GENETIC AND PHARMACOLOGICAL CHARACTERIZATION OF RAPID TOLERANCE TO THE ATAXIC EFFECTS OF ETHANOL IN MICE

By

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CERTIFICATE OF APPROVAL

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TABLE OF CONTENTS

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List of Tables

List of Figures

Abstract

Ethanol tolerance, a decrease in drug responsiveness with repeated administrations, is an important diagnostic criterion for alcoholism. Rapid tolerance develops within 8-24 hours of an initial ethanol exposure, and shares many similarities with chronic tolerance. The genetic contribution to rapid tolerance to ethanol-induced ataxia was estimated using a panel of inbred strains of mice. Strains differed significantly in the degree of rapid tolerance development, which had a narrow-sense heritability estimate of .11. Artificial selection was carried out to develop lines of mice which would show High (HRT) and Low (LRT) levels of Rapid Tolerance. A significant response to selection was seen in replicate 1 by the third selection generation, but no difference was found in replicate 2. Heritability estimates after the fourth generation were .25 for HRT-1 mice and .06 for LRT-1 mice. HRT-1 and LRT-1 mice also differed significantly in chronic tolerance development to 4 doses of ethanol. These studies provide evidence for a genetic contribution to rapid tolerance, and support a genetic link between rapid and chronic tolerance to ethanol's ataxic effects. HRT-1 and LRT-1 mice were tested for their sensitivity to the ataxic effects of MK-801, and for rapid tolerance to these effects. HRT-1 and LRT-1 mice did not differ in either of these measures. Further, the NMDA receptor antagonist MK-801 and partial agonist D-cycloserine failed to block or enhance rapid tolerance development in either the HRT or LRT lines. In genetically heterogeneous mice, the effect of MK-801 on motor learning on the accelerating rotarod was tested. MK-801 significantly impaired performance acutely, and prevented the retention of previous-training when tested drug-free the following day. Since MK-801

significantly impaired learning on the accelerating rotarod, HRT and LRT mice were tested to see whether the NMDA receptor drugs would influence tolerance development when they were forced to learn the task under the influence of ethanol. As was found in previously trained mice, in untrained HRT and LRT mice, neither MK-801 or D-cycloserine affected rapid tolerance development. These results suggest that modulation of the NMDA receptor system is insufficient to significantly affect rapid tolerance development in HRT and LRT mice, and suggest that HRT mice are able to develop rapid tolerance via an NMDA-independent mechanism.

Introduction

Alcoholism is a substantial problem in the United States, and costs the nation over $185 billion annually to cover expenses associated with health care, damaged property, and lost productivity (NIAAA). Nearly 15 million Americans (7% of the adult population) either abuse, or are dependent on alcohol. Since alcoholism affects such a large proportion of Americans, there is a significant push to better understand the mechanisms involved in the development of the disease, in order to devise better treatment and prevention strategies.

According to the Diagnostic Statistical Manual for Mental Disorders, 4th Edition, there are several key criteria that clinicians use to make an alcoholism diagnosis. They include: 1) loss of control over drinking behavior, 2) craving, 3) physical dependence, which is typically inferred from the observance of withdrawal signs when alcohol is removed from the body, and 4) tolerance to alcohol's physiological or psychological effects. It is generally thought that as an individual drinks, tolerance develops to alcohol's effects leading to increased, and sometimes uncontrolled drinking behavior. As the central nervous system adapts to the presence of alcohol (becomes tolerant), dependence develops, whereby the individual requires alcohol to maintain 'normal' functioning. Subsequently, if drinking is stopped, withdrawal signs appear as the individual re-adjusts to the absence of alcohol. Craving is a common withdrawal sign, which can result in a relapse of drinking behavior. Each of these diagnostic criteria may be mechanistically linked to any or all of the others; only through thorough examination of each will we have a better understanding about these symptoms, as well as the disease as a whole.

Much research has been done using human subjects in an attempt to elucidate the mechanisms involved in responses to ethanol. There are some ethical limitations, however, in the use of human subjects for investigating many of alcohol's effects. Few investigators would allow the administration of high doses of ethanol to alcohol naïve subjects, or to those which may be predisposed to developing certain alcoholism traits. In this regard, animal models have been, and continue to be very useful. Current rodent models provide well characterized genetic populations whose ethanol (as well as other drug) exposure can be carefully controlled.

Ethanol Tolerance

Tolerance, one of the important diagnostic criteria for alcoholism, can be defined as a decrease in responsiveness to ethanol upon continuous or repeated administration of the drug. It is demonstrated as a rightward shift in the dose-response curve for ethanol (Kalant et al., 1971). This can result from two separable processes. Dispositional tolerance develops through changes in the absorption, distribution, and/or elimination of ethanol in the body, leading to a decrease in the amount of ethanol left to act on a given target tissue. Hence, dispositional tolerance is also referred to as metabolic tolerance. Functional tolerance arises as the result of an attenuated sensitivity in the target tissues affected by ethanol. While both forms may play a role in increased ethanol intake in alcoholics, most research has focused on functional tolerance, because its regulation is likely under central nervous system control. The ethanol literature has divided functional tolerance into three subtypes, which are primarily distinguished by the rate at which they develop (Kalant et al., 1971; Lê et al., 1992). Chronic (or protracted) tolerance, which is

thought to be the most reflective of the changes seen in alcoholics, develops over days to weeks of intermittent or continuous exposure to ethanol. Clinically, tolerance research has mainly been focused on chronic tolerance, as withdrawal from alcohol in alcoholics is thought to be the result of physiological changes that occur during chronic tolerance development. In rodents, chronic tolerance has been shown to develop to the ataxic (Barbosa & Morato, 2000; Wu et al., 1993), hypothermic (Browman et al., 2000; Crabbe et al., 1982; Rustay et al., 2001), and hypnotic (Karcz-Kubicha & Liljequist, 1995) effects of ethanol. Chronic tolerance is typically studied in rodents by administering daily ethanol injections, using an ethanol-containing liquid diet, or by chronic ethanol inhalation.

Rapid tolerance describes the decreased sensitivity to ethanol that occurs on a shorter time frame compared to chronic tolerance; it is observed after a second ethanol administration given 8-24 hours after an initial dose. Rapid tolerance to ethanol's hypothermic effect was initially described by Crabbe et al. (1979), who showed that ethanol-induced hypothermia was significantly reduced when the animal was challenged again with ethanol at 24, but not 48 or 72 hours after an initial exposure. Bitrán and Kalant (1991) showed that rats treated with ethanol developed rapid tolerance to ethanol's ataxic effects within 8 hours of an initial ethanol exposure. Within this 8-24 hour period, ethanol is completely eliminated from the body; therefore, rapid tolerance is the reflection of adaptations that occur during the ethanol exposure, and potentially those that occur during the abstinence period between ethanol administrations. Rapid tolerance shares many characteristics with chronic tolerance (Khanna et al., 1991a; Khanna et al., 1991b; Wu et al., 1993), and in addition to the hypothermic (Crabbe et al., 1979; Khanna

et al., 1996) and ataxic (Bitrán & Kalant, 1991; Khanna et al., 1996) effects, will develop to ethanol's hypnotic effects (Karcz-Kubicha & Liljequist, 1995). Rapid tolerance is further discussed in later sections.

Acute functional tolerance (AFT), also known as within-session tolerance, develops within seconds to minutes of an initial ethanol exposure. It is specifically defined as tolerance which develops within a single exposure to ethanol. This type of tolerance was first described by Mellanby (1919) when he demonstrated that dogs showed less intoxication at a given blood ethanol concentration (BEC) on the descending limb of the blood alcohol curve than at the same BEC on the ascending limb. Several different methods have been developed to measure AFT. The practice of measuring sensitivity on the ascending limb of the BEC curve has become less common recently, due to the difficulty of getting accurate estimates of brain ethanol concentrations (BrEC) while ethanol is quickly being absorbed (but see Ponomarev & Crabbe, 2002). A common method involves testing mice with repeated doses of ethanol, each resulting in the inability to perform a particular behavior, and measuring BEC at each recovery of that ability (Erwin & Deitrich, 1996; Gallaher et al., 1982). In the Gallaher et al. and Erwin and Deitrich studies, mice were injected with a dose of ethanol which caused the mice to fall from a horizontal stationary dowel. Mice were tested until they regained the ability to balance on the dowel, at which time a blood sample was taken, followed by another smaller injection of ethanol. This second injection increased the BEC over the threshold for balancing on the dowel, again causing the mice to fall. Repeating the process resulted in the recovery of ability to balance on the dowel at successively higher BECs, demonstrating the development of AFT. With this method, BEC measurements

are taken at times when BEC and BrEC are at similar levels. A drawback with this method, compared to measuring BEC as ethanol is being absorbed, is that by assessing AFT only after the first recovery, there is no assessment of the AFT which develops between the ethanol administration and the first recovery. This leads to an underestimate of total AFT. Using the loss of righting reflex (LORR) procedure in mice, Ponomarev and Crabbe (2002) devised a method to accurately measure BEC at the LORR. By testing the mice at short intervals after ethanol administration, they were able to gain a better estimate of the total AFT which developed over the course of sequential ethanol administrations.

