 4 (with animals drinking more during Phase 5 than during Phase 3) treatment administration. A repeated measures ANOVA was also carried for all three drinking phases (Phase 1 *vs.* Phase 5) to assess whether a progressive reduction in alcohol ingestion occurred throughout the experiment. For clarity, statistical results are first presented for the alcohol consumption variable followed by statistical results for the ethanol preference ratio below.

Voluntary ethanol consumption (g/kg/day) was tested using within-line repeated measures ANOVAs by treatment to compare Phase 1 *vs.* Phase 3, Phase 3 *vs.* Phase 5, and cumulative treatment phase influences over the course of the experiment (tested as Phase 1 *vs.* Phase 5). In the WSP line, no significant differences in alcohol consumption were observed for any repeated measures comparison. In the WSR line, Phase 1 *vs.* Phase 3 comparisons revealed a main effect of the Phase 2 withdrawal experience(s) (Phase 1 > Phase 3, $F_{(1,53)}=4.71$, $p=0.034$). Results from the Phase 3 *vs.* Phase 5 analysis within WSR mice revealed a significant main effect of treatment (MW < SW, $F_{(1,53)}=5.19$, $p=0.027$) and strong trend for Phase 4 treatment(s) to reduce alcohol consumption (trend of Phase 3 < Phase 5, $F_{(1,53)}=3.96$, $p=0.052$). The overall analyses of Phase 1 *vs.* Phase 5 consumption in WSR mice revealed a trend towards an interaction between repeated withdrawal effects (cumulative phase effects) and treatment in the absence of main effects ($F_{(1,53)}=3.27$, $p=0.076$). WSP data did not show a repeated measures effect of treatment phases 2 or 4 on ethanol consumption, and therefore did not support hypothesis 2b. However, the WSR line's reduction in alcohol consumption following the Phase 2's ethanol vapor inhalation treatments did support hypothesis 2b (Phase 1 > Phase 3), and provided further support for a main effect of treatment in support of hypothesis 2a (MW < SW).

Finally, the alcohol preference ratio was examined to compare Phase 1 *vs.* Phase 3, Phase 3 *vs.* Phase 5, and Phase 1 *vs.* Phase 5. As was the case for the consumption measure, no significant effects of treatment or repeated measures were observed on the preference ratio index in the

WSP mice. When examining these variables in the WSR mice, a main effect of Phase 4 treatment was significant (WSR MW < WSR SW, $F_{(1,53)}$=7.75, p=0.007). When comparing Phase 1 *vs.* Phase 5 preference ratio, no significant findings or trends were observed. Hence, the preference ratio index provided additional support of treatment effects stated in hypothesis 2a.

In summary, none of the within-line analyses of the WSP mice supported hypotheses 2a or 2b. In the WSR mice, effects on ethanol consumption and preference ratio indices corroborated the significant influence of Phase 4 treatment in the WSR line, yielding strong support of hypothesis 2a. Phase 2 withdrawal treatments resulted in a significant decrease in Phase 3 post-withdrawal alcohol consumption in support of hypothesis 2b, but this was not observed for the preference ratio measure. Interestingly, the second round of withdrawal experiences (Phase 4) significantly affected voluntary ethanol consumption without showing a likewise effect on preference ratio measures—though this effect was opposite to what was predicted by hypothesis 2b (Phase 5 consumption increased relative to Phase 3). However, ethanol drinking during Phase 5 did not return to the degree of voluntary alcohol consumption observed in Phase 1 baseline values.

DISCUSSION

A kindling-like response of AWS symptoms by repeated alcohol detoxification has been observed clinically (Ballenger and Post, 1978). The phenomenon of withdrawal symptom kindling has more recently been extended to the observed exacerbation of ethanol withdrawal-precipitated HICs in C3H/He inbred mice (Becker and Hale, 1993; Becker, 1994). HIC susceptibility is a trait influenced by genetics and tends to inversely correlate with voluntary alcohol drinking (Crabbe, 1983; Metten *et al.*, 1998; Metten and Crabbe, 2005).

Herein, the effects of a history of repeated withdrawal on both behaviors was tested in the WSP and WSR lines. Experiment 1 asked if the effect of a history of withdrawal experience(s) (as produced by the MW and SW treatments) on the HIC response would be correlated with the typically divergent response to selection in the WSP and WSR lines. Experiment 2 addressed how repeated withdrawals from alcohol would influence voluntary ethanol drinking. In experiments 1 and 2, the SW treatment groups functioned as a control for the experience of multiple withdrawals, as the repetition of withdrawal was presumed to be necessary for the neural hyperexcitability underlying kindling. Evidence for either a kindling-like effect of persistent potentiation on HICs or a prolonged effect of repeated withdrawals on voluntary ethanol drinking would suggest that the respective neural processes underlying these responses during ethanol withdrawal are persistently affected by repeated withdrawals. The effects of kindling on both behaviors would suggest a pleiotropic influence of (some of the) genes fixed in the WSP and/or WSR lines on other measures of withdrawal. If subjects showed kindling effects on HICs but not for voluntary ethanol ingestion or *vice versa*, this would suggest that distinct neuromechanisms underlie each phenotype (*i.e.*, the responses are not genetically correlated), or that the time courses of the withdrawal effect on withdrawal-precipitated convulsions and free-choice alcohol drinking is different.

Experiment 1.

The HIC phenotype is influenced by the dose and duration of intoxication preceding withdrawal, as well as by genetics (Goldstein and Pal, 1971; Goldstein 1972a,b; 1973a,b; 1974) and a history of repeated withdrawal (Becker and Hale, 1993; Becker, 1994). WSP and WSR lines were artificially selected for extreme differences in their ethanol withdrawal HIC AUC25 scores following 72 hours of chronic ethanol intoxication (Crabbe *et al.*, 1983a,b; 1985). However, the difference in HIC sensitivity is not purely attributable to ethanol withdrawal, as drug- and withdrawal-naïve WSP mice demonstrate a proclivity to convulse during general handling and have higher baseline HIC scores than WSR mice (Crabbe *et al.*, 1993; Buckman and Meshul, 1997; Finn and Crabbe, 1999). Yet, their responsivity to alcohol- or benzodiazepine-induced withdrawal is reliably unique, with WSR mice being almost completely resistant to the influence of pharmacological agents that precipitate a large HIC reaction in WSP mice (Crabbe *et al.*, 1993). In addition, WSR mice are *more* sensitive to NMDA administration, which shows that the line differences are not because of a generally lower basal neuroexcitablility (Kosobud and Crabbe, 1993).

Typical HIC AUC25 line differences have also been observed across a range of ethanol treatments: following an acute 4-g/kg i.p. injection of EtOH in physiological saline (Kosobud and Crabbe, 1986; Roberts *et al.*, 1991), after chronic intoxication by 24-h of ethanol vapor inhalation (Finn and Crabbe, 1999), and in response to a 16-h ethanol vapor inhalation treatment as demonstrated herein. Specifically, experiment 1 results showed a 56-fold WSP:WSR difference in HIC responding among Phase 1 SW treatment groups (tested in hypothesis 1). When MW and SW groups were considered together, major main effects of line were still large (Phase 1 WSP:WSR ratio=41-fold and Phase 2 WSP:WSR ratio=92.6-fold; tested in hypothesis 3). The selected line differences were additionally corroborated in the two-way ANOVA by line and treatment that yielded a significant main effect of line (also hypothesis 3), and refuted by the absence of replicate-by-treatment interactions (tested in hypothesis 4). In all cases, the WSP line

had a vastly larger HIC AUC25 score than the WSR line, and within-line tests of replicate yielded similar responses (see Table 1 and Figure 4).

The manifestation of order-of-magnitude differences in EtOH withdrawal-precipitated HICs over a broad range of EtOH dosing underscores the robust influence of the genes underlying the HIC phenotype in these selected lines. The reliable responding of the WSP and WSR selected lines as a genetic model allowed the use of the 16-h EtOH:8-h air vapor inhalation paradigm with the confidence that the treatment would evoke neural hyperexcitability characteristic of withdrawal and necessary for withdrawal-induced kindling-like effects. This reliability allowed the use of an animal testing model with inherent face validity for the episodic drinking behavior observed clinically (16-h EtOH inhalation mimics near-constant binge drinking; 8-h air inhalation mimics a short-term abstinence during sleep).

The notion that kindling might occur—at least in WSP mice—was supported by previous findings. Increased intracellular Ca^{2+} concentrations underlie the generation/propagation of epileptiform activity causing seizures, and withdrawal severity in the WSP and WSR lines has been shown to coincide with the WSP line's upregulation of Ca^{2+} channels by up to 70% compared to WSR lines following short term, chronic ethanol exposure (unpublished observations of Feller DJ, Tso-Olivas DY, Savage DD, as referenced in Crabbe *et al.*, 1990a). Membrane preparations of whole brain samples show that WSR mice have more pre-ethanol treatment dihydropyridine-sensitive Ca^{2+} channel binding sites than WSP mice, but that WSP mice significantly upregulate these binding sites to surpass both pre- and post-ethanol WSR binding site concentrations following ethanol treatment (Brennan *et al.*, 1990). Because a greater Ca^{2+} conductance would result in an increased neural excitability, these findings suggest one source of the post-ethanol hyperexcitability that contributes to convulsion susceptibility in WSP mice following withdrawal (Brennan *et al.*, 1990). In studies of pre-synaptic glutamate immunoreactivity, WSP mice had greater labeling densities than WSR mice in hippocampal CA1 regions (Buckman and Meshul, 1997). These findings regarding Ca^{2+} differences support the notion of hyperexcitability that is intrinsic to withdrawal (Littleton, 1998; Becker, 1998). The manifestation of HIC kindling on the

second day of acute withdrawal in the WSP line (but not the WSR line) after receiving acute ethanol injections provides further support for the kindling concept (JC Crabbe, personal communication). Additionally, pentylenetetrazol (PTZ) treatment induced kindling in WSP mice, but not in WSR mice (Crabbe and Kosobud, 1986). (Note that the differential response to PTZ-induced convulsions may have been anomalous, as other convulsants active at the picrotoxin binding site of the $GABA_A$ receptor complex did not show such line-specific kindling-like effects; Kosobud and Crabbe, 1990). Therefore, it is in keeping with previous literature to hypothesize that repeated ethanol withdrawals would manifest potentiated HIC responses in the WSP mice. With regard to the effect of repeated withdrawals on the WSR line's resistance to ethanol-induced convulsions, it remains possible that repeated withdrawal experiences might either sensitize WSR mice to exhibit a larger HIC response or that the "resistant" genes fixed by artificial selection might serve to protect against a kindling-like potentiation of withdrawal-induced HICs.

WSP and WSR lines rank at almost completely different levels on the HIC scoring scale, involving different types of HIC seizures. WSR mice experience facial grimace at most and WSP exhibit tonic-clonic convulsions. As such, within-line comparisons were used to assess MW and SW treatment effects that would support the hypotheses of repeated withdrawal-precipitated HIC potentiation. Hypothesis 2a testing showed that the MW treatment group had significantly greater HIC AUC25 scores than the SW treatment group. The test of hypothesis 3 showed a main effect of treatment, where MW groups yielded higher HIC AUC25 scores during both phases. Within-line replicate analyses of treatment effects (hypothesis 4) demonstrated that replicates 1 and 2 had parallel treatment effects on HICs for Phase 1 and Phase 2. These results show that sensitization of convulsion severity occurred with the recurrent presentation of the ethanol withdrawal stimulus (MW groups) *versus* single presentation (SW groups) during both phases of experiment 1. Importantly, this effect was present in the WSP *and* WSR mice (both replicates of each line), demonstrating the augmentation of genetically determined HIC susceptibility in "prone" lines and increasing the HIC response in lines "resistant" to the acute or chronic effects of ethanol

withdrawal on the HIC response. Hence, this ethanol treatment manipulation can overcome the behavioral profile of the selected lines.

One criticism of this design is whether a true kindling-like effect was observed in MW groups—that is, does the potentiation observed suggest persistent long-term changes in the hyperexcitability of neural circuits? The use of a second phase in experiment 1 attempted to address whether a second SW or MW treatment cycle exacerbated the response observed after the first round of treatment. The cumulative effects of repeated withdrawal experiences for MW and SW groups, with MW groups experiencing a total of 6 intoxication:withdrawal cycles and SW groups experiencing 2 intoxication:withdrawal cycles by the end of the second phase of treatment, was tested with a repeated measures approach (hypothesis 2b). Significant effects of repeated withdrawal (dictated by the second application of the MW and SW treatments during Phase 2) were shown by repeated measures ANOVA tests in the WSP and WSR selected lines, but the direction of this effect was opposite: in WSP, Phase 1 < Phase 2, but Phase 1 > Phase 2 in WSR (see Figure 4 and Appendix Table 1). This significant effect remained stable in a one-way ANCOVA by treatment (D13D25BEC covariate), but the significance was eliminated in the WSR line under this hypothesis test. Taken together, experiment 1 demonstrated strong effects of repeated withdrawal in SW and MW groups over both phases of treatment, but the potentiation of Phase 2 seizures over Phase 1 seizures was robust in WSP mice only. This suggests that the effects of the Phase 1 treatment persisted over the 4-day scheduled respite between phases in WSP mice. Because a short term "kindling-like" response to the MW treatment as compared to the SW treatment was observed in both lines, it is possible that the effects were not persistent in the WSR line, precluding a "carry-over" repeated measures effect during Phase 2. The opposite direction of effect over the course of experiment 2 may be attributable to a protective effect of the genes fixed by artificial selection in the WSR line, possibly promoting a short-term tolerance.

Previous studies by HC Becker and colleagues have successfully demonstrated withdrawal-elicited potentiation of ethanol withdrawal HICs in C3H/He inbred mice, showing that the severity of HICs increased according to the number of intermittent detoxifications (withdrawals) in mice

subjected to the same duration of chronic ethanol vapor inhalation adjusted to obtain a target BEC (*e.g.*, dose) (Becker and Hale, 1993; Becker, 1994). EEG indices of withdrawal were later measured in the same strain and showed an increase in brief spindle episodes (BSE; a paroxysmal EEG patterns of neural firing) in repeatedly withdrawn mice not tested for HICs compared with multiply withdrawn mice tested for HICs (but not for EEG activity), indirectly suggesting a correlation of BSEs with HICs (Veatch and Becker, 2002). Later site-specific, intracranial measures of EEG-measured spike and sharp wave (SSW) showed increased excitability in the hippocampal CA3 region in multiple-withdrawn mice as compared to single-withdrawn mice (Duka *et al.*, 2004). The EEG sensitization to repeated ethanol withdrawal was observed to have a time course similar to peak HIC severity exhibited during withdrawal, giving further support to the definitive rendering of the withdrawal-induced HICs as seizures.

