Mechanisms of Rapid Adaptation to Hypnotic Effects of Ethanol in Mice: a Pharmacogenetic Approach.

by

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LIST OF ABBREVIATIONS

AFT = Acute Functional Tolerance

BEC = Blood Ethanol Concentration

BrEC = Brain Ethanol Concentration

CNS = Central Nervous System

DMSO = Dimethyl Sulfoxide

EPSP = Excitatory Postsynaptic Potential

EtOH = Ethanol

FHP = Family History Positive

FHN = Family History Negative

GABA = γ-Aminobutyric Acid

HAFT = High Acute Functional Tolerance

IS = Initial Sensitivity

i.p. = intraperitoneal

LAFT = Low Acute Functional Tolerance

LS = Long Sleep

LRR = Loss of Righting Reflex

NMDA = N-Methyl-D-Aspartate

NO = Nitric Oxide

RT = Rapid Tolerance

SS = Short Sleep.

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This part of the manuscript is dedicated to people who made a contribution to the work presented in this document. This section is a somewhat unique place of the dissertation. As my mentor (and full-time editor) Dr. John Crabbe will read it only after the whole document is bound, I will use this part of the manuscript to exercise my habit to place the definite and indefinite articles in the most unusual places or to fail placing them where they are expected.

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Abstract

Tolerance to ethanol (EtOH) appears in chronic, rapid and acute forms that depend on the amount and schedule of EtOH exposure as well as the behavioral measure of tolerance. Chronic tolerance is produced by repeated administration of EtOH over a period of days or weeks. Rapid tolerance (RT) can be observed in the response to a second dose of EtOH 24 hours after the first exposure. Acute tolerance appears within minutes after a single dose. Chronic tolerance is believed to be an important component of human alcoholism. The potential role of acute tolerance in alcohol abuse has also been suggested by a number of studies. It has been hypothesized that individuals who rapidly develop tolerance to alcohol's euphoric effects will drink substantial amounts of alcohol to maintain the effects, which could ultimately lead to the development of alcohol dependence. This suggested relationship between acute tolerance and alcohol abuse has received indirect support from studies on subjects with a positive family history for alcoholism, who were less intoxicated than family history negative controls, despite comparable blood alcohol concentrations. This low sensitivity in young men predicted alcoholism diagnoses later in life. The measures of sensitivity employed in these studies (subjective responses and body sway) most likely represent a combination of initial brain sensitivity and acute tolerance that rapidly develops during alcohol exposure. It is not clear how initial sensitivity and acute tolerance individually contribute to alcohol abuse. Human research faces a number of methodological and ethical problems that complicate dissociation of these two variables and investigation of their mechanisms independently. Therefore, animal models that offer reliably measured initial sensitivity and acute tolerance are desired. It is not clear how alcohol-related behaviors in rodents are related

to their prototypes monitored in human alcoholics. Therefore, it is important to study a variety of clearly defined measures of initial sensitivity and acute tolerance to different effects of EtOH. The present studies aimed to characterize initial sensitivity and acute functional tolerance (AFT) to sedative-hypnotic effects of EtOH in mice, using tools of behavioral pharmacology and classical genetics.

Loss of righting reflex (LRR) has been used historically to assess sensitivity to ethanol's hypnotic effects in mice. Because the traditional method of monitoring ethanol-induced sedation seemed to lack accuracy in estimating initial sensitivity, I began this project by developing a novel technique that improved detection of the onset of LRR, which resulted in a more accurate measure of initial brain sensitivity and increased magnitude of AFT. Initial characterization of AFT in genetically heterogeneous mice using the new method resulted in several observations that confirmed and extended previous knowledge about this phenomenon. Results showed that a) AFT developed in a dose-dependent fashion but not beyond a certain maximum value, b) AFT to ethanol-induced hypnosis could develop partially to a small subhypnotic dose, c) AFT to a subhypnotic dose develops within 10 minutes after ethanol exposure. We used these findings to develop behavioral paradigms for our pharmacological and genetic studies.

Several studies suggest involvement of different neurochemical systems in regulation of initial sensitivity and different forms of tolerance to ethanol, with data on GABA and NMDA receptor systems being most abundant. Therefore, we concentrated our efforts on investigation of the effects of different GABA and NMDA compounds on initial sensitivity and AFT. The NMDA receptor antagonist MK-801 inhibited the development of AFT in a dose-dependent manner but had no significant effects on initial

sensitivity in the dose range used, which implied the involvement of NMDA receptors and downstream calcium-dependent processes in regulation of AFT to EtOH-induced hypnosis. This finding was confirmed in another experiment that used a behavioral paradigm with a subhypnotic dose. On the other hand, two doses of the $GABA_B$ receptor agonist baclofen increased initial sensitivity but did not affect AFT. These findings suggest that initial sensitivity and AFT to ethanol-induced sedation are regulated by some different mechanisms that could be distinguished pharmacologically. The NMDA receptor partial agonist D-cycloserine and antagonist ifenprodil as well as the $GABA_A$ receptor antagonist picrotoxin did not affect either initial sensitivity or AFT in the dose range applied.

We further investigated the relationship between initial sensitivity and AFT using a panel of inbred mouse strains. Relationships between AFT and rapid tolerance as well as initial sensitivity and rapid tolerance were also studied. Three separate experiments that used mice of different sexes and ages were carried out to determine the reliability of the assessment of initial sensitivity and the two forms of tolerance. Strain mean values of initial sensitivity and AFT were intercorrelated across the three experiments. Correlations among the rapid tolerance values were not statistically significant, indicating that measures of rapid tolerance are somewhat sensitive to sex and age differences and/or environmental manipulation. Weak relationships among rapid tolerance values obtained in different experiments could also be an indication of relatively low heritability; heritability values of rapid tolerance were lower than those for initial sensitivity and AFT. Initial sensitivity, AFT and rapid tolerance did not correlate consistently, suggesting that these three domains are mainly influenced by different genetic mechanisms.

To summarize, this project resulted in the development of a novel behavioral technique to assess initial brain sensitivity and acute functional tolerance to the hypnotic effects of ethanol in the same animals. The first series of experiments determined the dose response and time course of this AFT. Pharmacological and genetic studies provided evidence that initial sensitivity, acute functional tolerance and rapid tolerance are regulated by some different mechanisms. The behavioral method developed and the knowledge obtained should be useful for future studies aimed to investigate potential contributions of initial sensitivity and AFT to alcohol abuse.

Introduction

Different forms of tolerance to ethanol

Ethanol (EtOH), the active ingredient of alcoholic beverages, affects many central processes including motor, sensory and cognitive functions. Acute administration of ethanol produces short-term stimulation followed by sedation. When abused, alcohol leads to a number of changes in the central nervous system (CNS), including cellular adaptation or tolerance to ethanol effects.

The term tolerance has two connotations. Initial or innate tolerance refers to the subject's ability to "tolerate" a given concentration of drug or, in other words, individual sensitivity. Acquired tolerance, on the other hand, is generally defined as a diminution of a drug effect after a period of administration of that drug. In a majority of studies, the term tolerance implies acquired tolerance, while, to avoid confusion the terms sensitivity or initial sensitivity are generally substituted for initial tolerance.

Tolerance can be classified into two main types, dispositional and functional (Kalant et al, 1971; Goldstein, 1983). Dispositional tolerance is seen when the drug becomes less effective after chronic use because there is less of it at the site of action. Changes in EtOH absorption, distribution, excretion and metabolism all contribute to dispositional tolerance. Functional tolerance implies an actual change in sensitivity of a specific tissue to a given drug concentration.

Another classification of tolerance is based on the time frame within which adaptation occurs. Chronic tolerance is produced by repeated administration of EtOH over a period of days or weeks. Rapid tolerance can be observed in the response to a

second dose of EtOH 24 hours after the first exposure, while acute tolerance appears within minutes to hours of one continuous drug exposure (Mellanby, 1919; Kalant et al. 1971; Crabbe et al, 1979; Khanna et al, 1996). Chronic and rapid tolerance are similar phenomenologically and methodologically (with the exception of when chronic tolerance is produced by continuous vapor inhalation or liquid diet) as they are measured after n discrete exposures to the drug, where n can range from 1 to infinity. Evidence suggests that these forms of tolerance are regulated by some common mechanisms. It is hypothesized that rapid tolerance is the initial step to the development of chronic tolerance (Khanna et al. 1991). One conceptual distinction between acute tolerance and the other two forms is that acute tolerance is measured when EtOH is still in the system, while tests for the rapid and chronic forms occur at a time when alcohol from the previous dose has been completely eliminated. Kalant and coworkers (1971) recognized this distinction by drawing a line between intrasessional adaptation for acute forms of tolerance and intersessional adaptation for chronic forms. While chronic tolerance to alcohol has long been a major focus of investigation in the scientific community, research on acute tolerance has just recently begun to uncover mechanisms underlying very rapid adaptation to EtOH.

The phenomenon of acute functional tolerance (AFT) was first described by Mellanby (1919), who had observed that dogs treated with a single dose of ethanol showed more motor impairment at a given blood ethanol concentration (BEC) on the rising portion of the BEC curve than at the same BEC on the falling portion. Initially, this report of the AFT phenomenon was met with criticism, as two objections were raised. First, as alcohol concentrations in the Mellanby experiment were determined in venous

blood, the differences in degrees of intoxication were thought to reflect arterio-venous differences in BEC during alcohol absorption. The second objection was that improved performance seen on the descending part of the BEC curve was due to continued practice that occurred during repeated testing, and thus acute tolerance was simply a learning artifact (Goldberg, 1943). Although subsequent work demonstrated that these factors can affect the measurement of AFT, an early study of LeBlanc and colleges (1975) eliminated these objections as the only possible explanations of the AFT phenomenon. In this study, rats were tested for EtOH-induced motor incoordination at different time points. First, rats were trained to walk on a motor-driven belt, with electric shock being a negative reinforcer (off the belt = shock). Then, each rat was injected with one dose of EtOH and tested for motor impairment (score = time off the belt) at one of three time points after injection. Brains were removed immediately after the test was completed and brain ethanol concentration (BrEC) was measured. The motor impairment scores were then plotted vs. BrEC and data for each time point were analyzed by separate linear regressions. Thus, three regression lines were obtained. AFT was demonstrated by a parallel shift to the right of the regression line. The regression line was progressively shifted towards higher BrEC with increasing time after alcohol administration. Measuring BrEC and testing animals only once eliminated the possible contribution of pharmacokinetic factors and intoxicated practice in such AFT. Since the work of LeBlanc and colleagues proved the reality of AFT as a pharmacodynamic phenomenon, acute tolerance and its role in alcohol abuse has been receiving increasing attention.

AFT studies with human subjects

Over the years a number of experimental designs have been employed to investigate the development of acute tolerance to alcohol in humans. Three basic experimental paradigms have been used to evaluate AFT. First, similarly to acute tolerance measured in the original study of Mellanby, AFT is defined as less functional impairment at the same or higher BEC following a single or two consecutive, closely-spaced administrations of EtOH. A second kind of procedure defines AFT as a greater BEC at the offset than that at the onset of intoxication, given the same level of impairment at each time. A third paradigm is similar to the first one. It employs repeated measurements and defines AFT as less intoxication at later time points while maintaining a steady-state BEC. BEC is often assessed from a breath sample. It has been shown that breath alcohol concentration (BreathAC) closely parallels those measured in arterial blood (for review, see Kalant, 1998).

A wide variety of tests have been used to demonstrate acute tolerance. AFT to ethanol effects have been shown on psychomotor performance, using reaction time and a composite motor score (Wilson et al, 1984); cognition, using numerical coding of letter charts (Vogel-Sprott, 1979) and arithmetical calculation (Hiltunen, 1997); as well as subjective responses (Martin and Moss, 1993). One of the most consistent findings of human research on acute tolerance is that AFT depends on the test employed to assess tolerance. For example, Wilson et al. (1984) showed that AFT to the impairing effects of alcohol can be measured on the dowel balancing, hand steadiness and reaction time tests, but not on a pursuit rotor task or body sway. Similarly, O'Connor and colleagues (1998)

studied effects of alcohol on subjective responses and reported indices of AFT in 3 of 15 items from Schuckit's (1984) Subjective High Assessment Scale. Some studies demonstrated that such factors as learning (Vogel-Sprott and Sdao-Jarvie, 1989), alcohol dose, and previous alcohol exposure (Hiltunen, 1997; Hiltunen et al., 2000) can also influence AFT. The effects of these factors on AFT will be discussed in detail in subsequent sections with examples from animal research.

The role of acute tolerance in alcohol abuse

Chronic tolerance is a well-known component of human alcoholism, as acquired tolerance is listed as one of the diagnostic criteria by the Diagnostic and Statistical Manual for Mental Disorders, Fourth Edition (DSM-IV). It is believed to be one of the determinants of the level of alcohol consumption, and thus to contribute to the maintenance or aggravation of alcohol dependence (Kalant, 1998). Alcohol dependence, the central concept of any definition of alcoholism, manifests itself upon withdrawal from alcohol as a series of behavioral and physiological disturbances.

A potential role for acute tolerance in alcohol abuse is not clear. It has been hypothesized that individuals who rapidly develop tolerance to alcohol's euphoric effects will drink substantial amounts of alcohol to maintain the effects, which could ultimately lead to the development of alcohol dependence (Kalant, 1998). This suggested relationship between acute tolerance and alcohol abuse received indirect support from studies of Schuckit and his colleagues who reported that subjects with a positive family history for alcoholism (FHP) were less intoxicated than family history negative (FHN)

controls, despite comparable blood alcohol concentrations (Schuckit, 1980). This low sensitivity in young men predicted alcoholism diagnoses later in life (Schuckit and Smith, 1996). After years of working with sons of alcoholics Schuckit and colleagues concluded that initial response or sensitivity to alcohol has a genetic basis and is an important predictor of future alcohol abuse liability. They acknowledged, however, that the measures of sensitivity employed in these studies (subjective responses and body sway) most likely represent a combination of initial brain sensitivity, acute tolerance that rapidly develops during alcohol exposure, and previous experience with alcohol.

The early work of Schuckit's group elicited additional interest in the alcohol research community as to the role of acute EtOH responses in alcohol abuse. In an attempt to find a psychobiological marker for alcoholism, a number of alcohol-challenge studies used Schuckit's approach and compared responses to acute administration of alcohol between FHP and FHN groups (for review, see Newlin and Thomson, 1990). Although none of these investigations was specifically designed to examine AFT, some studies provided indirect evidence that certain types of AFT could be linked to future alcohol abuse liability. Newlin and Thomson (1990) reviewed this work and reported that, compared to sons of non-alcoholics, sons of alcoholics tended to be more affected by alcohol during the first 30 minutes of exposure, that is, on the rising portion of the BEC curve, but showed a quicker recovery to baseline performance when measured at later time points on the falling part of the BEC curve. The authors suggested that FHP individuals are more sensitive and develop more AFT than FHN subjects. A recent study by Ramchandani et al. (1999) tested this hypothesis directly. Authors of this paper employed a so-called BreathAC clamping technique (O'Connor et al., 1998) to test FHP

and FHN individuals in a variety of tasks including subjective responses, eye movement tasks and EEG responses. EtOH (6%) was infused intravenously with a rate calculated individually for each subject to reach a target BreathAC (0.06%) within 20 min. The target BreathAC was then maintained for several hours. Subjects were tested on a battery of tasks three times: at the baseline before the infusion, between the 20^{th} and 60^{th} min, and between the 150^{th} and 180^{th} min of alcohol administration. Initial sensitivity to alcohol was estimated from differences between the second and the first tests, while acute adaptive responses were calculated as differences between the third and the second tests. Results of this study showed that, compared to the FHN individuals, FHP subjects were less sensitive to EtOH effects on subjective perceptions and more sensitive in the effect of EtOH to increase latency of volitional saccades. FHP subjects generally showed greater AFT than did FHN controls, who also showed some instances of acute sensitization, i.e., a gradual increase in sensitivity during EtOH exposure. In summary, the literature on human studies suggests that both initial sensitivity and acute tolerance are genetically regulated and may play certain roles in the future development of alcoholism.

It is not clear, however, how initial sensitivity and acute tolerance individually contribute to alcohol abuse. Human research faces a number of methodological and ethical problems that complicate dissociation of these two variables and investigation of their mechanisms independently. The most obvious complication is that it is virtually impossible to recruit human subjects absolutely naïve to alcohol, who are willing to ingest alcohol for an experiment. Thus, measures of initial sensitivity and acute tolerance in human research are contaminated with potential chronic or carry-over tolerance that

could develop during previous drinking episodes. It is also uncommon to use high doses of alcohol. Animal research showed that AFT develops in a dose-dependent fashion. By using small to moderate doses, human studies face the risk of not detecting some forms of tolerance that can be relevant to alcohol abuse. One methodological issue should also be mentioned. The slope of the rising BEC curves in human studies ranges from approximately 0.02 to 0.05 mg/ml/min, depending on the study (Wilson et al., 1984; O'Connor et al., 1998; Ramchandani et al., 1999). Several lines of evidence suggest that AFT to some EtOH effects in mice develop within minutes of EtOH exposure (Erwin and Deitrich, 1996; Gill and Deitrich, 1998) and, if measured in concentration units, with an estimated rate of 0.04 mg/ml/min (Erwin and Deitrich, 1996), which is greater than absorption rates in some human studies. Thus, it is likely that AFT to some effects of EtOH are simply not detected in human research because measures of initial sensitivity, on which measurements of AFT rely, may be contaminated with rapidly-developed tolerance.

The aforementioned problems could be partially or completely avoided by employing animal models that offer reliably measured initial sensitivity and acute tolerance. It is not clear how alcohol-related behaviors in rodents are related to their prototypes monitored in human alcoholics. Therefore, it is important to study a variety of clearly defined measures of initial sensitivity and acute tolerance to different effects of EtOH. One such model was utilized in the present project. The overall goal of this project was to investigate mechanisms of initial sensitivity and acute functional tolerance to sedative-hypnotic effects of EtOH in mice. The current study includes three basic parts; phenomenological, pharmacological and genetic. To facilitate the connection between

theoretical background and goals of the present project, each subsequent section of the Introduction will review literature most relevant to one of the three aforementioned aspects of adaptation to EtOH.

The phenomenon of AFT: Studies with experimental animals

Since the early 1970s, animal research on acute tolerance has generally focused on demonstrating the phenomenon of AFT to different alcohol effects and the role of genetic factors. Two types of experimental procedure have been used to define AFT in animals. According to the first type, AFT is defined as less functional impairment at the same or higher BEC following a single or two consecutive injections of EtOH. A second kind of procedure defines AFT as a greater BEC at the offset than that at the onset of intoxication, given the same level of behavioral impairment at each time. The second procedure has been more popular in recent years. A schematic diagram demonstrating principles of the second paradigm is shown in Figure 1. Initial sensitivity can also be determined in separate groups of animals as BrEC instead of BEC (Gallaher et al, 1996; Gehle and Erwin, 2000). Some investigators also use more than two subsequent doses of EtOH in an attempt to achieve AFT of a greater magnitude. In this serial recovery method, AFT is measured as a difference between maximum BEC at any recovery and either BEC at the first recovery (Gallaher et al., 1982) or BrEC at the time of initial loss of function (Gallaher et al, 1996). The 1982 study employed a stationary dowel task, while the 1996 paper reported data from a rotarod test.

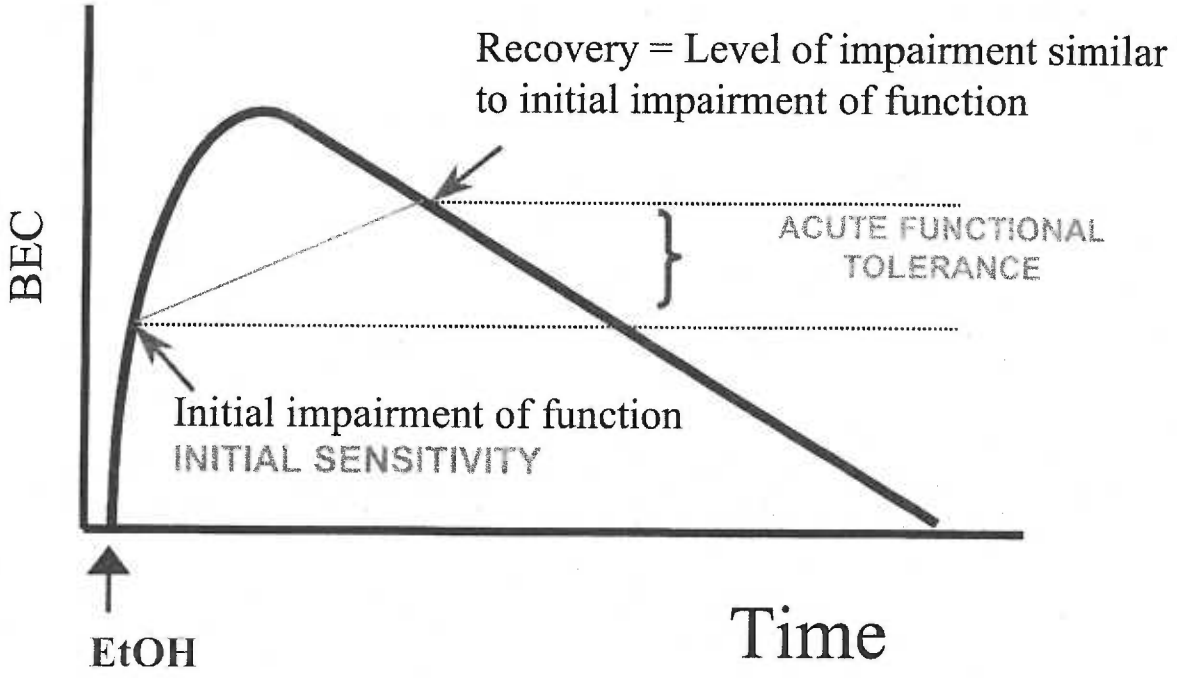

Figure 1. Paradigm used to assess initial sensitivity (IS) and acute functional tolerance (AFT) to impairing effects of EtOH. Curve indicates hypothetical blood EtOH concentration (BEC) after an impairing dose of EtOH, injected at the arrow. BEC at initial impairment of function is usually used to estimate IS (lower BEC = higher IS), while the difference between BEC at recovery of function and BEC at initial impairment is used to measure AFT.

The full extent of AFT can be assessed only when initial brain sensitivity is accurately measured. One of the assumptions of Kalant's theory is that tolerance develops in response to the degree of initial impairment caused by EtOH: that is, more sensitive animals should develop more tolerance. Over the years, researchers asked the question whether these two domains are regulated by similar mechanisms. The majority of studies that addressed this issue asked a more specific question, i.e., whether IS and AFT are influenced by some common genetic mechanisms. Results of these studies will be discussed later in the Introduction. The present project also investigated this relationship using the tools of behavioral pharmacology and classical genetics.

Factors influencing AFT. AFT can be influenced by a number of factors including EtOH dose, duration of EtOH exposure, practice during intoxication and previous alcohol experience. Dose- and time-dependency are two central concepts of any pharmacological effect. According to a theory of Kalant et al. (1971), AFT is not an exception and should be influenced by both factors. He also predicted that tolerance will develop to a certain maximum value that is determined by some inherent limit in the adaptive capacity of the organism. These predictions were generally confirmed by a number of studies that showed that both EtOH dose and duration of exposure were correlated positively with amount of tolerance developed (Keir and Deitrich, 1990; Gill and Deitrich, 1998) and that AFT ultimately reached a plateau of maximum possible tolerance (Gallaher et al., 1982; Gallaher et al., 1996; Erwin and Deitrich, 1996; Ponomarev and Crabbe, 2002). Another key notion of Kalant's theory is the concept that adaptive changes occur to a drug effect rather than to the drug itself. This means that AFT to a certain effect of EtOH depends on initial impairment and will not develop to a subthreshold dose that does not

cause the impairment. On the other hand, Radlow (1994) hypothesized that AFT depends on the drug itself rather than drug effects and will start to develop immediately after EtOH administration begins, regardless of the dose used. Radlow, however, could not provide direct evidence for his hypothesis. To my knowledge, this question has not been specifically addressed in any study and the notion has not yet been proven or disproven. In contrast to the relatively little attention paid to these competing hypotheses, relationships between initial impairment and subsequent AFT have been investigated rather extensively.

Practice while intoxicated and previous experience with alcohol can also affect the development of AFT in animals. Effects of these factors on AFT have been extensively studied by Kalant and his colleagues. For example, Le and Kalant (1992) reported that in rats tested repeatedly after EtOH, intoxication decreased more rapidly and to a greater extent than in rats tested just once. This facilitating effect of intoxicated practice was also demonstrated for chronic tolerance (Le et al., 1989).

Effects of previous EtOH experience on AFT can be seen as indications of the relationship between chronic and acute forms of tolerance. Such a relationship was also modeled in Kalant's theory. He predicted two possible interactions of acute and chronic tolerance. Using the paradigm depicted in Figure 1 as an example, the first possibility implies a progressive increase of both BEC at initial impairment of function and BEC at recovery of function with repeated administration of EtOH. According to this model, the rate of development of AFT (slope of the line between the two BECs) remains the same after each EtOH administration. The second model predicts that BEC at initial impairment will be the same, but the rate of AFT will rise on each successive drug

exposure, leading to a progressively higher BEC at recovery (Kalant et al., 1971). Both models predict that, with enough EtOH exposure, BEC at recovery will ultimately reach some maximum possible BEC, which is determined by the CNS's adaptive capacity. Data have generally supported the second rather than the first model (Kalant et al., 1978; Wu et al., 2001). For example, Wu and colleagues (2001) reported that daily injections of EtOH for six days did not influence initial sensitivity, but increased the magnitude and rate of development of AFT to EtOH-induced loss of balance in mice.

Role of behavioral endpoint in AFT. Acute tolerance has been shown to occur to many, but not all of the effects of EtOH. For example, Le and colleagues (1992) showed that under the same experimental conditions, AFT to the motor impairment but not to the anticonvulsant effects of EtOH can be demonstrated. Acute EtOH has biphasic effects on locomotor activity, stimulant at low doses and early time points, and depressant at higher doses (Phillips and Crabbe, 1991). As a CNS depressant, EtOH produces sedative-hypnotic effects on behavior, which are usually characterized by motor impairment, ranging from motor incoordination to loss of righting reflex (LRR). Because these effects can be measured easily and reliably, the majority of animal studies have chosen EtOH-induced sedation to investigate features of AFT. Acute tolerance to EtOH-induced sedation has been demonstrated using a variety of behavioral tasks and experimental paradigms (Gallaher et al., 1982; Le and Kalant, 1992; Erwin and Deitrich, 1996; Grieve and Littleton, 1979; Tabakoff and Ritzmann, 1979; Keir and Deitrich, 1990). I will describe three behavioral tests that have been most commonly used to study AFT in mice.

Stationary dowel. In this paradigm animals are trained to balance for 30 – 60 seconds on a wooden dowel suspended some distance above a bed of wood shavings.

They are then injected with a small dose of EtOH. In some studies, when the animal becomes intoxicated and falls from the dowel, BEC at the first fall is determined for a measure of initial sensitivity (BEC0), with lower ethanol levels indicating higher initial sensitivity. The animals are then tested repeatedly until they can remain on the dowel for 30 – 60 sec. Another blood sample is taken at the regain of balance and BEC is measured (BEC1). The animals are then usually given a second dose of EtOH and BEC at the second recovery is measured again (BEC2). Depending on the study, AFT is then defined as a difference between either BEC2 and BEC1 (Erwin and Deitrich, 1996) or BEC2 and BEC0 (Gehle and Erwin, 2000).