Relatedness of different forms of tolerance

There is evidence in the rodent literature that chronic and rapid tolerance share some common mechanisms. One method used to demonstrate that tolerance to different agents are under similar control is to determine whether animals that become tolerant to one treatment also show tolerance when challenged with another drug. This phenomenon is termed cross-tolerance. If the development of tolerance to ethanol invokes changes in the systems affected by ethanol, then drugs that work through similar mechanisms should also be better tolerated in the ethanol-tolerant individuals. Through pharmacological manipulations, Khanna et al. have shown similar cross-tolerance between ethanol and benzodiazepines in both rapid and chronic cross-tolerance paradigms (Khanna et al., 1991b). When tested for drug-induced ataxia and hypothermia, ethanol-treated rats did not show cross-tolerance to benzodiazepines, but benzodiazepine-treated rats did show

cross-tolerance to ethanol. This asymmetrical cross-tolerance was similar for both rapid and chronic forms of tolerance.

Similarly, drugs that have been shown to block or enhance the development of chronic tolerance also tend to modulate rapid tolerance (Kalant, 1996; Khanna et al., 1994a). In a series of studies, Barbosa and Morato showed that blockade of the N-methyl-d-aspartate (NMDA)-type glutamate receptor blocked the development of both rapid and chronic tolerance to the ataxic effects of ethanol in mice (Barbosa & Morato, 2000, 2001). In rats, rapid and chronic tolerance to ethanol have been shown be inhibited by NMDA receptor blockers in both ataxia and hypothermia paradigms (Khanna et al., 1994b; Khanna et al., 1991c). The results of pharmacological research on the relationship between rapid and chronic tolerance has led to the general agreement that rapid tolerance represents the initial induction of chronic tolerance. This is conceivable, as both rapid and chronic tolerance reflect intersessional adaptations. Rapid tolerance reflects changes that occur after only one abstinence period, and chronic tolerance is the result of *n* ethanol treatment sessions, where *n* can range from 2 to infinity (Kalant et al., 1971). AFT, which is strictly the result of intrasessional adaptation, may or may not share overlapping mechanisms with rapid and chronic tolerance.

There is evidence that AFT and rapid tolerance may not be mediated by the same processes. Khanna et al. (1992b), showed that while rapid tolerance to the ataxic and hypothermic effects of ethanol were blocked by NMDA receptor antagonists, AFT to the ataxic effects were not. In this AFT experiment, the authors used the BEC at recovery of motor function to look for the development of AFT. Significant AFT was found, as rats treated with higher doses of EtOH recovered at higher BECs than those treated with

lower doses; however, treatment with ketamine, a non-competitive NMDA channel blocker, did not affect AFT with this model. More recent work by the same group, however, suggests more similarity between the two types of tolerance. When Khanna and colleagues examined AFT as the relationship of BEC to the degree of impairment over time, they were able to see inhibition of AFT by NMDA receptor antagonists (Khanna et al., 2002). That is, NMDA antagonists slowed the development of AFT to the ataxic effects of ethanol in these rats. To this point, there is no clear mechanistic link between AFT and either rapid or chronic tolerance.

Importance of tolerance development

Tolerance is the behavioral or neuronal manifestation of the adaptations that occur with prolonged or repeated ethanol administrations. Clinically, this is a very important adaptation, as a diagnosis of alcoholism or alcohol abuse necessitates an observance of tolerance development which leads to an increased intake of ethanol. One who repeatedly administers ethanol for its subjective effects will likely experience tolerance development as a decrease in ethanol's physiological and psychological effects, necessitating increased intake to achieve the subjective effect to which he or she has grown accustomed (Kalant, 1996). Further, tolerance may develop to the aversive effects of high ethanol intake, allowing the individual to consume more ethanol with fewer side effects. Increased BECs in these individuals may increase the potential for heart and liver damage over extended drinking periods (Hoffman & Tabakoff, 1989; Kalant, 1996). AFT is thought to play a role in the continued intake of ethanol during a single drinking episode. If tolerance to the acute effects of ethanol (rewarding and/or aversive) were to

develop within a drinking session, it would allow for a greater intake of ethanol in that session. Intersessional tolerance development would also allow for a greater intake of ethanol, however this increased intake would be observable from one drinking bout to the next, and could potentially be influenced by the abstinence period between drinking bouts.

Researchers have also been interested in studying tolerance to different pharmacological agents because tolerance is an example of neuroplasticity. Tolerance reflects a compensatory response to the central effects of ethanol. Neuronal malleability is of interest to many researchers, from those who study learning and memory, to recovery from nervous system insult, to neuroregeneration. It is reasonable to think that the processes that allow an individual to develop tolerance to ethanol may share many similarities with those that underlie other instances of neuroplasticity. For these reasons, the study of ethanol tolerance may provide useful information to clinicians working with alcoholics, and also those working with individuals with learning disabilities and brain trauma patients.

Not only does tolerance represent an instance of neuroplasticity, but it also shares many characteristics with learning. Similar to the definition of tolerance, learning can be defined as a relatively stable change in behavior as a result of experience. These changes in behavior are thought to be reflective of CNS modifications that occur in response to changes in one's environment. In some respects, tolerance could be viewed as a specific example of learning. Ethanol tolerance results from the neuroadaptations elicited when ethanol repeatedly (or continuously) perturbs the normally functioning system.

Ethanol tolerance resembles learning in many respects. Both develop over repeated trials, with increased tolerance and learning seen with decreased time between trials (LeBlanc et al., 1976). That is, the greater the temporal proximity of the trials, the greater the tolerance development. Further, some of the neural substrates that seem to underlie learning have also been shown to play a role in ethanol tolerance. In particular, the NMDA-type glutamate receptor is important, and has been shown to mediate both long-term potentiation (LTP; an electrophysiological model of learning; Abraham & Mason, 1988; Silva et al., 1992) and also learning in different spatial (Heale & Harley, 1990; Ward et al., 1990) and non-spatial (Chiamulera et al., 1990; Venable & Kelly, 1990) tasks in both rats and mice. Drugs that modulate action at the NMDA receptor have also been shown to affect ethanol tolerance (details of the pharmacology of ethanol tolerance are introduced later). Another likeness is that many studies showing an affect of NMDA drugs on learning in the different spatial and non-spatial tasks, also show that the drugs do not affect the expression of learning if they are given after learning has occurred (Heale & Harley, 1990). Ethanol tolerance also does not seem to be affected by NMDA receptor drugs if it has already developed . Lastly, both learning and tolerance show decay over time when the stimuli leading to their development are stopped. Everyone is familiar with the concept of forgetting—that previously learned behaviors sometimes have to be relearned after a period of abstinence. Likewise, previously tolerant individuals will show a loss of tolerance when ethanol administration is ceased (Crabbe et al., 1979; LeBlanc et al., 1976). The time it takes for this loss to occur is dependent on the length of prior ethanol administration, and the paradigm used to measure tolerance (Khanna et al., 1992a; LeBlanc et al., 1976). These similarities suggest

that gaining some insight into the mechanisms of ethanol tolerance development may also lead to a better understanding of processes involved in learning. In fact, numerous reports have suggested that there is an important role for learning in the development of tolerance, which will be discussed in a later section.

Stimuli for tolerance development

A major focus in the study of ethanol tolerance has been on the necessary conditions for its development. Intuitively, in order for tolerance to develop (or at least in order to detect its development), an initial ethanol administration should result in some measurable perturbation in the system of investigation. Without this, there would be no stimulus for tolerance development. It is generally accepted that tolerance will develop as a function of the initial perturbation of the system (Kalant et al., 1971). That is, the greater the initial disturbance, the greater the tolerance development. One way to assess the relationship between sensitivity and tolerance development is to determine the correspondence between the two traits. If the initial disturbance is an important stimulus for tolerance development, sensitivity and tolerance should be highly correlated. There has been some debate in this regard, as some reports have shown a positive relationship between initial sensitivity and tolerance development (Crabbe et al., 1982; Khanna et al., 1985; San-Marina et al., 1989), while others have found little to no association between the two (Erwin & Deitrich, 1996; Lê & Kiianmaa, 1990). In the Crabbe et al. study, inbred strains of mice were tested for the hypothermic response to ethanol. After being injected with ethanol for 8 days, tolerance was assessed at the change in response on day 8 compared to day 1. They found that mice which were more sensitive on day 1 tended

to show greater tolerance to the hypothermic effects as measured on day 8. Similarly, San-Marina et al. tested the hypothermic effects of ethanol, but performed phenotypic correlations using genetically heterogeneous mice to examine the relationship between initial sensitivity and tolerance development. Like the Crabbe et al. study, they found that the more sensitive individuals tended to show greater tolerance than those that showed less of an initial response. Khanna et al. studied rat lines that were selectively bred for differences in sensitivity to the motor impairing effects of ethanol (Khanna et al., 1985). They showed that the most sensitive (MA; most affected) line developed greater chronic tolerance to these effects than did the less sensitive (LA; least affected) line. Interestingly, the two rat lines in these studies did not differ in tolerance to the hypothermic or hypnotic effects of ethanol, even though they were differentially sensitive on both measures.