Yet the genetics underlying differential response to convulsion susceptibility do not necessarily suggest that the WSP mice are "kindling"-prone or that WSR mice are "kindling"-resistant. WSP and WSR selected lines have never had electrophysiological or electroencephalographic (EEG) measurements taken while convulsing to definitively attribute the convulsion occurrence to epileptiform EEG activity, whereas "kindling" has traditionally described the increasing sensitivity of a electro- or chemo-convulsive stimulus to elicit seizures. Whereas EEGs measure overall changes in cortical electrical activity, they are not a good index for seizures that may be subcortical in origin. Implanting electrodes subcortically in regions important for seizure propagation (for example, the hippocampus or amygdala; McCown and Breese, 1990; Duka *et al.*, 2004; Westbrook, 2000) would address this, though it would be a mechanical challenge to measure HICs with this type of design. Regardless, Ballenger and Post (1978) concluded that the occurrence of withdrawal induced seizure *and* convulsions were affected by withdrawal-induced hyperexcitability and that individual withdrawal severity was highly correlated with the number of intoxication:withdrawal cycles in humans. However, Gonzalez (1998) noted that clinical publications on this topic often use the terms "kindling," "sensitization," and "potentiation" interchangeably, but experimental models involving the repeated treatment of intermittent EtOH

exposure and withdrawal cycles should be considered to demonstrate a "kindling-like" phenomenon unless electrographical indices showing typical patterns of seiziform activity are measured (Gonzalez, 1998). Hence, the outcome of experiment 1 supports a withdrawal-precipitated kindling-like phenomenon on HIC measures during ethanol withdrawal in the WSP selected line, but not in the WSR line.

Experiment 2.

The primary goals of experiment 2 were to examine the baseline voluntary alcohol intake of the WSP and WSR selected lines during Phase 1, to determine the effect of repeated withdrawals on the willingness to drink alcohol during Phases 3 and 5, and to assess the effect of repeated withdrawal treatments implemented during Phase 2 and Phase 4 on subsequent drinking phases. Given that voluntary drinking is a phenotype that has not been systematically manipulated in these selected lines, exploring the various environmental manipulations in this population would enrich the knowledge of how ethanol withdrawal might affect other behaviors. In addition, possibly divergent effects of an ethanol withdrawal history on free-choice alcohol drinking in a genetic model of convulsion susceptibility would suggest that the ethanol withdrawal-induced neurohyperexcitability has a broad influence on behavior.

Phase 1 "baseline" measurements of voluntary 10% alcohol drinking revealed that the WSR-1 replicate significantly drank 1.5 times more than the WSP-1 replicate using a two-bottle choice procedure (as calculated by the WSR:WSP consumption ratio). This effect was not upheld for the ethanol preference ratio measure (see Appendix Table 4). Previous findings on free-choice 10% alcohol consumption and the ethanol preference ratio showed a main effect of line, where WSR mice drank more 10% ethanol than WSP mice on both measures (Kosobud, *et al.*, 1988). However, much of this effect was attributable to replicate 2, as the WSR-2 line consumed much more 10% ethanol (and had a higher ethanol preference ratio) and the WSP-2 line had a slightly lower consumption compared to all other line-replicate groups (WSR-1, WSP-1, WSC-1, and WSC-2; WSC is the ethanol Withdrawal Seizure Control line, which has not been artificially

selected for ethanol withdrawal-induced HICs). Following up on this finding was not possible because only replicate 1 WSP and WSR mice were used in this experiment. The voluntary drinking in WSP and WSR mice was genetically correlated with HIC severity, where the WSR line drank more than the WSP line (Kosobud *et al.*, 1988). It is notable, however, that the difference in WSR-1 and WSP-1 drinking was very small for 10% ethanol, but differed more for 2.2% and 4.6% ethanol solutions (Kosobud *et al.*, 1988). The present study used a 10% ethanol solution because it produced the greatest overall effect, but using a different, lower concentration of alcohol might have prompted slightly higher consumption among the WSP mice.

A potential problem for attempting to interpret the volumetric (and volumetric-derived calculation of ethanol preference and consumption) is whether the animals had resumed regular eating and drinking habits after the MW and SW treatments, which was when the volumetric measurements were taken. Because weights returned to non-significantly different values during Phase 3 and Phase 5, it is likely that the mice were able to re-stabilize their ingestion patterns. As such, the different statistical results observed for alcohol consumption and the ethanol preference ratio may be attributable to relative volumes of fluid ingestion. This paper referred to both measures for the purpose of being thorough, and because results obtained by Kosobud *et al.* (1988) addressed both indices. However, alcohol consumption is often regarded as a better standard for measuring ethanol intake (JC Crabbe and JK Belknap, personal communication).

Within-line effects of the intermittent intoxication and withdrawal cycles were assessed to test whether voluntary drinking would decrease in response to the withdrawal experience(s) (hypothesis 2). This hypothesis was an extension of the afore-mentioned inverse relationship existing between withdrawal severity and baseline free-choice alcohol drinking, and was also intended to explore whether the relationship would be upheld after the mice had experienced a history of ethanol withdrawals. The WSP line was resistant to main effects of treatment on voluntary alcohol consumption and the 10% ethanol preference ratio, showing neither an increase nor decrease in ingestion patterns. This is not to say, however, that their ingestion patterns were stable (see figures 6 and 7). The lack of significance might be attributed to both groups showing

random fluctuations in alcohol ingestion that appear unrelated to drinking tube alternation (tube positions were switched every two days to avoid an effect of positional preference drinking; Bachmanov *et al.*, 2002). In the WSR line, however, the MW group was shown to ingest significantly less alcohol than the SW treatment group according to alcohol consumption and ethanol preference ratios during Phase 3 (following one 72-h phase of treatment presented during Phase 2) as well as for the Phase 5 ethanol preference ratio (following a second 72-h treatment during Phase 4). These results for the WSR line's drinking support hypothesis 2 and suggest that animals with the multiple withdrawal experiences (WSP MW and WSP SW groups) or the most severe treatment effects (WSR MW was possibly more severe than WSR SW) also drank less ethanol.

Repeated measures investigations queried the within-line effects of Phase 1 *vs.* Phase 3, Phase 3 *vs.* Phase 5, and Phase 1 *vs.* Phase 5 on the alcohol consumption and preference ratio indices. No effects of repeated withdrawal were observed on voluntary drinking measures in the WSP line. In the WSR line, mice were shown to significantly decrease their consumption after the first round of treatments, then gradually increase consumption during Phase 5 and restore voluntary consumption levels to that of pre-withdrawal, baseline measures (indexed by a lack of significant difference when comparing Phase 1 with Phase 5). These results refute the idea that the neural changes resulting from repeated withdrawal experiences would persistently influence voluntary drinking.

The clinical "self-medicating" hypothesis of alcoholism predicts a stable or increasing pattern of alcohol ingestion over time in order to avoid alcohol withdrawal symptoms. Though this hypothesis appears to stand at odds with the experimental inverse genetic correlation between withdrawal severity and voluntary ingestion, it is key to recognize that the inverse relationship is characteristic of *baseline* drinking (no history of withdrawal), and the self-medicating hypothesis describes the dependency of long-term drinkers. However, because the MW and SW treatments in this study create a history of intoxication and dependence in individual subjects, we explored whether the alcohol drinking behavior of the mice over time could be explained by the self-

medicating hypothesis. The absence of treatment effects in WSP mice might be explained by a "floor effect"-that is, the already low WSP alcohol ingestion baseline values of Phase 1 may have made it difficult to detect an increased aversion to alcohol or a further decrease in alcohol ingestion as predicted by hypothesis 2 during Phase 3 or Phase 5, if such an effect was present. The WSR line showed a decrease in consumption after the first round of treatment (Phase 1 > Phase 3), which supported the inverse relationship existing between withdrawal severity and free-choice alcohol drinking found in inbred strains and selected mouse lines (McClearn *et al.*, 1982; Metten *et al.*, 1998; Metten and Crabbe, 2005). However, voluntary alcohol drinking in after the second round of treatment showed a return to baseline (Phase 3 < Phase 5) suggestive of the clinical hypothesis. Perhaps the different response to the second round of treatments signals the beginning of a shift in the animals' long-term relationship with ethanol. It is also possible that the self-medicating aspect of alcoholism is something that is a psychological construct of humans where withdrawal induces craving or stimulates drinking as a means to cope with the symptoms contributing to the negative affect of withdrawal, and is not adequately addressed by the design of this study.

Interestingly, Becker and Lopez (2004; see also Lopez and Becker, 2005) demonstrated an increase in ethanol drinking following repeated EtOH dependence and withdrawal episodes in C57BL/6J inbred mice, a strain known for its predictably high alcohol intake (McClearn and Rodgers, 1959; Belknap *et al.*, 1993) and low HIC susceptibility (Crabbe *et al.*, 1983c). The effect was specific for free-choice alcohol drinking in these mice, as a withdrawal history did not potentiate HIC susceptibility (per HC Becker's personal communication with DA Finn). Once C57BL/6J mice had been trained to drink large volumes of alcohol with a modified sucrose fading technique, the repeated experience of chronic ethanol withdrawal increased and stabilized later alcohol ingestion compared to pre-withdrawal, post-training ethanol "baseline" volumes (Becker and Lopez, 2004; Lopez and Becker, 2005). In support of the persistence of withdrawal-induced effects on later drinking, Becker and Lopez (2004) saw withdrawal-induced alcohol drinking peak several days following repeated EtOH withdrawal. Although this study used the same SW *vs.*

MW treatments in the 16-h EtOH:8-h air inhalation procedure implemented in these studies, some distinct differences in methods exist. For one, mice were trained with sucrose fading until mice would voluntarily drink 15% ethanol, thus allowing ethanol to become a positive reinforcer before stimulating withdrawal. C57BL/6J mice were used in the study, and are models of stable high alcohol ingestion and 10% alcohol drinking preference (McClearn and Rodgers, 1959; Belknap *et al.*, 1993). Additionally, they are known to be resistant to one measure of withdrawal severity—HICs (Metten and Crabbe, 2005). Results similar to those of Lopez and Becker were observed in rats that were also trained to operantly respond for alcohol dosing (Roberts *et al.*, 1996). In both studies, the rodents began to ingest more alcohol with repeated withdrawal exposures, and irregular flux in daily drinking stabilized at volumes higher than those imbibed at baseline. Roberts additionally showed that the rats drank more, or "self-medicated," to avoid ethanol withdrawal symptoms (Roberts *et al.*, 1996).

Future directions.

This study confirmed the occurrence of a persistent, kindling-like neural excitation on ethanol withdrawal HICs in WSP mice, and showed partial support for the hypothesis that the experience of multiple alcohol withdrawals would attenuate voluntary drinking in WSR selected lines. Future studies for investigating the kindling-like potentiation of HICs should involve cranial or subcortical electrographical measurements to definitively attribute the phenomenon observed to kindling. The implementation of subcranial electrodes would have the advantage of testing for post-ictal activity in mice that score for motor disturbances on the HIC scoring scale, as well as for testing possible sub-threshold aberrances prior to handling or HIC elicitation. For distinguishing temporal effects in electrophysiological activity, free-moving mice with electrode placement could have their alcohol consummatory behavior measured with a lickometer to ascertain if the drinking reflects aberrant brain activity. Coupling electrographic measurements with a pharmacological intervention of chemotransmission (such as Ca^{2+} channel antagonists) would provide a link to determine the relative role of specific signaling mechanisms on withdrawal-induced hyperexcitability. Establishing ethanol as a positive reinforcer by training mice to operantly

respond for alcohol in limited access sessions before experiencing alcohol withdrawal is also desirable (Becker and Lopez, 2004; Lopez and Becker, 2005).

Studies using the brain tissue of kindled animals would be an additional approach for determining withdrawal-induced, site-specific up- and/or down-regulation of cellular proteins important to neural function. These studies could serve as a launching point for hypothesis generation, such as in the case of microarray studies. Ideally, the mice whose tissue has been harvested would have a history of repeated withdrawals and would have previously been measured for withdrawal symptom sensitivity and severity during and after withdrawal. The brain tissue preparations of these mice could also be used to assess differences in presynaptic transmitter density (as in the approach used by Buckman and Meshul, 1997), receptor binding, or receptor subunit composition, for example. Another whole-brain approach would be the histochemical examination of immediate early gene or inducible transcription factor induction. Immediate early genes histochemically reveal where changes in gene transcription have been initiated in response to a stimulus. This approach enables region-specific examination of activated brain regions, and of differential responses to treatment effects or line-specific changes following stimulus presentation. Prior research has supported differential c-*fos* activation to an ethanol challenge in inbred mice. Hitzemann and Hitzemann (1997) showed region-specific activation in the extended amygdala (part of the mesolimbic dopaminergic neural circuit known to mediate the experience of reward) that argues for different molecular mechanisms—and by extension, differential gene activation—in C57BL/6J and DBA/2J inbred strains. The differential cytochemistry underscores the fact that C57BL/6J alcohol-preferring mice and DBA/2J alcohol-avoiding mice have very different alcohol preference profiles, and also experience ethanol challenges differently. For example, C57BL/6J mice are somewhat HIC-resistant and DBA/2J mice are more HIC-sensitive by comparison. Both inbred strains were among the progenitors contributing to the gene pool of the HS/Ibg stock that served as a founding population for the WSP and WSR selected lines.

In summary, experiment 1 clearly showed that ethanol withdrawal severity increased as a function of short-term withdrawal history (*e.g.*, the number of intoxication:withdrawal cycles per phase). In the WSP line, withdrawal-induced hyperexcitability persisted over a 4-day respite preceeding the Phase 2 MW and SW treatments, supporting the occurrence of a kindling-like phenomenon. In experiment 2, the same administration of intoxication and withdrawal cycles was shown to affect 10% EtOH ingestion, but only in WSR mice and in a much less robust manner than kindling-like potentiation of HICs seen in experiment 1. In both experiments, the presence of significant effects of repeated withdrawal experiences supported the idea that the ethanol withdrawal-induced neural changes observed in each experiment were persistent.

REFERENCES

American Psychiatric Association (2000) Alcohol dependence, in Diagnostic and Statistical Manual of Mental Disorders – 4th edition, revised (DSM-IV-R), pp 213-214. American Psychiatric Association, Washington, D.C.

Bachmanov AA, Reed DR, Beauchamp GK, & Tordoff MG (2002) Food intake, water intake, and drinking spout side preference of 28 mouse strains. Behavior Genetics 32:435-443.

Ballenger JC, Post PM (1978) Kindling as a model for alcohol withdrawal syndromes. British Journal of Psychiatry 133:1-14.

Barkley-Levenson A, Cameron AJ, Crabbe JC (2005) Unpublished results on differences in WSP and WSR alcohol consumption.

Bayard M, McIntyre J, Hill KR, Woodside J (2004) Alcohol withdrawal syndrome. American Family Physician 69(6):1443-1450.

Becker HC (1994) Positive relationship between the number of prior ethanol withdrawal episodes and the severity of subsequent withdrawal seizures. Psychopharmacology (Berlin) 116:26-32.

Becker HC (1998) Kindling in alcohol withdrawal. Alcohol Health & Research World 22(1):25-33.

Becker HC (2000) Animal models in alcohol withdrawal. Alcohol Research & Health: Journal of the National Institute of Alcohol Abuse & Alcoholism: Journal of the National Institute of Alcohol Abuse & Alcoholism 24(2):105-113.