Rotating rod (rotarod). The rotarod is suspended a certain distance above a bed of wood shavings. It is attached to a motor that regulates its rotation speed. Mice are trained to a certain criterion, running on the rod at a constant or increasing speed of rotation. Procedures similar to those described for the stationary dowel test are usually used to determine initial sensitivity and AFT. Compared to the dowel test, the rotarod task generally requires more animal training to minimize performance variability that is usually present in a group of untrained animals. The rotarod procedure is sensitive to effects of intoxicated practice (Gill and Deitrich, 1998).

Loss of righting reflex (LRR). Traditionally, LRR is assessed by injecting the animal i.p. with a hypnotic dose of ethanol and then placing it on its back in a V-shaped trough when it loses its righting reflex. The animal stays in the trough until it regains its righting reflex. The criterion for the loss of righting was failure to right itself within a 30-60-sec period. Similarly, the animal was considered to have regained righting reflex when it could right itself twice within a 30- or 60-sec period, depending on the study (Tabakoff

and Ritzmann, 1979; McClearn and Kakihana, 1981). BEC or BrEC at initial LRR are often used as measures of initial sensitivity. AFT is again estimated as the difference between the initial BEC (or BrEC) and BEC (or BrEC) at recovery from hypnosis (Tabakoff and Ritzmann, 1979; Tabakoff et al., 1980).

Compared to the stationary dowel and rotarod tests, LRR has some advantages. First, righting reflex is a simple behavior present in virtually all animals, meaning no training is required to meet the behavioral criterion. When unimpaired, the animal will immediately right itself if placed in a supine position. In addition, because animals stay in a supine position throughout almost the whole experiment, possible effects of intoxicated practice on AFT become much less of an issue. Thus, when I became interested in the phenomenon of AFT and decided to investigate mechanisms underlying AFT, loss of righting reflex was my behavior of choice.

However, the traditional technique for the assessment of LRR has one shortcoming that limits its usage to study AFT. Behavioral neuroadaptation can be assessed to a full extent only when initial brain sensitivity is accurately measured. According to the traditional method, mice are manually restrained and observed for at least 30 sec after placing them on their backs (Tabakoff and Ritzmann, 1979). This makes it difficult to detect the precise moment of the initial loss of function, and hence to obtain the measure of initial sensitivity (Gill and Deitrich, 1998). Furthermore, because blood alcohol level is rising very rapidly after an i.p. injection, the 30-sec criterion pushes the initial blood sampling closer to the alcohol concentration plateau, thus diminishing chances to detect possible subsequent development of AFT. Several studies that used the traditional LRR method did fail to observe AFT in a variety of mouse genotypes

(Tabakoff et al., 1980; Crabbe and Kosobud, 1986), while others detected AFT only in some strains (Tabakoff and Ritzmann, 1979; Ritzmann and Tabakoff, 1980). It has been hypothesized that imprecise measurements of initial sensitivity have contributed to these results (Gill and Deitrich, 1998). Some studies avoided the problem of IS measurement by employing a between-subject assessment of AFT that did not depend on measurement of initial sensitivity (Belknap et al., 1977; Keir and Deitrich, 1990). In these studies, different groups of mice were injected with different doses of EtOH, and "sleep time" and BEC at recovery were measured. These variables were then plotted vs. EtOH dose and analyzed with linear regression. AFT was assessed from the slope of the regression lines, while initial sensitivity values were estimated as the intercepts of these plots. However, these studies have a potential problem of their own. Assessment of initial sensitivity and AFT with linear regression assumes linear development of AFT. Numerous studies have shown that development of AFT follows a curvilinear progression, with earlier portions of AFT developing much faster than parts of AFT at later time points (Gallaher et al, 1996; Erwin and Deitrich, 1996; Deitrich et al., 2000; Ponomarev and Crabbe, 2002). Because measurements of AFT based on BECs at recovery mainly capture development of the later portions of AFT, initial sensitivity measures estimated as intercepts of linear regression are likely to be lower than true values of initial brain sensitivity.

In an attempt to overcome the aforementioned problems, we have developed a novel method to assess the hypnotic sensitivity to EtOH (Ponomarev and Crabbe, 2002). The major differences between the new technique and the traditionally used method are a new apparatus and the manner in which animals are handled and scored for loss of

function. A cylindrical restrainer instead of a V-shaped trough is used to assess the loss and recovery of righting reflex. Our alternative of placing animals in the restrainers and slowly rotating them enables clear detection of the initial loss of upright posture and allows a shorter criterion for establishing LRR. The shorter criterion, in turn, results in lower BrEC and more sensitive values of initial brain response, which allows us to assess AFT to a full extent. Details of the novel technique are described in the Methods section.

We employed this novel method in the present project to investigate mechanisms of initial sensitivity and acute functional tolerance to the hypnotic effects of EtOH. First series of experiments examined effects of EtOH dose and time of EtOH exposure on the development of AFT.

Mechanisms of AFT: pharmacological interventions

Acquisition of AFT to intoxicating effects of EtOH is attributed to a rapid adaptation of neuronal processes, which causes a decreased response to the effects of EtOH on behavior (Deitrich et al., 2000). In fact, electrophysiological studies of the depressant effect of EtOH on the rate of neuronal firing have shown that acute neuronal tolerance develops in several brain structures thought to play a role in EtOH-induced sedation: cerebellum (Palmer et al., 1985; Pearson et al., 1997), medial septal area (Givens and Breese, 1990), and CA1 and CA3 regions of hippocampus (Ludvig et al., 2001). Whether these types of neuronal tolerance underlie AFT to EtOH-induced sedation should be addressed by further studies.

Despite considerable work on the phenomenon of AFT over recent years, little effort has been made to study the neurochemical mechanisms of AFT directly. One reason for the lack of such data may be inability of some behavioral paradigms to assess IS and AFT accurately in the same animals. One example is the traditional LRR method. Several lines of evidence indirectly suggest the involvement of different neurotransmitter systems in the regulation of AFT. Indirect evidence for such regulation can be obtained from at least three sources. The first is based on some electrophysiological studies showing rapid adaptation of some receptors to initial effects of EtOH. A second line of evidence is based on the assumption that all forms of tolerance are regulated by some common mechanisms, meaning that those neurochemical mechanisms thought to play a role in chronic tolerance may also affect AFT. Finally, studies that examined the effects of different drugs on duration of EtOH-induced LRR or "sleep time" might prove to be useful in generating hypotheses as to the mechanisms of AFT. It has long been recognized that LRR duration is influenced by both initial brain sensitivity, AFT that rapidly develops during the sleep time, and metabolic factors (Tabakoff and Ritzmann, 1979; Keir and Deitrich, 1990, Ponomarev and Crabbe, 2002). Thus, those pharmacological compounds that affect LRR duration may do so by influencing one, two or all three components that influence the sleep time. For example, those drugs that prolong duration of EtOH-induced LRR may decrease overall sensitivity (including initial brain sensitivity) and/or block the development of AFT, while compounds that shorten sleep time may have opposite effects on initial sensitivity and AFT.

Partial data supporting potential roles of different neurotransmitter systems in regulation of AFT are presented in Table 1. It is impossible to investigate all relevant

Table 1. Effects of manipulation of different neurotransmitter systems on duration of EtOH-induced LRR provide indirect evidence of potential involvement of these systems in regulation of AFT to EtOH-induced sedation.

Neurotransmitter system	Model	Effects on duration of EtOH-induced LRR	References
Glutamate	NMDA receptor antagonists	Increase	1
GABA	$GABA_A$ and $GABA_B$ receptor agonists	Increase	2
Serotonin	$5\text{-}HT_{1B}$ receptor knockout	Tend to be shorter than in controls Shorter than in controls	3 4
Dopamine	Apomorphine (agonist)	Increase in some genotypes	5
Nitric oxide (NO)	NO synthase inhibitor	Increase	6
Opioid	Naltrexone (antagonist)	Decrease	7
Norepinephrine	Alpha-2 receptor antagonist	Decrease	8

1. Daniell, 1990; 2. Martz et al., 1983; 3. Boehm et al., 2000; 4. Ponomarev and Crabbe, unpublished data; 5. Dudek et al., 1984; 6. Adams et al., 1994; 7. Kiianmaa et al., 1983; 8. Lister et al., 1989;

systems in one dissertation project. Therefore, I concentrated my efforts on the NMDA and GABA receptor systems, as they were supported most convincingly in the literature. Evidence has shown that excitatory NMDA and inhibitory GABA receptors are important sites of action of EtOH (Chandler et al., 1998). It has been suggested that acute intoxicating and incoordinating effects of EtOH are related to inhibition of NMDA receptor ion channels and potentiation of $GABA_A$ receptor ion channels (Crews et al., 1996). Research also suggested that $GABA_B$ receptors play an important role in modulation of EtOH effects (Shen et al., 1998; Yang et al., 2000). The rest of this section includes brief description of NMDA, $GABA_A$ and $GABA_B$ receptors as well as several examples showing that these receptor systems participate in processes associated with AFT.

NMDA and $GABA_A$ receptors are excitatory and inhibitory amino acid receptor complexes respectively, each of which comprises an ion channel and several modulatory sites. Both NMDA and $GABA_A$ receptors are composed of multiple subunit proteins assembled as heteromeric structures that exhibit distinct properties depending on the particular subunit composition (Chandler et al, 1998). Native NMDA receptors are usually a combination of NR1 and NR2 (NR2A-NR2D) subunits (Trujillo and Akil, 1995), while $GABA_A$ receptor compositions can include subunits of 7 families (alpha, beta, gamma, delta, epsilon, pi and rho) encoded by 18 genes (Mehta and Ticku, 1999). Activation of the competitive site on the NMDA receptor opens the ion channel and allows calcium to flow into the neuron. The channels are also permeable to sodium and potassium ions. Calcium entering the neuron can participate in several processes including activation of different protein kinases and the production of nitric oxide.

NMDA-receptor-mediated disruption of calcium influx has been shown to interfere with many processes that represent neural and behavioral plasticity, including neural development, learning, long-term potentiation, kindling, and rapid tolerance to EtOH (for review, see Trujillo and Akil, 1995). Agonists acting at the competitive site of the $GABA_A$ receptor complex increase Cl^- conductance across the membrane. This Cl^- influx leads to a rapid hyperpolarization of the cell, which accounts for GABA's inhibitory actions. $GABA_B$ receptors are inhibitory G-protein coupled receptors that exert their intracellular effects through inhibition of adenylate cyclase. Agonists acting at postsynaptic $GABA_B$ receptors activate potassium channels generating outward hyperpolarizing current, while presynaptic receptor activation can inhibit voltage-gated calcium channels, resulting in a decrease in neurotransmitter release (Frye and Fincher, 1996).

Two electrophysiological studies provided the most convincing evidence that changes in NMDA and $GABA_A$ receptor regulation might underlie AFT to EtOH. First, rapid desensitization (tolerance) of $GABA_A$ receptor to ETOH was reported by Allan and Harris (1987) who found that when mice were pretreated with EtOH 5 min to 1 hr prior to decapitation, EtOH no longer potentiated muscimol-stimulated ^{36}Cl- uptake in isolated cerebellar vesicles. In the second study, Grover and colleagues (1994) reported a case of acute neuronal tolerance, showing that application of EtOH to rat hippocampal slices led to an initial inhibition of NMDA-mediated EPSPs, which later recovered during the period of continuous EtOH exposure.

Research has also showed that rapid tolerance could be influenced by NMDA receptor agents (Khanna et al., 1997). For example, the NMDA partial agonist D-

cycloserine enhanced rapid tolerance to ethanol-induced motor incoordination in rats: small doses of EtOH on day 1 that previously did not lead to the development of rapid tolerance on day 2, did so after pretreatment with D-cycloserine (Khanna et al., 1995). The same group of investigators also reported that the NMDA receptor antagonist MK-801 blocked the development of rapid tolerance (Khanna et al., 1997).

Among a variety of chemicals that affect EtOH-induced sleep time, the NMDA, $GABA_A$, and $GABA_B$ receptor compounds play a central role. Generally, NMDA receptor antagonists and GABA receptor agonists prolong LRR duration, while $GABA_A$ antagonists shorten LRR duration. Among the former compounds are the NMDA NR2B subunit antagonist ifenprodil (Malinowska et al., 1999), the NMDA receptor channel blocker MK-801 (Daniell, 1990), the $GABA_A$ receptor agonist aminooxyacetic acid, and the $GABA_B$ agonist baclofen (Martz et al., 1983; Dudek and Phillips, 1989). The latter drugs include the $GABA_A$ antagonists, picrotoxin (Martz et al., 1983) and bicuculline (Phillips and Dudek, 1989). It is interesting to notice that effects of bicuculline on EtOH-induced hypnosis was strongly modulated by genotype, as this $GABA_A$ antagonist shortened duration of EtOH-induced LRR in some mouse genotypes but prolonged it in others (Phillips and Dudek, 1989; Dudek and Phillips, 1989).

The second part of the present project examined the effects of MK-801, D-cycloserine, ifenprodil, picrotoxin and baclofen on initial sensitivity and AFT to EtOH-induced sedation.

Mechanisms of AFT: Genetics

Inbred strains and selected lines. Studies of inbred strains of rodents and the development of selected lines are two classical approaches that have been utilized to study the genetic influence on alcohol-related traits. Inbred strains are developed by systematic inbreeding over 20 or more generations (Falconer and Mackay, 1996). Therefore, same-sex animals of any inbred strain are genetically identical. When several inbred strains are tested for a particular trait under carefully controlled environmental conditions, differences among strains represent genetic sources of phenotypic variance, whereas variability within strains is due to environmental effects or interaction between genetic and environmental influences. Heritability, defined broadly to include all sources of genetic variance, is estimated by comparing levels of variance within and among the inbred strains (Falconer and Mackay, 1996). A higher heritability is an indication of a greater genetic component that influences a specific trait. Testing inbred strains on a trait of interest gives us another advantage, one of studying genetic correlations between different traits, which, if significant, imply a common genetic etiology. Genetic correlation between the traits is assessed using Pearson's product moment correlation, with each strain's mean representing a single data point (Crabbe et al., 1990).

Another way to estimate genetic codetermination of two or more traits is to study animal lines selectively bred for high and low expression of the trait of interest. Usually, one or two pairs of the bidirectionally selected lines are established. Theoretically, during selection, genes affecting the trait will be fixed in a homozygous state, or at least increased in frequency, while the allelic frequencies of non-relevant genes should stay similar to those in the starting parental population. After several generations of selective breeding, additional phenotypic differences between lines may be discovered, which are

termed correlated responses to selection (Falconer and Mackay, 1996). If such differences are detected in all pairs of independently-derived replicated lines, it is commonly concluded that significant genetic correlation between the selected and correlated traits exists, implying a common genetic control of the two responses (Crabbe et al., 1990). Identification of traits that are genetically correlated with the selection response can be a powerful method for identifying potential mechanisms.

AFT is under genetic control. The fact that acute tolerance can be influenced by genetic factors has been known for a long time. Early work with inbred mouse strains showed that C57BL/6 mice developed more acute functional tolerance to the hypnotic effects of EtOH than DBA/2 animals (Grieve and Littleton, 1979; Tabakoff and Ritzmann, 1979). Using the 30-sec loss of righting criterion, these studies produced similar results despite employing different methods of EtOH administration, vapor inhalation for Grieve and Littleton and i.p. injection for Tabakoff and Ritzmann. However, later studies showed that these strain differences could be of different magnitude or even in the opposite direction, depending on the behavior tested. For example, these two strains did not differ in AFT to static dowel ataxia (Gehle and Erwin, 2000). In addition, Gallaher and colleagues (1996) reported a much greater AFT in the DBA/2J strain compared to C57BL/6J mice tested after a series of EtOH doses on a fixed-speed rotarod task. We also tested these two strains using our novel method based on the traditional LRR task. Similar to the Gallaher et al's results, DBA/2J mice in our study developed more AFT than C57BL/6J animals (Ponomarev and Crabbe, 2002). These results suggest not only that AFT is under genetic control, but also that AFT to different effects of EtOH can be influenced by different genes.

More convincing evidence for genetic regulation of AFT was provided by a selective breeding experiment. HAFT and LAFT lines of mice have been selectively bred in replicate (HAFT1, LAFT1, HAFT2 and LAFT2) for high and low AFT to EtOH-induced stationary dowel ataxia, respectively (Erwin and Deitrich, 1996). AFT was defined as the difference in BEC at regaining balance on a stationary dowel after two consecutive doses of EtOH. After 12 generations of selection, mean AFT scores of the HAFT lines were more than 4 times greater than those of the LAFT lines (Erwin et al., 2000). The HAFT and LAFT lines provide additional support for genetic heterogeneity of AFT to different EtOH effects. Despite drastic differences in the amount of AFT to dowel ataxia, these lines developed similar AFT to EtOH-induced motor impairment on a rotarod (Deitrich et al., 2000). Deitrich and colleagues suggested that different behavioral tasks employed to assess AFT are controlled by some different neuronal circuits that may, in turn, be regulated by different genes.

Genetic relationships between IS and AFT. Kalant and colleagues (1971) suggested a positive correlation between IS and acquired tolerance, which should be true for both acute and chronic forms. In fact, several studies using inbred mouse strains supported such a relationship between IS and chronic tolerance to EtOH-induced hypothermia (Moore and Kakihana, 1978; Crabbe et al., 1982; Crabbe et al., 1996) and ataxia (Crabbe et al., 1996). However, there is no clear picture as to whether IS and AFT are genetically related. Gallaher et al. (1996) showed the presence of some common genetic determinants for IS and AFT to EtOH-induced rotarod ataxia in the BXD RI mouse strains, with more sensitive strains developing more AFT. Contrary to this finding, Gehle and Erwin (2000) detected no genetic relationship between IS and AFT to

stationary dowel ataxia when means of 23 LSXSS recombinant inbred strains were correlated.

Selectively bred lines have also been used to study the genetic relationships between IS and AFT. Short-Sleep (SS) and Long-Sleep (LS) mice have been genetically selected for short and long duration of LRR after EtOH, respectively. Opposite to Kalant's prediction, the less sensitive SS mice developed more AFT to EtOH-induced LRR than the more sensitive LS animals (Keir and Deitrich, 1990). Despite substantial differences in AFT, the HAFT and LAFT lines did not differ in IS to the ataxic effects of EtOH (Deitrich et al, 2000). Thus, the genetic relationship between IS and AFT seems inconsistent. It is present only in some genotypes for some behavioral tasks, and sometimes the correlation is negative.

There are a number of problems that might lead to this inconsistency. First, measures of IS in some studies might have been estimated inaccurately, usually due to limitations in the behavioral paradigms. For example, studies that used the traditional LRR method might have faced a problem with determining IS precisely (Tabakoff and Ritzmann, 1979; Keir and Deitrich, 1990). Another problem is that calculation of AFT often relies on the value of IS (Tabakoff and Ritzmann, 1979; Gallaher et la., 1996), as BEC at initial LRR that characterizes this value is usually subtracted from another value of sensitivity at the time when AFT is developed [AFT = BEC (Sensitivity) at Time1 – BEC (IS) at initial LRR]. Thus, even if the second value of sensitivity is a random number, AFT will tend to correlate with IS, simply due to a mathematical dependency. This problem could be partially avoided by estimating IS and AFT from independent populations. To try to clarify the IS - AFT relationship further, it is desirable to test

additional populations of mice using additional EtOH-related tasks, on which IS and AFT can be clearly measured.

The final series of experiments investigated the genetic relationships among initial sensitivity, acute functional tolerance and rapid tolerance to EtOH-induced hypnosis. Three panels of inbred mouse strains were tested for hypnotic effects of an acute dose of EtOH using the procedure that assessed initial sensitivity and both forms of tolerance in the same animals.

Preliminary Study

This study was carried out to refine the novel technique for testing LRR and to demonstrate that AFT could be reliably measured in genetically heterogeneous mice. The procedure used in these experiments is schematically presented in Figure 2. We employed the loss of righting reflex, and used a modification of the serial recovery method previously described by Gallaher et al. (1982) for ethanol-induced ataxia. The bold line represents hypothetical blood (brain) ethanol concentration. Arrows represent injections of EtOH at the indicated doses

Nine male mice from the genetically heterogeneous Withdrawal Seizure-Control (WSC) stock (66-78 days of age) were placed in cylindric restrainers immediately after injection of 3 g/kg (20% v/v) EtOH. Restrainers were then gently turned 90 degrees every 2 seconds until mice were no longer able to right themselves within 5 seconds from a position on their back. A peri-orbital sinus blood sample (20 μl) was then obtained to measure BEC for an estimate of initial sensitivity and mice were placed back in the

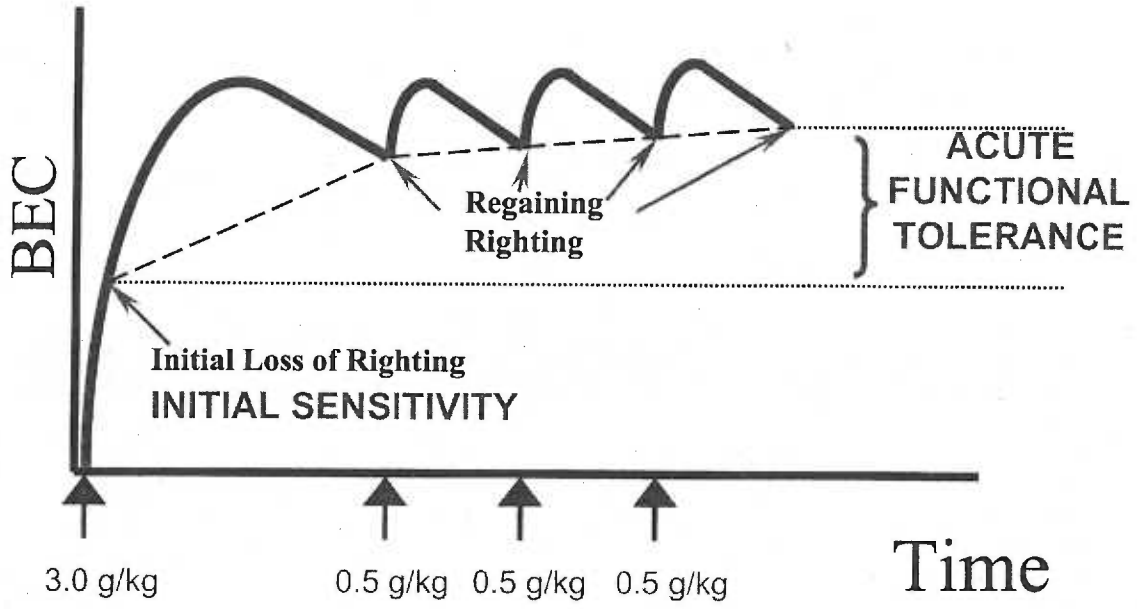

Figure 2. Paradigm used in the preliminary experiment. Curves indicate hypothetical blood EtOH concentration (BEC) after different doses of EtOH given at arrows. BEC at initial loss of righting reflex was used to estimate initial sensitivity (lower BEC = higher IS), while the difference between the highest BEC at any recovery from loss of righting reflex and BEC at initial loss of righting reflex was used to measure acute functional tolerance.

restrainers. Animals were then tested for the recovery of righting reflex at 5-10 min intervals. Animals were considered to regain righting reflex if they could right once within a 5-sec period or could not be placed on their backs after 8 turns of the restrainer. A "booster" injection of 0.5 g/kg EtOH was given upon each recovery to monitor the development of tolerance. Blood samples were also obtained at each recovery of righting reflex to quantify the time course and magnitude of AFT.

Despite rapidly rising EtOH concentrations during the first three minutes after injection, brain ethanol concentration (BrEC) can be reliably predicted and approximately equals BEC in a peri-orbital sinus blood sample taken 5-10 sec after initial LRR (Ponomarev and Crabbe, 2002). The average recovery time after initial and subsequent EtOH injections was 40-70 min. At the recovery time points, EtOH concentrations are changing very slowly and BEC approximately equals BrEC. Thus, using BEC estimates of BrEC allows us to measure initial sensitivity and functional tolerance to an acute exposure of EtOH in the same group of mice.

Figure 3 shows results of the testing. The dot at time zero indicates BEC at LRR – an estimate of initial sensitivity. Animals with higher BEC at LRR are considered to be less sensitive to the hypnotic effects of EtOH. The other four dots represent BEC at four successive recoveries of righting reflex. Repeated measures one-way ANOVA detected a main effect of BEC [$F(4,32)=21.0$, $p<0.001$]. The BECs at recoveries did not differ from each other but differed from the BEC at LRR. This result indicated that AFT developed to an almost full extent by the first recovery, and additional doses did not increase the magnitude of AFT significantly. Similar findings were reported in our subsequent study (Ponomarev and Crabbe, 2002), where C57BL/6J and DBA/2J strains also reached the

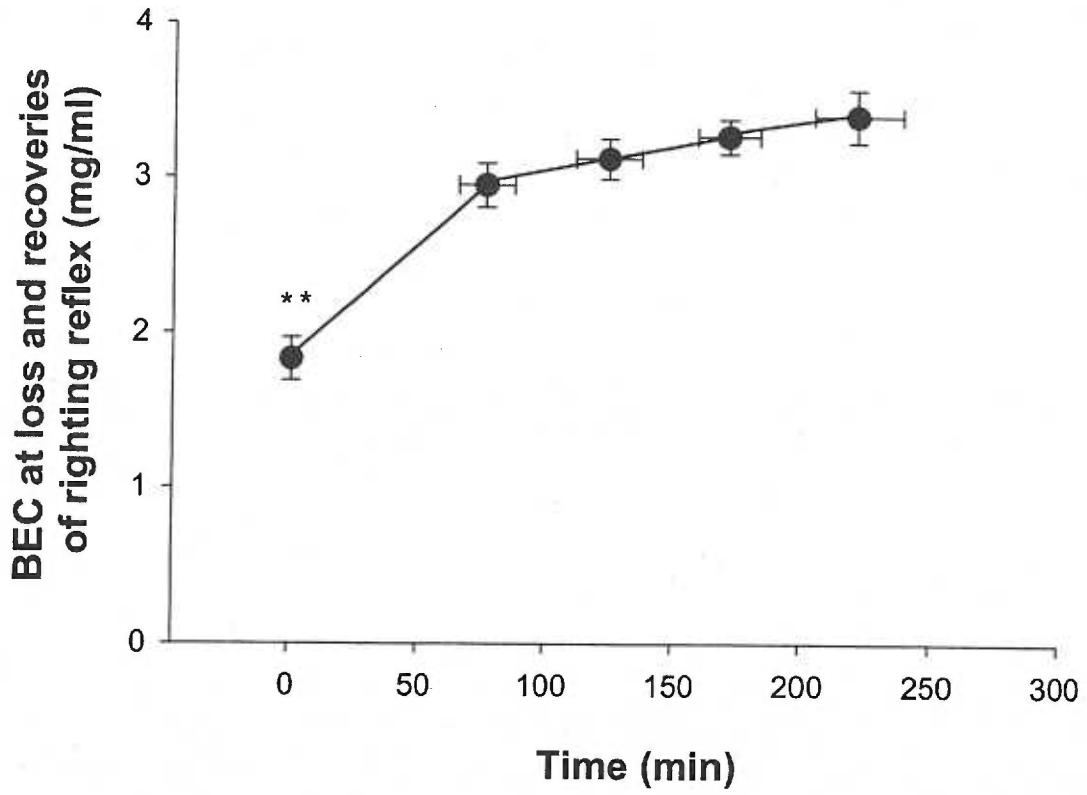

Figure 3. Results of the preliminary experiment. Means±SEM (n=9). Paradigm used for this experiment is shown in Figure 2. The dot at time zero indicates BEC at initial loss of righting reflex – an estimate of initial sensitivity. Lower BEC at loss of righting = higher initial sensitivity to the hypnotic effects of EtOH. The other four dots represent BEC at four successive recoveries of righting reflex. Asterisks indicate differences between BEC at loss of righting reflex and BECs at recoveries. ** = $p<0.01$

maximum BEC at recovery after the initial 3 g/kg dose. It is possible that additional doses did not increase the magnitude of AFT significantly because they did not result in a significant increase in BrEC compared to the peak following the initial injection. It is hypothesized that higher concentrations of brain ethanol will further increase the magnitude of AFT, because studies that used the traditional LRR method showed that mice injected with higher doses of ethanol recovered at higher BEC (Keir and Deitrich, 1990; Erwin et al., 2000). This hypothesis was tested in the first experimental part of this project.