The lack of differential tolerance to the hypothermic and hypnotic effects of ethanol in the MA and LA rats supports the results of other studies showing little to no relationship between initial sensitivity and tolerance development. Erwin and Deitrich were successfully able to breed lines of mice which develop significantly different levels of AFT to the ataxic effects of ethanol (Erwin & Deitrich, 1996) without changing ethanol sensitivity in the lines. Similarly, rat lines selected for sensitivity to the motor impairing effects of ethanol were shown not to differ in chronic tolerance to the same effect (Lê & Kiianmaa, 1990). Additionally, these rat lines were shown not to differ in sensitivity to the hypothermic effects of ethanol, however, they did develop tolerance differently to this effect.

There are several possible explanations for these discrepant results. First, species differences could explain some of the variation. Rats and mice show many differences in responses to ethanol, making some comparisons across species somewhat difficult. They could also partially be due to the behavioral response used to assess the ethanol effect. Individuals (or groups) which show high sensitivity on a particular measure have much more room to show an attenuation of the response with repeated administrations. Those that have low sensitivity have very little room to show tolerance. This is supported by the San-Marina et al. study (San-Marina et al., 1989), where the groups which were somewhat sensitive and those that were most sensitive both developed tolerance, but the maximum degree of tolerance reached in both groups was the same. The group which showed very little initial response did not develop significant chronic tolerance, perhaps due to the detection limits of the task. This could be looked at as a statistical issue; the farther an initial score is away from the population mean, the more likely it is to show up closer to the population mean with a repeated test. This regression to the mean could explain the proposed relationship between sensitivity and tolerance development. If an individual or group is scored as highly sensitive to ethanol, one might expect to see greater tolerance development in the more sensitive group just by chance, i.e., regression to the population mean with repeated tests.

Lastly, an important consideration in the interpretation of these studies is the apparent truth that high sensitivity on one task does not necessarily confer high sensitivity on another. Many of the aforementioned studies have shown differences in sensitivity among groups on one task, but have shown no difference on another, such as motor coordination and hypothermia. Even within a single domain such as "ataxia," it

has been shown that certain genotypes can be found to be differently sensitive on one task while show no difference on others (Boehm et al., 2000). The same argument could be made for tolerance to different ethanol effects as well. This possibility is addressed later in the introduction.

Another important stimulus for tolerance development (related to the initial disturbance) is ethanol dose. Typically, greater tolerance is seen with higher doses of ethanol. However, with repeated administrations of a low or high dose of ethanol, one might observe no tolerance development, while at intermediate doses there may be significant changes in post-ethanol performance (Barbosa & Morato, 2001). This may suggest an "inverted-U" dose-response for tolerance development, where greatest tolerance develops at intermediate doses. However, this lack of observable tolerance may be a reflection of the insensitivity of a given test to extreme doses of drug. At low doses, there may be no impairment on the task, disallowing any observable tolerance (a ceiling effect). At extremely high doses, tolerance may be developing, but the decreased sensitivity to ethanol that has developed may still be below the threshold of detection for the task (a floor effect). One way to avoid this confound while still examining the role of dose on tolerance development, is to give the different doses of ethanol on the first exposure, then challenge all dose groups with the same dose of ethanol that will elicit behavior within the sensitivity range of the task (Khanna et al., 1996; Khanna et al., 1995a). With this procedure, the groups differ only in the treatment they were given on the first ethanol exposure. Alternatively, one could treat several groups, each with a different dose of ethanol, then check for a right-ward shift in the dose-response curve by challenging animals within each group with different doses of ethanol.

Along with the impairment produced, another important stimulus for tolerance development is the total exposure to ethanol. The total exposure to a single bolus injection of ethanol is directly related to the dose administered. Bigger doses of ethanol will result in a longer total exposure to the drug due to ethanol's pseudo-linear elimination kinetics. However, if one is to prolong the exposure by giving smaller, booster injections over the course of the ethanol exposure, or by using a continuous intravenous infusion or ethanol inhalation, then tolerance should develop as a function of total ethanol exposure. There does appear to be an upper limit to the extent of tolerance development, whereby increased ethanol dose or exposure to ethanol does not result in increased tolerance (Gallaher et al., 1982; LeBlanc et al., 1976).

Role of learning in ethanol tolerance

Many studies have shown that there is an important contribution of learning to tolerance development. Functional tolerance in many cases is likely to be a combination of physiological tolerance, a true change in tissue sensitivity, and contingent (conditioned) tolerance, which develops as the result of learning associations between the unconditioned drug effects and the cues associated with them. Conditioned tolerance to ethanol's hypothermic effect has been shown to develop when rodents are tested repeatedly in the same testing environment (Lê et al., 1979b). By testing the animals in an identical setting, the entire testing procedure becomes predictive of the ethanol experience. It is thought that these cues are able to elicit a compensatory response in the animals that then counteracts ethanol's effects (reviewed by Siegel, 1989). This would appear as tolerance development, even if the previous ethanol administrations had not

caused any decreased sensitivity in the tissues where ethanol was acting. The influence of conditioning on tolerance development can be examined either by testing for tolerance development in a novel environment, or by challenging the subjects with vehicle instead of ethanol in the original test setting. If conditioning was explaining all the tolerance development (i.e., the tolerance was context dependent), then the subjects should show no tolerance development when tested in a novel environment (Lê et al., 1979b).

The influence of conditioning to ethanol tolerance in rats was examined by Mansfield and Cunningham (1980). These authors demonstrated that tolerance to the hypothermic effects of ethanol developed steadily over the course of several days of testing. They also found that rats challenged with ethanol in a context previously paired with a saline administration showed a much greater response to ethanol than those tested in an ethanol-paired environment, even though the groups had been given the same total amount of ethanol. This demonstrated that a proportion of the tolerance development was context-dependent. As an extreme example of this phenomenon, contextual conditioned tolerance has been demonstrated in the resistance to lethality produced by a high dose of heroin. Siegel et al. (1982) reported that when rats had been administered increasing, sub-lethal doses of heroin in a distinct environment, there was a substantial amount of tolerance development. This was evidenced by the fact that when the rats were given a high dose (lethal in naïve rats), those tested in the familiar environment had a much lower mortality rate than rats with the same amount of heroin experience, but which were tested in a non-familiar environment. The authors used this as a model of "overdose" among heroin users when they take the drug in unfamiliar environments.

Tolerance to the effects of ethanol can be also mediated at least in part by instrumental learning (Wolgin, 1989). This process entails the use of behavioral means to counteract the initial effects of ethanol intoxication. LeBlanc and colleagues have shown that rats given equal amounts of ethanol will differ in the amount of tolerance that develops to the inhibition of maze or motor performance by ethanol depending on whether or not they had intoxicated practice on the task. Rats were trained to perform in a spatial maze (LeBlanc et al., 1973) or on a treadmill motor coordination task (LeBlanc et al., 1976) before ethanol administration began. After thorough training, tests were performed to examine the effects of intoxicated practice on the development of tolerance to ethanol's inhibitory effects on the tasks. Rats that routinely received ethanol before testing showed greater tolerance to ethanol's effects than those given ethanol after practice on non-test days. Groups treated with ethanol after the practice trials did develop tolerance, but at a slower rate. The research group termed this intoxicated practice phenomenon "behaviorally augmented tolerance" (BAT). However, when the "after" group's ethanol treatment regimen was increased, they quickly reached the level of tolerance demonstrated by the BAT group. These experiments also revealed an upper limit for tolerance development. When the BAT group's ethanol dose was increased, they were unable to show greater tolerance. That is, they were not able to attain post-ethanol performance that equaled their pre-ethanol performance, even when their "behaviorally augmented tolerance" was combined with a "physiological tolerance" treatment regimen.

Khanna et al. (1997) also examined the role of BAT in tolerance to ethanol's ataxic effects. Using the tilt-plane test of ataxia in rats, they compared performance in

groups which received intoxicated practice to those which received only post-ethanol handling ("dummy testing"). They found that groups given intoxicated practice again developed greater tolerance, and this BAT was more susceptible to pharmacological blockade.

It is clear from the wealth of literature that tolerance to the effects of ethanol can arise from several different mechanisms. Metabolic tolerance can lead to a decreased amount of ethanol to act on certain tissues. Physiological tolerance may develop as individual cells, or systems of cells, compensate for the effects of ethanol. Further, the repeated application of ethanol in familiar settings may elicit compensatory responses by systems unaffected by ethanol which counteract ethanol's effects, resulting in an apparent decrease in the drug's effects. Lastly, the simple act of performing certain tasks under the influence of ethanol may lead to the development of behavioral strategies that act to reduce ethanol's effects. All of these processes will lead to a decrease in ethanol's effects. It should be noted that although they may all be mediated by separable processes, these changes are, in all likelihood, the end result of molecular and cellular adaptations in response to ethanol. These adaptations can be investigated through the application of pharmacological and genetic techniques (among others) to better understand the mechanisms behind them.

Pharmacology of Ethanol Tolerance

Early studies of the neurotransmission involved in the development of ethanol tolerance focused on chronic tolerance. Much of this focus was on the role of serotonin transmission in chronic tolerance to ethanol's ataxic and hypothermic effects. Frankel et

al. (1975) found that rats treated with p-cholorophenylalanine (p-CPA) to deplete whole brain serotonin showed decreased tolerance development compared to those with intact serotonin systems. A similar effect was seen in rats given lesions of the median raphe nucleus, an important source of forebrain serotonin (Lê et al., 1981a), suggesting a strong role of serotonin in tolerance development. This was further supported by the result that treatment with a tryptophan-rich diet, which effectively elevated serotonin levels, accentuated tolerance development in rats (Lê et al., 1979a).