Becker HC, Diaz-Granados JL, Hale RL (1997a) Exacerbation of ethanol withdrawal seizures in mice with a history of multiple withdrawal experience. Pharmacology Biochemistry and Behavior 57(1/2):179-183.

Becker HC, Diaz-Granados JL, Weathersby RT (1997b) Repeated ethanol withdrawal experience increases the severity and duration of subsequent withdrawal seizures in mice. Alcohol 14(4):319-326.

Becker HC, Hale RL (1993) Repeated episodes of ethanol withdrawal potentiate the severity of subsequent withdrawal seizures: an animal model of alcohol withdrawal "kindling." Alcoholism: Clinical & Experimental Research 17(1):94-98.

Becker HC, Littleton JM (1996) The alcohol withdrawal "kindling" phenomenon: clinical and experimental findings. Alcoholism: Clinical & Experimental Research 20(8):121A-124A.

Becker HC, Lopez MF (2004) Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice. Alcoholism: Clinical & Experimental Research 28(12):1829-1838.

Begleiter H, Porjesz B (2003) Alcoholism and human electrophysiology. Alcohol Research & Health: Journal of the National Institute of Alcohol Abuse & Alcoholism 27(2):153-160.

Begleiter H, Porjesz B (1999) What is inherited in the predisposition toward alcoholism? A proposed model. Alcoholism: Clinical & Experimental Research 23(7):1125-1135.

Belknap JK, Crabbe JC, Young ER (1993) Voluntary consumption of ethanol in 15 inbred mouse strains. Psychopharmacology (Berlin) 112(4):503-510.

Belknap JK, Richards SP, O'Toole LA, Helms ML, Phillips TJ (1997) Short-term selective breeding as a tool for QTL mapping: ethanol preference drinking in mice. Behavior Genetics 27:55-56.

Booth BM, Blow FC (1993) The kindling hypothesis: further evidence from a U.S. national study of alcoholic men. Alcohol & Alcoholism: International Journal of the Medical Council on Alcoholism 28(5):593-598.

Brennan CH, Crabbe JC, Littleton JH (1990) Genetic regulation of dihydropyridine-sensitive calcium channels in brain may determine susceptibility to physical dependence on alcohol. Neuropharmacology 29:429-432.

Brown ME, Anton RF, Malcolm R, Ballenger JC (1988) Alcohol detoxification and withdrawal seizures: clinical support for a kindling hypothesis. Biological Psychiatry 23:507-514.

Buck KJ, Metten P, Belknap JK, Crabbe JC (1997) Quantitative trait loci involved in genetic predisposition to acute alcohol withdrawal in mice. Journal of Neuroscience 17(10):3946-3955.

Buck KJ, Rademacher BLS, Metten P, Crabbe JC (2002) Mapping murine loci for physical dependence on ethanol. Psychopharmacology (Berlin) 160:398-407.

Buckman JF, Meshul CK (1997) Immunocytochemical analysis of glutamate and GABA in selectively bred mice. Brain Research 760:193-203.

Caetano R, Cunradi C (2002) Alcohol dependence: a public health perspective. Addiction 97:633-645.

Chester JA, Blose AM, Froehlich JC (2003) Further evidence of an inverse genetic relationship between innate differences in alcohol preference and alcohol withdrawal magnitude in multiple selectively bred rat lines. Alcoholism: Clinical & Experimental Research 27(3):377-387.

Chester JA, Price CS, Froehlich JC (2002) Inverse genetic association between alcohol preference and severity of alcohol withdrawal in two sets of rat lines selected for the same phenotype. Alcoholism: Clinical & Experimental Research 26(1):19-27.

Copeland NG, Jenkins NA, Gilbert DJ, Eppig JT, Maltais LJ, Miller JC, Dietrich WF, Weaver A, Lincoln SE, Steen RG, Stein LD, Nadeau JH, Lander ES (1993) A genetic linkage map of the mouse: current applications and future prospects. Science 262(5130):57-66.

Crabbe JC (1983) Sensitivity to ethanol in inbred mice: genotypic correlations among several behavioral responses. Behavioral Neuroscience 97(2):280-289.

Crabbe JC (1996) The 1996 RSA Distinguished Research Award Lecture: A genetic animal model of alcohol withdrawal. Alcoholism: Clinical & Experimental Research 20(8 Supplement):96A-100A.

Crabbe JC (1999a) Animal models in neurobehavioral genetics: methods for estimating genetic correlation, in Neurobehavioral Genetics: Methods and Applications (Mormede P, Jones BC eds), pp 121-138. CRC Press, Boca Raton, Florida.

Crabbe JC (1999b) Molecular genetics of addiction, in Neurobiology of Mental Illness (Charney DS, Nestler EJ, Bunney BS eds), pp 591-600. Oxford University Press, New York, New York.

Crabbe JC (1997) Where does alcohol act in the brain? Molecular Psychiatry 2:17-20.

Crabbe JC, Belknap JK (1992) Genetic approaches to drug dependence. Trends in Pharmacological Sciences 13(5):212-219.

Crabbe JC, Belknap JK (1998) Ethical consequences of mapping QTLs for complex human traits, in Molecular Dissection of Complex Traits (Paterson AH, ed), pp 279-285. CRC Press, Boca Raton, Florida.

Crabbe JC, Kosobud A (1986) Sensitivity and tolerance to ethanol in mice bred to be genetically prone or resistant to ethanol withdrawal seizures. Journal of Pharmacology and Experimental Therapeutics 239(2): 327-333.

Crabbe JC, Kosobud A, Young ER (1983a) Genetic selection for ethanol withdrawal severity: differences in replicate mouse lines. Life Sciences 33(10)955-962.

Crabbe JC, Kosobud A, Young ER (1983b) Peak ethanol withdrawal convulsions in genetically selected mice. Proceedings of the Western Pharmacology Society 26:201-204.

Crabbe JC, Kosobud A, Young ER, Tam BR, McSwigan JD (1985) Bidirectional selection for susceptibility to ethanol withdrawal seizures in *Mus musculus*. Behavior Genetics 15(6):521-536.

Crabbe JC, Merrill CM, Belknap JK (1993) Effect of acute alcohol withdrawal on sensitivity to pro- and anticonvulsant treatments in WSP mice. Alcoholism: Clinical and Experimental Research 17(6):1233-1239.

Crabbe JC, Merrill CM, Kim D, Belknap JK (1990a) Alcohol dependence and withdrawal: a genetic animal model. Annals of Medicine 22:259-263.

Crabbe JC, Phillips TJ (2004) Pharmacogenetic studies of alcohol self-administration and withdrawal. Psychopharmacology (Berlin) 174:539-560.

Crabbe JC, Phillips TJ, Kosobud A, Belknap JK (1990b) Estimation of genetic correlation: interpretation of experiments using selectively bred and inbred animals. Alcoholism: Clinical & Experimental Research 14(2):141-151.

Crabbe JC, Young ER, Janowsky J, Rigter H (1981) Pyrazole exacerbates handling-induced convulsions in mice. Neuropharmacology 20:605-609.

Crabbe JC, Young ER, Kosobud A (1983c) Genetic correlations with ethanol withdrawal severity. Pharmacology, Biochemistry & Behavior 18(Supplement 1):541-547.

Crawley JN (2000) Feeding and drinking, in What's Wrong with My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice (Crawley JN ed), pp 131-149. Wiley-Liss, New York, New York.

Deitrich RA, Dunwiddie TV, Harris RA, Erwin VG (1989) Mechanism of action of ethanol: initial central nervous system actions. Pharmacological Reviews 41(4):489-537.

De Witte Ph., Pinto E, Ansseau M, Verbanck P (2003) Alcohol and withdrawal: from animal research to clinical issues. Neuroscience and Biobehavioral Reviews 27:189-197.

Duka T, Gentry J, Malcolm R, Ripley TL, Borlikova G, Stephens DN, Veatch LM, Becker HC, Crews FT (2004) Consequences of multiple withdrawals from alcohol. Alcoholism: Clinical & Experimental Research 28(2):233-246.

Ellis FW, Pick JR (1969) Ethanol-induced withdrawal reactions in rhesus monkeys. Pharmacologist 11:256.

Essig CF, Lam RC (1968) Convulsions and hallucinatory behavior following alcohol withdrawal in the dog. Archives of Neurology 18:626-632.

Falconer DS, Mackay TFC (1996) Introduction to Quantitative Genetics, 4th edition. Pearson Education Limited, Essex, England.

Fehr C, Shirley RL, Metten P, Kosobud AEK, Belknap JK, Crabbe JC, Buck KJ (2004) Potential pleiotropic effects of Mpdz on vulnerability to seizures. Genes, Brain and Behavior 3:8-19.

Finn DA, Crabbe JC (1997) Exploring alcohol withdrawal syndrome. Alcohol Health and Research World 21(2):149-156.

Finn DA, Crabbe JC (1999) Chronic ethanol differentially alters susceptibility to chemically induced convulsions in Withdrawal Seizure-Prone and –Resistant mice. Journal of Pharmacology and Experimental Therapeutics 288(2):782-790.

Flannery BA, Roberts AJ, Cooney N, Swift RM, Anton RF, Rohsenow DJ (2001) The role of craving in alcohol use, dependence, and treatment. Alcoholism: Clinical & Experimental Research 25(2):299-308.

Freund G (1969) Alcohol withdrawal syndrome in mice. Archives of Neurology 21(3):315-320.

Gilbert (1992) Neurotoxicants and limbic kindling, in The Vulnerable Brain and Environmental Risks (Isaacson RL, Jensen KF eds), pp 173-192. Plenum Press, New York, New York.

Goddard GV, McIntyre DC, Leech CK (1969) A permanent change in brain function resulting from daily electrical stimulation. Experimental Neurology 25:295-330.

Goldstein DB (1972a) An animal model for testing effects of drugs on alcohol withdrawal reactions. Journal of Pharmacology and Experimental Therapeutics 183(1):14-22.

Goldstein DB (1972b) Relationship of alcohol dose to intensity of withdrawal signs in mice. Journal of Pharmacology and Experimental Therapeutics 180(2):203-215.

Goldstein DB (1973a) Convulsions elicited by handling: a sensitive method of measuring CNS excitation in mice treated with reserpine or convulsant drugs. Psychopharmacology (Berlin) 32:27-32.

Goldstein DB (1973b) Inherited differences in intensity of alcohol withdrawal reactions in mice. Nature 245:154-156.

Goldstein DB (1974) Rates of onset and decay of alcohol physical dependence in mice. Journal of Pharmacology and Experimental Therapeutics 190(2):377-383.

Goldstein DB, Pal N (1971) Alcohol dependence produced in mice by inhalation of ethanol: grading the withdrawal reaction. Science 172(980):288-290.

Gonzalez LP (1998) Electrophysiological changes after repeated alcohol withdrawal. Alcohol Health & Research World 22(1):34-37.

Hitzemann R (2000) Animal models of psychiatric disorders and their relevance to alcoholism. Alcohol Research & Health: Journal of the National Institute of Alcohol Abuse & Alcoholism 24(3):149-158.

Hitzemann B, Hitzemann R (1997) Genetics ethanol and the Fos response: a comparison of the C57BL/6J and DBA/2J inbred mouse strains. Alcoholism: Clinical & Experimental Research 21(8):1497-507.

Kalant H, LeBlanc AE, Gibbins RJ (1971) Tolerance to, and dependence on, some non-opiate psychotropic drugs. Pharmacological Reviews 23(3):135-191.

Koob GF (2004) A role for GABA mechanisms in the motivational effects of alcohol. Biochemical Pharmacology 68:1515-1525.

Koob GF, Le Moal M (1997) Drug abuse: hedonic homeostatic dysregulation. Science 278:52-58.

Koob GF, Rocio M, Carrera A, Gold LH, Heyser CJ, Maldonado-Irizarry C, Markou A, Parsons LH, Roberts AJ, Schulteis G, Stinus L, Walker JR, Weissenborn R, Weiss F (1998a) Substance dependence as a compulsive behavior. Journal of Psychopharmacology 12(1):39-48.

Koob GF, Sanna PP, Bloom FE (1998b) Neuroscience of addiction. Neuron 21:467-476.

Kopp C (2001) Locomotor activity rhythm in inbred strains of mice: implications for behavioral studies. Behavioral Brain Research 125:93-96.

Kosobud A, Bodor AS, Crabbe JC (1988) Voluntary consumption of ethanol in WSP, WSC and WSR selectively bred mouse lines. Pharmacology, Biochemistry & Behavior 29:601-607.

Kosobud A, Crabbe JC (1986a) 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 JC (1986b) Sensitivity and tolerance to ethanol in mice bred to be genetically prone or resistant to ethanol withdrawal seizures. Journal of Pharmacology and Experimental Therapeutics 239(2):327-333.

Kosobud A, Crabbe JC (1990) Genetic correlations among inbred strain sensitivities to convulsions induced by 9 convulsant drugs. Brain Research 526:8-16.

Kosobud A, Crabbe JC (1993) Sensitivity to N-methyl-D-aspartic acid-induced convulsions is genetically associated with resistance to ethanol withdrawal seizures. Brain Research 610(1):176-179.

Kosobud A, Cross SJ, Crabbe JC (1992) Neural sensitivity to pentylenetetrazol convulsions in inbred and selectively bred mice. Brain Research 592(1-2):122-128.

Kreek MJ, Koob GF (1998) Drug dependence: stress and dysregulation of brain reward pathways. Drug and Alcohol Dependence 51:23-47.

Littleton J (1998) Neurochemical mechanisms underlying alcohol withdrawal. Alcohol Health & Research World 22(1):13-24.

Lopez MF, Becker HC (2005) Effect of pattern and number of chronic ethanol exposures on subsequent voluntary ethanol intake in C57BL/6J mice. Psychopharmacology (Berlin) 181(4):688-696.

Lovinger DM, Crabbe JC (2005) Laboratory models of alcoholism: treatment target identification and insight into mechanisms. Nature Neuroscience 8(11):1471-1480.

Malcolm R, Herron JE, Anton RF, Roberts J, Moore J (2000) Recurrent detoxification may elevate alcohol craving as measured by the Obsessive Compulsive Drinking scale. Alcohol 20:181-185.

Martin SE (2001) The links between alcohol, crime and the criminal justice system: explanations, evidence and interventions. The American Journal on Addiction 10:136-158.

McClearn GE, Rodgers DA (1959) Differences in alcohol preference among inbred strains of mice. Quarterly Journal of Studies on Alcohol 20:691-695.

McClearn GE, Rodgers DA (1961) Genetic factors in alcohol preference of laboratory mice. Journal of Comparative and Physiological Psychology 54(2):116-119.

McClearn GE, Wilson JR, Petersen DR, Allen DL (1982) Selective breeding in mice for severity of the ethanol withdrawal syndrome. Substance and Alcohol Actions/Misuse 3:135-143.

McCown TJ, Breese GR (1990) Multiple withdrawals from chronic ethanol "kindles" inferior collicular seizure activity: evidence for kindling of seizures associated with alcoholism. Alcoholism: Clinical & Experimental Research 14(3):394-399.

McSwigan JD, Crabbe JC, Young ER (1984) Specific ethanol withdrawal seizures in genetically selected mice. Life Sciences 35(21):2119-2126.