In summary, the novel technique of testing EtOH-induced LRR was suitable to detect the development of AFT to EtOH. Moreover, the WSC mice proved to be a good model to study IS and AFT to the hypnotic effects of EtOH.

Hypotheses

An overall objective of the proposed studies was to investigate the phenomenological features and mechanisms of acute functional tolerance to the hypnotic effects of EtOH. A secondary goal of this project was to examine whether mechanisms of AFT are similar to those underlying IS and rapid tolerance. The first series of experiments (Hypotheses 1 and 2) took advantage of the novel LRR method, in which IS and AFT could be reliably measured in the same animals. Effects of EtOH dose on development of AFT and time course of AFT were studied. These experiments resulted in the development of the procedure that produced maximum or nearly maximum AFT. This AFT procedure, when repeated 24 hours later led to the development of between-session rapid tolerance. Subsequent experiments used this paradigm to ask the question whether initial sensitivity, AFT and rapid tolerance were influenced by similar or different mechanisms. The second series of experiments (Hypotheses 3 and 4) utilized a pharmacological approach to study mechanisms of AFT and to examine whether these mechanisms were similar to those regulating initial sensitivity and rapid tolerance. The final experiments (Hypothesis 5) used inbred mouse strains to study genetic relationships among initial sensitivity, AFT and rapid tolerance.

Hypothesis 1. AFT to EtOH-induced LRR develops in a dose-dependent manner.

This hypothesis is based on two theories. Kalant et al. (1971) suggested that magnitude of AFT is proportional to EtOH dose used, and that AFT develops only to those doses that produce initial impairment. Radlow (1994), on the other hand, suggested

that AFT is a time- but not dose-dependent process, and that AFT will start developing as soon as EtOH administration begins. We predict that the magnitude of AFT will depend on the dose applied. We also expect that this dependency will disappear when the magnitude of AFT reaches a certain maximum value (plateau). Compared to previous dose-response studies, a novel prediction is that AFT to EtOH-induced LRR will develop even to a dose that does not induce loss of function. Experiments 1 and 2 address this issue.

Hypothesis 2. AFT to EtOH-induced LRR develops within minutes after an EtOH injection.

Numerous data suggest that AFT to different effects of EtOH can develop within minutes to hours after an EtOH exposure (Mellanby 1919; LeBlanc et al., 1975; Keir and Deitrich, 1990; Gallaher et al., 1996; Gill and Deitrich, 1998). None of these studies was designed to evaluate the time course of the development of AFT directly. We predict that AFT to EtOH-induced sedation should be detectable within 5 – 10 minutes after exposure to EtOH, as suggested by time courses of acute neuronal tolerance (Palmer et al., 1985; Pearson et al., 1997; Givens and Breese, 1990; Ludvig et al., 2001). Experiments 2 and 3 address this issue.

Hypothesis 3. NMDA receptor compounds will affect both IS and AFT, but AFT to a greater extent.

As antagonists of central excitatory processes, the NMDA receptor antagonists increase general sensitivity to sedative-hypnotic drugs including EtOH (Daniell, 1990). In

fact, high doses of MK-801 by themselves can produce LRR in mice (Ponomarev and Crabbe, unpublished data). However, it is hypothesized that the NMDA receptor system is more sensitive to neuroadaptive processes that occur upon application of a relevant stimulus. This notion is supported by data on the involvement of the NMDA receptors in learning. Therefore, it is predicted that low doses of NMDA antagonists can block neuroadaptation to EtOH without affecting IS. We predict that the NMDA receptor antagonists MK-801 and ifenprodil will decrease AFT without increasing IS in the dose range applied. It is also expected that the NMDA receptor partial agonist D-cycloserine will enhance AFT, given that this compound enhanced some forms of RT. Experiments 4 - 8 address this issue.

Hypothesis 4. $GABA_A$ and $GABA_B$ receptor compounds will affect both IS and AFT.

GABA is a major inhibitory neurotransmitter. It is well know that relatively low doses of most GABA mimetics potentiate EtOH-induced sedation while higher doses can produce general anesthesia (Crews et al., 1996). Because previous data provided greater support for the involvement of NMDA receptors in regulation of AFT, as compared to GABA receptor systems, only two GABA drugs, a GABA agonist and a GABA antagonist were selected for these experiments. We predict that the $GABA_A$ antagonist picrotoxin will decrease while the $GABA_B$ agonist baclofen will increase general sensitivity to EtOH (including IS). It is not known whether IS and AFT to EtOH-induced hypnosis are affected equally by GABA receptor manipulations. Because picrotoxin and baclofen influenced duration of LRR, we expect that these compounds also affect acute

tolerance; picrotoxin should increase while baclofen should decrease AFT. Experiments 9 - 12 address this issue.

Hypothesis 5. IS, AFT and rapid tolerance share a common genetic component.

This hypothesis is based on the concept of Kalant (Kalant et al., 1971), who predicted that more sensitive animals will develop more tolerance. Thus, it is expected to find weak to moderate positive genetic correlations between IS and AFT as well as between IS and rapid tolerance. Given the assumption that AFT and rapid tolerance are regulated by some common mechanisms (San Marina et al., 1989), it is predicted to find these two variables to be correlated. Experiments 13, 14 and 15 address this issue.

Methods

Experimental procedures of this project did not require technical assistance and were carried out by the author of this manuscript. Blood and brain EtOH analyses were completed with the assistance of three technicians.

Subjects

Genetically heterogeneous Withdrawal Seizure-Control (WSC) male and female mice were used for experiments 1 through 12. This outbred stock was originally derived from intercrosses of eight inbred strains: A, AK, BALB/c, C3H, C57BL, DBA/2, Is/Bi, and RIII (McClearn and Kakihana, 1981). This colony is maintained at the Portland Department of Veterans Affairs Medical Center Veterinary Medical Unit. All WSC mice were 50 to 83 days old at the time of testing. Male and female mice of 21 isogenic genotypes were used for experiments 13 to 15. 20 inbred strains (129S1/SvIMJ, A/J, AKR/J, BALB/cByJ, BTBR+T tf/tf, C3H/HeJ, C57BL/6J, C57L/J, C58/J, CAST/Ei, DBA/2J, FVB/NJ, MOLF/Ei, NOD/LtJ, NZB/BINJ, PERA/Ei, PL/J, SJL/J, SM/J, SWR/J) and F1 hybrids of C57BL/6J × DBA/2J strains (B6D2F1/J) were obtained from the Jackson Laboratory, Bar Harbor, Maine. All 20 inbred strains were used in Experiment 15 only, while a subpanel of 8 strains (129S1/SvIMJ, A/J, BALB/cByJ, BTBR+T tf/tf, C3H/HeJ, C57BL/6J, DBA/2J, FVB/NJ) and the F1 hybrids were used in Experiments 13 and 14. These animals were allowed to acclimate to their new housing for at least one week upon arrival and were 70 to 115 days old at the time of testing. All mice were housed by strain, 3-5 animals per cage with food and water provided *ad*

libitum. Mice were maintained on a 12:12 hour light:dark cycle (lights on at 06:00). All experiments were initiated and completed between 08:30 and 18:00 hours.

Apparatus

Animals were tested in polycarbonate cylindric restrainers of our design, manufactured by a local company (Flair Plastic Products, Inc., Portland, Oregon). The restrainer is a hollow cylinder permanently attached to a squared base at one end and open at the other. After a mouse is placed inside the cylinder, the open end is shut with a sliding door through a gap located on the cylinder 6 mm from the open end. Both the squared base and the door contain round holes for ventilation. An adjustable plastic screw is located on the upper part of the door to tighten the door to the wall of the cylinder if necessary.

Restrainers of two sizes were used for bigger and smaller animals. Mice with body weight of 25 g or heavier were tested in restrainers with the following parameters: cylinder length (between base and door) – 100 mm, inner diameter of the cylinder – 44 mm, base side – 60 mm. Parameters for lighter mice were: cylinder length – 100 mm, inner diameter of the cylinder – 38 mm, base side – 55 mm. When placed in the restrainer, mice could easily turn around and had enough space to take one or two steps.

Drugs

All drugs were freshly mixed the morning of each experiment. MK-801 (dizocilpine), ifenprodil tartrate, D-cycloserine, picrotoxin and baclofen were purchased from Sigma. All drugs with the exception of ifenprodil were prepared in 0.9%

physiological saline. Ifenprodil tartrate was prepared in a 5% solution of dimethyl sulfoxide (DMSO) in saline. Doses are specified in the Methods for each experiment.

Ethanol (200 proof; Pharmco Products, Inc) was prepared 20% v/v in 0.9% physiological saline. Dose of EtOH was regulated by volume of EtOH solution injected (3.8 to 28.5 ml per kg body weight for 0.6 to 4.5 g/kg EtOH dose respectively).

Ethanol Assays

Procedures used to determine EtOH concentration in blood and brain were previously described in detail by Gallaher et al. (1996). Briefly, assays were carried out using a modification of the method of Roach and Creaven (1968). Brains were weighed and homogenized in an ice-cold mixture of $ZnSO_4$ (150 µl, 5%), $Ba(OH)_2$ (150 µl; 0.3 N) and water (brain weight x 1.5 – 300µl). The homogenate was centrifuged at 12,000 rpm for 10 min, and the supernatant was assayed using a gas chromatograph. For blood EtOH determination, a blood sample (20 µl) was added to a tube containing 50 µl $ZnSO_4$. An additional 50 µl $Ba(OH)_2$ and 300 µl water were added to the tube, followed by centrifugation at 12,000 rpm for 5 min. The supernatant was also assayed using gas chromatography. Sample peak area was referred to a standard curve derived from duplicates of 4 concentrations of ethanol in values bracketing the expected range. BrEC values were expressed as mg of EtOH per g of brain tissue, while BEC values had mg of EtOH / ml of blood units.

Although BrEC and BEC were expressed in different units, absolute values of ethanol concentration in these tissues should be comparable. One ml of blood weighs approximately 1 g (density of blood is just slightly greater than one). Therefore, BEC

values could also be expressed in mg/g. In addition, the water content in brain is similar to that in blood (approximately 79-81%). It is believed that EtOH is mainly distributed to tissues with higher water content (Goldstein, 1983). Thus, ethanol concentrations in brain and blood are expected to be similar when tissue equilibrium is reached.

Loss of Righting Reflex (LRR) Test

An animal was placed in a cylindric restrainer immediately after injection of a hypnotic dose of EtOH ip. The restrainer was then gently turned 90 degrees every 2-3 seconds. For the first few iterations of this procedure, mice immediately righted themselves. However, after approximately 10 to 30 such tests, mice would remain on their back after two successive 90 degree turns. Thus, the mouse was considered to have lost its righting reflex if it was no longer able to right itself within 5 seconds from a supine position. When that happened, the experimenter immediately started the timer. Latency to LRR was calculated as a time interval between onset of the injection and the end of the 5-sec cutoff period. A peri-orbital sinus blood sample (20 μl) was obtained as rapidly as possible for an estimate of initial sensitivity and the mouse was placed back in the restrainer. After practice, it was possible to obtain a sinus sample by 10 sec after LRR, and thereafter, efforts were made to keep this interval as regular as possible in all further studies. Mice remained in the restrainers throughout the experimental session. To eliminate the possibility of injections that missed the i.p. cavity, those mice that did not lose righting reflex within 2 min after the injection were excluded from the experiment. The rationale for this is discussed in Ponomarev and Crabbe (2002). Across all

experiments about 13% of animals were excluded. The percent of animals excluded did not appear to differ across treatment groups or across strains.

Animals were then tested for the recovery of righting reflex at 3-10 min intervals. Testing for recovery was similar to the procedure described above for the loss of righting reflex. Every testing episode began with the mouse being placed in an upright position. The restrainer was then again rotated 90 degrees every 2-3 seconds. Some of the mice could be placed on their back within the first 2 turns, while others were able to right themselves each time after a single 90 degree turn. Animals were considered to have regained righting reflex if they could either right themselves from a supine position within a 5-sec period or could not be placed on their backs after eight successive 90-degree turns of the restrainer. Duration of LRR – the time interval between the onset of LRR and recovery - was also registered.

General experimental procedures.

Mice were transferred to the experimental room in the morning of each experiment and left undisturbed for at least 1 hr. Mice were then weighed and placed in the cylindric restrainers for a 2-3 min habituation period. While in the restrainers, animals were turned 8 times to test their ability to maintain an upright position. All animals used in this project were able to maintain an upright position before EtOH administration. All data analyses were performed using the STATISTICA for Windows statistical package, version 5.1

Experimental procedures; Dose response and time course.

Experiment 1. Dose-response.

Separate groups of WSC mice (n=11-14 per group) were injected with 6 doses of EtOH (2.0, 2.5, 3.0, 3.5, 4.0, 4.5 g/kg), each mouse with one dose. They were then tested for the loss and recovery of righting reflex as described above and retro-orbital blood samples at LRR and recovery were taken. In addition, a third blood sample was taken 5 minutes after the injection. BEC at this time point represents the maximum EtOH concentration that could be reached after an i.p. injection (BECmax). Previous work has shown that BEC reaches plateau at 2-3 min after an i.p. injection and stays at the maximum level for at least 5 minutes (Gill and Deitrich, 1998; Ponomarev and Crabbe, 2002). BECs at the LRR were used as estimates of IS. Lower BECs indicated higher IS. An exploratory two-way analysis of variance (ANOVA) on all data was first carried out to detect AFT. Dose and Blood level were the two between-group factors, with Blood level having two levels: BEC at LRR and BEC at recovery. Effects of EtOH dose on each of the three variables (IS, BECmax, BEC at recovery) were estimated by separate one-way ANOVAs. Significant ANOVAs were followed by the Newman-Keuls post-hoc test.

Experiment 2. Effects of subhypnotic doses

The paradigm for this experiment is schematically shown in Figure 4. WSC mice (n=7-13 per group) were pretreated with a subhypnotic dose of EtOH (either 0, 0.6 or 1.1 g/kg) given 10 or 20 min before a higher, hypnotic dose that was calculated so that the total cumulative dose for each mouse was 3 g/kg. Thus, there were six groups. BEC was

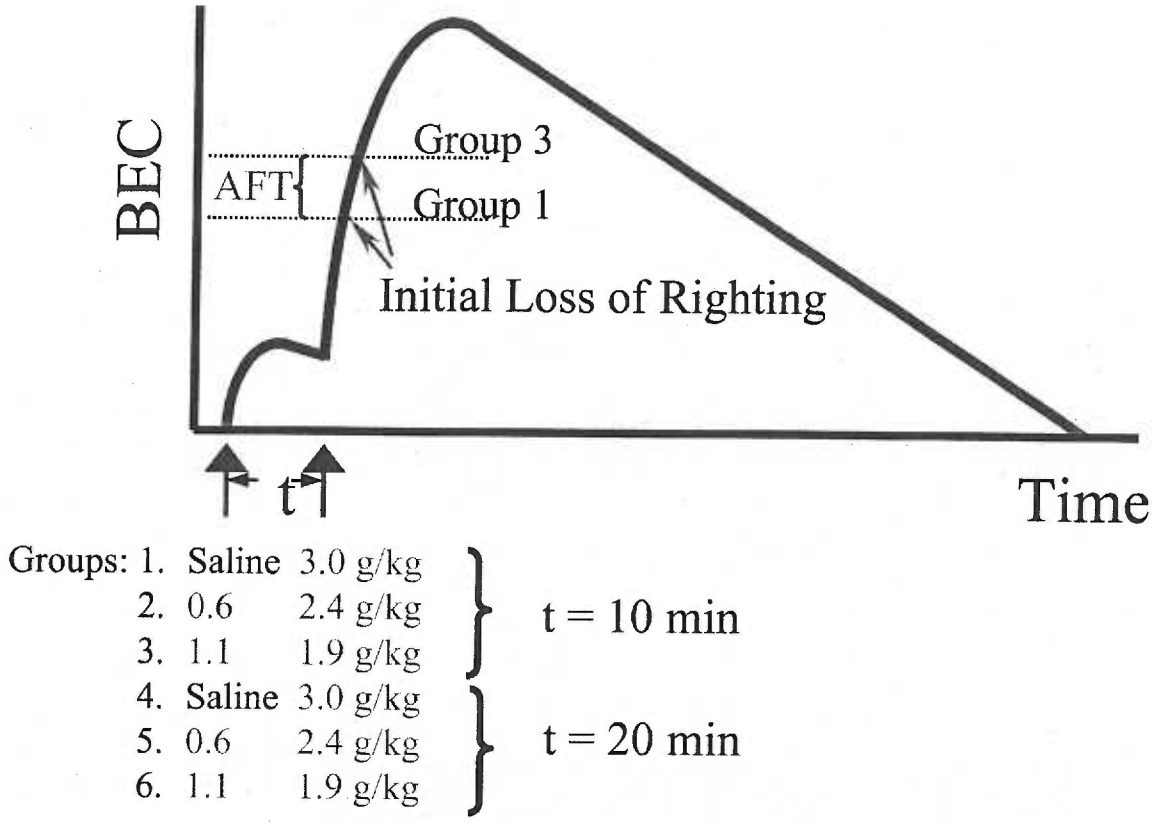

Figure 4. Procedure for Experiment 2. Curves indicate hypothetical blood EtOH concentration (BEC) after different subhypnotic and hypnotic doses of EtOH i.p. Dashed lines for Groups 1 and 3 were used as examples to demonstrate potential development of acute functional tolerance (AFT) to small subhypnotic doses. This procedure is described in detail in the Methods section.

measured at the initial LRR. In this paradigm, AFT can be seen as an increase in BEC at the LRR after pretreatment with EtOH, as compared to groups pretreated with saline (e.g. Group 3 vs. Group 1). Data were analyzed using a two-way (Dose x Time) ANOVA followed by the Newman-Keuls post-hoc test.

Experiment 3. Time course (BrEC)

The paradigm was similar to that used in Experiment 2. WSC mice (n=11-17 per group) were pretreated with either saline or a subhypnotic 1.3 g/kg dose of EtOH given 3, 6, 10 or 20 min before a hypnotic 3 g/kg dose (total of 8 groups). BrEC instead of BEC was measured at the LRR. Data from the four saline groups were first analyzed with a one-way ANOVA. Because there were no differences among the saline groups ($p>0.3$) data were collapsed across the groups. The combined Saline group and four EtOH groups were then analyzed by ANOVA followed by the Newman-Keuls post-hoc test.

Experimental procedures; Behavioral pharmacology.

Based on the first series of experiments, two behavioral paradigms were chosen for the pharmacological studies. These procedures are schematically shown and explained in Figure 5. Paradigm #1 used a hypnotic dose of EtOH. Initial sensitivity was estimated from BEC at LRR, and AFT was taken as the difference between BEC at loss and at recovery. This procedure was used for an exploratory purpose to identify those drugs that had statistically detectable effects on IS and/or AFT. Following injection of a drug (or saline) and a subhypnotic dose of EtOH (or saline) loss of righting reflex was

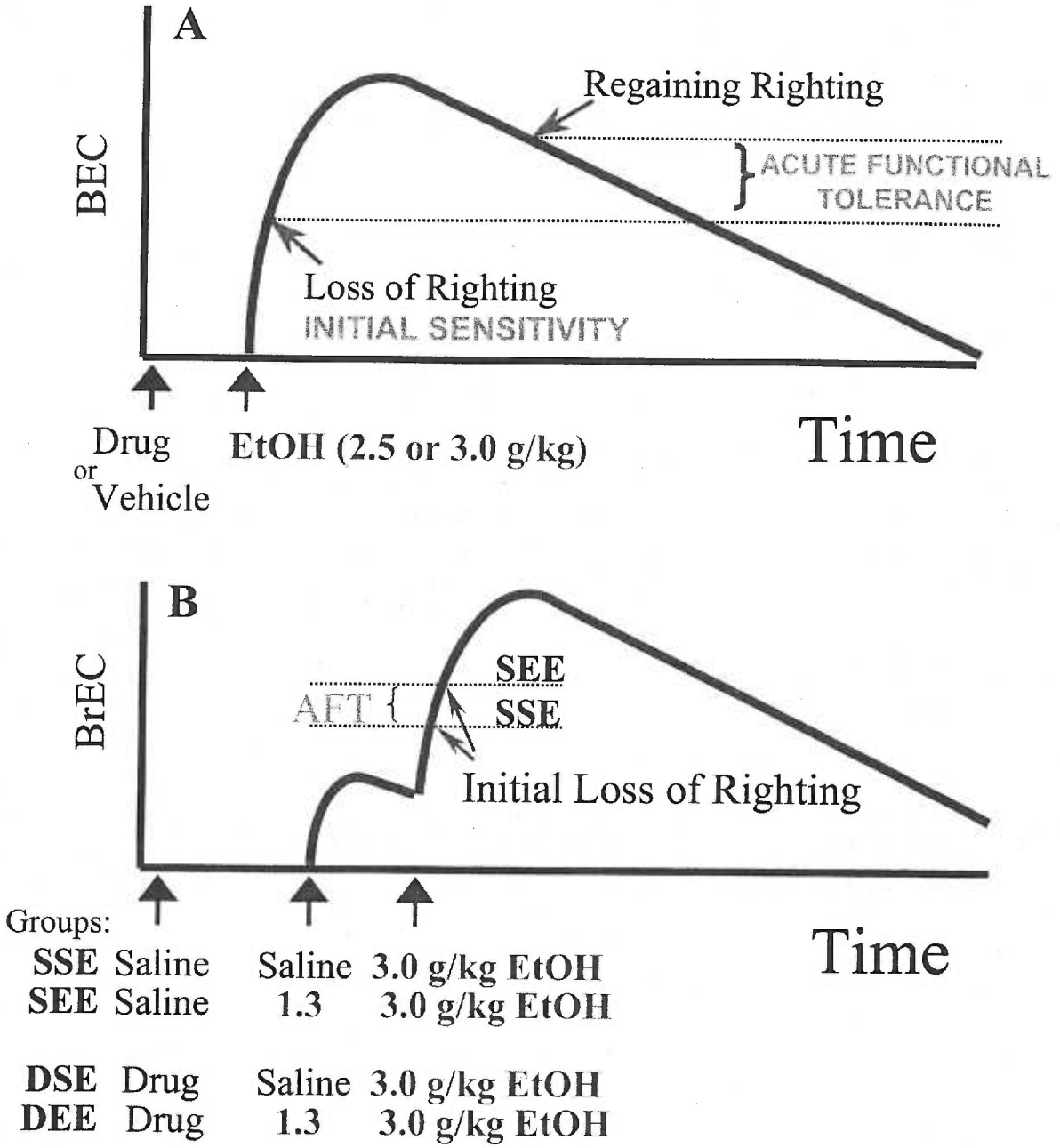

Figure 5. Curves indicate hypothetical blood (BEC) or brain (BrEC) EtOH concentration after different doses of EtOH i.p. (A) Paradigm #1. Mice were pretreated with either vehicle (5% DMSO in saline for ifenprodil and saline for other drugs) or one of five drugs (see Methods) 20-30 min before an i.p. injection of a hypnotic dose of EtOH. BECs at initial loss of righting and regain of righting were measured. BEC at initial loss of righting reflex was used to estimate initial sensitivity (IS) (lower BEC = higher IS), while the difference between BEC at recovery and BEC at initial loss of righting was used to measure acute functional tolerance (AFT). (B) Paradigm #2 was used to confirm findings from Paradigm #1. Mice were injected with either saline or one of three drugs 10-30 min before another injection of either saline or 1.3 g/kg EtOH. 20 min later all groups received 3.0 g/kg EtOH and BrEC at LRR was measured. AFT to the subhypnotic 1.3 g/kg dose was evident as the difference between saline-saline (SSE) and saline-EtOH (SEE) pretreated groups. The drug effect on IS could be assessed by comparing the SSE and drug-saline-pretreated (DSE) groups, while influence of the drug on AFT could be assessed by comparing the DSE and drug-EtOH-pretreated (DEE) groups.

determined after a 3rd injection, of a hypnotic dose of EtOH. The effects of these drugs were then confirmed or disconfirmed using Paradigm #2. In this procedure the same behavioral endpoint (initial LRR) was used to assess both IS and AFT. Time periods between injections of drugs and EtOH were chosen based on proposed maximum effects of these drugs on behavior (Martz et al., 1983; Daniell, 1990; Khanna et al., 1995; Khanna et al., 1997; Shen and Phillips, 1998; Malinowska et al., 1999).

Experiment 4. Effects of MK-801 on IS and AFT (Paradigm #1).

This experiment employed the NMDA receptor antagonist MK-801 to test the involvement of NMDA receptors in the mechanisms of IS and AFT to EtOH-induced hypnosis (hypothesis 3). Mice (n=6-13 per group) were pretreated with either saline or one of three doses of MK-801 (0.01, 0.1, 0.25 mg/kg) 20 min before an i.p. injection of 2.5 g/kg EtOH. Effects of MK-801 on LRR duration, IS and AFT were assessed with separate one-way ANOVAs followed by the Newman-Keuls post-hoc test.

Experiment 5. Effects of MK-801 on IS and AFT (Paradigm #2).

This experiment was used to confirm findings of Experiment 4. Saline or MK-801 (0.25 mg/kg) were given 30 min before a second injection of either saline or 1.3 g/kg EtOH. All four groups (n=10 per group) received a third injection of 3.0 g/kg EtOH, after which BrEC was measured at the LRR. Data were analyzed with one-way ANOVA followed by the Newman-Keuls post-hoc test.

Experiment 6. Effects of D-cycloserine on IS, AFT and rapid tolerance (Paradigm #1, Table 2).