Later, when members of the same group performed a series of experiments examining rapid tolerance, they found very similar results. Enhancement of serotonergic tone acted to increase rapid tolerance to ethanol's ataxic effects in rats, while decreasing serotonin decreased rapid tolerance (Khanna et al., 1994a). Interestingly, the group also found that NMDA receptor drugs were also quite effective at modulating rapid tolerance (Khanna et al., 1996; Khanna et al., 1993b; Khanna et al., 1991c). Non-competitive NMDA receptor antagonists (MK-801 and ketamine) were shown repeatedly to block rapid tolerance both to the hypothermic and ataxic effects of ethanol. To look at potential interactions between NMDA and serotonin systems in rapid tolerance, one study was done using pharmacological agents for both systems. Khanna and colleagues were able to replicate the finding that serotonin agonists enhanced rapid tolerance; however, MK-801 was able to block this enhancement (Khanna et al., 1994a). This suggested that the NMDA system may have a more important role in mediating rapid tolerance development. Since then, this group and others have published several reports showing that the NMDA receptor system is very important for the development of rapid and chronic tolerance to ethanol's ataxic (Khanna et al., 1997; Wu et al., 1993), and

hypothermic (Khanna et al., 1991c) effects in rats, and hypnotic (Karcz-Kubicha & Liljequist, 1995) and ataxic (Barbosa & Morato, 2000, 2001) effects in mice.

The γ-aminobutyric acid (GABA) system has also been implicated in ethanol tolerance development. The $GABA_B$ receptor agonist baclofen, and antagonists CGP36742 and CGP56433, respectively blocked and enhanced rapid tolerance measured by the accelerating rotarod in mice (Zaleski et al., 2001). Neurosteroids, which are potent positive modulators of the $GABA_A$ receptor, may also play a role in rapid and chronic tolerance to ethanol. Again using mice and the accelerating rotarod, Barbosa and Morato (Barbosa & Morato, 2000, 2001) showed that neurosteroid treatment facilitated both rapid and chronic tolerance to ethanol. Interestingly, the potentiating effect of pregnenolone sulfate was inhibited by the coadministration of MK-801. This provides more evidence that while other systems may be able to modulate ethanol tolerance, the NMDA system may have a prominent role.

Other neurochemical systems have been implicated in the process of ethanol tolerance and will briefly be introduced here. They include the neuropeptide vasopressin (Bitrán Speisky & Kalant, 1985; Hoffman & Tabakoff, 1989), and nitric oxide (NO) (Khanna et al., 1995b). Vasopressin has been shown to maintain tolerance after it has developed. NO is important in the development of rapid tolerance to the ataxic effects of ethanol. Interestingly, both the vasopressin system and NO are thought to play a role in learning, and may be involved with both the serotonin and NMDA receptor system in the development and maintenance of ethanol tolerance (Kalant, 1996).

Genetic approaches for studying ethanol tolerance

There are several approaches typically used to study the role of genetics in ethanol tolerance. They can be classified as either "bottom-up" or "top-down" approaches. Recently, the bottom-up approach has been dominated by molecular techniques used to produce mutant mice which are characterized by over- or under-expression of gene products thought to be important in the development of tolerance. These mouse models are then tested to examine the behavioral effects of the genetic manipulation. Boehm and colleagues (2003) have recently shown that fyn-kinase deficient mice do not develop AFT to the same extent as wild-type mice on the stationary dowel test of motor coordination. However, they did not differ from wild-type mice in AFT on the rotarod. Fyn-kinase is an important mediator of phosphorylation of NMDA receptors *in vivo*, and is thought to potentially mediate some of the adaptations to acutely administered ethanol (Yaka et al., 2003).

Top-down approaches often used include the analysis of inbred strains and selected lines. Inbred strain analysis allows for an estimation of the degree to which a behavior is under genetic control. Individuals within an inbred strain are essentially identical genetic replicates of one another. Therefore, any differences in a behavior within an inbred strain is, by definition, the result of environmental effects (e.g., diurnal, litter, and/or cage effects). Since each inbred strain represents a separate genetic population, significant differences among a panel of inbred strains suggest that genetic make-up influences the expression of the behavior. Many researchers have used inbred strain analysis for detecting genetic influences in ethanol-related traits. Chronic tolerance to the hypothermic effects of ethanol in mice was shown to be significantly influenced by

genotype (Crabbe et al., 1982). More recently, inbred strains of mice have been shown to differ in AFT to the ethanol's ataxic (Kirstein & Tabakoff, 2001) and hypnotic effects (Ponomarev & Crabbe, 2002).

Another strategy using inbred strains is the analysis of genetic correlation. These analyses provide information on the genetic relatedness among traits. By testing a panel of inbred strains on multiple behavioral traits, one can gain some understanding of which traits share similar genetic control. If two traits show a similar strain distribution (i.e., are highly correlated), it is likely that the two traits have some underlying mechanisms in common.

By examining several inbred strains for ethanol tolerance, it is possible to estimate the genetic contribution to the trait. Heritability (h^2) is the term given to the degree to which a trait can be passed from parent to offspring. While the most direct measure of this would come from breeding studies (see later section), Hegmann and Possidente (1981) have provided a framework for estimating narrow-sense heritability using inbred strains. Narrow-sense heritability is defined as the degree of genetic determination for a trait (Falconer & Mackay, 1996), and its estimation is derived by first determining the proportion of trait variance that is accounted for by differences among strains (genetic variance, V_G). The ratio of the genetic variance to the total phenotypic variance (V_P) for the trait is calculated for the estimation of narrow-sense heritability. The greater the genetic variance and lower the total phenotypic variance, the higher the narrow-sense heritability estimate. From a behavioral genetics standpoint, heritability is an important consideration, as artificial selection studies will only be successful for those traits which are heritable (Crabbe, 1999).

Artificial selection is another common method used for studying the genetic contribution to behavioral traits. Selective breeding has been used throughout history as a means of generating plants and animals which display high or low levels of a phenotype of interest. Its basic premise is simple: select the "best" subjects of the group to produce the offspring for the next generation. The criterion for what is "best" is completely up to the experimenter (beauty is in the eye…of the experimenter!). Hence, artificial selection is akin to natural selection (survival of the fittest), with the exception that the experimenter is responsible for determining which animals are "fittest." The selection pressure applied by the experimenter causes shifts in the allelic frequencies in the population to more resemble those in the selected parents (Falconer & Mackay, 1996). Over successive generations of selective breeding, the resulting population can differ quite greatly from the original starting population. The rate at which the change is seen, as well as the magnitude of the change, is dependent on several things, such as the degree to which the trait is under genetic control, how many genes contribute to the trait, the genetic variability in the starting population, the degree of inbreeding, and any environmental constraints that may limit further response to selection (Crabbe, 1999). A significant response to artificial selection provides evidence for a role of genetics in expression of the phenotype. The realized heritability for the trait can be estimated by dividing the response to selection by the cumulative selection differential, or amount of selection pressure applied to the population (Falconer & Mackay, 1996). Selection pressure refers to the difference in the means of the selected parents from the mean of the total population, and is typically expressed in standard deviation units.

Artificial selection has been a particularly popular method for examining ethanol-related traits. Early selective breeding for ethanol preference drinking in rats was done in the 1940s by Mardones, producing the UChA (alcohol avoiding) and UChB (alcohol drinking) lines (Mardones, 1960) and in the 1960s by Eriksson, termed AA (Alko Alcohol) and ANA (Alko Non-Alcohol) lines (Eriksson, 1968). Since then, numerous others have selected additional rat and mice lines that differ in ethanol drinking (P & NP: Lumeng et al., 1977); HAD & LAD: Li et al., 1993; HAP & LAP: Grahame et al., 1999), sensitivity to ethanol's hypothermic (HOT & COLD: Crabbe et al., 1987a), ataxic (AT & ANT: Eriksson & Rusi, 1981), and locomotor stimulant (FAST & SLOW: Crabbe et al., 1987b) effects, as well as chronic ethanol withdrawal (WSP & WSR: Crabbe et al., 1983). Recently, lines of mice have been selected for high and low degrees of AFT to ethanol's ataxic effects (HAFT & LAFT: Erwin & Deitrich, 1996). The artificial selection approach is very useful for performing mechanistic studies of the selected trait. Presumably, the response to selection is the result of concurrent changes in the biological systems mediating the trait. Examining the selected lines for differences in these systems can lead to a better understanding of the mechanisms behind the behavior.

Selected lines are also useful for testing correlated responses to selection (Crabbe, 1999). If selection results in changes in the biological systems mediating the selection trait, other behaviors influenced by these systems should also be expected to be differentially expressed in the selected lines. Similar to correlational studies with inbred strains, this provides evidence that the traits share a common genetic control, and that the genes mediating the responses are exhibiting pleiotropy (Crabbe et al., 1990).