Metten P, Crabbe JC (1996) Dependence and withdrawal, in Pharmacological Effects of Ethanol on the Nervous System (Deitrich RA, Erwin VG eds), pp 269-290. CRC Press, Boca Raton, Florida.

Metten P, Crabbe JC (2005) Alcohol withdrawal severity in inbred mouse (*Mus musculus*) strains. Behavioral Neuroscience 119(4):911-925.

Metten P, Phillips TJ, Crabbe JC, Tarantino LM, McClearn GE, Plomin R, Erwin VG, Belknap JK (1998) High genetic susceptibility to ethanol withdrawal predicts low ethanol consumption. Mammalian Genome 9:983-990.

Nevo I, Hamon M (1995) Neurotransmitter and neuromodulatory mechanisms involved in alcohol abuse and alcoholism. Neurochemistry International 26(4):305-336.

Phillips TJ, Belknap JK, Hitzemann RJ, Buck KJ, Cunningham CL, Crabbe JC (2002) Harnessing the mouse to unravel the genetics of human disease. Genes, Brain and Behavior 1:14-26.

Phillips TJ, Crabbe JC, Metten P, Belknap JK (1994) Localization of genes affecting alcohol drinking in mice. Alcohol: Clinical and Experimental Research 18:931-941.

Plomin R, DeFries JC, McClearn GE, McGuffin P (2001) Behavioral Genetics, 4th edition. Worth Publishers, New York, NEW YORK.

Post RM, Ballenger JC, Putnam FW, Bunney WE (1983) Carbamazepine in alcohol withdrawal syndromes: relationship to the kindling model. Journal of Clinical Psychopharmacology 3(3):204-205.

Roberts AJ, Chu HP, Crabbe JC, Keith LD (1991) Differential modulation by the stress axis of ethanol withdrawal seizure expression in WSP and WSR mice. Alcoholism: Clinical & Experimental Research 15(3):412-417.

Roberts AJ, Cole M, Koob GF (1996) Intra-amygdala muscimol decreases operant ethanol self-administration in rats. Alcoholism: Clinical & Experimental Research 20(7):1289-98.

Ryabinin AE, Bachtell RK, Heinrichs SC, Lee S, Rivier C, Olive MF, Mehmert KK, Camarini R, Kim JA, Koenig HN, Nannini MA, Hodge CW, Roberts AJ, Koob GF (2002) The corticotrophin-releasing factor/urocortin system and alcohol. Alcoholism: Clinical & Experimental Research 26(5):714-722.

Shirley RL, Walter NA, Reilly MT, Fehr C, Buck KJ (2004) *Mpdz* is a quantitative trait gene for drug withdrawal seizures. Nature Neuroscience 7(7):699-700

Spanagel R (2001) Recent animal models of alcoholism. Alcohol Research & Health: Journal of the National Institute of Alcohol Abuse & Alcoholism: Journal of the National Institute of Alcohol Abuse & Alcoholism 24(2):124-131.

Stephens DN, Brown G, Duka T, Ripley TL (2001) Impaired fear conditioning but enhanced seizure sensitivity in rats given repeated experience of withdrawal from alcohol. European Journal of Neuroscience 14:2023-2031.

Tabakoff B, Hoffman PL (2000) Animal models in alcohol research. Alcohol Research & Health: Journal of the National Institute of Alcohol Abuse & Alcoholism 24(2):77-84.

Veatch LM, Becker HC (2002) Electrographic and behavioral indices of ethanol withdrawal sensitization. Brain Research 946:272-282.

Veatch LM, Gonzalez LP (1999) Repeated ethanol withdrawal delays development of focal seizures in hippocampal kindling. Alcoholism: Clinical & Experimental Research 23(7):1145-1150.

Veatch LM, Gonzalez LP (1996) Repeated ethanol withdrawal produces site-dependent increases in EEG spiking. Alcoholism: Clinical and Experimental Research 20:262-267.

Veatch LM, Gonzalez LP (1997) Chronic ethanol retards kindling of hippocampal area CA3. NeuroReport 8:1903-1906.

Westbrook GL (2000) Seizures and epilepsy, in Principles of Neural Science, 4th Edition (Kandel ER, Schwartz JH, Jessell TM eds), pp 910-935. McGraw Hill Health Professions Division, New York, New York.

APPENDIX TABLES

Variable	Hypothesis	Description	Factor	n	df	R^2	F-value	p-value	Effect
D0 AGE ANOVAs		WSP vs. WSR	LINE	144	1,142	0.34	73.86	0.000	WSP > WSR
		MW (TX-grouped)	LINE	79	1, 77	0.38	47.61	0.000	WSP > WSR
		SW (TX-grouped)	LINE	65	1, 63	0.29	25.33	0.000	WSP > WSR
		MW vs. SW	TX	144	1, 142	0.00	0.65	0.422	
		WSP (LINE-grouped)	TX	98	1, 96	0.00	0.05	0.818	
		WSR (LINE-grouped)	TX	46	1, 44	0.00	0.00	0.972	
		2-way ANOVA	LINE	144	1, 140	0.34	68.17	0.000	WSP > WSR
			TX	144	1, 140	0.34	0.03	0.874	
			LINE*TX	144	1, 140	0.34	0.02	0.887	
Day 0 Body Weight ANOVAs		WSP vs. WSR	LINE	144	1, 142	0.04	5.38	0.022	WSP < WSR
		MW (TX-grouped)	LINE	79	1, 77	0.04	3.03	0.086	
		SW (TX-grouped)	LINE	65	1, 63	0.04	2.84	0.097	
		MW vs. SW	TX	144	1, 142	0.00	0.30	0.583	
		WSP (LINE-grouped)	TX	98	1, 96	0.00	0.38	0.542	
		WSR (LINE-grouped)	TX	46	1, 44	0.01	0.52	0.475	
		2-way ANOVA	LINE	144	1, 140	0.04	5.79	0.017	WSP < WSR
			TX	144	1, 140	0.04	0.83	0.364	
			LINE*TX	144	1, 140	0.04	0.05	0.824	
Phase 1 Body Weight ANOVAs		WSP vs. WSR	LINE	144	1, 142	0.06	8.79	0.004	WSP < WSR
		MW (TX-grouped)	LINE	79	1, 77	0.10	8.13	0.006	WSP < WSR
		SW (TX-grouped)	LINE	65	1, 63	0.05	3.35	0.072	
		MW vs. SW	TX	144	1, 142	0.04	5.26	0.023	MW < SW
		WSP (LINE-grouped)	TX	98	1, 96	0.07	6.77	0.011	MW < SW
		WSP-1	TX	47	1, 45	0.06	2.81	0.101	
		WSP-2	TX	51	1, 49	0.07	3.94	0.053	
		WSP, SW	REP	49	1, 47	0.02	0.85	0.362	
		WSR (LINE-grouped)	TX	46	1, 44	0.03	1.36	0.250	
		WSR-1	TX	22	1, 20	0.00	0.03	0.870	
		WSR-2	TX	24	1, 22	0.16	4.09	0.055	
		WSR, SW	REP	16	1, 14	0.67	28.82	0.000	1 > 2
		2-way ANOVA	LINE	144	1, 140	0.11	10.32	0.002	WSP < WSR
			TX	144	1, 140	0.11	5.67	0.019	MW < SW
			LINE*TX	144	1, 140	0.11	0.27	0.602	
Phase 1 Body Weight ANCOVAs (Covariate: Day 0 Weight)		*WSP vs. WSR*	*LINE*	*144*	*1, 141*	*0.79*	*3.63*	*0.059*	
			D0 Wt	*144*	*1, 141*	*0.79*	*490.57*	*0.000*	*WSP < WSR*
		MW (TX-grouped)	*LINE*	*79*	*1, 76*	*0.87*	*9.89*	*0.002*	*WSP < WSR*
			D0 Wt	*79*	*1, 76*	*0.87*	*434.51*	*0.000*	*WSP < WSR*
		SW (TX-grouped)	*LINE*	*65*	*1, 62*	*0.74*	*0.53*	*0.469*	
			D0 Wt	*65*	*1, 62*	*0.74*	*167.87*	*0.000*	*WSP < WSR*
		MW vs. SW	*TX*	*144*	*1, 141*	*0.81*	*15.99*	*0.000*	*MW < SW*
			D0 Wt	*144*	*1, 141*	*0.81*	*560.95*	*0.000*	*MW < SW*
		WSP (LINE-grouped)	*TX*	*98*	*1, 95*	*0.77*	*17.06*	*0.000*	*MW < SW*
			D0 Wt	*98*	*1, 95*	*0.77*	*288.69*	*0.000*	*MW < SW*
		WSR (LINE-grouped)	*TX*	*46*	*1, 43*	*0.92*	*2.60*	*0.114*	
			D0 Wt	*46*	*1, 43*	*0.92*	*468.19*	*0.000*	*MW < SW*
		2-way ANCOVA	*LINE*	*144*	*1, 139*	*0.82*	*5.32*	*0.023*	*WSP < WSR*
			TX	*144*	*1, 139*	*0.82*	*11.94*	*0.001*	*MW < SW*
			*LINE*TX*	*144*	*1, 139*	*0.82*	*2.54*	*0.113*	
			D0 Wt	*144*	*1, 139*	*0.82*	*544.32*	*0.000*	
Phase 2 Body Weight ANOVAs		WSP vs. WSR	LINE	144	1, 142	0.05	7.88	0.006	WSP < WSR
		MW (TX-grouped)	LINE	79	1, 77	0.07	5.52	0.021	WSP < WSR
		SW (TX-grouped)	LINE	65	1, 63	0.09	6.48	0.013	WSP < WSR
		MW vs. SW	TX	144	1, 142	0.06	9.70	0.002	MW < SW
		WSP (LINE-grouped)	TX	98	1, 96	0.08	8.78	0.004	MW < SW
		WSP-1	TX	47	1, 45	0.15	7.71	0.008	MW < SW
		WSP-2	TX	51	1, 49	0.06	2.86	0.097	
		WSP, SW	REP	49	1, 47	0.01	0.62	0.436	
		WSR (LINE-grouped)	TX	46	1, 44	0.10	4.74	0.035	MW < SW
		WSR-1	TX	22	1, 20	0.05	1.14	0.298	
		WSR-2	TX	24	1, 22	0.31	10.09	0.004	MW < SW
		WSR, SW	REP	16	1, 14	0.46	11.90	0.004	1 > 2
		2-way ANOVA	LINE	144	1, 140	0.14	11.52	0.009	WSP < WSR
			TX	144	1, 140	0.14	12.08	0.001	MW < SW
			LINE*TX	144	1, 140	0.14	0.08	0.778	
Phase 2 Body Weight ANCOVAs (Covariate: Day 0 Weight)		*WSP vs. WSR*	*LINE*	*144*	*1, 141*	*0.53*	*2.60*	*0.109*	
			D0 Wt	*144*	*1, 141*	*0.53*	*142.15*	*0.000*	*WSP < WSR*
		MW (TX-grouped)	*LINE*	*79*	*1, 76*	*0.64*	*2.39*	*0.126*	
			D0 Wt	*79*	*1, 76*	*0.64*	*121.63*	*0.000*	*WSP < WSR*
		SW (TX-grouped)	*LINE*	*65*	*1, 62*	*0.46*	*3.43*	*0.069*	
			D0 Wt	*65*	*1, 62*	*0.46*	*42.83*	*0.000*	*WSP < WSR*
		MW vs. SW	*TX*	*144*	*1, 141*	*0.57*	*15.77*	*0.000*	*MW < SW*
			D0 Wt	*144*	*1, 141*	*0.57*	*164.40*	*0.000*	*MW < SW*
		WSP (LINE-grouped)	*TX*	*98*	*1, 95*	*0.48*	*11.33*	*0.001*	*MW < SW*
			D0 Wt	*98*	*1, 95*	*0.48*	*71.34*	*0.000*	*MW < SW*
		WSR (LINE-grouped)	*TX*	*46*	*1, 43*	*0.79*	*9.84*	*0.003*	*MW < SW*
			D0 Wt	*46*	*1, 43*	*0.79*	*139.97*	*0.000*	*MW < SW*
		2-way ANCOVA	*LINE*	*144*	*1, 139*	*0.58*	*5.47*	*0.021*	*WSP < WSR*
			TX	*144*	*1, 139*	*0.58*	*16.32*	*0.000*	*MW < SW*
			*LINE*TX*	*144*	*1, 139*	*0.58*	*0.03*	*0.861*	
			D0 Wt	*144*	*1, 139*	*0.58*	*149.95*	*0.000*	

Appendix Table 1: ANOVA and ANCOVA results for experiment 1. Abbreviations: D=Day; EtOH=ethanol; BEC=blood ethanol concentration; WSP=ethanol Withdrawal Seizure Prone selected line; WSR=ethanol Withdrawal Seizure Resistant selected line; TX=treatment; MW=multiple withdrawal treatment; SW=single withdrawal treatment; REP=replicate; *=by; "()" show order of effect with a one-tailed test.