The experimental paradigm is presented in Table 2. Groups (n=10-14 per group) were named according to their respective treatment on day 1. Group SS received two doses of saline, SE was treated with saline and then EtOH, and DS and DE received combinations of D-cycloserine and saline or D-cycloserine and EtOH respectively. Two doses of D-cycloserine were used, 10 and 100 mg/kg, thereby resulting in two DS and two DE groups. Effects of D-cycloserine on initial sensitivity and AFT were assessed by comparison of EtOH-treated groups (SE and DE) on day 1. Statistical analysis was similar to that used in Experiment 4. All groups were treated with EtOH on day 2. BEC at LRR and BEC at recovery on day 2 were analyzed with separate one-way ANOVAs followed by Newman-Keuls post-hoc test. Significantly greater BECs of the SE group, compared to SS animals was an indication of rapid tolerance development. The magnitude and direction of differences between the DS and DE groups reflected the effects of DC on rapid tolerance.

Experiment 7. Effects of MK-801 on rapid tolerance (Paradigm #1, Table 2).

The experimental paradigm is presented in Table 2. Measurement of rapid tolerance and statistical analysis were similar to that for Experiment 6 (n=8-12 per group). Development of rapid tolerance was also assessed by additional one-way ANOVAs on data of the SS and SE groups combined across Experiments 6 and 7.

Table 2. Drugs, doses and schedule for Experiments 6 and 7.

Experiment	**Group****	**Day 1**		**Day 2**
		Time 0 min	Time 20-30 min*	
6, 7	SS	Saline	Saline Yoked control	All groups were injected with 3 g/kg EtOH and LRR was tested.
6, 7	SE	Saline	EtOH, 3 g/kg Test for LRR (IS and AFT)	
7	MS	MK-801 (0.1 mg/kg)	Saline Yoked control	
7	ME	MK-801 (0.1 mg/kg)	EtOH, 3 g/kg Test for LRR (IS and AFT)	
6	DS	D-cycloserine (10, 100 mg/kg)	Saline Yoked control	
6	DE	D-cycloserine (10, 100 mg/kg)	EtOH, 3 g/kg Test for LRR (IS and AFT)	

* Time is chosen on the basis of published data as to the proposed maximum effects of the drugs (30 min for D-cycloserine and 20 min for MK-801). ** Groups were named according to their respective treatment on day 1.

Experiment 8. Effects of ifenprodil on IS and AFT (Paradigm #1).

Mice (n=9-13 per group) were pretreated with either vehicle or one of two doses of ifenprodil (1, 10 mg/kg) 20 min before an i.p. injection of 3 g/kg EtOH. Vehicle was a 5% solution of DMSO in saline. Statistical analysis was similar to that used in Experiment 4.

Experiment 9. Effects of picrotoxin on IS and AFT (Paradigm #1).

Mice (n=7-9 per group) were pretreated with either saline or one of two doses of picrotoxin (1, 2 mg/kg) 20 min before an i.p. injection of 3 g/kg EtOH. Statistical analysis was similar to that used in Experiment 4.

Experiment 10. Effects of picrotoxin on IS and AFT (Paradigm #2).

This experiment was used to confirm findings of Experiment 9. The procedure and statistical analysis were similar to those used in Experiment 5 (n=10 per group). The dose of picrotoxin used was 2 mg/kg.

Experiment 11. Effects of baclofen on IS and AFT (Paradigm #1).

Mice (n=6-9 per group) were pretreated with either saline or one of two doses of baclofen (2, 4 mg/kg) 20 min before an i.p. injection of 2.5 g/kg EtOH. Statistical analysis was similar to that used in Experiment 4.

Experiment 12. Effects of baclofen on IS and AFT (Paradigm #2).

This experiment was used to confirm findings of Experiment 11. The procedure and statistical analysis were similar to those used in Experiment 5 (n=10 per group). The dose of baclofen used was 4 mg/kg.

Experimental procedures; Behavioral genetics.

Because it was impossible to obtain sufficient numbers of animals of a certain age from all strains of interest at the same time, the genetics portion of the project was accomplished with three independent experiments. Table 3 explains the details of subjects and variables tested.

Experiment 13. Genetics of IS and AFT.

Mice of 9 isogenic genotypes (n=6-9 per genotype) were injected with 3 g/kg EtOH and tested for IS and AFT on day 1 only. IS was estimated as BEC at LRR (lower BEC = higher IS), while the difference between BEC at recovery and BEC at LRR was used to measure AFT. The genetic contribution of each variable was assessed with one-way ANOVA, where Genotype was the between-subjects factor. Heritability values (h^2) for each variable were calculated as SSbetween/SStotal from ANOVA.

Experiment 14. Genetics of IS, AFT and rapid tolerance.

Mice of 9 isogenic genotypes (n=4-6 per genotype per group) were tested. One half of the subjects were injected with 3 g/kg EtOH and tested for IS and AFT (EtOH groups), while the other half were injected with saline on day 1 (Saline groups). All mice

Table 3. Strains and variables for Experiments 13-15

Experiment #	Panels of strains	Sex/age	N per strain	Variables tested*
13	1. Subpanel of 9 genotypes	Female/ 70-80 day old	6-9	IS, AFT
14	2. Subpanel of 9 genotypes	Male/female/ 115 day old	4-6	IS, AFT, RTwithin, RTbetween
15	3. Full panel of 20 strains	Male/female/ 70 day old	3-6	IS, AFT, RTwithin

* IS and AFT were measured on day 1. IS was estimated as BEC at LRR (lower BEC = higher IS), while the difference between BEC at recovery and BEC at LRR was used to measure AFT. RTwithin for BEC at LRR and BEC at recovery was calculated as the difference between the corresponding measures obtained on days 2 and 1. RTbetween was calculated similarly from values obtained on day 2. The strain mean value of either BEC at LRR or BEC at recovery of the group treated with saline on day 1 and EtOH on day 2 was subtracted from each subject's value of the corresponding variable of the group treated with EtOH on both days.

received 3 g/kg EtOH on day 2. Values of RTwithin for BEC at LRR and BEC at recovery were calculated as the difference between the corresponding measures obtained on days 2 and 1. RTbetween was calculated similarly from values obtained on day 2. Strain mean values of either BEC at LRR or BEC at recovery of the Saline groups were subtracted from each subject's value of the corresponding variable of the EtOH groups. Thus, there were 2 RTwithin and 2 RTbetween variables. Each variable was analyzed with separate one-way ANOVAs, and heritability values were then estimated.

Experiment 15. Genetics of IS, AFT and rapid tolerance.

Mice of 20 inbred strains received 3 g/kg EtOH on two consecutive days. Measures of IS, AFT and 2 measures of RTwithin were obtained. Heritability values were again estimated from ANOVA.

Eight strains were used in all three experiments. Reliability of IS, AFT and 2 RTwithin measurements was assessed by genetic correlations among the three panels. Genetic correlations were estimated using Pearson's product moment correlation, with each strain's mean representing a single data point. Thus, 12 correlation coefficients were obtained, 3 for each variable. In addition, we calculated genetic correlations among the four variables (IS, AFT and 2 RTwithin measures) using data combuned across all three experiments; that is, using means of 20 inbred strains and B6D2F1 hybrids.

Results

Dose response and time course.

Results of Experiments 1 - 3 showed that a) AFT developed in a dose-dependent fashion but not beyond a certain maximum value and b) AFT developed very rapidly and approached its maximum value for a certain dose by the 10^{th} min after EtOH administration began.

Experiment 1. Dose response.

BEC at initial LRR did not differ among the groups [$F(5,71)=1.2$; $p=0.34$], indicating that IS does not depend on EtOH dose (Table 4). Results are shown in Figure 6. A two-way ANOVA with Dose and Blood level (two levels: BEC at LRR and BEC at recovery) as between-group factors detected a main effect of Dose [$F(5,71)=5.2$; $p<0.001$] and a main effect of Blood level [$F(1,71)=186$; $p<0.001$], with the latter finding indicating development of AFT to different doses. The Dose x Blood level interaction did not reach statistical significance [$F(5,71)=1.9$; $p=0.1$], suggesting that AFT development is rather dose-independent. However, ANOVA with multiple factors is rather insensitive to detect interactions between factors that have multiple levels, especially when some of those levels are not different from each other (Pedhazur, 1982). For example the Dose factor has 6 dose levels, with the highest four doses appearing not different from each other, thereby "contaminating" the interaction analysis with additional variance. Reducing the number of levels increases the chances of detecting a significant

Table 4. BEC at initial LRR (lower BEC = higher IS) after several doses of EtOH.

Means±SEM. No group differences were detected by ANOVA.

EtOH dose (g/kg)	2.0	2.5	3.0	3.5	4.0	4.5
BEC at LRR (mg/ml)	2.26 ± 0.10	2.34 ± 0.19	2.61 ± 0.16	2.58 ± 0.14	2.67 ± 0.11	2.45 ± 0.15

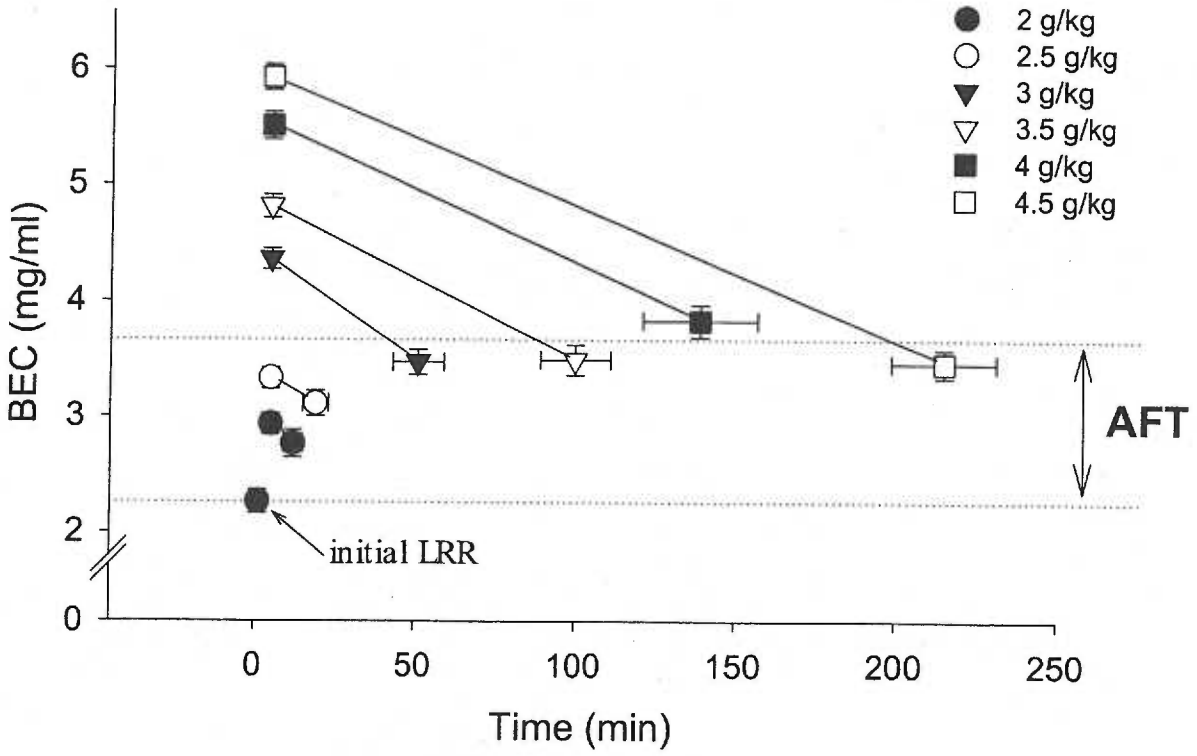

Figure 6. Dose response for AFT. Means±SEM (n=11-14 per group). Symbol at time zero shows an example of BEC at initial loss of righting reflex (LRR) after a 2 g/kg dose (other values not shown; see Table 4). Symbols vertically arranged at the 5 min time point represent maximum BECs after i.p. injections of different doses. The other symbols are BECs at recovery from loss of righting reflex. The lower dotted line demonstrates an approximate level of initial sensitivity, while the upper dotted line models an average recovery plateau. The distance between the lines reflects the magnitude of acute functional tolerance (AFT) developed.

interaction. For example, including only 2 doses in the analysis (2.0 and 4.0 g/kg) resulted in a significant Dose x Blood level interaction [$F(1,21)=10.9$; $p<0.01$] that indicated that magnitude of AFT depends on EtOH dose. To clarify effects of Dose on IS and AFT, the two-way ANOVA was followed by a number of one-way ANOVA for each dependent variable.

A one-way ANOVA on BEC at recoveries showed a main effect of dose [$F(5,71)=8.7$; $p<0.001$] (Figure 6). The post-hoc analysis revealed differences between the 2 g/kg group and the 5 highest-dose groups as well as between the 2.5 g/kg group and each of the two highest dose groups. There were no significant differences among the 4 highest dose groups. These findings along with the IS data suggest that AFT develops in a dose-dependent manner, but not linearly with dose, reaching its near maximum value after a 3 g/kg dose. Results of this experiment also showed that different doses of EtOH produced different maximum BECs [$F(5, 71)=132.0$; $p<0.001$] (Figure 6).

Experiment 2. Effects of subhypnotic doses.

The only significant effect detected by a two-way (Dose x Time) ANOVA was a main effect of Dose [$F(2,57)=7.0$; $p<0.01$] (Figure 7). Post-hoc analysis showed that BEC at LRR values after each of the EtOH doses were significantly greater than values after saline pretreatment, but did not differ from each other, indicating that AFT developed to subhypnotic doses of EtOH. Results of the first two experiments combined showed that the magnitude of AFT is generally proportional to the dose (Figure 8). Results of Experiment 2 suggested that AFT developed within the first 10 min of EtOH

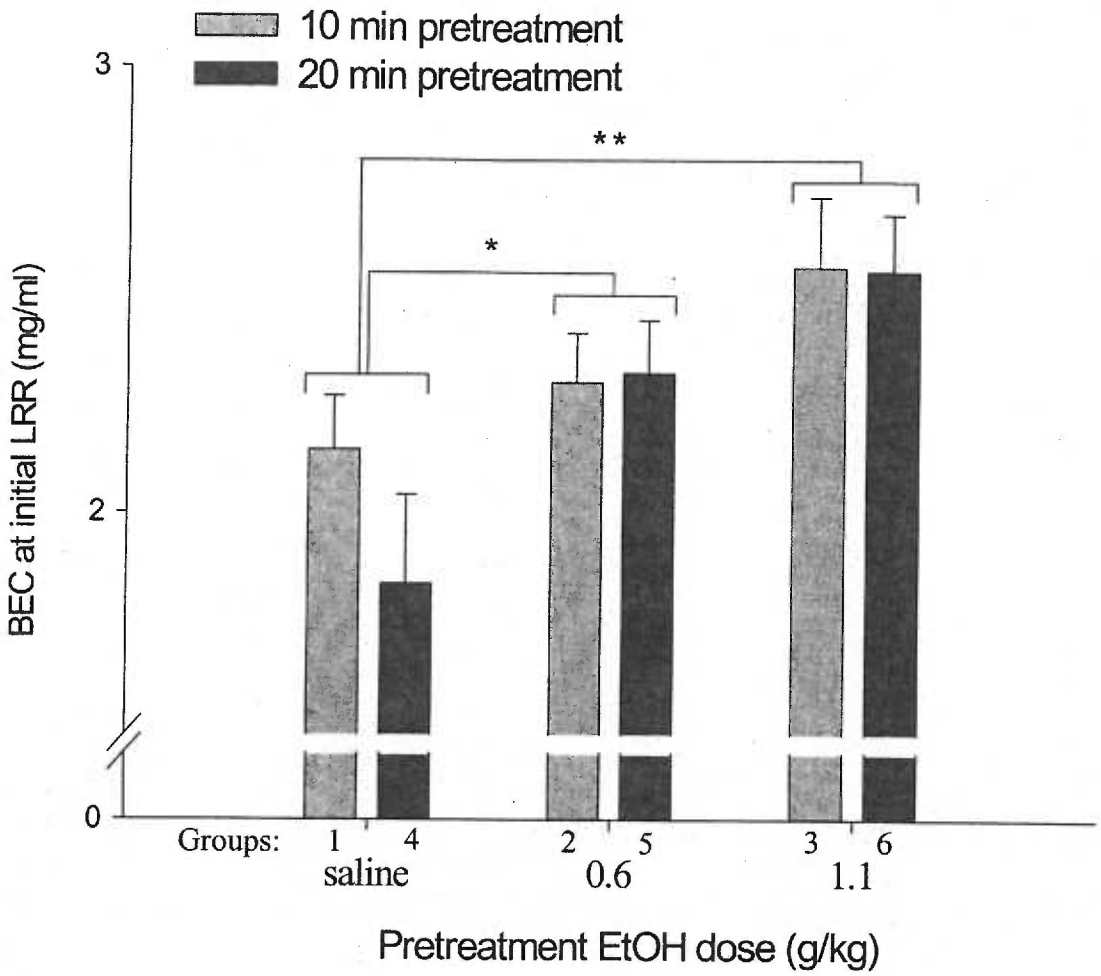

Figure 7. Development of acute functional tolerance to two subhypnotic doses of EtOH. Means±SEM (n=7-13 per group). Groups 1 to 6 were treated according to the procedure shown in Figure 4. Asterisks indicate significant differences between EtOH-pretreated and saline groups detected by ANOVA as a main effect of Dose and by post-hoc analysis. * = $p<0.05$; ** = $p<0.01$

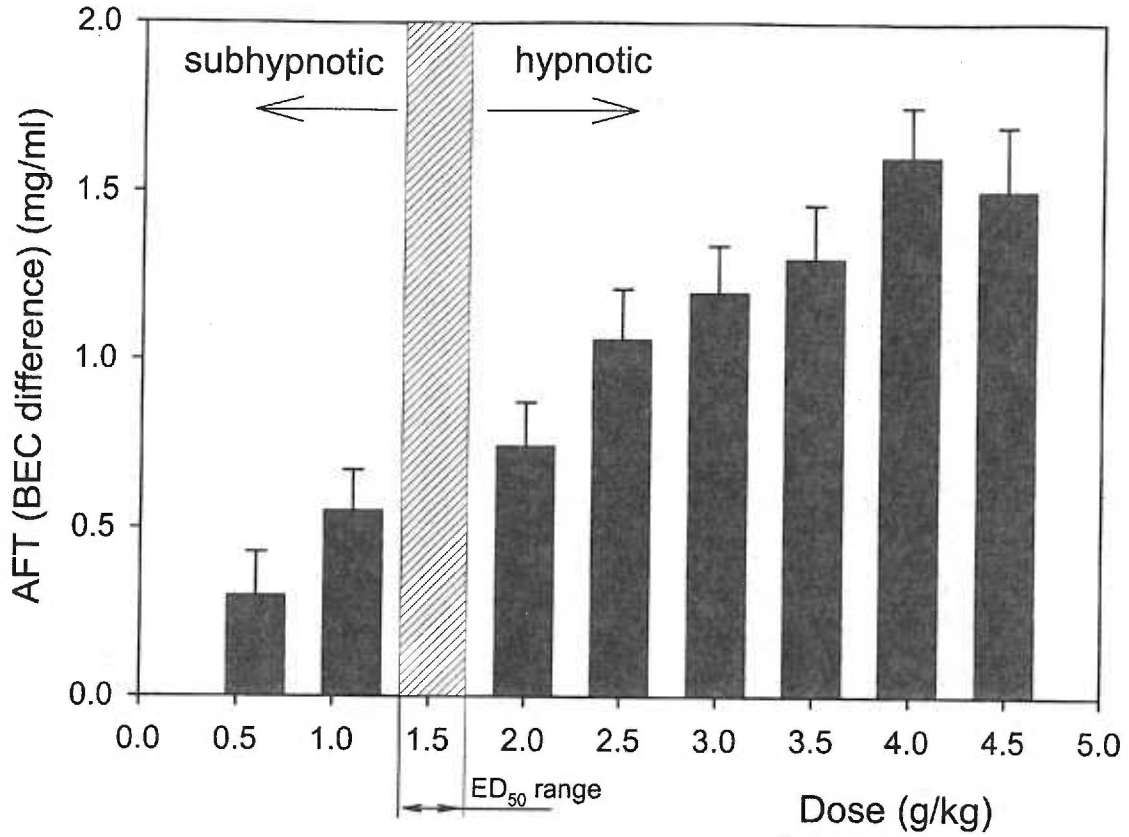

Figure 8. Calculated acute functional tolerance (AFT) values for different doses of EtOH (solid bars), derived from data in Table 4 and Figures 6 and 7. The striped bar shows an estimated range of doses that may or may not result in loss of righting reflex in WSC mice (ED_{50} ranges from 1.3 to 1.7 g/kg). The two solid bars on the left are AFT values for the two subhypnotic doses used in Experiment 2. They are calculated as differences between individual values of EtOH-pretreated animals and average values of saline-pretreated groups (see fig. 6). Solid bars to the right of the striped bar show AFT values based on Experiment 1. These values are calculated as differences between BEC at recovery and BEC at loss of righting + 0.28 mg/ml. The 0.28 value indicates the approximate increase in BEC for 10 sec during the very rapid absorption phase after a 2.0 g/kg dose, given that the average absorption rate for this dose = 0.028 mg/ml/sec. This absorption rate was reported by Gallaher et al., (1996) for C57BL/6J and DBA/2J mice and was confirmed in our laboratory for WSC mice (Ponomarev and Crabbe, unpublished data). Because BEC at loss of righting reflex was measured with a delay of approximately 10 sec from the onset of loss of righting reflex, the values of AFT from Experiment 1 were adjusted by adding the 0.28 minimum value. Results indicate that the magnitude of AFT appears to be proportional to the dose regardless of the effects this dose exerts on behavior (subhypnotic vs. hypnotic).

administration. To refine the time course of AFT development, Experiment 3 was carried out.

Experiment 3. Time course.

Results of this experiment confirmed and further extended findings of Experiment 2 as to the time course of AFT. A one-way ANOVA revealed a main effect of group [$F(4,57)=7.9$; $p<0.001$] (Figure 9). Post-hoc analysis detected differences between the saline and the 10-min EtOH groups as well as the saline and the 20-min EtOH groups. Differences between the saline group and each of the other two groups did not reach statistical significance. The 10-min and the 20-min EtOH groups did not differ from each other. Thus, AFT to a subhypnotic dose of EtOH appeared to develop fully by the 10^{th} min of EtOH exposure.

Behavioral pharmacology

Results of Experiments 4-12 suggested that IS and AFT to EtOH-induced hypnosis are, in part, regulated by different mechanisms; the NMDA receptor antagonist MK-801 dose-dependently inhibited AFT but did not affect IS, while the $GABA_B$ receptor agonist baclofen increased IS but did not affect AFT. MK-801 also blocked the development of RT, which suggests the involvement of NMDA receptors in regulation of both acute and rapid forms of tolerance.

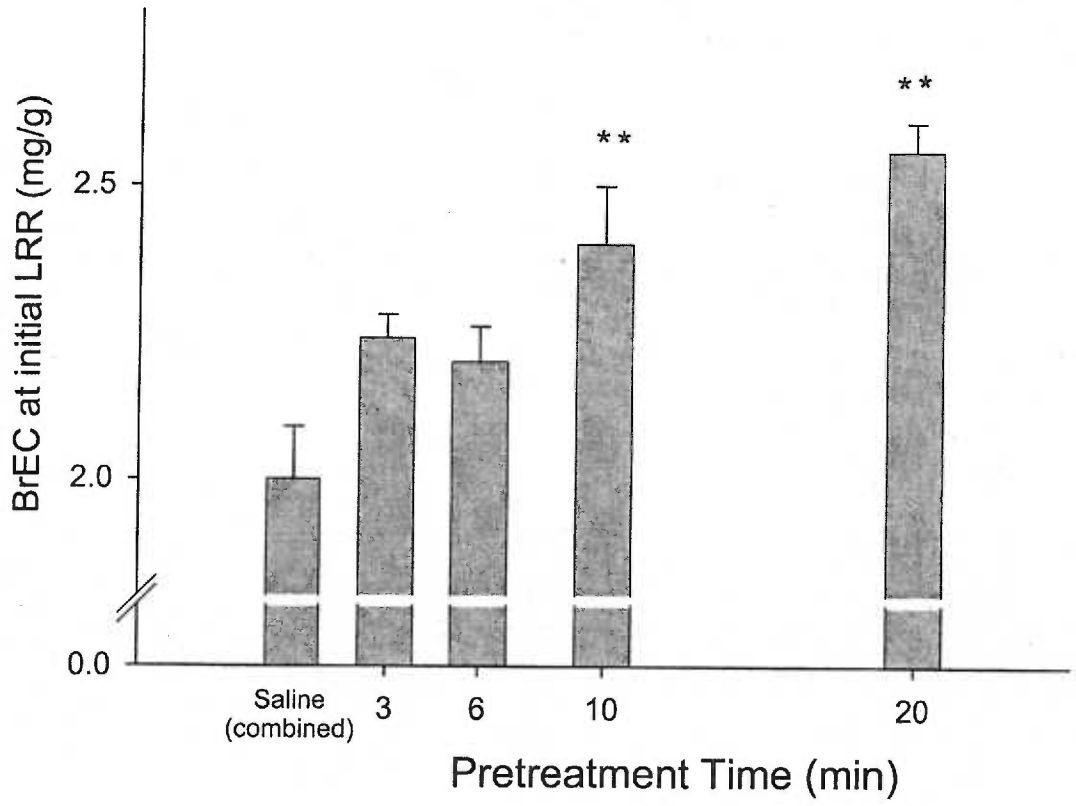

Figure 9. Development of acute functional tolerance to a subhypnotic (1.3 g/kg) dose of EtOH, followed by a 3 g/kg dose. Means±SEM (n=11-17 per group). Groups were treated according to a procedure similar to that shown in Figure 4. Asterisks indicate significant differences between EtOH-pretreated groups and saline group. ** = $p<0.01$

Experiment 4. Effects of MK-801 on IS and AFT (Paradigm #1).

Results are shown in Figure 10. ANOVA detected a main effect of drug dose on LRR duration [$F(3,30)=17.8$; $p<0.001$]. Groups pretreated with the two highest doses of the drug had longer duration of EtOH-induced LRR. IS was not significantly affected by the drug [$F(3,30)=0.5$; $p=0.66$]. On the other hand, AFT was significantly influenced by MK-801 [$F(3,30)=4.2$; $p=0.01$], with the two highest dose pretreatment groups having lower AFT values, as compared to saline-pretreated controls. Experiment 5 was carried out to confirm these findings.

Experiment 5. Effects of MK-801 on IS and AFT (Paradigm #2).

A one-way ANOVA revealed a main effect of group [$F(3,36)=4.2$; $p=0.01$] (Figure 11). BrEC values of the SE group were greater than those of the SS group, indicating development of AFT to a subhypnotic pretreatment dose ($p<0.05$). The MS and ME groups did not differ, showing inhibiting effects of MK-801 pretreatment on AFT. The SS and MS groups also did not differ, indicating no significant effect of MK-801 on IS. Results of this experiment confirmed the findings of Experiment 4: that is, MK-801 inhibited acquisition of AFT, but did not affect IS to EtOH-induced sedation.

Experiment 6a. Effects of D-cycloserine on IS and AFT (Paradigm #1, Table 2).

The two doses of D-cycloserine used in this study did not affect either "sleep time", IS or AFT (all $p>0.25$) (Figure 12). Therefore, this drug was not tested using Paradigm #2.