An important consideration for the study of ethanol tolerance is whether tolerance to a particular effect of ethanol necessarily confers tolerance on another measure. Previously mentioned studies using both rats and mice have provided evidence that while animals may show differences in tolerance development to the hypothermic effects of ethanol, they may or may not show differences when tested on a given measure of ataxia (or vice versa). Selected lines are particularly useful for determination of the relationship between tolerance on different tests. As mentioned earlier, Erwin and Deitrich produced selected lines of mice that develop high and low levels of AFT when tested for ethanol's ataxic effects on the static dowel (Erwin & Deitrich, 1996). AFT was measured as the difference in BEC at the second recovery compared to the first recovery. High (HAFT) and Low (LAFT) AFT mice have been extensively characterized to examine whether there was task and drug specificity for the development of tolerance in these mice, and to see whether the mice would differ in other forms of tolerance. HAFT and LAFT mice were tested for their development of AFT to the hypnotic and hypothermic effects of ethanol and were found not to differ in either test (Erwin et al., 2000). We have tested the HAFT and LAFT mice for the development of AFT on a different measure of ataxia, the fixed-speed rotarod (Rustay et al., 2001). We found that HAFT mice developed more AFT on this measure, but the lines did not differ in chronic tolerance to ethanol's effects in the grid test of ataxia, or hypothermia. HAFT and LAFT mice were also tested to see whether chronic administration of ethanol would affect subsequent development of AFT in the lines. Wu et al. (2001) showed that chronic ethanol did increase the degree of AFT, however it was increased to the same degree in both HAFT and LAFT mice. These results suggest that the genes mediating the development of AFT to the ataxic effects are

not completely overlapping with those mediating AFT to the hypnotic and hypothermic effects, or with those mediating chronic tolerance to these effects.

On the surface these results may seem somewhat surprising. Considering, though, that different tasks are likely mediated partially or completely via different neural substrates, makes them sound more reasonable. The determination of these substrates is one goal of selected line and inbred strain studies. These approaches allow the determination of the genetic relatedness of different traits, and may eventually (through follow-up mechanistic studies) allow us to parse out the biological differences among genotypes that contribute to variations in performance. We have recently published data showing that among a set of inbred strains, acquisition, maximal performance and sensitivity to ethanol on the accelerating rotarod is highly dependent on genetic differences among strains (Rustay et al., 2003a). Using the same panel of strains, we are currently examining numerous other tests of ataxia to provide data on the relatedness of the different tasks. By selecting mice for different forms of ethanol tolerance and on multiple tasks, we can begin to assess how the different forms are related. Further, we may be able to show that different forms of tolerance are more related when tested with some tests than when tested with others.

The work reviewed here demonstrates the complexity of studies on the development of ethanol tolerance. Results can be influenced by the duration of ethanol exposure, ethanol dose, contextual conditioning, behavioral compensation, as well as by the specific test modality (e.g., hypothermia, ataxia, hypnosis) and genetic background of the subjects. While it is clear that AFT is influenced by genetics, few data are available on the genetics of rapid tolerance. Further, tests of the relatedness of acute, rapid and

chronic tolerance are scant, and most have been done through pharmacological manipulation. Genetic analysis of these relationships can add another level of understanding. AFT has been investigated in this regard with the generation of the HAFT and LAFT mice. Artificial selection for other forms of tolerance will increase our understanding further through correlated response and pharmacologic studies in the selected mice. Research on the role of certain neurotransmitter systems in rapid tolerance have provided evidence for an involvement of NMDA receptors. If NMDA receptors are important, their function should be modulated in mouse lines selectively bred for differences in rapid tolerance.

Rationale and Hypotheses

Chapter 1: The genetic contribution to rapid tolerance

The initial set of experiments that follows used two separate genetic models to provide an estimation of the genetic contribution to rapid tolerance to the ataxic effects of ethanol. There have been studies examining the genetics of AFT and chronic tolerance, but little done in this regard for rapid tolerance. First, inbred strain analysis was used to estimate the genetic contribution to rapid tolerance as measured by the accelerating rotarod. Based on this estimation, artificially selected lines were developed to produce lines which showed high (HRT) or low (LRT) levels of rapid tolerance. Once developed, these selected lines provided a useful genetic model for investigating the correlation between rapid and chronic tolerance. This relationship has been investigated using pharmacological manipulations (Khanna et al., 1991b; Wu et al., 1993), but not through the use of behavioral genetics.

Hypotheses

I predicted that rapid tolerance would be under significant genetic control, and hence, inbred strains would differ in their development of rapid tolerance. Further, I hypothesized that this genetic contribution would be supported by the successful artificial selection of high and low rapid tolerance lines of mice. I also predicted that HRT mice would develop greater chronic tolerance than LRT mice, suggesting similar genetic control of rapid and chronic tolerance.

Chapter 2: Pharmacology of rapid tolerance

The second set of experiments was designed to explore the mechanisms behind rapid tolerance to ethanol's ataxic effects via pharmacological manipulation. Using the HRT and LRT mice, I sought to show that the NMDA receptor system is important in rapid tolerance, in accordance with much of the literature. It was thought that a response to selection may arise due to the role of the NMDA receptor in rapid tolerance development. To test this, I tested the selected lines for sensitivity to the ataxic effect of MK-801, a non-competitive NMDA receptor blocker, and for the development of rapid tolerance to this effect. HRT mice were then tested to see whether rapid tolerance was inhibited by MK-801. Additionally, both HRT and LRT mice were tested with D-cycloserine, a partial agonist at the glycine site on the NMDA receptor, for its ability to potentiate rapid tolerance.

An additional set of experiments was aimed at investigating the role of NMDA receptors in motor learning, and the potential importance of this in relation to tolerance to

ethanol's ataxic effects. Genetically heterogeneous mice were treated with MK-801 to determine its effects on motor learning using the accelerating rotarod. MK-801's effects on rapid tolerance were also examined using HRT mice that were forced to learn the task under the influence of ethanol, as opposed to being thoroughly trained on the accelerating rotarod prior to ethanol exposure.

Hypotheses

I predicted that MK-801 and D-cycloserine would significantly inhibit and enhance, respectively, the development of rapid tolerance in HRT and LRT mice. This would support a role of NMDA receptors in rapid tolerance. If the NMDA receptor system were important in rapid tolerance development, I would expect that HRT and LRT mice would also differ in sensitivity to NMDA receptor drugs. Therefore, I hypothesized that HRT mice would be more sensitive to the ataxic effects of MK-801 than LRT mice. NMDA receptor antagonists have been shown to impair learning in many tasks (Heale & Harley, 1990; Ward et al., 1990). I predicted that MK-801 would also impair acquisition of accelerating rotarod performance. The learned component to tolerance development has been shown to be sensitive to pharmacological blockade (Khanna et al., 1997). As a result, I predicted that MK-801 would inhibit tolerance development when mice are forced to learn the task under the influence of ethanol.

Chapter 1: The genetic contribution to rapid tolerance

Introduction

Alcoholism or alcohol abuse affects nearly 15 million American adults. Tolerance, an attenuated response to ethanol after previous exposure, is one of the key diagnostic criteria for alcoholism. Tolerance can take several different forms, primarily distinguished by the time course of development (Kalant et al., 1971; Lê et al., 1992). Chronic tolerance develops over the course of days to weeks of repeated administration or continuous exposure to ethanol, and therefore may be more reflective of changes seen during the development of alcoholism. Acute functional tolerance (AFT), also known as within-session tolerance, is that which develops in seconds to minutes during a single exposure to ethanol. Rapid tolerance develops within 8-24 hours of an initial ethanol administration (Crabbe et al., 1979), and shares many characteristics with chronic tolerance (Bitrán & Kalant, 1993; Kalant, 1996; Khanna et al., 1991b; Khanna et al., 1992c; Khanna et al., 1994b). Because of its similarities with chronic tolerance, rapid tolerance has become a popular model for studying the mechanisms of tolerance development. Gaining insight into the mechanisms behind tolerance development could potentially help design new intervention and treatment strategies for alcoholism.

It is clear from studies in rodents that genetics plays a significant role in the development of ethanol tolerance (Browman et al., 2000; Crabbe et al., 1982; Erwin & Deitrich, 1996; Gallaher et al., 1996; Rustay et al., 2001). Isogenic populations, such as inbred strains, are useful for estimating the genetic contribution to certain behaviors. When tested under identical laboratory conditions, differences within an inbred strain are

thought to be the result of environmental influences, while differences among a panel of inbred strains are, by definition, due to differences in genotype (Crabbe et al., 1990; Hegmann & Possidente, 1981). Using inbred strains of mice, Ponomarev and Crabbe (submitted) have recently shown that the degree of AFT to ethanol's sedative effects differs among strains. Further, Erwin and Deitrich (1996) used artificial selection to produce lines of mice that differ in the degree to which they develop AFT to ethanol's ataxic effects. Successful artificial selection also provides evidence that the trait is under significant genetic control. Selected lines are also useful to study the genetic relatedness of particular traits. Lines that have been selected for a particular trait can be tested to see whether they differ on other traits as well. It is thought that correlated responses to selection arise principally due to pleiotropic gene effects.