Variable	Hypothesis	Description	Factor	n	df	R^2	*F*-value	*p*-value	Effect
Phase 1		WSP *vs.* WSR	LINE	138	1, 136	0.08	11.72	0.001	WSP > WSR
D3BEC		MW (TX-grouped)	LINE	75	1, 73	0.23	22.25	0.000	WSP > WSR
ANOVAs	1a	SW (TX-grouped)	LINE	63	1, 61	0.00	0.22	0.642	
		MW *vs.* SW	TX	138	1, 136	0.00	0.16	0.686	
	2a	WSP (LINE-grouped)	TX	93	1, 91	0.03	3.24	0.075	
		WSP-1	TX	46	1, 44	0.01	0.63	0.430	
		WSP-2	TX	47	1, 45	0.06	2.67	0.109	
		WSP (LINE-grouped)	REP	93	1, 91	0.05	5.16	0.025	1 < 2
	1b	WSP, SW	REP	48	1, 46	0.02	1.01	0.320	
	4	WSP (LINE-grouped)	REP	93	1, 89	0.10	5.35	0.023	1 < 2
			TX	93	1, 89	0.10	3.25	0.075	
			REP*TX	93	1, 89	0.10	1.05	0.309	
	2a	WSR (LINE-grouped)	TX	45	1, 43	0.26	14.94	0.000	MW < SW
		WSR-1	TX	22	1, 20	0.40	13.58	0.001	MW < SW
		WSR-2	TX	23	1, 21	0.11	2.56	0.125	
		WSR (LINE-grouped)	REP	45	1, 43	0.00	0.08	0.785	
	1b	WSR, SW	REP	15	1, 13	0.13	1.96	0.185	
	4	WSR (LINE-grouped)	REP	45	1, 41	0.32	0.49	0.488	
			TX	45	1, 41	0.32	15.05	0.000	MW < SW
			REP*TX	45	1, 41	0.32	3.55	0.067	
	3	2-way ANOVA	LINE	138	1, 134	0.15	7.16	0.008	WSP > WSR
			TX	138	1, 134	0.15	1.40	0.238	
			LINE*TX	138	1, 134	0.15	11.42	0.001	
Phase 1		WSP *vs.* WSR	LINE	144	1, 142	0.82	661.73	0.000	WSP > WSR
HIC AUC25		MW (TX-grouped)	LINE	79	1, 77	0.94	1132.19	0.000	WSP > WSR
ANOVAs	1a	SW (TX-grouped)	LINE	65	1, 63	0.71	155.62	0.000	WSP > WSR
		MW *vs.* SW	TX	144	1, 142	0.00	0.08	0.774	
	2a	WSP (LINE-grouped)	TX	98	1, 96	0.19	21.92	0.000	MW > SW
		WSP-1	TX	47	1, 45	0.27	16.45	0.000	MW > SW
		WSP-2	TX	51	1, 49	0.17	9.86	0.003	MW > SW
		WSP (LINE-grouped)	REP	98	1, 96	0.17	19.63	0.000	1 < 2
	1b	WSP, SW	REP	49	1, 47	0.23	13.65	0.001	1 < 2
		WSP (LINE-grouped)	REP	98	1, 94	0.36	23.90	0.000	1 < 2
			TX	98	1, 94	0.36	26.82	0.000	MW > SW
			REP*TX	98	1, 94	0.36	1.81	0.182	
		WSR (LINE-grouped)	TX	46	1, 44	0.03	1.49	0.229	
		WSR-1	TX	22	1, 20	0.03	0.56	0.462	
		WSR-2	TX	24	1, 22	0.12	3.08	0.093	
		WSR (LINE-grouped)	REP	46	1, 44	0.17	8.85	0.005	1 < 2
	1b	WSR SW	REP	16	1, 14	0.01	0.10	0.753	
		WSR (LINE-grouped)	REP	46	1, 42	0.26	5.43	0.025	1 < 2
			TX	46	1, 42	0.26	1.49	0.229	
			REP*TX	46	1, 42	0.26	3.48	0.069	
		2-way ANOVA	LINE	144	1, 140	0.86	751.21	0.000	WSP > WSR
			TX	144	1, 140	0.86	10.65	0.001	MW > SW
			LINE*TX	144	1, 140	0.86	8.32	0.005	
Phase 1		*WSP vs. WSR*	*LINE*	*138*	*1, 135*	*0.82*	*558.16*	*0.000*	*WSP > WSR*
HIC AUC25			*D3BEC*	*138*	*1, 135*	*0.82*	*0.73*	*0.393*	
ANCOVAs		*MW (TX-grouped)*	*LINE*	*75*	*1, 72*	*0.94*	*789.50*	*0.000*	*WSP > WSR*
(Covariate: D3BEC)			*D3BEC*	*75*	*1, 72*	*0.94*	*0.33*	*0.567*	
		SW (TX-grouped)	*LINE*	*63*	*1, 60*	*0.70*	*141.27*	*0.000*	*WSP > WSR*
			D3BEC	*63*	*1, 60*	*0.70*	*0.06*	*0.809*	
		MW vs. SW	*TX*	*138*	*1, 135*	*0.08*	*0.01*	*0.936*	
			D3BEC	*138*	*1, 135*	*0.08*	*11.94*	*0.001*	
		WSP (LINE-grouped)	*TX*	*93*	*1, 90*	*0.18*	*19.09*	*0.000*	*MW > SW*
			D3BEC	*93*	*1, 90*	*0.18*	*0.00*	*0.962*	
		WSP (LINE-grouped)	*REP*	*98*	*1, 90*	*0.16*	*16.74*	*0.000*	*1 < 2*
			D3BEC	*93*	*1, 90*	*0.16*	*0.04*	*0.841*	
	4	*WSP (LINE-grouped)*	*REP*	*93*	*1, 88*	*0.36*	*22.19*	*0.000*	*1 < 2*
			TX	*93*	*1, 88*	*0.36*	*25.26*	*0.000*	*MW > SW*
			*REP*TX*	*93*	*1, 88*	*0.36*	*1.36*	*0.248*	
			D3BEC	*93*	*1, 88*	*0.36*	*1.08*	*0.302*	
	2a	*WSR (LINE-grouped)*	*TX*	*45*	*1, 42*	*0.18*	*7.53*	*0.009*	*MW > SW*
			D3BEC	*45*	*1, 42*	*0.18*	*6.26*	*0.016*	
		WSR (LINE-grouped)	*REP*	*45*	*1, 42*	*0.19*	*8.25*	*0.006*	*1 < 2*
			D3BEC	*45*	*1, 42*	*0.19*	*2.00*	*0.165*	
	4	*WSR (LINE-grouped)*	*REP*	*45*	*1, 40*	*0.37*	*5.02*	*0.031*	*1 < 2*
			TX	*45*	*1, 40*	*0.37*	*7.10*	*0.011*	*MW > SW*
			*REP*TX*	*45*	*1, 40*	*0.37*	*2.99*	*0.091*	
			D3BEC	*45*	*1, 40*	*0.37*	*4.93*	*0.032*	
	3	*2-way ANCOVA*	*LINE*	*138*	*1, 133*	*0.85*	*653.29*	*0.000*	*WSP > WSR*
			TX	*138*	*1, 133*	*0.85*	*10.10*	*0.002*	*MW > SW*
			*LINE*TX*	*138*	*1, 133*	*0.85*	*6.58*	*0.011*	
			D3BEC	*138*	*1, 133*	*0.85*	*0.01*	*0.909*	

Appendix Table 1: ANOVA and ANCOVA results for experiment 1, continued.

Variable	Hypothesis	Description	Factor	n	df	R^2	F-value	p-value	Effect
Phase 2		WSP *vs*. WSR	LINE	142	1, 140	0.01	1.64	0.202	
D10BEC		MW (TX-grouped)	LINE	77	1, 75	0.02	1.26	0.265	
ANOVAs		SW (TX-grouped)	LINE	65	1, 63	0.00	0.13	0.715	
		MW *vs*. SW	TX	142	1, 140	0.00	0.40	0.530	
	2a	WSP (LINE-grouped)	TX	97	1, 95	0.00	0.00	0.974	
		WSP-1	TX	46	1, 44	0.00	0.03	0.873	
		WSP-2	TX	51	1, 49	0.00	0.01	0.914	
		WSP (LINE-grouped)	REP	97	1, 95	0.05	4.68	0.033	1 > 2
		WSP, SW	REP	49	1, 47	0.06	2.90	0.095	
	4	WSP (LINE-grouped)	REP	97	1, 93	0.05	4.59	0.035	1 > 2
			TX	97	1, 93	0.05	0.00	0.965	
			REP*TX	97	1, 93	0.05	0.04	0.848	
	2a	WSR (LINE-grouped)	TX	45	1, 43	0.01	0.51	0.480	
		WSR-1	TX	22	1, 20	0.03	0.60	0.447	
		WSR-2	TX	23	1, 21	0.01	0.14	0.715	
		WSR (LINE-grouped)	REP	45	1, 43	0.00	0.01	0.921	
		WSR, SW	REP	16	1, 14	0.00	0.06	0.816	
	4	WSR (LINE-grouped)	REP	45	1, 41	0.01	0.01	0.922	
			TX	45	1, 41	0.01	0.48	0.491	
			REP*TX	45	1, 41	0.01	0.00	0.957	
	3	2-way ANOVA	LINE	143	1, 138	0.02	1.10	0.297	
			TX	143	1, 138	0.02	0.46	0.500	
			LINE*TX	143	1, 138	0.02	0.41	0.521	
Phase 2		WSP *vs*. WSR	LINE	144	1, 142	0.83	679.85	0.000	WSP > WSR
HIC AUC25		MW (TX-grouped)	LINE	79	1, 77	0.95	1396.36	0.000	WSP > WSR
ANOVAs		SW (TX-grouped)	LINE	65	1, 63	0.72	159.12	0.000	WSP > WSR
		MW *vs*. SW	TX	144	1, 142	0.00	0.23	0.634	
	2a	WSP (LINE-grouped)	TX	98	1, 96	0.23	29.19	0.000	MW > SW
		WSP-1	TX	47	1, 45	0.32	21.19	0.000	MW > SW
		WSP-2	TX	51	1, 49	0.19	11.46	0.001	MW > SW
		WSP (LINE-grouped)	REP	98	1, 96	0.09	9.86	0.002	1 < 2
		WSP, SW	REP	49	1, 47	0.13	6.86	0.012	1 < 2
		WSP (LINE-grouped)	REP	98	1, 94	0.33	12.19	0.001	1 < 2
			TX	98	1, 94	0.33	32.21	0.000	MW > SW
			REP*TX	98	1, 94	0.33	1.04	0.310	
	2a	WSR (LINE-grouped)	TX	98	1, 96	0.08	4.00	0.052	(MW > SW)
		WSR-1	TX	22	1, 20	0.07	1.47	0.239	
		WSR-2	TX	24	1, 22	0.14	3.65	0.069	
		WSR (LINE-grouped)	REP	46	1, 44	0.13	6.47	0.015	1 < 2
		WSR SW	REP	16	1, 14	0.08	1.27	0.278	
	4	WSR (LINE-grouped)	REP	46	1, 42	0.25	4.04	0.051	
			TX	46	1, 42	0.25	4.16	0.048	MW > SW
			REP*TX	46	1, 42	0.25	2.52	0.120	
	3	2-way ANOVA	LINE	144	1, 140	0.87	819.98	0.000	WSP > WSR
			TX	144	1, 140	0.87	14.00	0.000	MW > SW
			LINE*TX	144	1, 140	0.87	11.41	0.001	
Phase 2		*WSP vs. WSR*	*LINE*	*142*	*1, 139*	*0.83*	*646.31*	*0.000*	*WSP > WSR*
HIC AUC25			*D10BEC*	*142*	*1, 139*	*0.83*	*0.18*	*0.668*	
ANCOVAs		*MW (TX-grouped)*	*LINE*	*77*	*1, 74*	*0.95*	*1318.99*	*0.000*	*WSP > WSR*
(Covariate: D10BEC)			*D10BEC*	*77*	*1, 74*	*0.95*	*2.38*	*0.127*	
		SW (TX-grouped)	*LINE*	*65*	*1, 63*	*0.72*	*158.09*	*0.000*	*WSP > WSR*
			D10BEC	*65*	*1, 63*	*0.72*	*0.43*	*0.517*	
		MW vs. SW	*TX*	*142*	*1, 139*	*0.02*	*0.34*	*0.561*	
			D10BEC	*142*	*1, 139*	*0.02*	*1.88*	*0.172*	
		WSP (LINE-grouped)	*TX*	*97*	*1, 94*	*0.23*	*28.43*	*0.000*	*MW > SW*
			D10BEC	*97*	*1, 94*	*0.23*	*0.10*	*0.749*	
		WSP (LINE-grouped)	*REP*	*97*	*1, 94*	*0.11*	*11.12*	*0.001*	*1 < 2*
			D10BEC	*97*	*1, 94*	*0.11*	*0.99*	*0.322*	
	4	*WSP (LINE-grouped)*	*REP*	*97*	*1, 92*	*0.34*	*13.19*	*0.000*	*1 < 2*
			TX	*97*	*1, 92*	*0.34*	*31.29*	*0.000*	*MW > SW*
			*REP*TX*	*97*	*1, 92*	*0.34*	*0.94*	*0.335*	
			D10BEC	*97*	*1, 92*	*0.34*	*1.22*	*0.273*	
		WSR (LINE-grouped)	*TX*	*45*	*1, 43*	*0.34*	*6.72*	*0.013*	*MW > SW*
			D10BEC	*45*	*1, 43*	*0.34*	*17.14*	*0.000*	
		WSR (LINE-grouped)	*REP*	*45*	*1, 42*	*0.35*	*7.48*	*0.009*	*1 < 2*
			D10BEC	*45*	*1, 42*	*0.35*	*15.64*	*0.000*	
		WSR (LINE-grouped)	*REP*	*45*	*1, 40*	*0.49*	*5.54*	*0.024*	*1 < 2*
			TX	*45*	*1, 40*	*0.49*	*7.88*	*0.008*	*MW > SW*
			*REP*TX*	*45*	*1, 40*	*0.49*	*2.85*	*0.099*	
			D10BEC	*45*	*1, 40*	*0.49*	*21.32*	*0.000*	
		2-way ANCOVA	*LINE*	*142*	*1, 137*	*0.87*	*788.55*	*0.000*	*WSP > WSR*
			TX	*142*	*1, 137*	*0.87*	*13.58*	*0.000*	*MW > SW*
			*LINE*TX*	*142*	*1, 137*	*0.87*	*11.07*	*0.001*	
			D10BEC	*142*	*1, 137*	*0.87*	*0.27*	*0.606*	

Appendix Table 1: ANOVA and ANCOVA results for experiment 1, continued.

Variable	Hypothesis	Description	Factor	n	df	R^2	F-value	p-value	Effect
D3D10AVGBEC		WSP *vs.* WSR	**TX**	**144**	**1, 142**	**0.07**	**11.27**	**0.001**	**MW < SW**
ANOVAs		WSP (LINE-grouped)	TX	98	1, 96	0.02	1.77	0.187	
		WSR (LINE-grouped)	**TX**	**46**	**1, 44**	**0.14**	**7.44**	**0.009**	**MW < SW**
Repeated Measures	2b	WSP (LINE-grouped)	**TX**	**98**	**1, 96**	**0.22**	**27.61**	**0.000**	**Phase 1 < Phase 2**
ANOVAs			**Repeated Withdrawal**	**98**	**1, 96**	**0.25**	**32.83**	**0.000**	**Phase 1 < Phase 2**
Phase 1 & Phase 2			Repeated Withdrawal * TX	98	1, 96	0.03	2.99	0.087	(Phase 1 < Phase 2)
HIC AUC25	2b	WSR (LINE-grouped)	TX	46	1, 44	0.07	3.09	0.086	(Phase 1 > Phase 2)
			Repeated Withdrawal	**46**	**1, 44**	**0.13**	**6.59**	**0.014**	**Phase 1 > Phase 2**
			Repeated Withdrawal * TX	46	1, 44	0.00	0.00	0.998	
Repeated Measures		*WSP vs. WSR*	***TX***	***144***	***1, 141***	***0.82***	***653.45***	***0.000***	***Phase 1 < Phase 2***
ANCOVAs			*D3D10AVGBEC*	*144*	*1, 141*	*0.00*	*0.17*	*0.680*	
(Covariate=D3D10AVGBEC)			***Repeated Withdrawal***	***144***	***1, 141***	***0.09***	***13.38***	***0.000***	***Phase 1 < Phase 2***
Phase 1 & Phase 2			***Repeated Withdrawal * TX***	***144***	***1, 141***	***0.16***	***26.96***	***0.000***	***Phase 1 < Phase 2***
HIC AUC25			***Repeated Withdrawal * D3D10AVGBEC***	***144***	***1, 141***	***0.06***	***8.23***	***0.005***	***Phase 1 < Phase 2***
	2b	*WSP (LINE-grouped)*	***TX***	***98***	***1, 95***	***0.22***	***27.40***	***0.000***	***Phase 1 < Phase 2***
			D3D10AVGBEC	*98*	*1, 95*	*0.00*	*0.14*	*0.706*	
			Repeated Withdrawal	***98***	***1, 95***	***0.18***	***21.04***	***0.000***	***Phase 1 < Phase 2***
			Repeated Withdrawal * TX	***98***	***1, 95***	***0.05***	***4.96***	***0.028***	***Phase 1 < Phase 2***
			Repeated Withdrawal * D3D10AVGBEC	***98***	***1, 95***	***0.10***	***10.35***	***0.002***	***Phase 1 < Phase 2***
	2b	*WSR (LINE-grouped)*	***TX***	***46***	***1, 43***	***0.16***	***8.38***	***0.006***	***Phase 1 > Phase 2***
			D3D10AVGBEC	***46***	***1, 43***	***0.17***	***8.82***	***0.005***	***Phase 1 > Phase 2***
			Repeated Withdrawal	*46*	*1, 43*	*0.01*	*0.51*	*0.477*	
			*Repeated Withdrawal * TX*	*46*	*1, 43*	*0.00*	*0.00*	*0.973*	
			*Repeated Withdrawal * D3D10AVGBEC*	*46*	*1, 43*	*0.00*	*0.01*	*0.933*	

Appendix Table 1: ANOVA and ANCOVA results for experiment 1, continued.