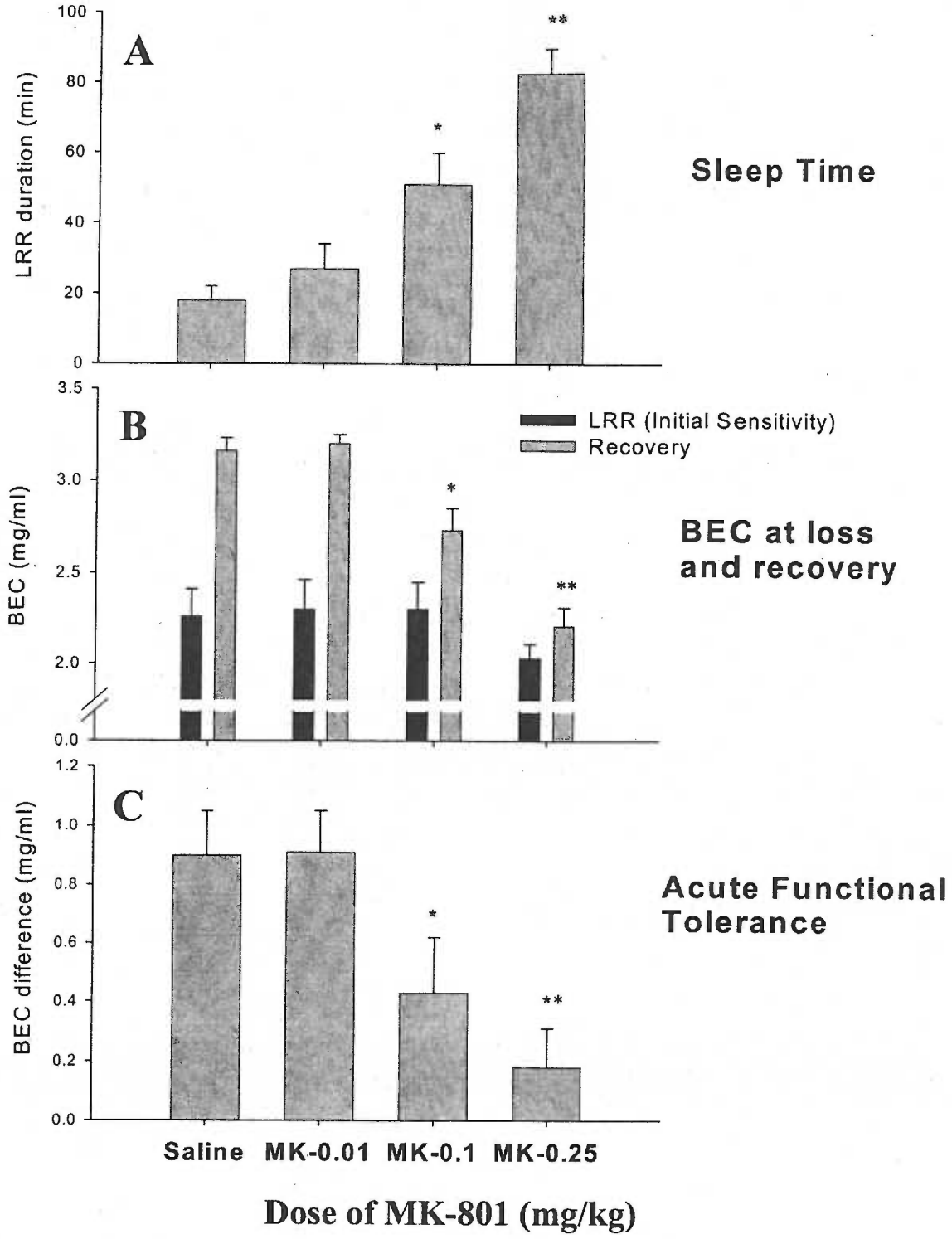

Figure 10. Effects of MK-801 on (A) "sleep time", (B) initial sensitivity and recovery, and (C) acute functional tolerance. Means±SEM (n=6-13 per group). Groups were pretreated with either saline or different doses of the drug and then injected with 2.5 g/kg EtOH 20 min later. Asterisks indicate significant differences between drug-pretreated and saline groups. * = $p<0.05$; ** = $p<0.01$

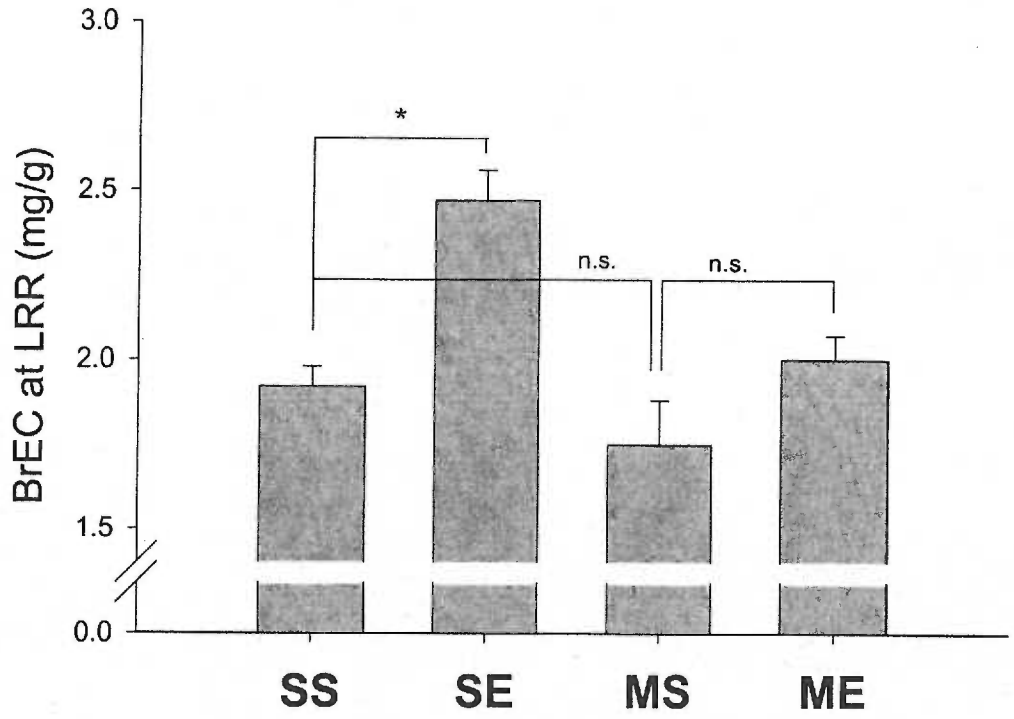

Figure 11. Effects of MK-801 on initial sensitivity and acute functional tolerance (AFT). Means±SEM (n=10 per group). Groups were treated according to the procedure shown in Figure 5B. A small subhypnotic dose of EtOH (1.3 g/kg) resulted in development of AFT to EtOH-induced loss of righting reflex (SS vs SE groups). Pretreatment with MK-801 (0.25 mg/kg) blocked this AFT (MS vs ME) but did not affect initial sensitivity (SS vs MS). * = $p<0.05$; n.s. (non significant) = $p>0.05$

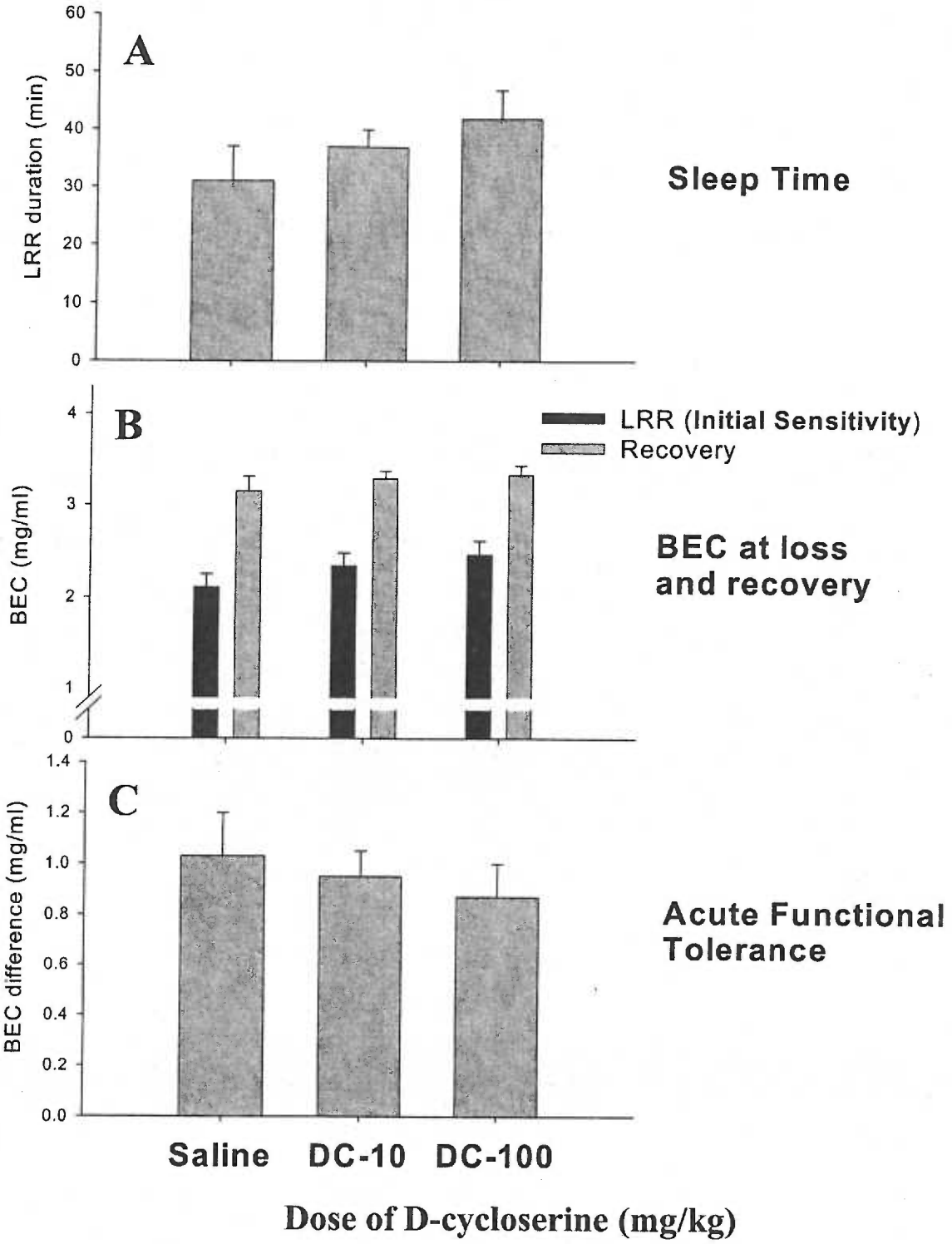

Figure 12. Effects of D-cycloserine on (A) "sleep time", (B) initial sensitivity and recovery, and (C) acute functional tolerance. Means±SEM (n=10-14 per group). Groups were pretreated with either saline or different doses of the drug and then injected with 3.0 g/kg EtOH 30 min later. No group differences were found for any variable.

Experiment 7. Effects of MK-801 on rapid tolerance (Paradigm #1, Table 2).

MK-801 blocked the development of rapid tolerance to the BEC at LRR (Figure 13A). This effect was detected by ANOVA [$F(3,34)=3.2$, $p=0.03$], followed by a post-hoc analysis as no difference between MS and ME groups ($p=0.8$), compared to significant differences between SS and SE groups ($p=0.03$). This effect of MK-801 was also evident as a trend to block rapid tolerance to the recovery BEC [$F(3, 34)=2.1$; $p=0.1$]. This experiment confirmed previous findings of Khanna et al (1997) that pretreatment with MK-801 on day 1 blocked the development of rapid tolerance measured on day 2.

Experiment 6b. Effects of D-cycloserine on rapid tolerance (Paradigm #1, Table 2).

Animals that were tested for IS and AFT in Experiment 6a were tested for rapid tolerance on day 2. Rapid tolerance was assessed on day 2, when all groups (see Table 2) received 3 g/kg EtOH (Figure 13B). Pretreatment with D-cycloserine on day 1 did not affect rapid tolerance to EtOH-induced hypnosis measured on day 2. This experiment failed to confirm previous findings of Khanna et al (1995), who showed that pretreatment with D-cycloserine on day 1 enhanced the development of rapid tolerance in rats. The differential results of this experiment and the 1996 study could be based on using a different species and/or behavioral paradigms.

Because rapid tolerance was not statistically detected in 3 out of 4 cases in two experiments, I decided to pool data from the SS and SE groups from the two experiments and analyze them separately. When data were collapsed over the two experiments (6b and

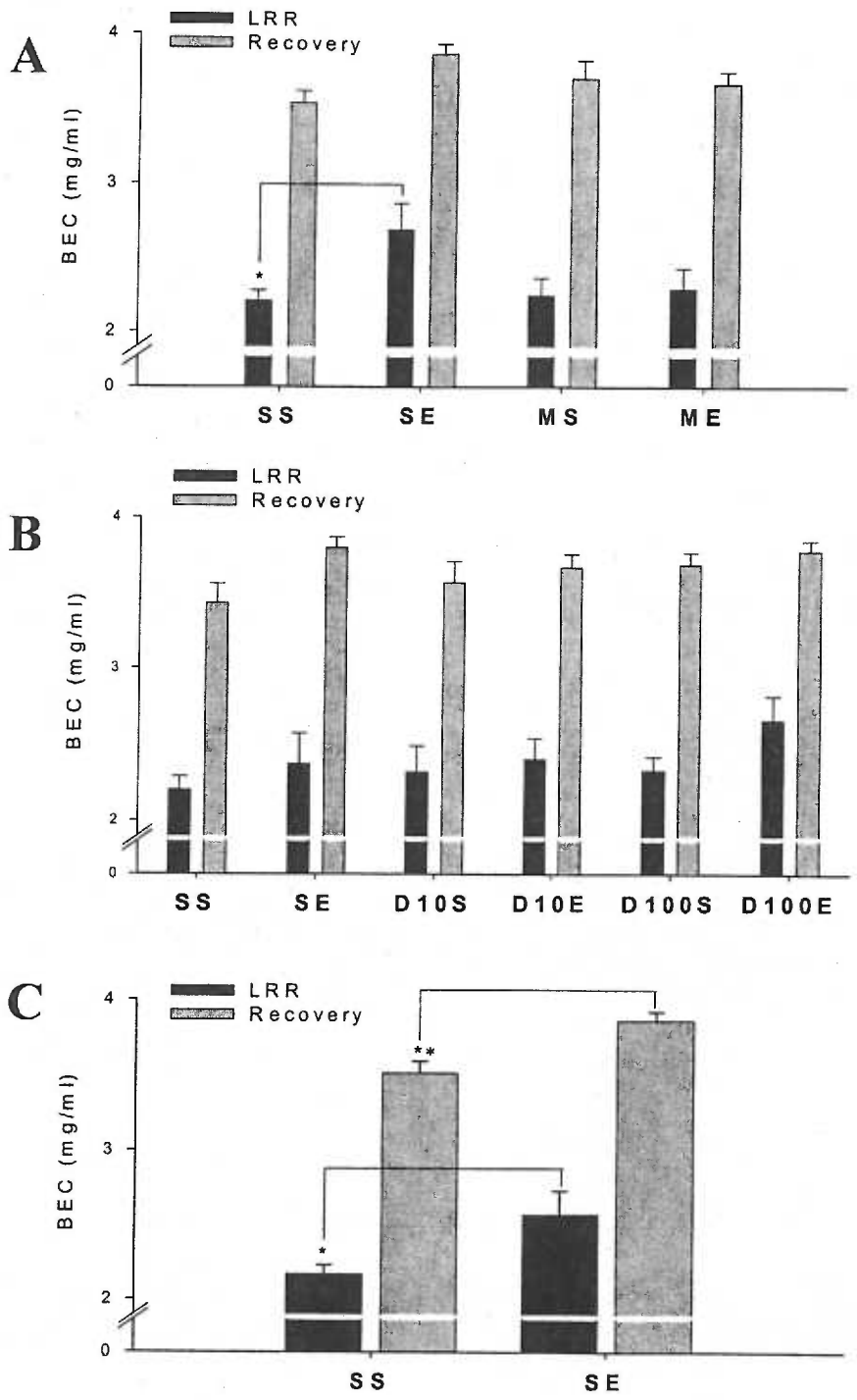

Figure 13. Effects of (A) MK-801 and (B) D-cycloserine on rapid tolerance. Means±SEM (n=8-12 per group). Groups were treated according to the two-day procedure described in Table 2. Data shown are from day 2, when all groups were injected with a 3 g/kg EtOH dose. Groups are marked according to their treatment on day 1; two doses of saline (SS), saline and EtOH (SE), a dose of one of the drugs and saline (MS, D10S, D100S), and drug plus EtOH (ME, D10E, D100E). (C). Data of the SS and SE groups from the two experiments (A and B) were combined. Asterisks indicate development of rapid tolerance. * = $p<0.05$; ** = $p<0.01$

7) statistically detectable rapid tolerance was seen (Figure 13C), as animals treated with two consecutive injections of saline on day 1 (SS) lost righting reflex and recovered at lower BECs compared to animals that received a combination of saline and EtOH (SE) [$F(1,36)=5.9$; $p=0.02$ for LRR, $F(1,36)=12.1$; $p<0.01$ for recovery].

Experiment 8. Effects of ifenprodil on IS and AFT (Paradigm #1).

The two doses of ifenprodil used in this study did not affect either "sleep time", IS or AFT (all $p>0.21$) (Figure 14). This drug was not tested using Paradigm #2. These data did not support the previous finding of Malinowska et al. (1999) who reported that a 10 mg/kg dose of ifenprodil prolonged the duration of EtOH-induced LRR in Swiss Webster mice. The differential results of this experiment and the 1999 study could be based on using a different mouse genotype and/or behavioral paradigms.

Experiment 9. Effects of picrotoxin on IS and AFT (Paradigm #1).

Picrotoxin shortened EtOH-induced duration of LRR [$F(2,20)=3.2$; $p=0.05$] (Figure 15A). However, BEC at recovery was not significantly affected by the pretreatment of picrotoxin [$F(2,20)=2.2$; $p=0.14$], which indicated that the GABA antagonist might influence EtOH metabolism. Picrotoxin did not have significant effects on either IS or AFT (all $p>0.09$). There was a slight trend for the drug to increase IS (decrease BEC at LRR) (Figure 15B) and a stronger trend to increase AFT (Figure 15C). Because of these trends and because the former trend was in contrast to my predictions, effects of picrotoxin on IS and AFT were examined using Paradigm #2.

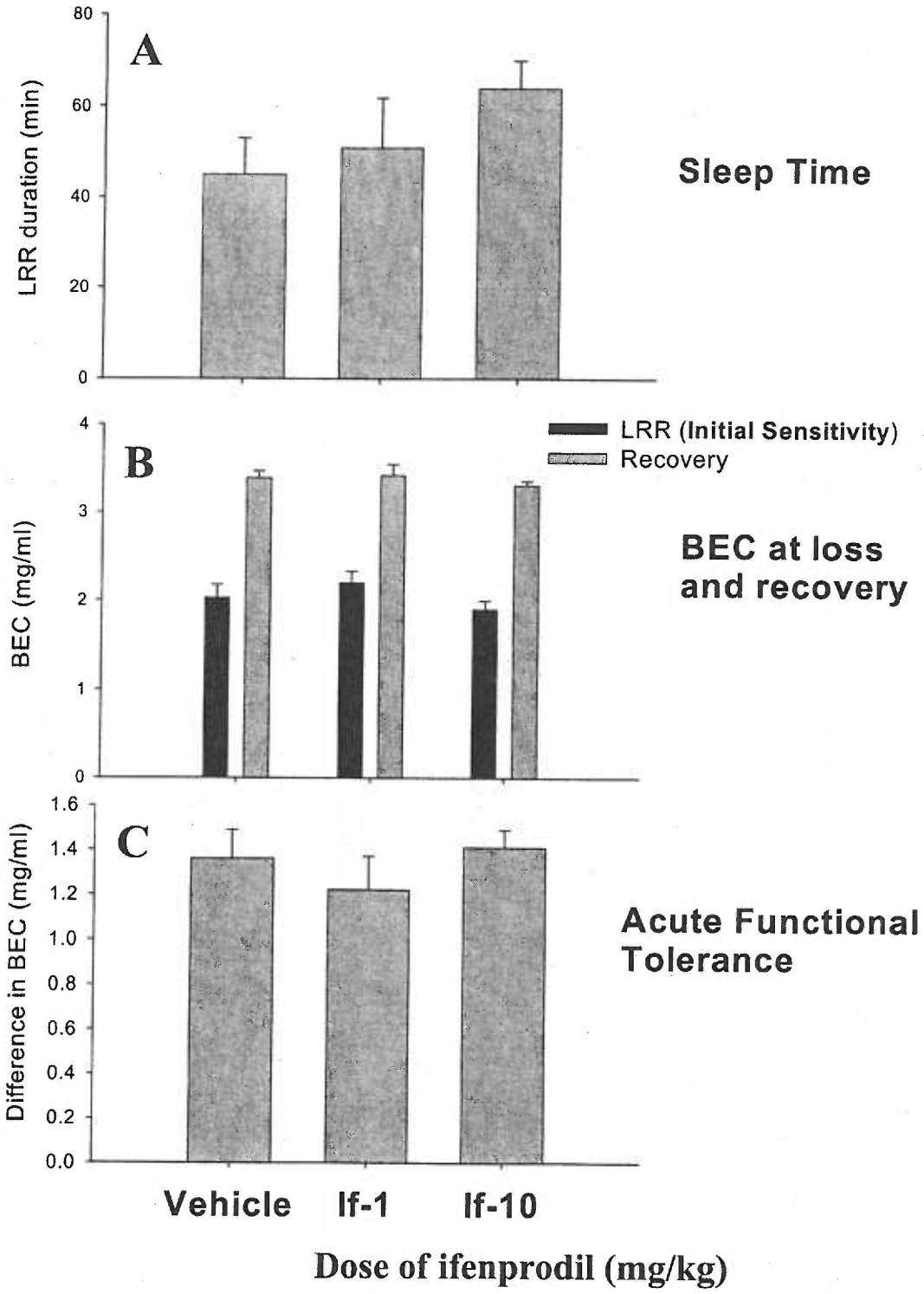

Figure 14. Effects of ifenprodil on (A) "sleep time", (B) initial sensitivity and recovery, and (C) acute functional tolerance. Means±SEM (n=9-13 per group). Groups were pretreated with either vehicle or different doses of the drug and then injected with 3.0 g/kg EtOH 20 min later. No group differences were found for any variable.

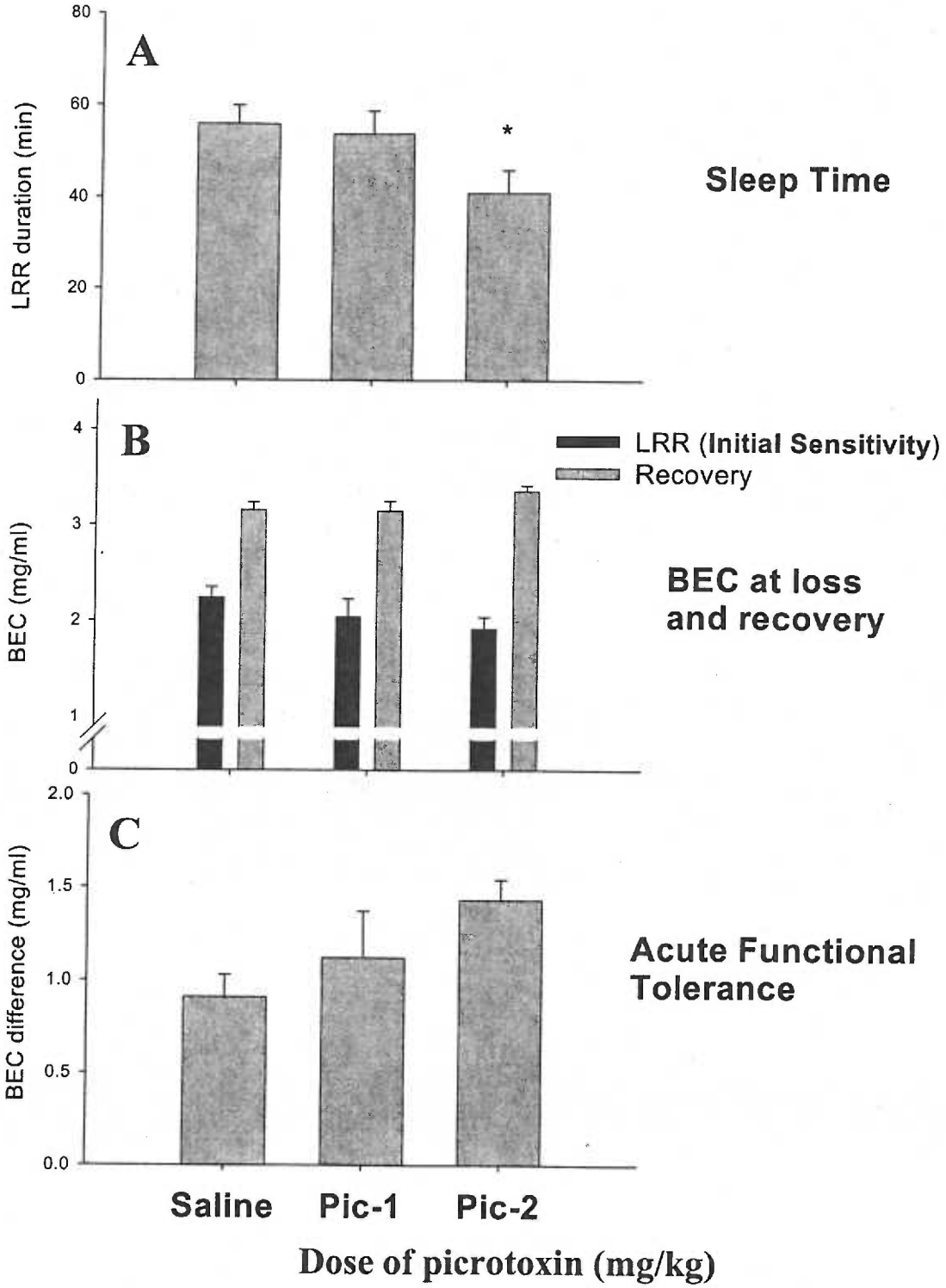

Figure 15. Effects of picrotoxin on (A) "sleep time", (B) initial sensitivity and recovery, and (C) acute functional tolerance. Means±SEM (n=7-9 per group). Groups were pretreated with either vehicle or different doses of the drug and then injected with 3.0 g/kg EtOH 20 min later. Asterisk indicates a significant difference between the group pretreated with 2 mg/kg of the drug and the saline group. * = $p<0.05$

Experiment 10. Effects of picrotoxin on IS and AFT (Paradigm #2).

An overall ANOVA detected a main effect of group [$F(3,36)=7.0$; $p<0.001$] (Figure 16). A small subhypnotic dose of EtOH resulted in development of AFT to EtOH-induced LRR, as the SE group lost righting reflex at greater BrEC than the SS mice ($p=0.01$). Pretreatment with picrotoxin did not affect either IS [no difference between SS and PS groups ($p>0.4$)] or AFT, as the PE group was different from the PS group ($p=0.01$), and the magnitude of this difference was similar to that of the SS – SE difference. Based on these two experiments, it could be concluded that the two doses of picrotoxin used have a low capacity, if any to modulate the hypnotic effects of EtOH.