The current studies were designed to determine the genetic contribution to rapid tolerance to the ataxic effects of ethanol through the use of inbred strain analysis and the development of short term selected lines. Artificial selection was used to provide a useful animal model for studies examining the specific mechanisms involved in the trait. Further, we wanted to examine the relationship between rapid and chronic tolerance by comparing mice selected for high and low rapid tolerance in an extended, chronic tolerance paradigm. While numerous reports have taken a pharmacological approach to compare the two (Barbosa & Morato, 2001; Bitrán & Kalant, 1993; Khanna et al., 1992c; Khanna et al., 1994b), a genetic approach has not yet been reported.

Materials and Methods

Husbandry. Mice were housed in either the Veterinary Medical Unit at the Department of Veterans Affairs Medical Center (Portland, OR), or in the Oregon Health & Science University (OHSU) Department of Comparative Medicine vivarium (Portland, OR). All mice were housed 1-6 per cage at 20-22°C, with food and water freely available except during test procedures. Mice were kept on a 12 hour light:dark cycle (lights on at 0600) and testing occurred between 0800 and 1600. All procedures were approved by either the OHSU or Department of Veterans Affairs Medical Center Institutional Animal Care and Use Committees in accordance with NIH guidelines for the care and use of laboratory animals.

Rapid tolerance. Mice were tested on the AccuRotor Rota Rod (Accuscan Instruments, Columbus, OH). The apparatus was modified to have a 63 cm fall height and 6.35 cm diameter dowel (Rustay et al., 2003b). On a pretraining day, all mice were given 10 consecutive acquisition trials on the ARR (accelerated at a rate of 20 rpm/min), with a 30 sec ITI. The following day (Test Day 1), mice were given 3 baseline trials, immediately given an injection of 2.5 g/kg EtOH (20% v/v, i.p.) and were placed in individual holding cages. After 30 min, mice were given 3 more tests on the ARR. Twenty-four hours later (Test Day 2), all mice were tested exactly as they were on Test Day 1. For all tests, average latency to fall for trials 2 and 3 of each test session was recorded as a measure of motor coordination, with increased latency to fall corresponding with increased coordination. Rapid tolerance was defined as an increased latency to fall after ethanol on day 2 compared to day 1. A "tolerance score" was computed for each animal by

subtracting the average post-EtOH latency to fall on Test Day 1 from that on Test Day 2. Greater positive tolerance scores corresponded with greater tolerance development.

Inbred Strains. Male and female mice from 7 inbred strains (129S1/SvImJ, A/J, BALB/cByJ, BTBR/T+$^{tf/tf}$, C3H/HeJ, C57BL/6J, and DBA/2J) were purchased from The Jackson Laboratory at 5 weeks of age, and tested between 8 and 10 weeks of age for the development of rapid tolerance on the ARR (see above). The experiment was completed in 2 passes. In the first pass (n = 6/strain), mice were given 2 days of training (with 10 trials on each day) 1 week prior to ethanol testing. In the second pass (n = 12/strain), mice were given just 1 day of training (10 trials) on the day prior to ethanol testing.

Artificial Selection. Male and female genetically heterogeneous mice (HS/Npt, n = 47-49/sex) were obtained from Dr. Robert Hitzemann and used as the starting population for artificial selection. These mice were developed from the systematic intercross of 8 different inbred strains (A/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6J, CBA/J, DBA/2J, LP/J) for over 40 generations at the start of selective breeding (Hitzemann et al., 1994). All mice were 60-80 days old at the start of testing. The starting population was tested for rapid tolerance development with 10 acquisition trials on the training day followed by two consecutive days of ethanol testing (see above). The 10 highest scoring males and 10 highest scoring females were randomly assigned to form 10 breeding pairs. Five breeding pairs each were then assigned to start two sets of selected lines, the High Rapid Tolerance lines (HRT-1 and HRT-2). Similarly, the lowest scoring males and females were used to form the Low Rapid Tolerance lines (LRT-1 and LRT-2). Replicate lines

were started to provide two separate genetic experiments in order to be confident that any response to selection was due to the artificial selection pressure itself, as opposed to random drift in gene frequency. In successive generations, individual selection was performed within each line; as in the initial generation, brother-sister mating was avoided whenever possible. Individual selection was chosen over within-family selection in order to maximize the initial response to selection. By selecting the best scoring individuals regardless of family, we are able to maximize the selection pressure in each generation. Individual selection can potentially increase the inbreeding coefficient in each generation by not explicitly keeping families separate, as is done using within-family selection. Nevertheless, we decided to use individual selection because with a short-term selection study (4-5 generations), the effects of inbreeding in this procedure are likely to be minimal (Falconer & Mackay, 1996). Because no response to selection was seen in either replicate after 2 generations of breeding (see Results), the EtOH dose was increased to 2.75 g/kg, and mice from both the 1st and 2nd litters were used as the pool from which breeding pairs were selected in generations 3-5.

Chronic tolerance. Naive male and female HRT-1 and LRT-1 mice from selection generation 5 (S_5) were tested for the development of chronic tolerance on the ARR (n = 9-12 per line and dose). Mice were trained on the ARR with 10 consecutive trials at 20 rpm/min on the day before the first ethanol exposure. For the 5 days following training, mice were given 3 baseline trials, followed by an injection of 2.25, 2.5, 2.75 or 3.0 g/kg EtOH. Thirty min later, mice were tested for post-EtOH performance. Tolerance was assessed as the increase in post-EtOH performance across the 5 days of testing.

Drugs. Ethanol (200 proof, Pharmco, Brookfield, CT) and physiological saline (0.9%) were used to make 20% (v/v) ethanol solutions. Injections were given intraperitoneally (i.p.), with volume adjusted according to body weight.

Statistics. All statistics were calculated using Systat 10 (Chicago, IL). Rapid tolerance was determined using a within-subject design. ANOVA was used to look for differences among inbred strains, selected lines and dose groups, with day as a repeated measure for chronic tolerance. One-sample t-tests were used to determine which strains developed significant rapid tolerance. Differences were considered significant at $p<.05$. The narrow-sense heritability estimate for rapid tolerance in the inbred strains was made by dividing the genetic variance (V_G; variance accounted for by the Strain variable) by the total phenotypic variance (V_P) (Falconer & Mackay, 1996). Realized heritability estimates for the selected lines were calculated, as suggested by Falconer and Mackay (1996) by dividing the response to selection (R) by the cumulative selection differential (S) for generations 1-4 of selective breeding.

BEC determination. Blood ethanol concentrations (BEC) were determined by gas chromatography (Model 6890N, Agilent Technologies, Palo Alto, CA). Mice (12-13/line) were injected with 2.75 g/kg ethanol and placed in individual holding cages. After 30 min, mice were gently restrained and 20 µl of blood was taken from the tip of the tail. Fifty µl of $ZnSO_4$, 50 µl of $Ba(OH)_2$, and 300 µl of dH_2O were added to the

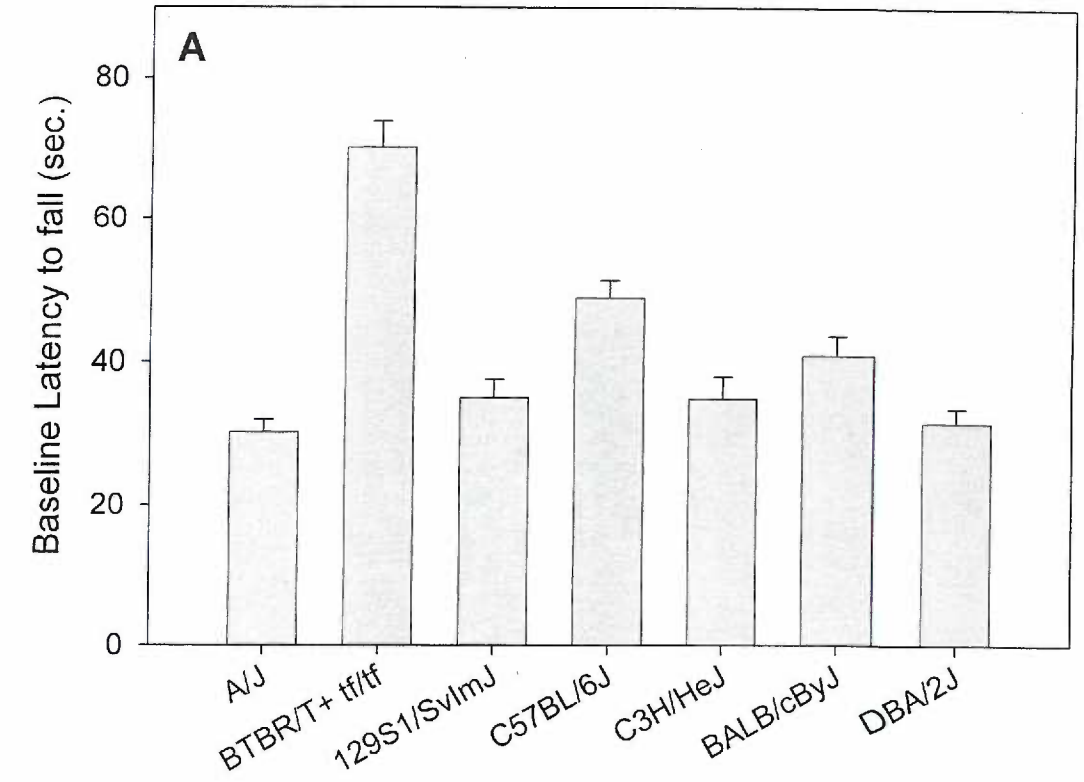
samples, which were centrifuged at 12,000 rpm for 5 min. The resulting supernatant was removed and analyzed compared to a standard ethanol concentration curve