Selected Line	Replicate	Treatment	Statistic	D0 Age	D3BEC	Phase 1 HIC AUC25 Scores									
						Desc Stats	H1-ANOVA	H2a-ANOVA	H2a-ANCOVA	H2b-ANOVA	H2b-ANCOVA	H3-ANOVA	H3-ANCOVA	H4-ANOVA	H4-ANCOVA
			n	98	93	98									
			Mean	63.39	1.36	94.74						94.74	94.34		
WSP			SEM	0.79	0.05	2.45						1.85	1.96		
			n	46	45	46									
			Mean	53.33	1.07	2.30						2.11	1.96		
WSR			SEM	0.28	0.05	0.50						2.83	2.98		
			n	69	68	69									
			Mean	59.14	1.19	57.77								57.52	58.95
	1		SEM	0.88	0.05	5.35								5.74	5.61
			n	75	70	75									
			Mean	61.12	1.34	72.07								72.22	69.31
	2		SEM	0.99	0.07	5.75								5.53	5.55
			n	79	75	79									
			Mean	59.68	1.25	66.25						53.94	53.77	65.94	64.35
		MW	SEM	0.91	0.06	5.81						2.12	2.21	5.36	5.31
			n	65	63	65									
			Mean	60.77	1.28	63.95						42.91	42.52	63.80	63.91
		SW	SEM	0.99	0.05	5.29						2.63	2.76	5.90	5.79
			n	47	46	47									
			Mean	62.21	1.24	84.40									
WSP	1		SEM	1.01	0.05	3.70									
			n	51	47	51									
			Mean	64.47	1.48	104.27									
WSP	2		SEM	1.19	0.09	2.63									
			n	22	22	22									
			Mean	52.59	1.08	0.86									
WSR	1		SEM	0.46	0.10	0.39									
			n	24	23	24									
			Mean	54.00	1.05	3.63									
WSR	2		SEM	0.27	0.06	0.81									
			n	49	45	49									
			Mean	63.57	1.46	105.13		105.13	104.77	105.13	104.98	105.13	104.67		
WSP		MW	SEM	1.14	0.08	2.34		3.14	3.39	3.14	3.16	2.61	2.86		
			n	49	48	49									
			Mean	63.20	1.27	84.36	84.36	84.36	83.99	84.36	84.51	84.36	84.01		
WSP		SW	SEM	1.11	0.07	3.77	3.30	3.14	3.28	3.14	3.16	2.61	2.69		
			n	30	30	30									
			Mean	53.33	0.94	2.75		2.75	3.23	2.75	3.03	2.75	2.88		
WSR		MW	SEM	0.35	0.05	0.68		0.62	0.60	0.62	0.62	3.34	3.59		
			n	16	15	16									
			Mean	53.31	1.33	1.47	1.47	1.47	0.10	1.47	0.94	1.47	1.04		
WSR		SW	SEM	0.46	0.10	0.66	5.77	0.85	0.89	0.85	0.87	4.57	4.81		
			n	37	36	37									
			Mean	58.73	1.12	60.95								60.95	63.79
	1	MW	SEM	1.21	0.07	8.13								7.82	7.74
			n	32	32	32									
			Mean	59.63	1.27	54.09								54.09	54.10
	1	SW	SEM	1.31	0.06	6.76								8.41	8.12
			n	42	39	42									
			Mean	60.52	1.37	70.93								70.93	64.90
	2	MW	SEM	1.34	0.10	8.29								7.34	7.40
			n	33	31	33									
			Mean	61.88	1.30	73.52								73.52	73.72
	2	SW	SEM	1.49	0.09	7.84								8.28	8.25
			n	23	22	7.8									
			Mean	62.57	1.28	97.65									
WSP	1	MW	SEM	1.41	0.08	3.48									
			n	24	24	24									
			Mean	61.88	1.20	71.71	71.71								
WSP	1	SW	SEM	1.46	0.06	5.30	4.79								
			n	26	23	26									
			Mean	64.46	1.63	111.75									
WSP	2	MW	SEM	1.75	0.13	2.58									
			n	25	24	25									
			Mean	64.48	1.34	96.50	96.50								
WSP	2	SW	SEM	1.65	0.12	4.17	4.70								
			n	14	14	14									
			Mean	52.43	0.87	0.64									
WSR	1	MW	SEM	0.59	0.08	0.18									
			n	8	8	8									
			Mean	52.88	1.45	1.25	1.25								
WSR	1	SW	SEM	0.77	0.16	1.04	0.97								
			n	16	16	16									
			Mean	54.13	0.99	4.59									
WSR	2	MW	SEM	0.31	0.08	1.08									
			n	8	7	8									
			Mean	53.75	1.19	1.69	1.69								
WSR	2	SW	SEM	0.53	0.07	0.88	0.97								

Appendix Table 2: Descriptive statistics for Day 0 Weight, D3BEC, and Phase 1 HIC AUC25 values for experiment 1 (kindling-like potentiation of handling-induced convulsions). D3BEC represents the blood ethanol concentrations (BECs; BEC singular) immediately preceding ethanol-withdrawal handling-induced convulsion scoring (third and final day of MW or SW treatment application). Area under the curve (AUC) scores reflect the composite HIC scoring for 25 hours of ethanol withdrawal. "Adjusted values" refer to the least squares mean and corresponding standard error calculated for ANOVA or ANCOVA analyses. See text for additional information. Additional abbreviations: D=day; H=hypothesis; WSP=ethanol Withdrawal Seizure-Prone selected line; WSR=ethanol Withdrawal Seizure-Resistant selected line; MW=multiple withdrawal treatment; SW=single withdrawal treatment.

Selected Line	Replicate	Treatment	Statistic	D10BEC	Phase 2 HIC AUC25 Scores									D3&D10 Mean BEC
					Desc Stats	H2a-ANOVA	H2a-ANCOVA	H2b-ANOVA	H2b-ANCOVA	H3-ANOVA	H3-ANCOVA	H4-ANOVA	H4-ANCOVA	
			n	97	98									98
			Mean	1.38	101.95					101.95	101.91			1.37
WSP			SEM	0.05	2.64					1.93	1.96			0.04
			n	45	46									46
			Mean	1.27	1.09					0.89	0.89			1.17
WSR			SEM	0.08	0.32					2.96	3.01			0.05
			n	68	69									69
			Mean	1.41	63.91							63.61	61.91	1.31
	1		SEM	0.05	5.89							6.28	6.31	0.04
			n	74	75									75
			Mean	1.28	75.09							75.10	76.85	1.30
	2		SEM	0.06	6.27							6.05	6.07	0.04
			n	77	79									79
			Mean	1.32	71.61					58.02	58.01	71.38	71.86	1.29
		MW	SEM	0.07	6.38					2.21	2.27	5.87	5.90	0.05
			n	65	65									65
			Mean	1.37	67.45					44.82	44.79	67.33	66.90	1.33
		SW	SEM	0.04	5.67					2.75	2.78	6.46	6.41	0.03
			n	46	47									47
			Mean	1.48	93.68									1.37
WSP	1		SEM	0.07	3.84									0.05
			n	51	51									51
			Mean	1.28	109.57									1.37
WSP	2		SEM	0.06	3.33									0.05
			n	22	22									22
			Mean	1.27	0.30									1.18
WSR	1		SEM	0.08	0.11									0.06
			n	23	24									24
			Mean	1.26	1.81									1.16
WSR	2		SEM	0.14	0.56									0.07
			n	48	49									49
			Mean	1.38	114.51	114.51	114.55	114.51	114.89	114.51	114.49			1.42
WSP		MW	SEM	0.08	2.34	3.29	3.36	3.29	3.30	2.73	2.78			0.06
			n	49	49									49
			Mean	1.38	89.39	89.39	89.39	89.39	89.01	89.39	89.32			1.32
WSP		SW	SEM	0.05	4.02	3.29	3.32	3.29	3.30	2.73	2.76			0.04
			n	29	30									30
			Mean	1.22	1.53	1.53	1.41	1.53	1.82	1.53	1.53			1.08
WSR		MW	SEM	0.12	0.46	0.38	0.30	0.38	0.35	3.49	3.60			0.06
			n	16	16									16
			Mean	1.34	0.25	0.25	0.11	0.25	-0.30	0.25	0.25			1.33
WSR		SW	SEM	0.05	0.11	0.52	0.40	0.52	0.48	4.77	4.82			0.06
			n	36	37									37
			Mean	1.39	67.74							67.74	65.81	1.28
	1	MW	SEM	0.09	8.93							8.56	8.62	0.07
			n	32	32									32
			Mean	1.44	59.47							59.47	58.01	1.35
	1	SW	SEM	0.05	7.46							9.20	9.18	0.04
			n	41	42									42
			Mean	1.26	75.01							75.01	77.91	1.30
	2	MW	SEM	0.09	9.12							8.03	8.11	0.07
			n	33	33									33
			Mean	1.30	75.18							75.18	75.79	1.30
	2	SW	SEM	0.07	8.40							9.06	9.00	0.05
			n	22	23									23
			Mean	1.49	108.74									1.41
WSP	1	MW	SEM	0.12	2.82									0.09
			n	24	24									24
			Mean	1.47	79.25									1.34
WSP	1	SW	SEM	0.06	5.65									0.05
			n	26	26									26
			Mean	1.28	119.62									1.42
WSP	2	MW	SEM	0.09	3.38									0.08
			n	25	25									25
			Mean	1.29	99.12									1.31
WSP	2	SW	SEM	0.08	5.08									0.06
			n	14	14									14
			Mean	1.23	0.39									1.05
WSR	1	MW	SEM	0.12	0.15									0.07
			n	8	8									8
			Mean	1.35	0.13									1.40
WSR	1	SW	SEM	0.06	0.13									0.08
			n	15	16									16
			Mean	1.22	2.53									1.10
WSR	2	MW	SEM	0.21	0.78									0.10
			n	8	8									8
			Mean	1.33	0.38									1.26
WSR	2	SW	SEM	0.09	0.18									0.07

Appendix Table 3: Descriptive statistics for D10BEC, Phase 2 HIC AUC25 values, and the average of D3 and D10 BEC values used for repeated measures analyses in experiment 1 (kindling-like potentiation of handling-induced convulsions). D10BEC represents the blood ethanol concentrations (BECs; BEC singular) immediately preceding ethanol-withdrawal handling-induced convulsion scoring (third and final day of MW or SW treatment application). Area under the curve (AUC) scores reflect the composite HIC scoring for 25 hours of ethanol withdrawal. "Adjusted values" refer to the least squares mean and corresponding standard error calculated for ANOVA or ANCOVA analyses. See text for additional information. Additional abbreviations: D=day; H=hypothesis; WSP=ethanol Withdrawal Seizure-Prone selected line; WSR=ethanol Withdrawal Seizure-Resistant selected line; MW=multiple withdrawal treatment; SW=single withdrawal treatment.

Variable	Hypothesis	Description	Factor	n	df	R^2	*F*-value	*p*-value	Effect
D0 AGE		WSP *vs.* WSR	LINE	94	1, 92	0.01	0.99	0.323	
ANOVAs		MW (tx-grouped)	LINE	39	1, 37	0.00	0.11	0.738	
		SW (tx-grouped)	LINE	55	1, 53	0.00	0.00	0.963	
		MW *vs.* SW	TX	94	1, 92	0.00	0.08	0.773	
		WSP (LINE-grouped)	TX	45	1, 43	0.01	0.24	0.627	
		WSR (LINE-grouped)	TX	49	1, 47	0.02	0.79	0.379	
		2-way ANOVA	LINE	94	1, 90	0.01	0.92	0.339	
			TX	94	1, 90	0.01	0.09	0.768	
			LINE*TX	94	1, 90	0.01	0.06	0.813	
Phase 0		WSP *vs.* WSR	LINE	94	1, 92	0.03	3.11	0.081	(WSP < WSR)
Body Weight		MW (tx-grouped)	**LINE**	**45**	**1, 43**	**0.11**	**5.48**	**0.024**	**WSP < WSR**
ANOVAs		SW (tx-grouped)	LINE	49	1, 47	0.00	0.09	0.760	
		MW *vs.* SW	TX	94	1, 92	0.03	2.69	0.104	
		WSP (LINE-grouped)	TX	39	1, 37	0.00	0.00	0.998	
		WSR (LINE-grouped)	TX	55	1, 53	0.07	3.87	0.054	(MW > SW)
		2-way ANOVA	LINE	94	1, 90	0.08	3.27	0.074	(WSP < WSR)
			TX	94	1, 90	0.08	1.84	0.178	
			LINE*TX	94	1, 90	0.08	1.85	0.177	
Phase 1		WSP *vs.* WSR	LINE	94	1, 92	0.00	0.29	0.589	
Body Weight		MW (tx-grouped)	LINE	45	1, 43	0.01	0.63	0.433	
ANOVAs		SW (tx-grouped)	LINE	49	1, 47	0.00	0.01	0.944	
		MW *vs.* SW	TX	94	1, 92	0.02	1.42	0.236	
		WSP (LINE-grouped)	TX	38	1, 36	0.00	0.07	0.797	
		WSR (LINE-grouped)	TX	55	1, 53	0.03	1.77	0.190	
		2-way ANOVA	LINE	94	1, 90	0.02	0.29	0.591	
			TX	94	1, 90	0.02	1.09	0.300	
			LINE*TX	94	1, 90	0.02	0.40	0.527	
Phase 1	1	WSP *vs.* WSR	LINE	94	1, 92	0.03	3.02	0.086	(WSP < WSR)
Baseline		MW (tx-grouped)	LINE	45	1, 43	0.02	1.06	0.309	
Voluntary		SW (tx-grouped)	LINE	49	1, 47	0.04	1.84	0.181	
EtOH		MW *vs.* SW	TX	94	1, 92	0.01	0.90	0.344	
Consumption		WSP (LINE-grouped)	TX	39	1, 37	0.02	0.87	0.357	
ANOVAs		WSR (LINE-grouped)	TX	55	1, 53	0.00	0.23	0.632	
		2-way ANOVA	LINE	94	1, 90	0.04	2.84	0.096	(WSP < WSR)
			TX	94	1, 90	0.04	0.88	0.351	
			LINE*TX	94	1, 90	0.04	0.06	0.800	
Phase 1	1	WSP *vs.* WSR	LINE	94	1, 92	0.01	0.52	0.475	
Baseline		MW (tx-grouped)	LINE	45	1, 43	0.00	0.01	0.939	
Voluntary		SW (tx-grouped)	LINE	49	1, 47	0.02	0.75	0.390	
10% EtOH		MW *vs.* SW	TX	94	1, 92	0.00	0.31	0.577	
Preference		WSP (LINE-grouped)	TX	39	1, 37	0.46	0.55	0.546	
Ratio		WSR (LINE-grouped)	TX	55	1, 53	0.00	0.00	0.958	
ANOVAs		2-way ANOVA	LINE	94	1, 90	0.01	0.44	0.507	
			TX	94	1, 90	0.01	0.39	0.534	
			LINE*TX	94	1, 90	0.01	0.31	0.579	

Appendix Table 4: ANOVA and ANCOVA results for experiment 2. Abbreviations: D=Day; EtOH=ethanol; BEC=blood ethanol concentration; WSP=ethanol Withdrawal Seizure Prone selected line; WSR=ethanol Withdrawal Seizure Resistant selected line; TX=treatment; MW=multiple withdrawal treatment; SW=single withdrawal treatment; REP=replicate; *=by; "()" show order of effect with a one-tailed test.