Experiment 11. Effects of baclofen on IS and AFT (Paradigm #1).

The two doses of baclofen significantly prolonged EtOH-induced "sleep time" [$F(2,20)=14.2$; $p<0.001$], lowered BEC at recovery [$F(2,20)=11.9$; $p<0.001$] and increased IS [$F(2,20)=6.6$; $p<0.01$], but did not affect AFT [$F(2,20)=0.3$; $p=0.77$] (Figure 17). These findings together indicate that the $GABA_B$ receptor agonist generally enhanced the hypnotic effect of EtOH without influencing the rapid adaptation to this effect. To confirm this conclusion Experiment 12 was carried out.

Experiment 12. Effects of baclofen on IS and AFT (Paradigm #2).

ANOVA detected a main effect of group [$F(3,36)=12.0$; $p<0.001$] (Figure 18). Development of AFT was induced by a subhypnotic dose [SE group > SS group ($p<0.01$)]. Pretreatment with baclofen enhanced the hypnotic effects of EtOH, as both groups pretreated with baclofen had lower BrEC at LRR, compared to their respective

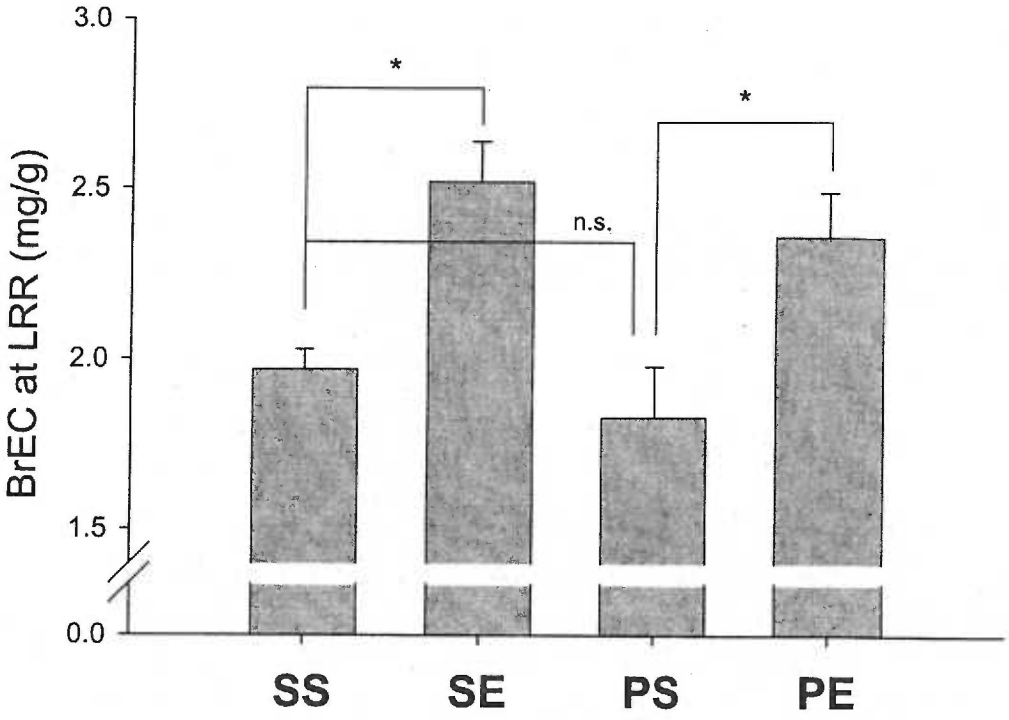

Figure 16. Effects of picrotoxin on initial sensitivity and acute functional tolerance (AFT). Means±SEM (n=10 per group). Groups were treated according to the procedure shown in Figure 5B. A small subhypnotic dose of EtOH (1.3 g/kg) resulted in development of AFT to EtOH-induced loss of righting reflex (SS vs SE groups). Pretreatment with picrotoxin (2 mg/kg) did not affect either initial sensitivity (SS vs PS) or AFT (PS vs PE). * = $p<0.05$; n.s. (non significant) = $p>0.05$

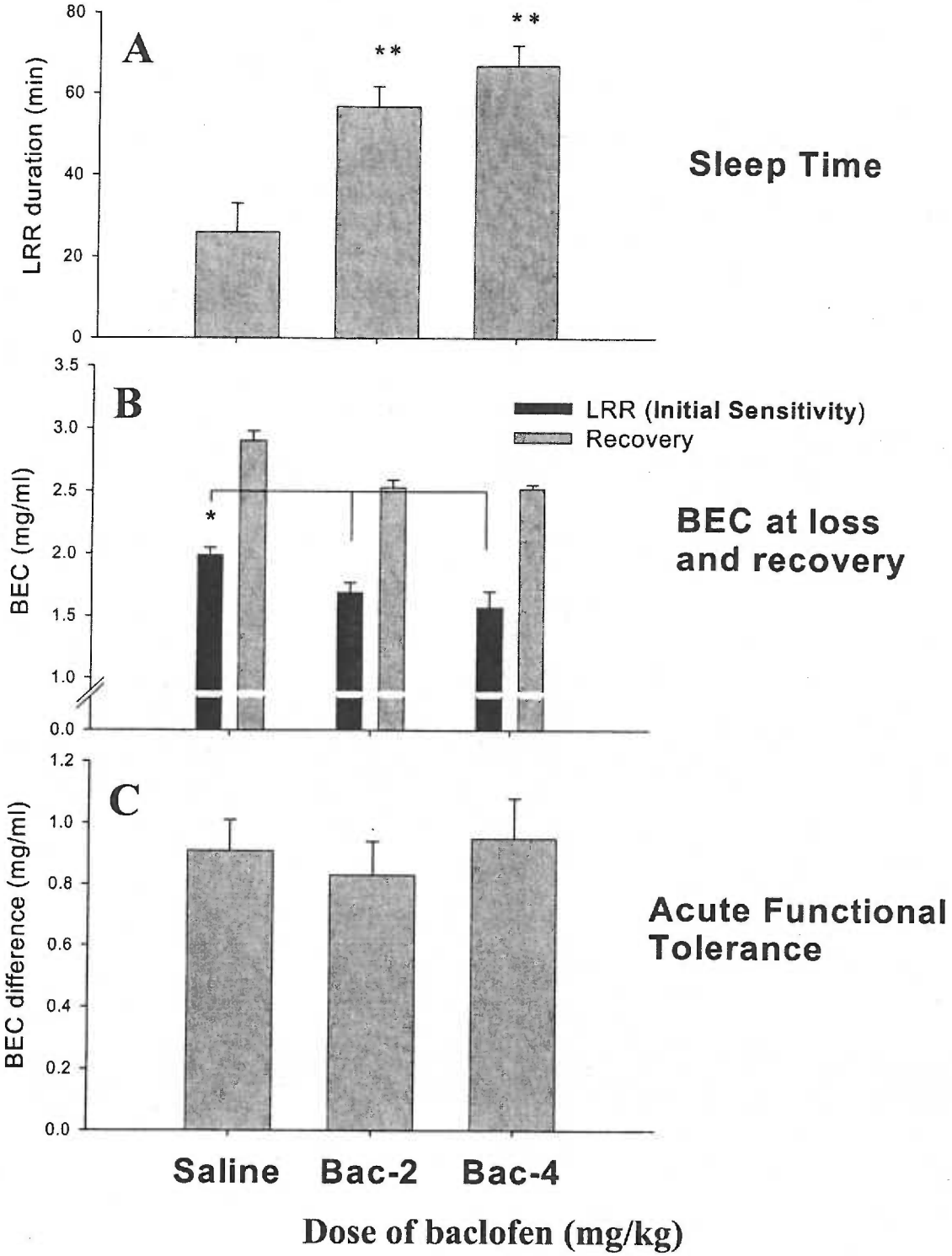

Figure 17. Effects of baclofen on (A) "sleep time", (B) initial sensitivity and recovery, and (C) acute functional tolerance. Means±SEM (n=6-9 per group). Groups were pretreated with either vehicle or different doses of the drug and then injected with 2.5 g/kg EtOH 20 min later. Asterisks indicate significant differences between drug-pretreated groups and saline group. * = $p<0.05$; ** = $p<0.01$

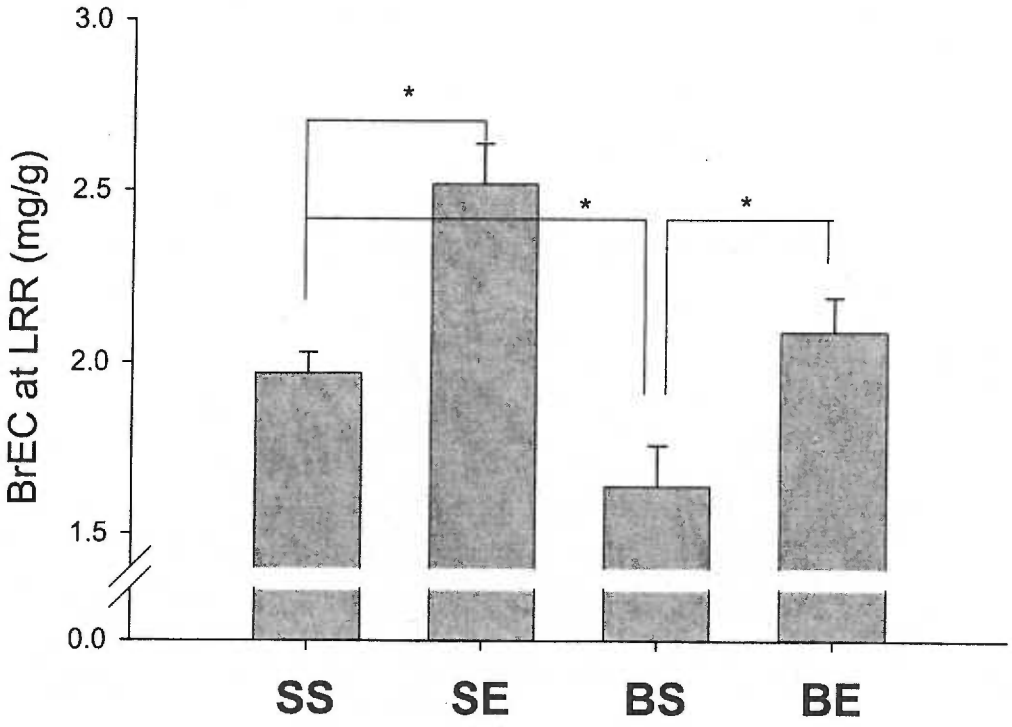

Figure 18. Effects of baclofen on initial sensitivity and acute functional tolerance (AFT). Means±SEM (n=10 per group). Groups were treated according to the procedure shown in Figure 5B. A small subhypnotic dose of EtOH (1.3 g/kg) resulted in development of AFT to EtOH-induced loss of righting reflex (SS vs SE groups). Pretreatment with baclofen (4 mg/kg) increased initial sensitivity (BS < SS), but did not affect AFT (BE-BS = SE-SS). * = $p<0.05$

groups pretreated with saline [BS < SS ($p=0.03$) and BE < SE ($p<0.01$)]. Baclofen did not affect AFT, as the BE animals had greater BrEC at LRR than BS mice ($p=0.01$), and the magnitude of this difference was similar to that of the SS – SE difference. Results of this experiment confirmed findings of Experiment 11: that is, baclofen increased IS to EtOH-induced sedation but did not affect rapid adaptation to this effect of EtOH.

Behavioral genetics

The genetic experiments of this project used inbred mouse strains as a tool to study the genetic component underlying AFT and to investigate whether AFT, IS and rapid tolerance are genetically related. Both IS and AFT to EtOH-induced hypnosis had rather high heritability values, while similar values for rapid tolerance variables were within low to moderate range. Correlational analysis suggested some common genetic determinants for IS and AFT and virtually no genetic association between IS and rapid tolerance as well as between AFT and rapid tolerance.

Experiment 13. Genetics of IS and AFT (panel of strains 1 from Table 3).

Overall ANOVAs on IS [$F(8,56)=2.2$; $p=0.04$] and AFT [$F(8,56)=7.7$; $p<0.01$] (Figure 19) revealed differences among strains. Heritability values were 0.24 for IS and 0.53 for AFT.

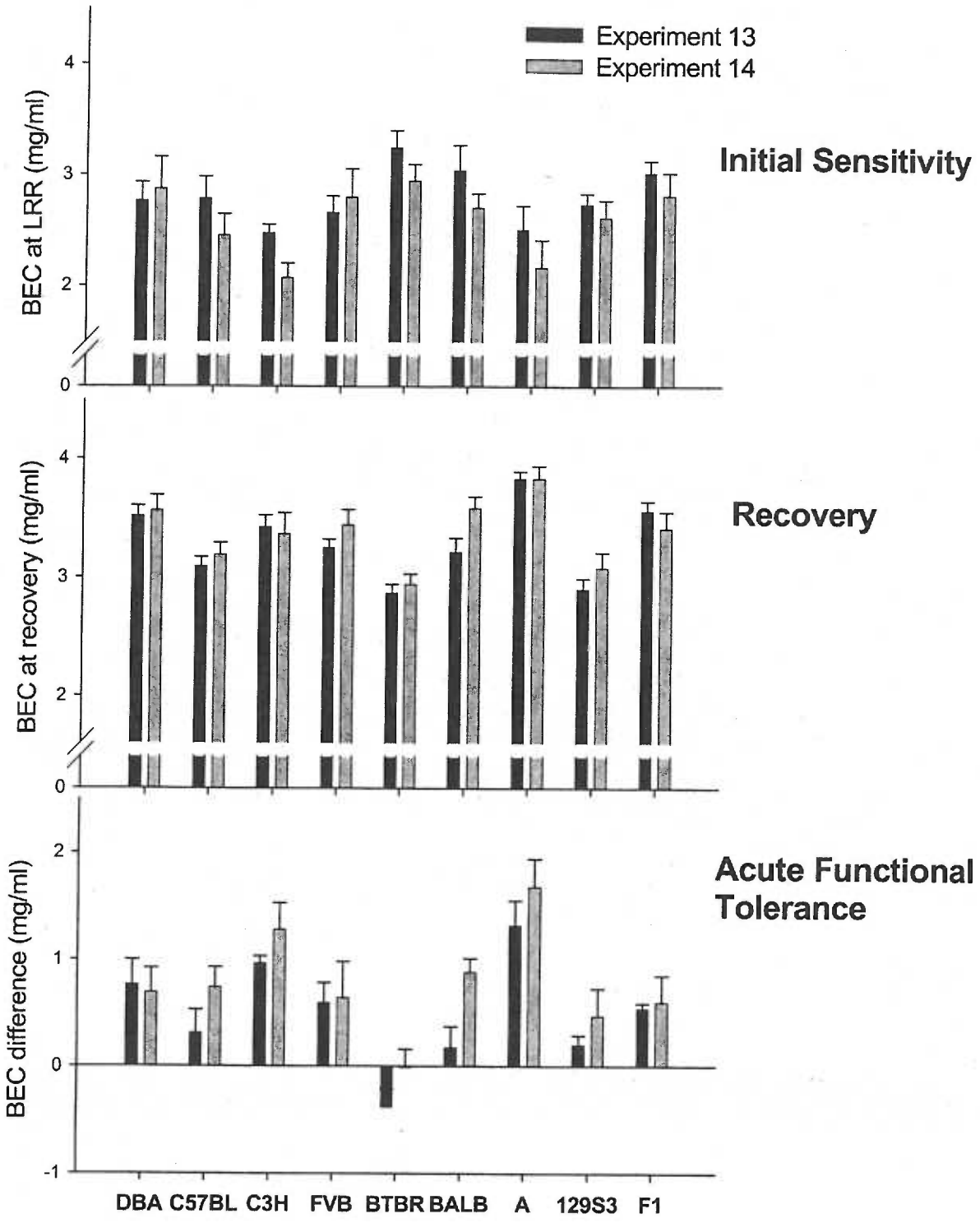

Figure 19. Initial sensitivity, blood EtOH concentration (BEC) at recovery and acute functional tolerance of 8 inbred mouse strains and B6D2F1 hybrids that were common for two genetic experiments. Means±SEM (n=4-9 per bar). All mice were injected with 3 g/kg EtOH. Details of experimental designs are presented in Table 3.

Experiment 14. Genetics of IS, AFT and rapid tolerance (panel of strains 2 from Table 3).

Overall ANOVAs on IS [$F(8,40)=3.7$; $p<0.01$] and AFT [$F(8,40)=6.2$; $p<0.01$] (Figure 19) revealed differences among strains. Heritability values were 0.44 for IS and 0.55 for AFT. Strains did not differ in either rapid tolerance values (RTwithin or RTbetween, see Table 3 for details) measured as differences between BEC at LRR values (all $p>0.6$) (Figure 20, upper panel). On the other hand, ANOVA on rapid tolerance variables assessed at recovery level showed a main effect of strain both for RTwithin [$F(8,35)=2.3$; $p=0.05$] and for RTbetween [$F(8,35)=2.2$; $p=0.05$] (Figure 20, lower panel). Heritability values ranged from 0.04 for RTwithin at LRR to 0.34 for RTwithin at recovery.

Experiment 15. Genetics of IS, AFT and rapid tolerance (panel of strains 3 from Table 3).

There were differences among 20 strains on both IS [$F(19,75)=4.1$; $p<0.01$] and AFT [$F(19,75)=2.5$; $p<0.01$] (Table 5). Heritability values were 0.51 for IS and 0.39 for AFT. Strains did not significantly differ in either RTwithin at LRR or RTwithin at recovery (all $p>0.1$), with heritability values being 0.31 and 0.22 respectively.

Only eight strains were used in all three experiments. We tested reliability of IS, AFT and rapid tolerance measurements by calculating genetic correlations among the three panels of strains (Experiments 13, 14, 15) using means of these strains. For this number of strains ($df=6$), the critical value is $r>0.71$ for $p<0.05$. Correlation coefficients for IS were mostly significant, ranging from 0.54 to 0.75. They were also high for AFT ranging from 0.77 to 0.90, thereby indicating that our new behavioral procedure results in reliable assessment of IS and AFT. On the other hand correlations among different rapid

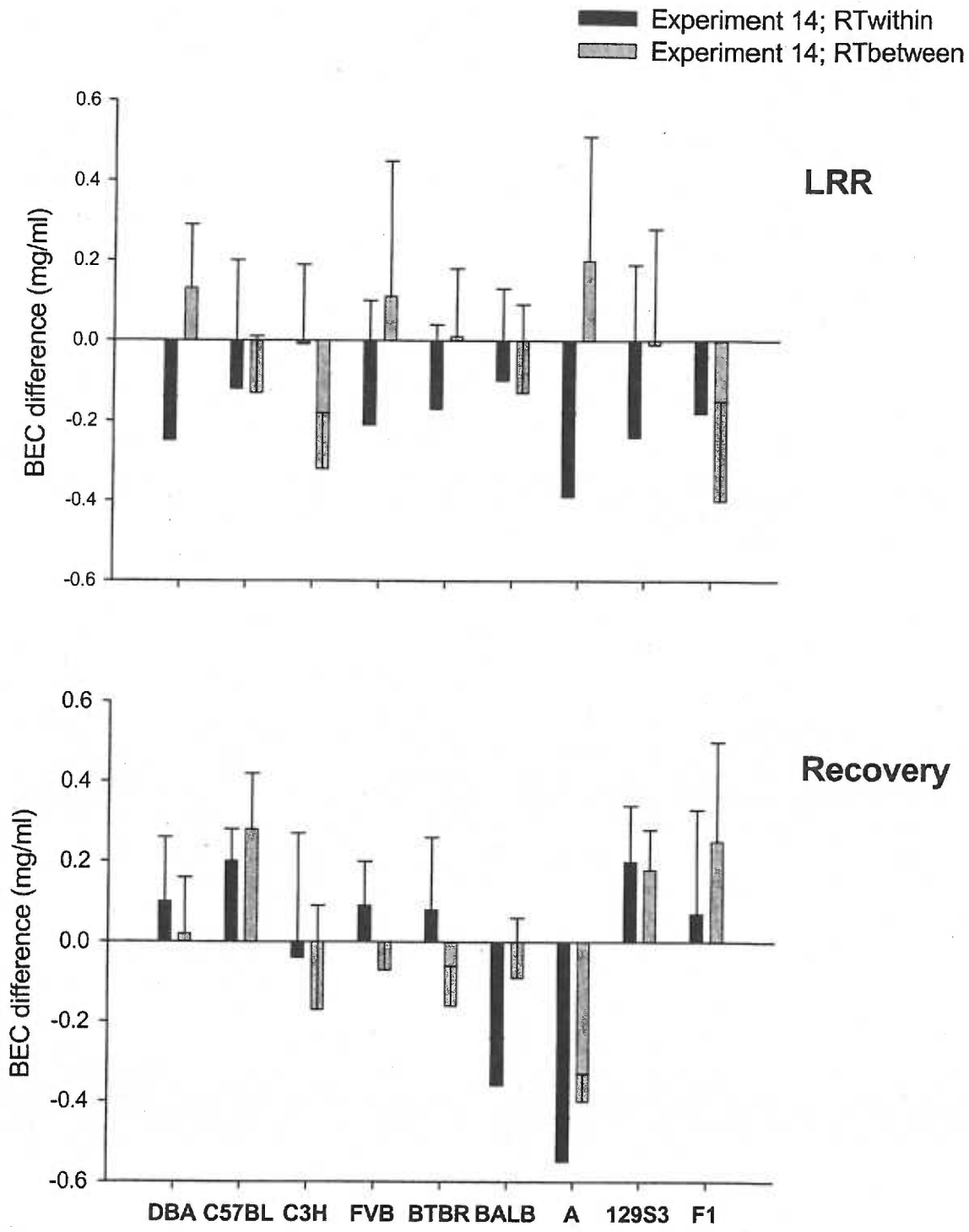

Figure 20. Rapid tolerance values (RTwithin and RTbetween) of 8 inbred mouse strains and B6D2F1 hybrids tested in Experiment 14. Means±SEM (n=3-9 per bar). Details of experimental designs and calculation of rapid tolerance values are presented in Table 3.

Table 5. Values of IS, BEC at recovery, AFT, RTwithin to LRR and RTwithin to recovery for 20 inbred strains tested in Experiment 15. All tolerance values represent within-subject differences. Means±SEM.

Strain	BEC at LRR (IS) (mg/ml)	BEC at Recovery (mg/ml)	AFT (mg/ml)	Rapid tolerance to LRR (mg/ml)	Rapid tolerance to recovery (mg/ml)
129S3*	2.58 ± 0.09	2.94 ± 0.11	0.40 ± 0.11	-0.02 ± 0.26	0.17 ± 0.09
A*	2.29 ± 0.14	3.65 ± 0.06	1.50 ± 0.14	-0.34 ± 0.15	-0.32 ± 0.14
BALB*	2.92 ± 0.11	3.60 ± 0.19	0.52 ± 0.13	-0.07 ± 0.13	-0.30 ± 0.09
BTBR*	3.00 ± 0.10	3.11 ± 0.13	-0.05 ± 0.12	-0.24 ± 0.14	0.09 ± 0.11
C3H*	2.32 ± 0.07	3.58 ± 0.22	1.11 ± 0.11	0.02 ± 0.19	-0.21 ± 0.23
C57BL*	2.69 ± 0.11	2.89 ± 0.16	0.39 ± 0.14	-0.09 ± 0.19	0.24 ± 0.10
DBA2*	2.87 ± 0.09	3.38 ± 0.10	0.62 ± 0.12	-0.1 ± 0.20	0.07 ± 0.12
FVB*	2.78 ± 0.11	3.41 ± 0.37	0.58 ± 0.10	-0.28 ± 0.17	0.09 ± 0.10
SJL	1.74 ± 0.15	2.95 ± 0.37	1.20 ± 0.38	0.28 ± 0.30	0.08 ± 0.30
SWR	2.76 ± 0.17	3.48 ± 0.08	0.73 ± 0.23	-0.21 ± 0.16	0.26 ± 0.23
NOD	2.59 ± 0.21	3.34 ± 0.10	0.75 ± 0.24	-0.19 ± 0.33	0.33 ± 0.31
SM	2.56 ± 0.27	2.71 ± 0.04	0.15 ± 0.26	0.06 ± 0.34	0.32 ± 0.10
AKR	2.47 ± 0.14	3.05 ± 0.09	0.58 ± 0.16	0.42 ± 0.23	0.28 ± 0.12
C57L	2.89 ± 0.18	3.38 ± 0.14	0.49 ± 0.09	-0.11 ± 0.09	-0.11 ± 0.13
C58	2.47 ± 0.12	3.47 ± 0.06	1.00 ± 0.09	-0.57 ± 0.15	0.06 ± 0.17
CAST	3.32 ± 0.16	3.40 ± 0.08	0.08 ± 0.19	-0.73 ± 0.41	0.11 ± 0.08
PL	2.35 ± 0.26	3.24 ± 0.29	0.89 ± 0.45	0.68 ± 0.48	0.32 ± 0.14
NZB	2.02 ± 0.08	2.65 ± 0.16	0.62 ± 0.16	-0.37 ± 0.18	-0.14 ± 0.05
MOLF	2.87 ± 0.14	3.39 ± 0.05	0.53 ± 0.19	0.18 ± 0.15	-0.09 ± 0.07
PERA	2.96 ± 0.11	3.57 ± 0.05	0.61 ± 0.12	-0.21 ± 0.20	-0.10 ± 0.11

* Values of these strains are for the combined data set (across experiments 13, 14 and 15)

tolerance variables ranged from (–0.51) to 0.58 (all not significant), suggesting that either this procedure is not very suitable to assess rapid tolerance, or that rapid tolerance, compared to the IS and AFT measures, is much more sensitive to age, sex and/or other differences that existed among the three panels. Weak relationships among rapid tolerance values obtained in different experiments could also be an indication of relatively low heritability.

To develop a better estimate of genetic correlations among IS, AFT and rapid tolerance variables, data were collapsed across all three experiments. Thus, means of 20 inbred strains and B6D2F1 hybrids were included in the analysis. Significant genetic correlation was found between initial sensitivity and AFT ($r=0.67$; $p<0.01$), while neither rapid tolerance variable correlated significantly with either IS or AFT (all $p>0.08$) (Table 6). This finding should be interpreted with caution because these two variables are mathematically related. That is AFT = BEC at recovery – BEC at LRR. BEC at LRR is inversely related to IS. Thus, the correlation between IS and AFT reflects not only the relationship between these variables, but also the effects of a third variable (in our case BEC at recovery) that is also correlated with AFT (Table 6). Usually, if effects of a third variable are suspected, calculating a partial correlation is recommended. A partial correlation removes the effects of a third variable from the relationship between the variables of interest. However, in closed equations like the one shown above, the partial correlation between IS and AFT will be equal to 1.00, because AFT will completely depend on the IS measurement when effects of BEC at recovery are "partialed out". Another potential solution to avoid this mathematical dependency would be calculating the correlation between IS and BEC at recovery. Because BEC at recovery is included in

Table 6. Genetic correlations calculated from a combined data set: 20 inbred strains and B6D2F1 hybrids.