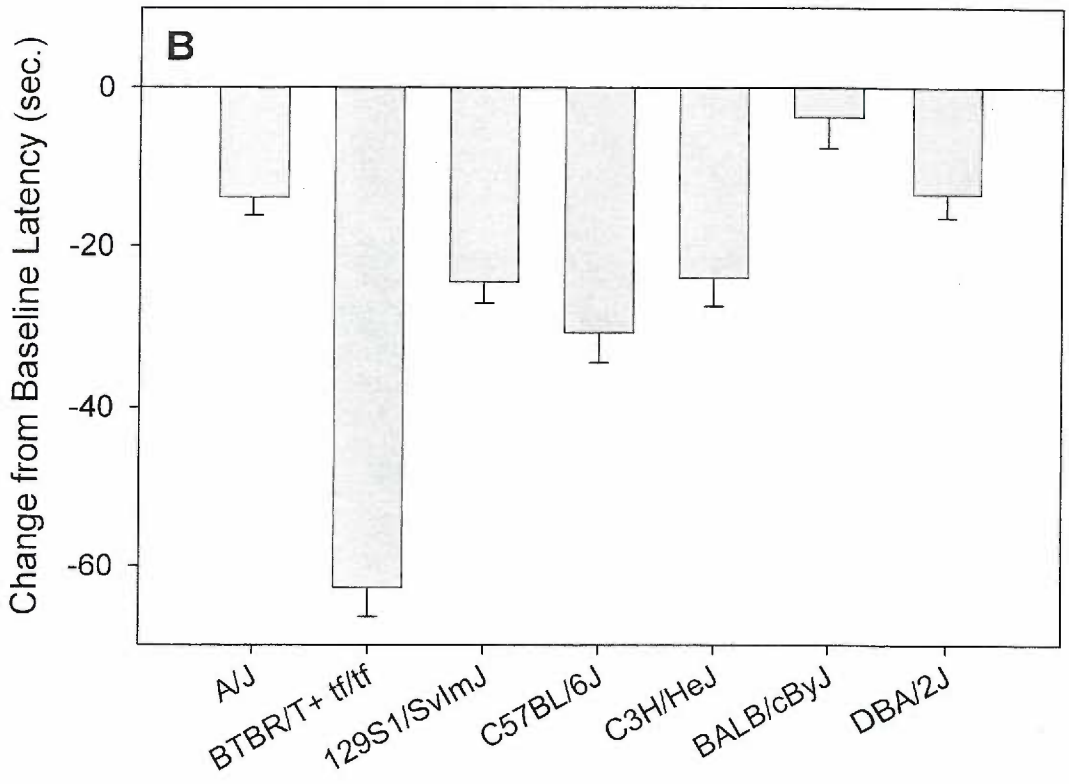
Results

There were no significant differences between sexes in any of the experiments. As a result, all analyses were collapsed on sex.

Inbred strains. Baseline performance on test day 1 was assessed by 2-way ANOVA (Strain X Pass) to examine whether the different training regimens given to each pass affected baseline performance. This analysis revealed a significant effect of Strain [$F(6,111)=24.6$, $p<.001$], but no significant effect of Pass or Strain X Pass interaction [$F's<1.1$, $p's>.41$]. As the different training regimens did not affect baseline performance on the first day of testing, all analyses are collapsed on Pass. Because there was a significant difference in baseline performance among strains (Fig 1A), ethanol sensitivity was assessed by the change from baseline performance after ethanol. One-way ANOVA (Strain) of Day 1 change from baseline performance showed a significant effect of Strain [$F(6,118)=34.3$, $p<.001$], suggesting that strains differed in their sensitivity to 2.5 g/kg EtOH (Fig 1B). The change in post-ethanol performance from day 1 to day 2 was used to assess rapid tolerance (Fig 2). One-way ANOVA (Strain) of the tolerance scores showed a significant effect of Strain [$F(6,118)=2.3$, $p<.05$]. Mice from the 129S1/SvImJ, C3H/HeJ, BALB/cByJ, and DBA/2J strains showed significant rapid tolerance development ($p<.05$), while A/J, BTBR/T+$^{tf/tf}$ and C57BL/6J did not. DBA/2J mice developed the most rapid tolerance.

Figure 1. (A) Baseline performance of inbred strains determined immediately before ethanol administration on day 1. Bars represent means ± SEM for the latency to fall in sec. (B) Sensitivity to ethanol in inbred strains. Values represent means ± SEM for the change from baseline latency to fall 30 min after 2.5 g/kg ethanol.


A
Baseline Latency to fall (sec.)
80
60
40
20
0
A/J
BTBR/T+ tf/tf
129S1/SvImJ
C57BL/6J
C3H/HeJ
BALB/cByJ
DBA/2J


B
Change from Baseline Latency (sec.)
0
-20
-40
-60
A/J
BTBR/T+ tf/tf
129S1/SvImJ
C57BL/6J
C3H/HeJ
BALB/cByJ
DBA/2J

Narrow-sense heritability was estimated by the proportion of variance accounted for by the Strain variable to the total phenotypic variance (see Methods). This resulted in a narrow-sense heritability estimate of .11, suggesting that 11% of the total phenotypic variance in rapid tolerance development was due to genetic differences among strains.

Artificial Selection. Figure 3 shows the distribution of rapid tolerance scores in the starting population of 96 male and female HS/Npt mice. Mice showed a normal distribution with a mean of 11.2 sec and a standard deviation of 11.2 (Kolmogorov-Smirnov test, two-tailed, p=.68). High and low scoring mice from this population were chosen for the start of selective breeding.

The response to selection for the first 4 generations of selective breeding in both replicates is shown in figure 4. In neither replicate was a significant response to selection seen by S_1 or S_2. Close examination of performance in S_2 showed that mice in both replicates were significantly impaired after the first administration of ethanol; however, all groups were developing nearly complete tolerance. For example, HRT-1 mice had day 1 baseline and post-ethanol latencies of 42.8 ± 1.8 and 33.6 ± 2.4 sec, and day 2 baseline and post-ethanol latencies of 47.2 ± 2.6 and 48.5 ± 3.1 sec, demonstrating that on day 2, mice had recovered any ability they had lost on day 1. We thought that this apparent ceiling effect may have hindered our ability to see differences among individuals, hence preventing the selection of the most appropriate breeders. Therefore, starting with S_3, the selection dose of ethanol was increased to 2.75 g/kg to try to eliminate this ceiling effect. Also, to increase selection differential, both 1st and 2nd litters