Variable	Hypothesis	Description	Factor	n	df	R^2	*F*-value	*p*-value	Effect
Phase 2		WSP *vs.* WSR	LINE	94	1, 92	0.21	25.01	0.000	WSP < WSR
Body Weight		MW (TX-grouped)	LINE	45	1, 43	0.23	12.63	0.001	WSP < WSR
ANOVAs		SW (TX-grouped)	LINE	49	1, 47	0.24	15.04	0.000	WSP < WSR
		MW *vs.* SW	TX	94	1, 92	0.06	6.01	0.016	MW < SW
		WSP (LINE-grouped)	TX	39	1, 37	0.10	4.13	0.049	MW < SW
		WSR (LINE-grouped)	TX	55	1, 53	0.08	4.59	0.037	MW < SW
		2-way ANOVA	LINE	94	1, 90	0.28	27.55	0.000	WSP < WSR
			TX	94	1, 90	0.28	8.24	0.005	MW < SW
			LINE*TX	94	1, 90	0.28	0.00	0.959	
Phase 2		WSP *vs.* WSR	LINE	93	1, 91	0.02	2.33	0.131	
D13BEC		MW (TX-grouped)	LINE	44	1, 42	0.16	8.17	0.007	WSP < WSR
ANOVAs		SW (TX-grouped)	LINE	49	1, 47	0.01	0.30	0.584	
		MW *vs.* SW	TX	93	1, 91	0.10	9.79	0.002	MW < SW
	2a	WSP (LINE-grouped)	TX	38	1, 36	0.00	0.10	0.758	
	2a	WSR (LINE-grouped)	TX	55	1, 53	0.16	10.35	0.002	MW > SW
		2-way ANOVA	LINE	93	1, 89	0.17	2.78	0.099	WSP < WSR
			TX	93	1, 89	0.17	7.03	0.009	MW > SW
			LINE*TX	93	1, 89	0.17	5.92	0.017	
Phase 3		WSP *vs.* WSR	LINE	94	1, 92	0.00	0.45	0.503	
Body Weight		MW (TX-grouped)	LINE	45	1, 43	0.02	0.72	0.399	
ANOVAs		SW (TX-grouped)	LINE	49	1, 47	0.00	0.00	0.986	
		MW *vs.* SW	TX	94	1, 92	0.00	0.02	0.899	
		WSP (LINE-grouped)	TX	39	1, 37	0.01	0.22	0.641	
		WSR (LINE-grouped)	TX	55	1, 53	0.00	0.25	0.617	
		2-way ANOVA	LINE	94	1, 90	0.01	0.49	0.486	
			TX	94	1, 90	0.01	0.00	0.991	
			LINE*TX	94	1, 90	0.01	0.46	0.500	
Phase 3		WSP *vs.* WSR	LINE	94	1, 92	0.00	0.02	0.894	
Voluntary		MW (TX-grouped)	LINE	45	1, 43	0.04	1.57	0.217	
EtOH Consumption		SW (TX-grouped)	LINE	49	1, 47	0.03	1.48	0.230	
ANOVAs		MW *vs.* SW	TX	94	1, 92	0.01	1.01	0.318	
	2a	WSP (LINE-grouped)	TX	39	1, 37	0.01	0.38	0.541	
		WSR (LINE-grouped)	TX	55	1, 53	0.07	4.07	0.049	MW < SW
		2-way ANOVA	LINE	94	1, 90	0.04	0.01	0.942	
			TX	94	1, 90	0.04	0.49	0.485	
			LINE*TX	94	1, 90	0.04	2.98	0.087	
Phase 3		*WSP vs. WSR*	*LINE*						
Voluntary			*D13BEC*						
EtOH Consumption		*MW (TX-grouped)*	*LINE*						
ANCOVAs			*D13BEC*						
(Covariate: D13BEC)		*SW (TX-grouped)*	*LINE*						
			D13BEC						
		MW vs. SW	*TX*						
			D13BEC						
		WSP (LINE-grouped)	*TX*						
			D13BEC						
	2a	***WSR (LINE-grouped)***	*TX*	*55*	*1, 52*	*0.07*	*4.01*	*0.050*	(MW < SW)
			D13BEC	*55*	*1, 52*	*0.07*	*0.18*	*0.674*	
		WSP (LINE-grouped)	*TX*						
			D13BEC						
		2-way ANOVA	LINE						
			TX						
			LINE*TX						
			D13BEC						
Phase 3		WSP *vs.* WSR	LINE	94	1, 92	0.00	0.00	0.994	
Voluntary		MW (TX-grouped)	LINE	45	1, 43	0.03	1.44	0.237	
10% EtOH Preference Ratio		SW (TX-grouped)	LINE	49	1, 47	0.02	0.85	0.360	
ANOVAs		MW *vs.* SW	TX	94	1, 92	0.02	1.84	0.178	
	2a	WSP (LINE-grouped)	TX	39	1, 37	0.00	0.05	0.818	
		WSR (LINE-grouped)	TX	55	1, 53	0.07	4.18	0.046	MW < SW
		2-way ANOVA	LINE	94	1, 90	0.04	0.00	0.964	
			TX	94	1, 90	0.04	1.18	0.280	
			LINE*TX	94	1, 90	0.04	2.12	0.148	
Phase 3		*WSP vs. WSR*	*LINE*						
Voluntary			*D13BEC*						
10% EtOH Preference Ratio		*MW (TX-grouped)*	*LINE*						
ANCOVAs			*D13BEC*						
(Covariate: D13BEC)		*SW (TX-grouped)*	*LINE*						
			D13BEC						
		MW vs. SW	*TX*						
			D13BEC						
		WSP (LINE-grouped)	*TX*	*38*	*1, 35*	*0.28*	*0.00*	*0.993*	
			D13BEC	*38*	*1, 35*	*0.28*	*13.48*	*0.001*	
	2a	***WSR (LINE-grouped)***	*TX*	*55*	*1, 52*	*0.08*	*2.94*	*0.092*	(MW < SW)
			D13BEC	*55*	*1, 52*	*0.08*	*0.12*	*0.733*	
		WSP (LINE-grouped)	*TX*						
			D13BEC						
		2-way ANOVA	LINE						
			TX						
			LINE*TX						
			D13BEC						

Appendix Table 4: ANOVA and ANCOVA results for experiment 2, continued.

Variable	Hypothesis	Description	Factor	n	df	R^2	*F*-value	*p*-value	Effect
Phase 4		WSP *vs.* WSR	LINE	94	1, 92	0.20	23.60	0.000	WSP < WSR
Body Weight		MW (TX-grouped)	LINE	45	1, 43	0.17	8.95	0.005	WSP < WSR
ANOVAs		SW (TX-grouped)	LINE	49	1, 47	0.26	16.72	0.000	WSP < WSR
		MW *vs.* SW	TX	94	1, 92	0.04	0.04	0.068	(MW < SW)
		WSP (LINE-grouped)	TX	39	1, 37	0.03	1.11	0.299	
		WSR (LINE-grouped)	TX	55	1, 53	0.07	4.00	0.051	(MW < SW)
		2-way ANOVA	LINE	94	1, 90	0.25	24.70	0.000	WSP < WSR
			TX	94	1, 90	0.25	4.38	0.039	MW < SW
			LINE*TX	94	1, 90	0.25	0.25	0.617	
Phase 4		WSP *vs.* WSR	LINE	93	1, 91	0.02	1.52	0.221	
D25BEC		MW (TX-grouped)	LINE	44	1, 42	0.03	1.31	0.259	
ANOVAs		SW (TX-grouped)	LINE	49	1, 47	0.01	0.28	0.600	
		MW *vs.* SW	TX	93	1, 91	0.01	1.35	0.248	
	2a	WSP (LINE-grouped)	TX	39	1, 37	0.03	1.20	0.281	
	2a	WSR (LINE-grouped)	TX	54	1, 52	0.00	0.24	0.624	
		2-way ANOVA	LINE	93	1, 89	0.04	1.70	0.195	
			TX	93	1, 89	0.04	1.72	0.193	
			LINE*TX	93	1, 89	0.04	0.73	0.396	
Phase 5		WSP *vs.* WSR	LINE	94	1, 92	0.08	7.94	0.006	WSP < WSR
Body Weight		MW (TX-grouped)	LINE	45	1, 43	0.08	3.84	0.057	(WSP < WSR)
ANOVAs		SW (TX-grouped)	LINE	49	1, 47	0.08	3.86	0.055	(WSP < WSR)
		MW *vs.* SW	TX	94	1, 92	0.01	1.09	0.299	
		WSP (LINE-grouped)	TX	39	1, 37	0.01	0.27	0.604	
		WSR (LINE-grouped)	TX	55	1, 53	0.01	0.73	0.397	
		2-way ANOVA	LINE	94	1, 90	0.09	7.72	0.007	WSP < WSR
			TX	94	1, 90	0.09	0.90	0.345	
			LINE*TX	94	1, 90	0.09	0.03	0.858	
Phase 5		WSP *vs.* WSR	LINE	94	1, 92	0.02	1.54	0.218	
Voluntary		MW (TX-grouped)	LINE	45	1, 43	0.02	0.67	0.416	
EtOH Consumption		SW (TX-grouped)	LINE	49	1, 47	0.02	1.10	0.301	
ANOVAs		MW *vs.* SW	TX	94	1, 92	0.04	4.28	0.041	MW < SW
	2a	WSP (LINE-grouped)	TX	39	1, 37	0.02	0.94	0.338	
	2a	WSR (LINE-grouped)	TX	55	1, 53	0.07	3.85	0.055	MW < SW
		2-way ANOVA	LINE	94	1, 90	0.06	1.67	0.200	
			TX	94	1, 90	0.06	3.99	0.049	MW < SW
			LINE*TX	94	1, 90	0.06	0.20	0.658	
Phase 5		*WSP vs. WSR*	*LINE*						
Voluntary			*D13BEC*						
EtOH Consumption		*MW (TX-grouped)*	*LINE*						
ANCOVAs			*D13BEC*						
(Covariate: D13BEC)		*SW (TX-grouped)*	*LINE*						
			D13BEC						
		MW vs. SW	*TX*						
			D13BEC						
		WSP (LINE-grouped)	*TX*						
			D13BEC						
		WSR (LINE-grouped)	*TX*						
			D13BEC						
		WSP (LINE-grouped)	*TX*						
			D13BEC						
		2-way ANOVA	LINE						
			TX						
			LINE*TX						
			D13BEC						
Phase 5		WSP *vs.* WSR	LINE	94	1, 92	0.00	0.01	0.904	
Voluntary		MW (TX-grouped)	LINE	45	1, 43	0.00	0.01	0.904	
10% EtOH Preference Ratio		SW (TX-grouped)	LINE	49	1, 47	0.00	0.02	0.883	
ANOVAs		MW *vs.* SW	TX	94	1, 92	0.05	5.26	0.024	MW < SW
	2a	WSP (LINE-grouped)	TX	39	1, 37	0.03	1.28	0.265	
	2a	WSR (LINE-grouped)	TX	55	1, 53	0.09	5.25	0.026	MW < SW
		2-way ANOVA	LINE	94	1, 90	0.05	0.03	0.853	
			TX	94	1, 90	0.05	4.97	0.028	MW < SW
			LINE*TX	94	1, 90	0.05	0.00	0.954	
Phase 5		*WSP vs. WSR*	*LINE*						
Voluntary			*D13BEC*						
10% EtOH Preference Ratio		*MW (TX-grouped)*	*LINE*						
ANCOVAs			*D13BEC*						
(Covariate: D13BEC)		*SW (TX-grouped)*	*LINE*						
			D13BEC						
		MW vs. SW	*TX*						
			D13BEC						
		WSP (LINE-grouped)	*TX*						
			D13BEC						
		WSR (LINE-grouped)	*TX*						
			D13BEC						
		WSP (LINE-grouped)	*TX*						
			D13BEC						
		2-way ANOVA	LINE						
			TX						
			LINE*TX						
			D13BEC						

Appendix Table 4: ANOVA and ANCOVA results for experiment 2, continued.