VARIABLE	BEC at LRR (IS)	BEC at recovery	AFT	RT at LRR
BEC at recovery	0.39			
AFT	**-0.67**	**0.42**		
RT at LRR	-0.39	-0.27	0.16	
RT at recovery	0.02	-0.40	-0.34	0.29

Critical value for df=19: $r>0.42$ for $p<0.05$. Significant correlations are marked in bold. AFT = acute functional tolerance; IS – initial sensitivity is inversely related to BEC at LRR. The negative correlation between BEC at LRR and AFT indicates development of greater AFT in more sensitive animals; RT = rapid tolerance.

the calculation of AFT, the correlation between IS and BEC at recovery could be looked at as the relationship between IS and that part of AFT unaccountable for by IS. The correlation between BEC at LRR and BEC at recovery was 0.39 (p=0.08) (Figure 21), which represented a trend for more sensitive animals (that is those having lower BEC at LRR) tending to recover at lower BEC. Influence analysis suggested that this trend appeared to be influenced by two strains: NZB/BINJ and SJL/J. The genetic correlation recalculated after removal of the NZB/BINJ strain mean was 0.25, and it was even lower (0.08), when the second strain mean was also removed. These findings indicate instability of genetic relationship between initial sensitivity and BEC at recovery.

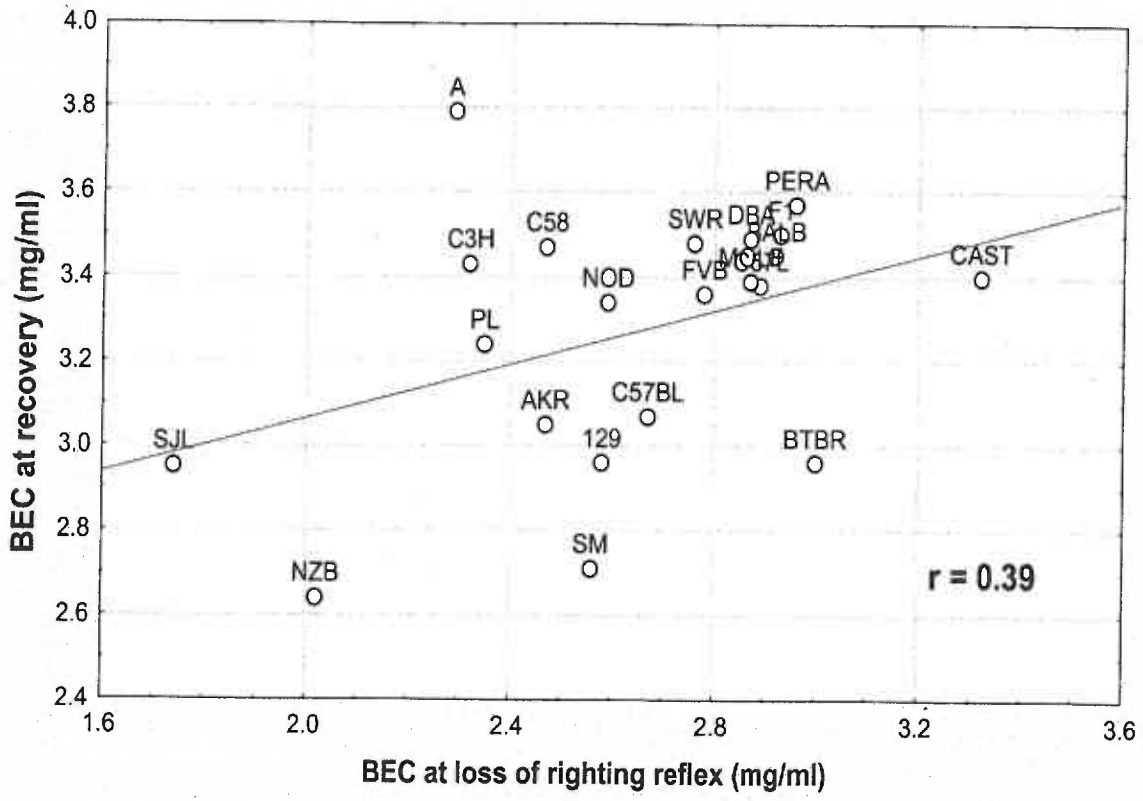

Figure 21. Genetic correlation between BEC at loss of righting reflex and BEC at recovery. Each data point identifies the mean of each genotype. Line represents least square linear regression; r = 0.39, p=0.08. This trend indicates that more sensitive animals tend to recover at lower BEC.

Discussion

AFT: Dose response and time course.

The phenomenon of acute functional tolerance (AFT) to EtOH has been demonstrated in humans and animals. Compared to human subjects, animal models have several advantages including direct investigation of neurochemical and genetic mechanisms underlying AFT. It is important to utilize models in which AFT is clearly defined. The present project employed a novel method of measuring initial sensitivity and AFT to EtOH-induced sedation in the same mice.

Theories. The first series of experiments (Experiments 1-3) explored phenomenological features of AFT; effects of dose and time on the development of AFT were examined. Results showed that AFT to EtOH-induced LRR is both dose- and time-dependent. These experiments were based on two somewhat contrasting theories. The first theory by Kalant and colleges (1971) includes two central postulates: 1) Alcohol induces AFT after a threshold concentration for alcohol effect is crossed; 2) Magnitude of AFT is proportional to the difference between the maximum BrEC that can be reached after a certain EtOH dose and the threshold BrEC for IS, implying dose-dependent development of AFT. On the other hand, Radlow (1994) argued against these concepts and offered an alternative theory of the AFT phenomenon. There are two basic assumptions of this theory: 1) AFT is a linear process with time. It will start at a value of zero when alcohol administration begins and will increase by an equal quantity during each unit of time that there is alcohol in the organism's system; 2) The slope of this linear

function is a measure of magnitude of the AFT. Radlow's assumptions imply that the development of AFT depends on the passage of time only and will start immediately after EtOH administration begins, regardless of the dose used. I will discuss the results of the first three experiments in the context of these theoretical notions.

Data. Results of Experiment 1 showed that different doses of EtOH produced different maximum BECs. Magnitude of AFT increased proportionally with dose, but only to a certain maximum value that did not change with further dose increases. The dose-response and the presence of the AFT plateau supported the second postulate of Kalant's theory that also predicted the existence of some maximum AFT value presumably determined by adaptive capacity of the organism. However, in contrast to Kalant's first postulate, and in partial support of Radlow's first assumption, Experiment 2 demonstrated that AFT to EtOH-induced hypnosis also develops to small subhypnotic doses. The doses used in this experiment did not induce loss of righting, but were sufficient to raise BEC at initial LRR when a higher hypnotic dose was given 10 or 20 minutes later. This increase in BEC at LRR was produced in a dose-dependent and time-independent fashion. Results of Experiment 2 suggest that adaptation to a certain dose of EtOH develops very rapidly and approaches its capacity for this dose by the 10^{th} min after EtOH administration begins. Experiment 3 extended this conclusion with the demonstration of the time-dependent development of AFT. Results showed that a subhypnotic dose of EtOH resulted in a statistically detectable AFT by the 10^{th} and 20^{th} min, but not by the 3^{rd} or 6^{th} minutes after the injection, with the magnitude of AFT at the 10^{th} and 20^{th} minutes being similar. Data from Experiment 1 also suggest that AFT to

hypnotic doses develops within the same time frame, as most animals that received a 2.0 or 2.5 g/kg dose recovered from LRR within 10-20 minutes of EtOH exposure.

Kalant's prediction that the magnitude of AFT will be correlated with EtOH dose has been supported by a number of studies (Keir and Deitrich, 1990; Erwin and Deitrich, 1996) including Experiment 1 of this project. However, there is one potential problem with the experimental paradigms employed in these studies. In experiments with high doses of EtOH, where AFT is measured as the difference between EtOH concentrations at the onset and offset of impairment, the "Time" variable cannot be controlled, and hence, studied independently from the "Dose" variable. There is a high correlation between the dose of EtOH and the time EtOH stays in the system. Radlow (1994) argued that the dose effect is seen because higher doses lead to a longer EtOH exposure, implying that the passage of time, and not the dose influence the magnitude of AFT. The design of Experiment 2 allowed us to investigate effects of time and dose on AFT using the same paradigm. Contrary to the Radlow's assumption, the magnitude of AFT that developed to a subhypnotic dose of EtOH was proportional to the dose, but not to the passage of time.

Implications. The concept of linearity of AFT suggested by Radlow raises the question whether AFT is mechanistically homogeneous, that is, whether presumably linear development of AFT is regulated by a single mechanism or by a set of related mechanisms. This question can be considered from different perspectives. For example, it is possible that acute tolerance to different effects of EtOH is controlled by the same neural circuit that involves the same brain structures. To evaluate this possibility directly, concurrent measurements of neuronal activity and behavior would be necessary. While

these kind of studies are yet to be carried out, indirect evidence suggests that different neuronal circuits are involved in acquisition of AFT to different EtOH effects. This evidence is provided by some studies that employed different behavioral assays to assess ethanol-induced motor incoordination. One such study by Deitrich and colleagues (2000) was introduced earlier in the manuscript. The authors of that paper tested SS, LS, HAFT, and LAFT mice for EtOH sensitivity and tolerance using different modifications of the rotarod and stationary dowel tests. Opposite to the authors' predictions, HAFT and LAFT mice genetically selected for differences in dowel AFT developed similar tolerance on the rotarod task, while SS and LS mice known to differ dramatically in AFT to EtOH-induced hypnosis showed similar differences on the rotarod, but not on the dowel test. Deitrich and his colleagues suggested that this genetic dissociation between measures of AFT is mediated by different neuronal circuits that are required for different behavioral endpoints used to assess AFT.

The question of mechanisms underlying AFT can also be considered in a "temporal" dimension, that is based on the analysis of the time courses of AFT to different effects of EtOH. While none of the previous AFT investigations specifically studied the issue of time course, data from those studies suggest that AFT to different EtOH effects have different time courses. Two basic patterns of AFT development can usually be detected. When the dependent variable indexing intoxication is plotted vs. time, the AFT curves resemble either a linear or a curvilinear sigmoidal function. A linear development of AFT is usually of slow rate and may or may not reach the adaptive capacity plateau within the time period AFT is assessed. For example, LS mice repeatedly tested on the rotarod after a 2 g/kg dose during a 30-min time period showed

gradual but very slow improvement of performance that was about 25% of the 200-sec criterion at the end of the 30-min testing period (Deitrich et al., 2000). This pattern could also be seen in LS and LAFT mice tested on the stationary dowel task (Erwin and Deitrich, 1996; Deitrich et al., 2000). When the sigmoidal fit is evident, a large portion of AFT develops within minutes after ethanol administration with a subsequent rapid decrease in the rate of development and eventual attainment of a plateau. This pattern is seen for some recombinant inbred mouse strains tested on the rotarod (Gallaher et al, 1996) and SS and HAFT mice tested on the stationary dowel (Erwin and Deitrich, 1996; Deitrich et al., 2000) tasks. The sigmoidal shape of the AFT curve is also evident in our study (Figure 6). Most sigmoidally-shaped AFT curves can be roughly broken into two linear phases: a very sharp increase in magnitude within the first 10 to 30 min of EtOH exposure and a more slowly-rising development (if any) after that. Data from Figure 6 could be used as an example to model the two-phase development of AFT. Thus, the very rapid phase includes BEC at initial LRR and recoveries after 2.0 and 2.5 g/kg, while the slow phase incorporates the other four recovery points, and is mainly represented by a slight increase in recovery values from 3.0 and 3.5 g/kg doses to 4.0 g/kg dose.

It would probably be safe to say that the very rapid adaptation seen in the first 30 minutes of sigmoidally-curved AFT depends on preexisting cellular conditions and should be gene expression – independent. Immediate early gene mRNA expression has been detected as early as 5 min after a stimulus. mRNA accumulates and reaches peak values at 30-40 min post-stimulation; *de novo* protein synthesis follows mRNA expression and peaks at 1-2 hours after stimulation (Chandler et al., 1999; Morgan and Curran, 1991; Dr. Andrey Ryabinin, personal communication). These time frames

suggest that AFT detected within the first 10-30 min after EtOH administration does not depend on *de novo* protein synthesis. Protein phosphorylation is suggested to play a major role in the very rapid AFT. Data supporting this suggestion will be discussed in subsequent sections. Whatever portion of AFT develops beyond the 30-60 min time frame is most likely regulated by a mixture of mechanisms that are both independent of and dependent on EtOH-induced gene expression. The role of *de novo* protein synthesis in development of AFT has not been extensively investigated. One study by Bitran and Kalant (1993) showed that the protein synthesis inhibitor anisomycin completely blocked development of rapid tolerance, but exhibited only a trend to inhibit AFT to EtOH-induced motor impairment. This tendency was rather substantial at later time points, as rats pretreated with anisomycin and tested 50 min after 1.7 g/kg EtOH showed twice as low performance values on the moving belt task as saline-pretreated animals (40 sec off belt compared to 20 sec respectively). This finding suggests that *de novo* protein synthesis may be involved in acquisition of the later portions of AFT.

Returning to our discussion of the effects of dose and time on AFT, it is tempting to speculate that the two phases of sigmoidally-shaped AFT are differentially affected by these two variables. Based on the first two recovery points of Figure 6 and the subhypnotic dose data (Figures 7 and 9), it would be logical to conclude that the rapid AFT to all the doses used develops within 10-20 minutes after an EtOH injection, with the magnitude being proportional to the dose. This phase of AFT appears to be dose-dependent and relatively time-independent. This dose dependency and the rapid AFT are seen only until the maximum BEC at the 5 min time point crosses the recovery plateau (top dashed line in Figure 6). It is possible that the different brain structures involved in

regulation of righting reflex are differentially sensitive to EtOH; that is, EtOH doses that inhibit one brain area may have no effect on another. The magnitude of the rapid-phase AFT may be proportional to the level of inhibition in the brain. When dose (BECmax) is high enough to affect all brain areas involved in righting reflex regulation, the rapid-phase AFT attains its plateau. That is, if these brain regions are not further affected by higher doses, magnitude of AFT to EtOH-induced LRR is no longer increasing. In contrast to the rapid-phase AFT, the slow-phase AFT does not appear to be affected by dose, as mice recovered at similar BEC after different doses ranging from 3.0 g/kg and higher. A slight increment in BEC at recovery between the 3.0 and 4.0 g/kg doses might indicate involvement of EtOH-induced gene expression that probably depends on a passage of time since EtOH injection rather than EtOH dose.

The enhancing effects of intoxicated practice have been reported for both acute (Gill and Deitrich, 1998) and chronic forms of tolerance (for review, see Le and Mayer, 1996). It is interesting to notice that acquisition of AFT in different genotypes is differentially affected by intoxicated practice. For example, Gill and Deitrich (1998) showed that performance of SS mice on EtOH-induced rotarod ataxia did not depend on the number of practice sessions during intoxication, while LS mice developed additional tolerance that was proportional to the increase in intoxicated practice sessions. It is not unreasonable to suggest that some genotypes such as the LS and LAFT selectively bred lines may lack mechanisms underlying the very rapid phase of AFT. In this case, the development of AFT in these lines would be following a slow linear progression, the earlier phases of which could be influenced by intoxicated practice only. Thus, the competing theories of Kalant and Radlow could perhaps be reconciled by the presence or

absence of the very rapid phase of AFT in some genotypes and/or measurements of AFT. To differentiate mechanisms underlying different phases of AFT, it is necessary to study each phase separately, limiting the effects of other variables. I believe that AFT to EtOH-induced sedation, as I have chosen to measure it, most likely represents the very rapid phase. Because AFT was assessed within 40 to 50 minutes of EtOH exposure and practice of the recovery from LRR did not exist or was very limited, the effects of EtOH-induced gene expression and intoxicated practice could be virtually ruled out.

Based on the first series of experiments, two behavioral paradigms were chosen for the pharmacological and genetic studies. The first paradigm used a single 3.0 g/kg dose to calculate AFT as BEC difference between recovery and loss of righting reflex. This procedure offers a number of advantages. First, it is a simple one-dose procedure, compared to the two- or multiple-dose paradigms used in the majority of previous studies. Second, this dose resulted in a near maximum but not the maximum magnitude of AFT, which gave us the advantage of detecting both increases and decreases in magnitude after administration of the NMDA and GABA drugs. This procedure also has two possible shortcomings. First, there is potentially a difference between mechanisms of loss and recovery from righting reflex, which might be differentially affected by the NMDA and GABA drugs. This concern was raised by a number of investigators who suggested that EtOH-induced LRR and recovery from LRR are two differentially regulated behavioral endpoints (Keir and Deitrich, 1990). Therefore, AFT calculated as difference between the two BECs may not represent true neuroadaptation to EtOH. Although the question of potential mechanistic differences between these two behavioral endpoints has not yet been investigated, this concern should be taken into consideration

by AFT studies. A second potential shortcoming of Paradigm #1 is that there could be unequal concentrations of the NMDA and GABA drugs at the loss and recovery measured at different time points after drug injection. These issues were resolved by the second paradigm, in which a 1.3 g/kg subhypnotic dose was used to induce AFT. In this procedure the same behavioral endpoint (initial LRR) was used to assess both IS and AFT, with all measurements being taken at the same time after injection of the drugs, thus limiting the possibility of unequal drug concentrations or different drug effects. This combination of the two paradigms should be useful for investigation of effects of different pharmacological agents on IS and AFT to EtOH-induced sedation, with Paradigms #1 and #2 having been used for exploratory and confirmatory purposes respectively. In addition to the aforementioned advantages of the combination of paradigms used, the novel method used to assess LRR allowed us to measure initial sensitivity and AFT in the same group of animals after a single injection of EtOH.

AFT: Behavioral pharmacology

NMDA receptors. MK-801 reduced the magnitude of AFT in a dose-dependent fashion but did not have a significant effect on IS, while D-cycloserine and ifenprodil did not affect either variable in the dose ranges used. The three drugs bind to different sites on NMDA receptor complex, thereby producing different effects on calcium influx. MK-801 is the only one of the three drugs that directly blocks the receptor channel and prevents influx of calcium. The effects of MK-801 suggest that some calcium-dependent

intracellular processes mediate acquisition of AFT. Protein phosphorylation may be one such process.

NMDA receptor function is enhanced by tyrosine kinases and reduced by tyrosine phosphatases (Chandler et al., 1998). A recent study by Miyakawa et al. (1997) suggested the involvement of NMDA receptor tyrosine phosphorylation by the tyrosine kinase Fyn in AFT to EtOH-induced sedation. Fyn phosphorylates the NR2 subunit of NMDA receptors, which can potentially modulate the electrophysiological function of the receptor, including its sensitivity to EtOH (Chandler et al., 1998). Miyakawa and colleagues reported that Fyn-deficient mice did not show acute tolerance to NMDA receptor-mediated EPSPs and exhibited enhanced sensitivity to the hypnotic effects of EtOH measured by duration of LRR. In contrast to the Fyn kinase data, mice lacking the gene that codes for another kinase, PKCγ, had a shorter duration of EtOH-induced LRR, compared to control animals (Harris et al., 1995). PKC phosphorylation of the NR1 subunit of NMDA receptor can also regulate channel function (Chandler et al., 1998). These two studies suggest that protein phosphorylation plays an important role in mediating acute effects of EtOH as well as rapid adaptation to these effects.

It is possible that protein phosphorylation affects general sensitivity to EtOH (including IS) but not AFT. Unfortunately, studies that investigate the role of protein phosphorylation in acute effects of EtOH lack a proper behavioral assessment of AFT. A combination of the two behavioral paradigms described above should be suitable for such studies. The role of different protein kinases in AFT to EtOH can be examined using both pharmacological and genetic approaches by utilizing specific kinase/phosphatase

inhibitors and different transgenic mouse models, such as "knockout", "knockdown", "knockin", etc.

NMDA-stimulated synthesis of nitric oxide (NO) may also be involved in regulation of AFT. NMDA receptor-dependent calcium entry can stimulate the production and liberation of NO (Trujillo and Akil, 1995). NO is an intracellular messenger that can trigger a number of biochemical events through stimulation of cGMP production (Chandler et al., 1998; Adams and Cicero, 1998). Several lines of evidence suggest that NO can mediate various effects of EtOH. For example, a study of Adams et al. (1994) showed that an inhibitor of nitric oxide synthase prolonged duration of EtOH-induced LRR, while experiments of Khanna et al. (1993) demonstrated that the same inhibitor impaired rapid tolerance to motor-incoordinating effects of EtOH in rats. These findings indicate that formation of nitric oxide may play a role in the development of acute and rapid tolerance to EtOH. The role of different NMDA receptor subunits in AFT could also be studied using transgenic techniques. Several "knockout" and overexpression models of NMDA receptor subunits are available for behavioral testing.

$GABA_A$ receptors. The only $GABA_A$ receptor compound used in this study was a non-competitive antagonist picrotoxin. Picrotoxin was chosen for this study for its ability to shorten the duration of EtOH-induced LRR in mice (Martz et al., 1983). This effect was confirmed using the first paradigm of this experimental series. However, neither BEC at recovery nor IS values were significantly affected by the pretreatment of picrotoxin, which indicated that the GABA antagonist might influence EtOH metabolism. Although picrotoxin tended to increase both IS and AFT in Paradigm #1, Paradigm #2 did not confirm these trends.

Based on these experiments, it could be concluded that drugs acting at the picrotoxin site have a low modulatory capacity (if any) for the hypnotic effects of EtOH. However, this conclusion should be taken with caution for several reasons. First, the two doses used in this study, 1.0 and 2.0 mg/kg, might be at the low end of the potential dose-response curve for picrotoxin. We did not test higher doses because they could cause spontaneous life-threatening convulsions (Crabbe et al., unpublished data). A second reason was the potentially differential effects of some convulsants on behavior with and without EtOH in the system. CNS excitation caused by picrotoxin can result in a preconvulsive state, which in turn may impair locomotor activity and perhaps the ability to regain righting reflex when tested shortly after the injection of EtOH. On the other hand, picrotoxin counteracts the inhibitory actions of EtOH (i.e., is an analeptic), thereby leading to an early recovery. Finally, modulatory capacity of the drugs acting at the picrotoxin site could be influenced by genetic background. A study by Phillips and Dudek (1989) showed that bicuculline shortened EtOH-induced "sleep time" in SS mice but produced an opposite effect in LS animals. Thus, $GABA_A$ agonists appear to be more suitable compounds for the investigation of the role of $GABA_A$ receptors in IS and AFT to EtOH-induced hypnosis.

Despite the negative results with picrotoxin, the role of $GABA_A$ receptors in mechanisms of rapid adaptation to EtOH should be further investigated. Protein phosphorylation might mediate the potential role of these receptors in AFT, as PKC has been shown to phosphorylate $GABA_A$ receptors (Chandler et al., 1998). Similar to the proposed NMDA receptor studies, a pharmacogenetic approach could also be employed to investigate $GABA_A$ receptors involvement in IS and AFT to EtOH-induced hypnosis.

Testing $GABA_A$ agonists and different receptor subunit transgenic models using our behavioral paradigms is one of the keys to this approach. Several studies investigated the role of $GABA_A$ subunits in acute actions of EtOH using "knockout" and overexpression models. The γ2 subunit appears to be a strong candidate for the involvement in AFT regulation. Overexpression of this subunit in the brain reduced a portion of AFT measured as BEC differences between the second and first recoveries from EtOH-induced ataxia on the dowel (Wick et al., 2000). No initial sensitivity measures were reported in this study. Therefore, conclusions as to the effects of this overexpression on AFT could not be drawn without testing these mice using a procedure that assesses AFT to a full extent. Deficits of delta, alpha6 and beta3 $GABA_A$ receptor subunits did not appear to modulate the hypnotic effects of EtOH (Quinlan et al., 1998; Homanics et al., 1997; Mihalek et al., 2001).

$GABA_B$ receptors. One of the major findings of our pharmacological experiments is that baclofen increased IS but did not affect AFT to EtOH-induced hypnosis, which suggests that $GABA_B$ receptors do not play a major role in regulation of AFT. The effects of baclofen on EtOH-induced sedation may be mediated by cerebellar Purkinje neurons that have low sensitivity to EtOH. Yang et al (2000) reported that baclofen increased sensitivity of these neurons to EtOH enhancement of GABA inhibition, measured as decrease in cell firing in anesthetized rats. In addition, both behavioral and electrophysiological data suggest that the cerebellum plays an important role in mediating EtOH's sedative effects (Seiger et al., 1983; Pearson et al., 1997).

Conclusions from pharmacological studies. Results of pharmacological experiments fully supported hypothesis 3, but only partly supported hypothesis 4. My

summary of the pharmacological data on IS and AFT includes three general statements. 1) The effects of the NMDA receptor ion channel blocker MK-801 suggest the involvement of these receptors and calcium influx-associated processes, in particular, in the regulation of AFT to EtOH-induced sedation. 2) $GABA_B$ receptors are unlikely to play a major role in this regulation. 3) To my knowledge, this is the first study that demonstrated a pharmacological dissociation of the mechanisms underlying IS and AFT.

We have also tested the effects of MK-801 and D-cycloserine on the development of rapid tolerance to EtOH-induced sedation. Previous studies showed that these drugs produced different effects on rapid tolerance to EtOH-induced intoxication in rats; MK-801 blocked acquisition of tolerance, while D-cycloserine enhanced its development (Khanna et al., 1995; Khanna et al., 1997). In our study, experiments showed that rapid tolerance to EtOH-induced hypnosis develops in WSC mice. We found that MK-801 blocked rapid tolerance detected as the BEC at LRR, while the anticipated enhancement of rapid tolerance by D-cycloserine was not seen. The lack of detectable effects of D-cycloserine might be due to a ceiling effect, and if a higher dose of EtOH had been used, the group pretreated with D-cycloserine on day 1 and treated with EtOH on both days might have recovered at a greater BEC on day 2. The lack of expected effect could also result from the use of a different species and/or different behavioral paradigms. The results of the MK-801 experiments suggest that AFT and rapid tolerance have some common mechanisms related to activation of the NMDA receptors. Future studies should identify those calcium-dependent processes that might mediate these two forms of tolerance.

AFT: Behavioral genetics

The genetic experiments of this project used inbred mouse strains as a tool to study the genetic influences underlying AFT and to investigate whether AFT, IS and rapid tolerance are genetically related. Before asking specific questions about particular genes involved in AFT regulation we need to know that the trait we measure is variable and that this variation is heritable. AFT values varied among animals of different strains. This variation was not due to environmental effects only. Calculated heritability values for AFT were rather high for a behavioral variable ranging from 0.39 to 0.55, which would practically assure success for a selective breeding experiment, had this selection for AFT been initiated. For comparison, realized heritability values for AFT scores in HAFT and LAFT selected lines were 0.04 and 0.26 respectively. Despite these low values, 12 generations of selective breeding resulted in more than 4-fold line AFT score difference (Erwin et al., 2000).

A future selective breeding project could take advantage of the more heritable AFT measured by our behavioral technique. The advantage of selectively bred lines in research on genetics of EtOH-related behavior has been demonstrated with a number of selective breeding experiments in mice and rats. Correlated line differences in neurochemical traits imply the involvement of these neurochemical mechanisms in regulation of the genetically selected behavior (Crabbe et al., 1990). For example, SS mice had 20-30% more NMDA receptor binding sites in hippocampus and cerebral cortex than LS animals (Velardo et al., 1998), implying potential involvement of NMDA receptors in mediating sensitivity and/or AFT to EtOH-induced sedation. No difference

between selected lines could also result in valuable implications. For example, HAFT and LAFT lines did not differ in AFT to pentobarbital-induced loss of balance, despite drastic differences in a similar measure of AFT to EtOH-induced ataxia (Erwin et al., 2000), which suggests that $GABA_A$ receptors do not play a major role in regulation of AFT. These two findings generally agree with the results of pharmacological experiments of the present project.

In our study, IS was also highly heritable, demonstrating a high degree of genetic influence. On the other hand, rapid tolerance measures had low to moderate heritability. This finding is not surprising. Rapid tolerance is a somewhat more complex variable than AFT or IS and is thought to be influenced by not only pharmacological mechanisms but also by different forms of learning. All these factors may interact to increase within-strain variability, thereby decreasing heritability values. Despite a considerable degree of variability within strains and among replicates, some genotypes showed some consistency across the two experiments that obtained rapid tolerance values. For example, C57BL/6J and 129S1/SvIMJ strains exhibited positive values for rapid tolerance, while most values of BALB/cByJ and A/J mice were negative. The negative values indicate development of sensitization to EtOH. The pharmacological mechanisms of this phenomenon are not known. It is possible that the "sensitized" strains are more sensitive to EtOH's toxic effects, which might result in slight deterioration in general health, and subsequent increase of sensitivity to EtOH-induced sedation on day 2.

Studies that attempt to map genes influencing a certain trait have to rely on the paradigm that assesses this trait. Reliability of behavioral measures has always been an issue in genetic studies mapping quantitative trait loci (QTL). In our study, reliability

estimated by genetic correlations among three panels of strains varied among IS, AFT and rapid tolerance. Our behavioral procedure resulted in reliable assessment of initial sensitivity and acute functional tolerance, but not rapid tolerance. One of the primary goals of studying inbred strains is to detect those genotypes that exhibit considerable differences on the trait of interest. These strains could later be used to generate recombinant inbred (RI) strains – a powerful tool for QTL mapping. In the present study A/J and C3H/HeJ strains exhibited the greatest AFT, while the magnitude of AFT of the BTBR, C57BL/6J and 129S1/SvIMJ genotypes was the lowest. These strains would be most suitable for a QTL project. In fact, panels of RI strains exist for C57BL/6J x A/J and C57BL/6J x C3H/HeJ crosses. LS and SS mice as well as LS x SS RI strains would also be suitable for QTL studies, because these selected lines also differ in AFT. QTLs for EtOH-induced "sleep time" and AFT to EtOH-induced loss of balance have been mapped to several murine chromosomes. (Markel et al., 1997; Radcliffe et al., 2000; Gehle and Erwin, 2000). Conducting a QTL project using the present measurement of AFT and comparing newly discovered QTLs with those previously mapped would be one direction for future research. The QTL project could be followed by the development of congenic mouse lines that would narrow down QTL regions and make selection of candidate genes within those regions more feasible.

Correlational analysis only partially supported Hypothesis 5 of this project. Genetic correlations among IS, AFT and rapid tolerance variables suggested a weak genetic relationship between IS and AFT and virtually no consistent genetic associations between either IS and rapid tolerance or AFT and rapid tolerance. There is no consistency in the literature as to the genetic relationship between initial sensitivity and AFT. One

possible reason for this inconsistency is mathematical dependency of IS and AFT measures. Studies that estimate AFT on the basis of IS usually find a positive genetic correlation between IS and AFT; that is, more sensitive animals tend to develop greater AFT (Gallaher et al., 1996; present project), while a study of Gehle and Erwin (2000), which used a partial measure of AFT, which was independent of IS did not report such genetic association. There is no simple solution to the problem of mathematical dependency. It would probably be more useful to investigate the domains of initial sensitivity and acute functional tolerance separately (that is, without interpreting direct correlation), and then compare mechanisms underlying each domain.

It should be noticed that measures of rapid tolerance in our genetic experiments had low heritability and low reliability. These findings alone could be responsible for the lack of genetic associations between IS and rapid tolerance as well as AFT and rapid tolerance. It is surprising that only a few strains showed consistent positive values of rapid tolerance. On the other hand, genetically heterogeneous WSC mice developed reliably measured rapid tolerance. It is possible that inbreeding in general may affect mechanisms underlying rapid tolerance, and that a combination of different alleles is required for reliable display of this kind of neuroadaptation. It is also possible that low statistical power (small sample size) contributed to low estimates of heritability and reliability. Discovery of the genes affecting rapid tolerance could be initiated by a QTL project employing strains that exhibit opposite rapid tolerance responses.

Inbred mouse strains represent a powerful tool for genetic analysis of behavior. Genetic correlation between the behavior of interest and a brain function implies the involvement of this function in regulation of this behavior (Crabbe et al., 1990). One

example of such an analysis is investigation of the relationship between cerebellar functions and EtOH sensitivity. Genetic correlations between Purkinje neuron inhibition by EtOH and EtOH-induced LRR (Spuhler et al., 1982) as well as between cAMP accumulation in cerebellar cells and EtOH-induced ataxia (Kirstein and Tabakoff, 2001) suggest the importance of the cerebellum in mediating acute effects of EtOH. It is important that inbred strain data from different studies are collected and organized in a single database available to the scientific community. This would provide the advantage of studying behavioral genetics using multivariate approaches and would advance our understanding of genetic regulation of behavior. The Mouse Phenome Project organized by the Jackson Laboratory (Bar Harbor, Maine) has recently been initiated to create such a database (http://www.jax.org/phenome).

To conclude this section of discussion, I would like to point out that a combination of several genetic techniques is required to pinpoint those genetic mechanisms underlying EtOH-related traits. The recent development of cDNA microarray technologies has been met with enthusiasm by scientific communities in different fields. High-density DNA microarrays allow researchers to quickly quantify changes in expression of thousands of genes in a parallel manner. This approach in combination with classical genetic as well as transgenic approaches could be a key to success in research on the genetics of alcoholism. For example, a combination of LSxSS RI – based QTLs and gene expression profiles in HAFT and LAFT mice has recently been employed by a research group at the University of Colorado Health Sciences Center to reduce the list of candidate genes underlying AFT to EtOH-induced loss of balance (Kirstein et al., 2002; Dr. Boris Tabakoff, personal communication). I believe it will only

be a matter of time until the majority of EtOH-related traits including AFT to EtOH-induced hypnosis are investigated using a combination of classical genetic and advanced high-technology techniques.

Summary and Future Directions

This project introduces a new behavioral method that employs loss of righting reflex to assess initial sensitivity (IS) and acute functional tolerance (AFT) to EtOH-induced hypnosis. This method has several advantages over the traditionally used procedure, including a more accurate estimation of IS and AFT values. AFT to EtOH-induced sedation assessed by the novel technique developed in a dose- and time-dependent fashion. The first series of experiments suggests that AFT is not a homogenous phenomenon. The very rapid portion of AFT develops within 10-20 minutes after EtOH administration, with the magnitude of AFT being proportional to the dose used. The slow-phase AFT is likely to be dose-independent and may be influenced by EtOH-induced gene expression and intoxicated practice. Pharmacological experiments suggest the involvement of NMDA receptors and downstream calcium-dependent processes in regulation of the very rapid AFT. $GABA_B$ receptors appear to enhance the hypnotic effects of acute EtOH, but are unlikely to play a major role in regulation of AFT. Evidence suggests that IS, AFT and rapid tolerance to EtOH-induced sedation are likely to be regulated by mainly independent mechanisms, although some common genetic and pharmacological influences on those three domains have been detected.

Future research will use concurrent measurements of neuronal activity and behavior to start pinpointing neural circuits underlying rapid adaptation to EtOH-induced sedation. A combination of behavioral, pharmacological, classical genetic, transgenic, and novel high-technology genetic approaches will be necessary for successful identification of the mechanisms of this adaptation. A proper assessment of IS and AFT will be required for this investigation. The knowledge obtained from animal research should be useful for human studies that will investigate the potential roles of IS and AFT in alcohol abuse.

References

Adams ML, Meyer ER, Sewing BN and Cicero TJ (1994) Effects of nitric oxide-related agents on alcohol narcosis. *Alcoholism: Clinical & Experimental Research* 18:969-75

Adams ML and Cicero TJ (1998) Alcohol intoxication and withdrawal: The role of nitric oxide. *Alcohol* 16:153-158

Allan AM and Harris RA (1987) Acute and chronic ethanol treatments alter GABA receptor-operated chloride channels. *Pharmacology, Biochemistry & Behavior* 27(4):665-70

Belknap JK, Belknap ND, Berg JH and Coleman R (1977) Preabsorptive vs. postabsorptive control of ethanol intake in C57BL/6J and DBA/2J mice. *Behavior Genetics* 7:413-425

Bitran M and Kalant H (1993) Effect of anisomycin on the development of rapid tolerance to ethanol-induced motor impairment. *Pharmacology, Biochemistry & Behavior* 45:225-8

Boehm SL II, Schafer GL, Phillips TJ, Browman KE and Crabbe JC (2000) Sensitivity to ethanol-induced motor incoordination in 5-HT(1B) receptor null mutant mice is task-dependent: implications for behavioral assessment of genetically altered mice. *Behavioral Neuroscience* 114(2):401-9

Chandler LJ, Harris RA and Crews FT (1998) Ethanol tolerance and synaptic plasticity. *Trends in Pharmacological Sciences* 19(12):491-5

Crabbe JC, Rigter H, Uijlen J and Strijbos C (1979) Rapid development of tolerance to the hypothermic effect of ethanol in mice. *Journal of Pharmacology & Experimental Therapeutics* 208(1):128-33

Crabbe JC, Janowsky JS, Young ER, Kosobud A, Stack J and Rigter H (1982) Tolerance to ethanol hypothermia in inbred mice: genotypic correlations with behavioral responses. *Alcoholism: Clinical & Experimental Research* 6:446-58

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

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

Crabbe JC, Phillips TJ, Gallaher EJ, Crawshaw LI and Mitchell SR (1996) Common genetic determinants of the ataxic and hypothermic effects of ethanol in BXD/Ty recombinant inbred mice: genetic correlations and quantitative trait loci. *Journal of Pharmacology & Experimental Therapeutics* 277:624-32

Crews FT, Morrow AL, Criswell H and Breese G (1996) Effects of ethanol on ion channels. *International Review of Neurobiology,* 39:283-367

Daniell LC (1990) The noncompetitive N-methyl-D-aspartate antagonists, MK-801, phencyclidine and ketamine, increase the potency of general anesthetics. *Pharmacology, Biochemistry & Behavior* 36:111-5

Deitrich RA, Bludeau P and Erwin VG (2000) Phenotypic and genotypic relationships between ethanol tolerance and sensitivity in mice selectively bred for initial

sensitivity to ethanol (SS and LS) or development of acute tolerance (HAFT and LAFT). *Alcoholism: Clinical & Experimental Research* 24:595-604

Dudek BC, Abbott ME, Garg A and Phillips TJ (1984) Apomorphine effects on behavioral response to ethanol in mice selectively bred for differential sensitivity to ethanol. *Pharmacology, Biochemistry & Behavior*, 20:91-4

Dudek BC and Phillips TJ (1989) Genotype-dependent effects of GABAergic agents on sedative properties of ethanol. *Psychopharmacology*, 98:518-523

Erwin VG and Deitrich RA (1996) Genetic selection and characterization of mouse lines for acute functional tolerance to ethanol. *Journal of Pharmacology & Experimental Therapeutics* 279:1310-7

Erwin VG, Gehle VM and Deitrich RA (2000) Selectively bred lines of mice show response and drug specificity for genetic regulation of acute functional tolerance to ethanol and pentobarbital. *Journal of Pharmacology & Experimental Therapeutics* 293(1):188-95

Falconer DS and Mackay TFC: (1996) Introduction to Quantitative Genetics, Fourth Edition. Longman, London

Frye GD and Fincher A (1996) Sensitivity of postsynaptic GABAB receptors on hippocampal CA1 and CA3 pyramidal neurons to ethanol. *Brain Research,* 735:239-48

Gallaher EJ, Parsons LM and Goldstein DB (1982) The rapid onset of tolerance to ataxic effects of ethanol in mice. *Psychopharmacology* 78:67-70

Gallaher EJ, Jones GE, Belknap JK and Crabbe JC (1996) Identification of genetic markers for initial sensitivity and rapid tolerance to ethanol-induced ataxia using

quantitative trait locus analysis in BXD recombinant inbred mice. *Journal of Pharmacology & Experimental Therapeutics* 277(2):604-12

Gehle VM and Erwin VG (2000) The genetics of acute functional tolerance and initial sensitivity to ethanol for an ataxia test in the LSxSS RI strains. *Alcoholism: Clinical & Experimental Research* 24(5):579-87

Gill K and Deitrich RA (1998) Acute tolerance to the ataxic effects of ethanol in short-sleep (SS) and long-sleep (LS) mice. *Psychopharmacology* 136(1):91-8

Givens BS and Breese GR (1990) Electrophysiological evidence that ethanol alters function of medial septal area without affecting lateral septal function. *Journal of Pharmacology & Experimental Therapeutics* 253:95-103

Goldberg L (1943) Quantitative studies on alcohol tolerance in man. *Acta Physiologica Scandinavica* (suppl 16) 5:1-126

Goldstein DB (1983) Pharmacology of Alcohol. Oxford University Press, New York

Grieve SJ and Littleton JM (1979) Age and strain differences in the rat of development of functional tolerance to ethanol by mice. *Journal of Pharmacy & Pharmacology* 31:696-700

Grover CA, Frye GD and Griffith WH (1994) Acute tolerance to ethanol inhibition of NMDA-mediated EPSPs in the CA1 region of the rat hippocampus. *Brain Research* 642(1-2):70-6

Harris RA, McQuilkin SJ, Paylor R, Abeliovich A, Tonegawa S and Wehner JM (1995) Mutant mice lacking the gamma isoform of protein kinase C show decreased behavioral actions of ethanol and altered function of gamma-aminobutyrate type A

receptors. *Proceedings of the National Academy of Sciences of the United States of America,* 92:3658-62

Hiltunen AJ (1997) Acute alcohol tolerance in cognitive and psychomotor performance: influence of the alcohol dose and prior alcohol experience. *Alcohol* 14:125-30

Hiltunen AJ, Saxon L, Skagerberg S and Borg S (2000) Acute tolerance during intravenous infusion of alcohol: comparison of performance during ascending and steady state concentrations--a pilot study. *Alcohol* 22:69-74

Homanics GE, Ferguson C, Quinlan JJ, Daggett J, Snyder K, Lagenaur C, Mi ZP, Wang XH, Grayson DR and Firestone LL (1997) Gene knockout of the alpha6 subunit of the gamma-aminobutyric acid type A receptor: lack of effect on responses to ethanol, pentobarbital, and general anesthetics. *Molecular Pharmacology* 51:588-96

Kalant H, LeBlanc AE and Gibbins RJ (1971) Tolerance to, and dependence on, some non-opiate psychotropic drugs. *Pharmacological Reviews* 23(3):135-91

Kalant H, LeBlanc AE, Gibbins RJ and Wilson A (1978) Accelerated development of tolerance during repeated cycles of ethanol exposure. *Psychopharmacology* 60(1):59-65

Kalant H (1998) Research on tolerance: what can we learn from history? *Alcoholism: Clinical & Experimental Research* 22(1):67-76

Khanna JM, Kalant H, Shah G and Weiner J (1991) Rapid tolerance as an index of chronic tolerance. *Pharmacology, Biochemistry & Behavior* 38:427-32

Khanna JM, Morato GS, Shah G, Chau A and Kalant H (1993) Inhibition of nitric oxide synthesis impairs rapid tolerance to ethanol. *Brain Research Bulletin* 32:43-47

Khanna JM, Morato GS, Chau A and Shah G (1995) D-cycloserine enhances rapid tolerance to ethanol motor incoordination. *Pharmacology, Biochemistry & Behavior* 52(3):609-14

Khanna JM, Chau A and Shah G (1996) Characterization of the Phenomenon of rapid tolerance to ethanol. *Alcohol* 13(6):621-8

Khanna JM, Shah G and Chau A (1997) Effect of NMDA antagonists on rapid tolerance to ethanol under two different testing paradigms. *Pharmacology, Biochemistry & Behavior* 57(4):693-7

Keir WJ and Deitrich RA (1990) Development of central nervous system sensitivity to ethanol and pentobarbital in short- and long-sleep mice. *Journal of Pharmacology & Experimental Therapeutics* 254:831-835

Kiianmaa K, Hoffman PL and Tabakoff B (1983) Antagonism of the behavioral effects of ethanol by naltrexone in BALB/c, C57BL/6, and DBA/2 mice. *Psychopharmacology* 79:291-4

Kirstein SL and Tabakoff B (2001) Genetic correlations between initial sensitivity to Ethanol and brain cAMP signaling in inbred and selectively bred mice. *Alcoholism: Clinical & Experimental Research* 25:791-9

Kirstein SL, Davidson KL, Ehringer MA and Tabakoff B (2002) Quantitative trait loci affecting initial sensitivity and acute functional tolerance to ethanol-induced ataxia and brain cAMP signaling in BXD RI mice. *Journal of Pharmacology & Experimental Therapeutics* 302:???-???

Le AD, Kalant H and Khanna JM (1989) Roles of intoxicated practice in the development of ethanol tolerance. *Psychopharmacology* 99:366-70

Le AD and Kalant H (1992) Influence of intoxicated practice on the development of acute tolerance to the motor impairment effect of alcohol. *Psychopharmacology* 106:572-576

Le AD, Mana M, Quan B and Kalant H (1992) Differential development of acute tolerance to the motor impairment and anticonvulsant effects of ethanol. *Psychopharmacology* 109:107-11

Le AD and Mayer JM (1996) Aspects of alcohol tolerance in humans and experimental animals. In Pharmacological effects of ethanol on the nervous system, ed by Deitrich RA and Erwin VG, pp 251-268, CRC press, Inc., Boca Raton, Florida

LeBlanc AE, Kalant H and Gibbins RJ (1975) Acute tolerance to ethanol in the rat. *Psychopharmacologia* 41:43-46

Lister RG, Durcan MJ, Nutt DJ and Linnoila M (1989) Attenuation of ethanol intoxication by alpha-2 adrenoceptor antagonists. *Life Sciences* 44:111-9

Ludvig N, George MA, Tang HM, Gonzales RA and Bungay PM (2001) Evidence for the ability of hippocampal neurons to develop acute tolerance to ethanol in behaving rats. *Brain Research* 900:252-60

Malinowska B, Napiorkowska-Pawlak D, Pawlak R, Buczko W and Gothert M (1999) Ifenprodil influences changes in mouse behaviour related to acute and chronic ethanol administration. *European Journal of Pharmacology* 377(1):13-19

Markel PD, Bennett B, Beeson M, Gordon L and Johnson TE (1997) Confirmation of quantitative trait loci for ethanol sensitivity in long-sleep and short-sleep mice. *Genome Research* 7:92-9

Martin CS and Moss HB (1993) Measurements of acute tolerance to alcohol inhuman subjects. *Alcoholism: Clinical & Experimental Research* 17:211-216

Martz A, Deitrich RA and Harris RA (1983) Behavioral evidence for the involvement of gamma-aminobutyric acid in the actions of ethanol. *European Journal of Pharmacology* 89(1-2):53-62

McClearn GE and Kakihana R (1981) Selective breeding for ethanol sensitivity: Short-sleep and long-sleep mice. In Development of Animal Models as Pharmacogenetic Tools, ed by McClearn GE, Deitrich RA and Erwin VG, pp147-159, NIAAA, Rockville, Maryland

Mehta AK and Ticku MK (1999) An update on $GABA_A$ receptors. *Brain Research - Brain Research Reviews* 29:196-217

Mellanby E (1919) Alcohol: its absorption into and disappearance from the blood under different conditions. National Research Council (Great Britain) Special Report Series No 31

Mihalek RM, Bowers BJ, Wehner JM, Kralic JE, VanDoren MJ, Morrow AL and Homanics GE (2001) GABA(A)-receptor delta subunit knockout mice have multiple defects in behavioral responses to ethanol. *Alcoholism: Clinical & Experimental Research* 25:1708-18

Miyakawa T, Yagi T, Kitazawa H, Yasuda M, Kawai N, Tsuboi K and Niki H (1997) Fyn-kinase as a determinant of ethanol sensitivity: relation to NMDA-receptor function. *Science* 278:698-701

Moore JA and Kakihana R (1978) Ethanol-induced hypothermia in mice: influence of genotype on development of tolerance. *Life Sciences* 23:2331-2337

Morgan JI and Curran T (1991) Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. *Annual Review of Neuroscience* 14:421-451

Newlin DB and Thomson JB (1990) Alcohol challenge with sons of alcoholics: a critical review and analysis. *Psychological Bulletin* 108:383-402

O'Connor S, Morzorati S, Christian J and Li TK (1998) Clamping breath alcohol concentration reduces experimental variance: application to the study of acute tolerance to alcohol and alcohol elimination rate. *Alcoholism: Clinical & Experimental Research* 22:202-10

Palmer MR, Basile AS, Proctor WR, Baker RC and Dunwiddie TV (1985) Ethanol tolerance of cerebellar purkinje neurons from selectively outbred mouse lines: in vivo and in vitro electrophysiological investigations. *Alcoholism: Clinical & Experimental Research* 9:291-6

Pedhazur EJ (1982) Multiple regression in behavioral research. The Dryden Press, Fort Worth, Texas

Pearson BJ, Donatelli DP, Freund RK and Palmer MR (1997) Differential development and characterization of rapid acute neuronal tolerance to the depressant effects of ethanol on cerebellar Purkinje neurons of low-alcohol-sensitive and high-alcohol-sensitive rats. *Journal of Pharmacology & Experimental Therapeutics* 280(2):739-46

Phillips TJ and Dudek BC (1989) Modification of ethanol effects by bicuculline: genotype-dependent responses and inheritance. *Psychopharmacology* 98:549-555

Phillips TJ and Crabbe JC (1991) Behavioral studies of genetic differences in alcohol action. In: Crabbe JC and Harris RA (eds). The genetic basis of alcohol and drug actions, pp. 25-104, Plenum Press, New York

Ponomarev I and Crabbe JC (2002) A novel method to assess initial sensitivity and acute functional tolerance to hypnotic effects of ethanol. *Journal of Pharmacology & Experimental Therapeutics* 302:257-263

Quinlan JJ, Homanics GE and Firestone LL (1998) Anesthesia sensitivity in mice that lack the beta3 subunit of the gamma-aminobutyric acid type A receptor. *Anesthesiology* 88:775-80

Radcliffe RA, Bohl ML, Lowe MV, Cycowski CS and Wehner JM (2000) Mapping of quantitative trait loci for hypnotic sensitivity to ethanol in crosses derived from the C57BL/6 and DBA/2 mouse strains. *Alcoholism: Clinical & Experimental Research* 24:1335-42

Radlow R (1994) A quantitative theory to acute tolerance to alcohol. *Psychopharmacology* 114:1-8

Ramchandani VA, O'Connor S, Blekher T, Kareken D, Morzorati S, Nurnberger J Jr. and Li TK (1999) A preliminary study of acute responses to clamped alcohol concentration and family history of alcoholism. *Alcoholism: Clinical & Experimental Research* 23:1320-30

Ritzman RF and Tabakoff B (1980) Strain differences in the development of acute tolerance to ethanol. In Biological Effects of Ethanol, ed by H. Begleiter, pp. 197-209, Plenum Publishing Co., New York

San-Marina A, Khanna JM and Kalant H (1989) Relationship between initial sensitivity, acute tolerance and chronic tolerance to ethanol in a heterogeneous population of Swiss mice. *Psychopharmacology* 99:450-7

Schuckit MA (1980) Self-rating of alcohol intoxication by young men with and without family histories of alcoholism. *Journal of Studies on Alcohol* 41(3):242-249

Schuckit MA (1984) Subjective responses to alcohol in sons of alcoholics and control subjects. *Archives of General Psychiatry* 41:879-884

Schuckit MA and Smith TL (1996) An 8-year follow-up of 450 sons of alcoholic and control subjects. *Archives of General Psychiatry* 53:202-10

Shen EH, Dorow J, Harland R, Burkhart-Kasch S and Phillips TJ (1998) Seizure sensitivity and GABAergic modulation of ethanol sensitivity in selectively bred FAST and SLOW mouse lines. *Journal of Pharmacology & Experimental Therapeutics* 287:606-15

Shen EH and Phillips TJ (1998) MK-801 potentiates ethanol's effects on locomotor activity in mice. *Pharmacology, Biochemistry & Behavior* 59(1):135-43

Seiger A, Sorensen SM and Palmer MR (1983) Cerebellar role in the differential ethanol sensitivity of long sleep and short sleep mice. *Pharmacology, Biochemistry & Behavior* 18 Suppl 1:495-9

Spuhler K, Hoffer B, Weiner N and Palmer M (1982) Evidence for genetic correlation of hypnotic effects and cerebellar Purkinje neuron depression in response to ethanol in mice. *Pharmacology, Biochemistry & Behavior* 17:569-78

Tabakoff B and Ritzmann RF (1979) Acute tolerance in inbred and selected lines of mice. *Drug & Alcohol Dependence* 4(1-2):87-90

Tabakoff B, Ritzmann RF, Raju TS, Deitrich RA (1980) Characterization of acute and chronic tolerance in mice selected for inherent differences in sensitivity to ethanol. *Alcoholism: Clinical & Experimental Research* 4:70-73

Trujillo KA and Akil H (1995) Excitatory amino acids and drugs of abuse: a role for N-methyl-D-aspartate receptors in drug tolerance, sensitization and physical dependence. *Drug & Alcohol Dependence* 38:139-54

Velardo MJ, Simpson VJ and Zahniser NR (1998) Differences in NMDA receptor antagonist-induced locomotor activity and [3H]MK-801 binding sites in short-sleep and long-sleep mice. *Alcoholism: Clinical & Experimental Research* 22:1509-15

Vogel-Sprott M (1979) Acute recovery and tolerance to low doses of alcohol: differences in cognitive and motor skills performance. *Psychopharmacology* 61:287-291

Vogel-Sprott M and Sdao-Jarvie K (1989) Learning alcohol tolerance: the contribution of response expectancies. *Psychopharmacology* 98:289-296

Wick MJ, Radcliffe RA, Bowers BJ, Mascia MP, Luscher B, Harris RA and Wehner JM (2000) Behavioural changes produced by transgenic overexpression of gamma2L and gamma2S subunits of the GABAA receptor. *European Journal of Neuroscience* 12:2634-8

Wilson JR, Erwin VG, McClearn GE, Plomin R, Johnson RC, Ahern FM and Cole RE (1984) Effects of ethanol: II. Behavioral sensitivity and acute behavioral tolerance. *Alcoholism: Clinical & Experimental Research* 8:366-374

Wu PH, Tabakoff B, Szabo G and Hoffman PL (2001) Chronic ethanol exposure results in increased acute functional tolerance in selected lines of HAFT and LAFT mice. *Psychopharmacology* 155:405-12

Yang X, Criswell HE and Breese GR (2000) Ethanol modulation of gamma-aminobutyric acid (GABA)-mediated inhibition of cerebellar Purkinje neurons: relationship to GABAb receptor input. *Alcoholism: Clinical & Experimental Research* 24:682-90