Variable	Hypothesis	Description	Factor	n	df	R^2	*F*-value	*p*-value	Effect
Repeated Measures ANOVAs Phase 1 vs. Phase 3 EtOH Consumption (g/kg)	2b	WSP (LINE-grouped)	TX	39	1, 37	0.03	1.14	0.293	
			Repeated Withdrawal	39	1, 37	0.00	0.07	0.793	
			Repeated Withdrawal * TX	39	1, 37	0.00	0.01	0.905	
	2b	WSR (LINE-grouped)	TX	55	1, 53	0.01	0.50	0.484	
			Repeated Withdrawal	**55**	**1, 53**	**0.08**	**4.71**	**0.034**	**Phase 1 > Phase 3**
			Repeated Withdrawal * TX	**55**	**1, 53**	**0.09**	**5.54**	**0.022**	
Repeated Measures ANCOVAs (Covariate=D13AVGBEC) Phase 1 vs. Phase 3 EtOH Consumption (g/kg)	*2b*	*WSP (LINE-grouped)*	*TX*	*38*	*1, 35*	*0.02*	*0.63*	*0.431*	
			D13D25AVGBEC	***38***	***1, 35***	***0.31***	***15.93***	***0.000***	
			Repeated Withdrawal	*38*	*1, 35*	*0.02*	*0.78*	*0.383*	
			*Repeated Withdrawal * TX*	*38*	*1, 35*	*0.01*	*0.38*	*0.544*	
			*Repeated Withdrawal * D13D25AVGBEC*	*38*	*1, 35*	*0.02*	*0.75*	*0.391*	
	2b	*WSR (LINE-grouped)*	*TX*	*55*	*1, 52*	*0.02*	*1.13*	*0.293*	
			D13D25AVGBEC	*55*	*1, 52*	*0.02*	*1.06*	*0.307*	
			Repeated Withdrawal	*55*	*1, 52*	*0.00*	*0.00*	*0.998*	
			*Repeated Withdrawal * TX*	*55*	*1, 52*	*0.05*	*2.96*	*0.091*	
			*Repeated Withdrawal * D13D25AVGBEC*	*55*	*1, 52*	*0.02*	*1.16*	*0.286*	
Repeated Measures ANOVAs Phase 1 vs. Phase 3 10% EtOH Preference Ratio	2b	WSP (LINE-grouped)	TX	39	1, 37	0.01	0.40	0.530	
			Repeated Withdrawal	39	1, 37	0.00	0.09	0.762	
			Repeated Withdrawal * TX	39	1, 37	0.00	0.15	0.705	
	2b	WSR (LINE-grouped)	TX	55	1, 53	0.03	1.41	0.241	
			Repeated Withdrawal	55	1, 53	0.04	2.40	0.127	
			Repeated Withdrawal * TX	55	1, 53	0.07	4.00	0.506	
Repeated Measures ANCOVAs (Covariate=D13AVGBEC) Phase 1 vs. Phase 3 10% EtOH Preference Ratio	*2b*	*WSP (LINE-grouped)*	*TX*	*38*	*1, 35*	*0.01*	*0.35*	*0.556*	
			D13D25AVGBEC	***38***	***1, 35***	***0.33***	***16.98***	***0.000***	
			Repeated Withdrawal	*38*	*1, 35*	*0.03*	*0.99*	*0.326*	
			*Repeated Withdrawal * TX*	*38*	*1, 35*	*0.01*	*0.36*	*0.555*	
			*Repeated Withdrawal * D13D25AVGBEC*	*38*	*1, 35*	*0.02*	*0.84*	*0.366*	
	2b	*WSR (LINE-grouped)*	*TX*	*55*	*1, 52*	*0.03*	*1.77*	*0.189*	
			D13D25AVGBEC	*55*	*1, 52*	*0.01*	*0.39*	*0.535*	
			Repeated Withdrawal	*55*	*1, 52*	*0.01*	*0.67*	*0.415*	
			*Repeated Withdrawal * TX*	*55*	*1, 52*	*0.03*	*1.38*	*0.245*	
			*Repeated Withdrawal * D13D25AVGBEC*	*55*	*1, 52*	*0.05*	*2.88*	*0.096*	
Repeated Measures ANOVAs Phase 3 vs. Phase 5 EtOH Consumption (g/kg)	2b	WSP (LINE-grouped)	TX	39	1, 37	0.00	0.04	0.844	
			Repeated Withdrawal	39	1, 37	0.00	0.00	0.947	
			Repeated Withdrawal * TX	39	1, 37	0.07	2.78	0.104	
	2b	WSR (LINE-grouped)	**TX**	**55**	**1, 53**	**0.09**	**5.19**	**0.027**	**MW < SW**
			Repeated Withdrawal	55	1, 53	0.07	3.96	0.052	(Phase 3 < Phase 5)
			Repeated Withdrawal * TX	55	1, 53	0.00	0.01	0.940	
Repeated Measures ANCOVAs (Covariate=D25AVGBEC) Phase 3 vs. Phase 5 EtOH Consumption (g/kg)	*2b*	*WSP (LINE-grouped)*	*TX*	*39*	*1, 36*	*0.00*	*0.09*	*0.767*	
			D13D25AVGBEC	*39*	*1, 36*	*0.01*	*0.35*	*0.556*	
			Repeated Withdrawal	*39*	*1, 36*	*0.02*	*0.56*	*0.458*	
			*Repeated Withdrawal * TX*	*39*	*1, 36*	*0.08*	*3.14*	*0.085*	
			*Repeated Withdrawal * D13D25AVGBEC*	*39*	*1, 36*	*0.02*	*0.63*	*0.433*	
	2b	*WSR (LINE-grouped)*	***TX***	***54***	***1, 51***	***0.08***	***4.50***	***0.039***	
			D13D25AVGBEC	*54*	*1, 51*	*0.01*	*0.77*	*0.384*	
			Repeated Withdrawal	*54*	*1, 51*	*0.00*	*0.01*	*0.932*	
			*Repeated Withdrawal * TX*	*54*	*1, 51*	*0.00*	*0.00*	*0.975*	
			*Repeated Withdrawal * D13D25AVGBEC*	*54*	*1, 51*	*0.00*	*0.22*	*0.641*	
Repeated Measures ANOVAs Phase 3 vs. Phase 5 10% EtOH Preference Ratio	2b	WSP (LINE-grouped)	TX	39	1, 37	0.01	0.29	0.595	
			Repeated Withdrawal	39	1, 37	0.01	0.42	0.520	
			Repeated Withdrawal * TX	39	1, 37	0.08	3.17	0.083	
	2b	WSR (LINE-grouped)	**TX**	**55**	**1, 53**	**0.13**	**7.75**	**0.007**	**MW < SW**
			Repeated Withdrawal	55	1, 53	0.02	0.87	0.354	
			Repeated Withdrawal * TX	55	1, 53	0.00	0.07	0.789	
Repeated Measures ANCOVAs (Covariate=D25AVGBEC) Phase 3 vs. Phase 5 10% EtOH Preference Ratio	*2b*	*WSP (LINE-grouped)*	*TX*	*39*	*1, 36*	*0.01*	*0.37*	*0.545*	
			D13D25AVGBEC	*39*	*1, 36*	*0.01*	*0.25*	*0.617*	
			Repeated Withdrawal	*39*	*1, 36*	*0.01*	*0.19*	*0.666*	
			*Repeated Withdrawal * TX*	*39*	*1, 36*	*0.09*	*3.53*	*0.068*	
			*Repeated Withdrawal * D13D25AVGBEC*	*39*	*1, 36*	*0.02*	*0.59*	*0.448*	
	2b	*WSR (LINE-grouped)*	***TX***	***54***	***1, 51***	***0.12***	***6.70***	***0.013***	***MW < SW***
			D13D25AVGBEC	*54*	*1, 51*	*0.01*	*0.74*	*0.395*	
			Repeated Withdrawal	*54*	*1, 51*	*0.00*	*0.16*	*0.692*	
			*Repeated Withdrawal * TX*	*54*	*1, 51*	*0.00*	*0.06*	*0.809*	
			*Repeated Withdrawal * D13D25AVGBEC*	*54*	*1, 51*	*0.01*	*0.45*	*0.504*	

Appendix Table 4: ANOVA and ANCOVA results for experiment 2, continued.

Variable	Hypothesis	Description	Factor	n	df	R^2	*F*-value	*p*-value	Effect
D13D25AVGBEC		WSP *vs.* WSR	TX	94	1, 92	0.00	0.01	0.924	
ANOVAs		WSP (LINE-grouped)	TX	39	1, 37	0.04	1.62	0.210	
		WSR (LINE-grouped)	TX	55	1, 53	0.13	7.78	0.007	MW > SW
Repeated Measures	2b	WSP (LINE-grouped)	TX	39	1, 37	0.00	0.02	0.890	
ANOVAs			Repeated Withdrawal	39	1, 37	0.00	0.05	0.818	
Phase 1 vs. Phase 5			Repeated Withdrawal * TX	39	1, 37	0.05	2.08	0.157	
EtOH Consumption (g/kg)	2b	WSR (LINE-grouped)	TX	55	1, 53	0.01	0.67	0.415	
			Repeated Withdrawal	55	1, 53	0.00	0.08	0.782	
			Repeated Withdrawal * TX	55	1, 53	0.06	3.27	0.076	
Repeated Measures	*2b*	*WSP (LINE-grouped)*	*TX*	*39*	*1, 36*	*0.00*	*0.04*	*0.835*	
ANCOVAs			*D13D25AVGBEC*	*39*	*1, 36*	*0.00*	*0.13*	*0.717*	
(Covariate=D13D25AVGBEC)			*Repeated Withdrawal*	*39*	*1, 36*	*0.07*	*2.72*	*0.108*	
Phase 1 vs. Phase 5			*Repeated Withdrawal * TX*	*39*	*1, 36*	*0.08*	*3.31*	*0.077*	
EtOH Consumption (g/kg)			*Repeated Withdrawal * D13D25AVGBEC*	*39*	*1, 36*	*0.08*	*3.19*	*0.082*	
	2b	*WSR (LINE-grouped)*	*TX*	*55*	*1, 52*	*0.02*	*1.05*	*0.309*	
			D13D25AVGBEC	*55*	*1, 52*	*0.01*	*0.54*	*0.464*	
			Repeated Withdrawal	*55*	*1, 52*	*0.02*	*0.93*	*0.338*	
			*Repeated Withdrawal * TX*	*55*	*1, 52*	*0.03*	*1.69*	*0.199*	
			*Repeated Withdrawal * D13D25AVGBEC*	*55*	*1, 52*	*0.02*	*1.19*	*0.280*	
Repeated Measures	2b	WSP (LINE-grouped)	TX	39	1, 37	0.00	0.12	0.727	
ANOVAs			Repeated Withdrawal	39	1, 37	0.00	0.01	0.909	
Phase 1 vs. Phase 5			Repeated Withdrawal * TX	39	1, 37	0.05	2.07	0.159	
10% EtOH Preference Ratio	2b	WSR (LINE-grouped)	TX	55	1, 53	0.03	1.69	0.199	
			Repeated Withdrawal	55	1, 53	0.00	0.23	0.637	
			Repeated Withdrawal * TX	55	1, 53	0.04	2.31	0.134	
Repeated Measures	*2b*	*WSP (LINE-grouped)*	*TX*	*39*	*1, 36*	*0.00*	*0.15*	*0.698*	
ANCOVAs			*D13D25AVGBEC*	*39*	*1, 36*	*0.00*	*0.06*	*0.804*	
(Covariate=D13D25AVGBEC)			*Repeated Withdrawal*	*39*	*1, 36*	*0.07*	*2.73*	*0.107*	
Phase 1 vs. Phase 5			*Repeated Withdrawal * TX*	*39*	*1, 36*	*0.08*	*3.26*	*0.079*	
10% EtOH Preference Ratio			*Repeated Withdrawal * D13D25AVGBEC*	*39*	*1, 36*	*0.08*	*3.08*	*0.088*	
	2b	*WSR (LINE-grouped)*	*TX*	*55*	*1, 52*	*0.04*	*2.29*	*0.136*	
			D13D25AVGBEC	*55*	*1, 52*	*0.01*	*0.72*	*0.400*	
			Repeated Withdrawal	*55*	*1, 52*	*0.02*	*1.03*	*0.314*	
			*Repeated Withdrawal * TX*	*55*	*1, 52*	*0.02*	*0.99*	*0.324*	
			*Repeated Withdrawal * D13D25AVGBEC*	*55*	*1, 52*	*0.03*	*1.45*	*0.234*	
Repeated Measures	2b	WSP (LINE-grouped)	TX	39	1, 37	0.00	0.05	0.833	
ANOVAs			Repeated Withdrawal	39	2, 74	0.00	0.05	0.952	
Phase 1 vs. Phase 3			Repeated Withdrawal * TX	39	2, 74	0.02	1.38	0.259	
vs. Phase 5	2b	WSR (LINE-grouped)	TX	55	1, 53	0.03	1.82	0.184	
EtOH Consumption (g/kg)			Repeated Withdrawal	55	2, 106	0.02	2.24	0.112	
			Repeated Withdrawal * TX	**55**	**2, 106**	**0.03**	**3.16**	**0.046**	
Repeated Measures	*2b*	*WSP (LINE-grouped)*	*TX*	*39*	*1, 36*	*0.00*	*0.00*	*0.982*	
ANCOVAs			*D13D25AVGBEC*	*39*	*1, 36*	*0.02*	*0.81*	*0.373*	
(Covariate=D13D25AVGBEC)			*Repeated Withdrawal*	*39*	*2, 72*	*0.03*	*1.89*	*0.158*	
Phase 1 vs. Phase 3			*Repeated Withdrawal * TX*	*39*	*2, 72*	*0.03*	*1.87*	*0.162*	
vs. Phase 5			*Repeated Withdrawal * D13D25AVGBEC*	*39*	*2, 72*	*0.03*	*2.24*	*0.114*	
EtOH Consumption	*2b*	*WSR (LINE-grouped)*	*TX*	*55*	*1, 52*	*0.04*	*1.98*	*0.165*	
			D13D25AVGBEC	*55*	*1, 52*	*0.00*	*0.20*	*0.660*	
			Repeated Withdrawal	*55*	*2, 204*	*0.00*	*0.71*	*0.494*	
			*Repeated Withdrawal * TX*	*55*	*2, 204*	*0.01*	*1.61*	*0.204*	
			*Repeated Withdrawal * D13D25AVGBEC*	*55*	*2, 204*	*0.01*	*1.21*	*0.302*	
Repeated Measures	2b	WSP (LINE-grouped)	TX	39	1, 37	0.00	0.02	0.888	
ANOVAs			Repeated Withdrawal	39	2, 74	0.00	0.11	0.894	
Phase 1 vs. Phase 3			Repeated Withdrawal * TX	39	2, 74	0.02	1.52	0.226	
vs. Phase 5	2b	WSR (LINE-grouped)	TX	55	1, 53	0.06	3.65	0.061	(MW < SW)
10% EtOH Preference Ratio			Repeated Withdrawal	55	2, 106	0.01	1.08	0.343	
			Repeated Withdrawal * TX	55	2, 106	0.02	2.03	0.136	
Repeated Measures	*2b*	*WSP (LINE-grouped)*	*TX*	*39*	*1, 36*	*0.00*	*0.07*	*0.786*	
ANCOVAs			*D13D25AVGBEC*	*39*	*1, 36*	*0.01*	*0.44*	*0.513*	
(Covariate=D13D25AVGBEC)			*Repeated Withdrawal*	*39*	*2, 72*	*0.03*	*2.34*	*0.104*	
Phase 1 vs. Phase 3			*Repeated Withdrawal * TX*	*39*	*2, 72*	*0.03*	*2.22*	*0.116*	
vs. Phase 5			*Repeated Withdrawal * D13D25AVGBEC*	*39*	*2, 72*	*0.03*	*2.48*	*0.091*	
10% EtOH Preference	*2b*	*WSR (LINE-grouped)*	*TX*	*55*	*1, 52*	*0.06*	*3.35*	*0.073*	*(MW < SW)*
			D13D25AVGBEC	*55*	*1, 52*	*0.00*	*0.03*	*0.864*	
			Repeated Withdrawal	*55*	*2, 104*	*0.01*	*0.98*	*0.380*	
			*Repeated Withdrawal * TX*	*55*	*2, 104*	*0.01*	*0.77*	*0.464*	
			*Repeated Withdrawal * D13D25AVGBEC*	*55*	*2, 104*	*0.02*	*1.72*	*0.184*	

Appendix Table 4: ANOVA and ANCOVA results for experiment 2, continued.