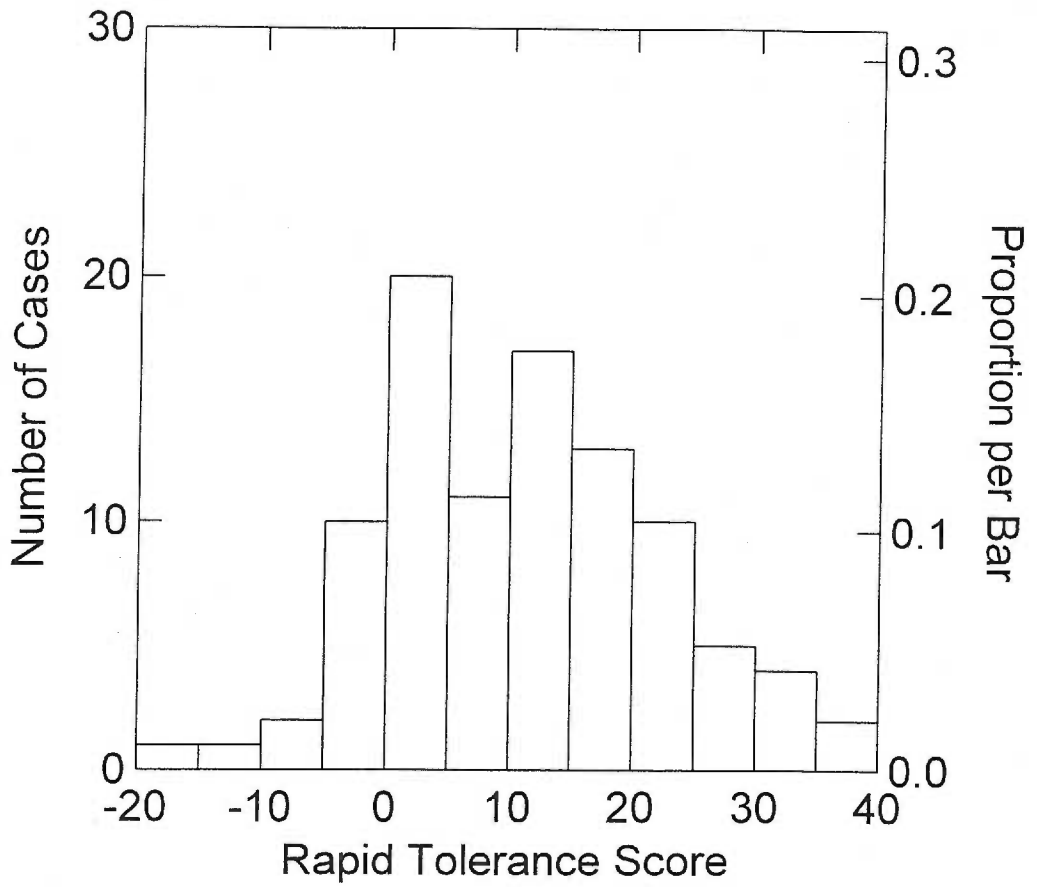
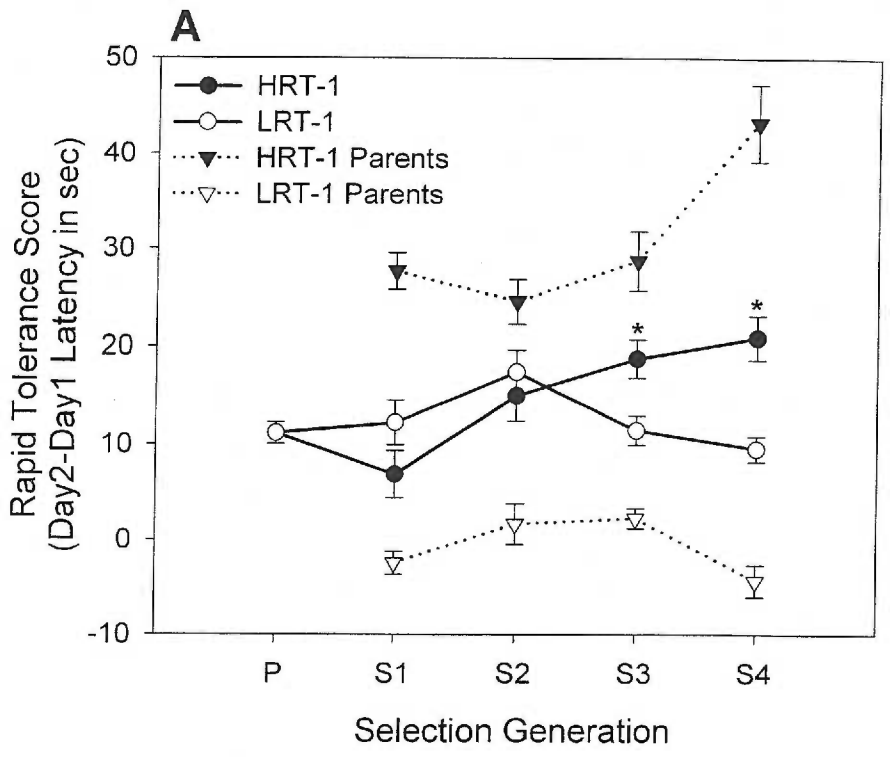
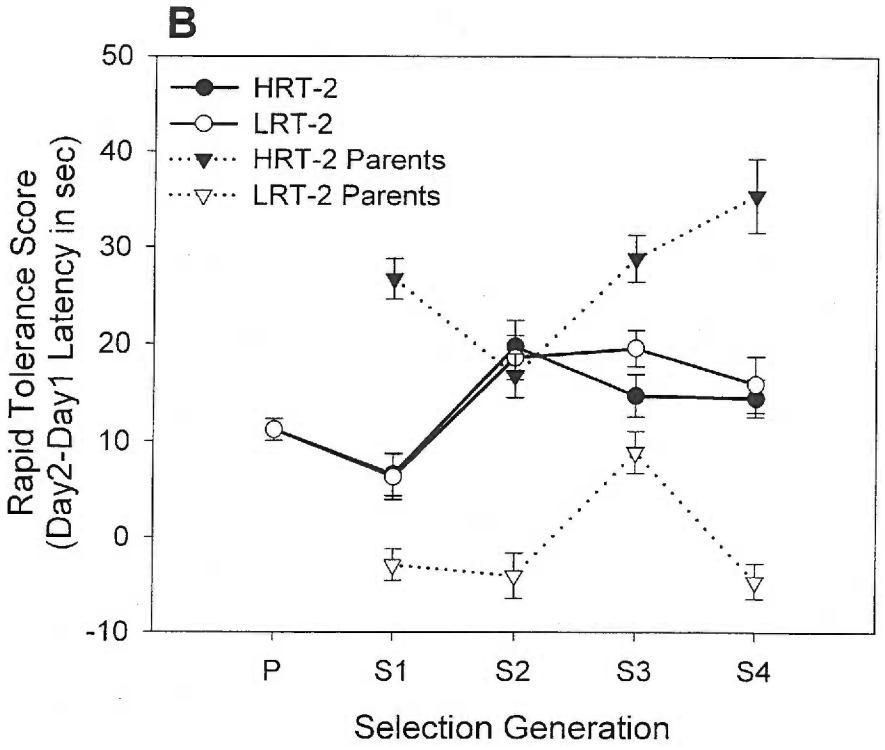


Figure 3. Distribution of tolerance scores in HS/Npt mice used for the starting population for the selection of HRT and LRT mice. The distribution was not significantly different from normal, with a mean of 11.2 sec and a standard deviation of 11.2.

Figure 4. Solid lines: Parental generation scores (P), and response to selection and selection pressure in replicate 1 (A) and replicate 2 (B) for High and Low Rapid Tolerance development. Replicate 1 showed a significant response to selection in selection generation 3 (S_3) that was still evident in S_4 (*, $p<.05$ vs LRT-1). Replicate 2 never showed a significant response to selection. Dotted Lines: The means of the selected parents are also shown to demonstrate the amount of selection pressure applied for each generation. For example, the HRT-1 parents' score, shown as a filled triangle above S_3, represents the average score of mice selected for breeding from S_2 HRT-1 mice shown as a filled circle above S_2. The difference between these values is the selection differential (S) for that generation (see Table 1).


A
HRT-1
LRT-1
HRT-1 Parents
LRT-1 Parents
Rapid Tolerance Score
(Day2-Day1 Latency in sec)
50
40
30
20
10
0
-10
P
S1
S2
S3
S4
Selection Generation
*
*


B
HRT-2
LRT-2
HRT-2 Parents
LRT-2 Parents
Rapid Tolerance Score
(Day2-Day1 Latency in sec)
50
40
30
20
10
0
-10
P
S1
S2
S3
S4
Selection Generation

were tested to increase the pool of mice from which we could select the breeders for subsequent generations (see Table 1).

Generations 3 and 4 resulted in a significant divergence between HRT and LRT mice in replicate 1, but not in replicate 2. Two-way ANOVA (Line X Replicate) for the tolerance scores in S_3 resulted in a significant effect of Line [$F(1,174)=5.35$, $p<.03$] with HRTs developing more tolerance than LRTs. There was also a significant Line X Replicate interaction [$F(1,174)=8.82$, $p<.01$]. This interaction is the result of the response to selection in replicate 1, but not in replicate 2. By S_4, HRT-1 mice developed twice as much tolerance as LRT-1 mice with means and SEM of 20.9 ± 2.2 and 9.5 ± 1.3 sec, respectively. HRT-2 and LRT-2 mice developed 14.4 ± 1.9 and 15.8 ± 2.9 sec of rapid tolerance, respectively. The response to selection in replicate 1 was more unidirectional than bidirectional, as evidenced by the realized heritability calculations performed separately for each line. Realized heritabilities (see Methods) were .25 and .06 for HRT-1 and LRT-1 mice, respectively, demonstrating a greater response to selection in HRT-1 than in LRT-1 mice.

Because we saw a significant response to selection in only replicate 1, we made the decision to discontinue the HRT-2 and LRT-2 lines. While we believe that we would have seen a response in replicate 2 mice eventually (see Discussion), the successful response in replicate 1 provided us with a pair of lines that significantly differed in rapid tolerance development. As a result, the remainder of the results describe the particular responses in HRT-1 and LRT-1 mice only.

Table 1.

Line		n	*i*	S	Cum. S	R/Gen	R
HRT-1:	P	96	1.47	16.48	16.48	--	--
	S_1	37	1.18	17.68	34.16	-4.31	-4.31
	S_2	29	1.00	13.76	47.92	8.11	3.80
	S_3	63	1.58	24.48	72.40	3.77	7.57
	S_4	43	1.28	18.85	91.25	2.14	9.71
LRT-1:	P	96	-1.22	-13.65	-13.65	--	--
	S_1	26	-0.90	-10.58	-24.23	1.06	1.06
	S_2	33	-1.16	-15.12	-39.35	5.14	6.20
	S_3	67	-1.29	-15.78	-55.13	-5.93	0.27
	S_4	80	-1.11	-12.96	-68.09	-1.92	-1.55
HRT-2:	P	96	1.39	15.53	15.53	--	--
	S_1	25	0.92	10.13	25.66	-4.59	-4.59
	S_2	20	0.75	9.13	34.79	13.12	8.53
	S_3	47	1.40	20.75	55.54	-5.03	3.50
	S_4	37	--	--	--	-0.54	2.96
LRT-2:	P	96	-1.26	-14.07	-14.07	--	--
	S_1	26	-0.87	-10.35	-24.42	-4.86	-4.86
	S_2	23	-0.89	-9.66	-34.08	12.24	7.38
	S_3	75	-1.51	-24.16	-58.24	0.99	8.37
	S_4	18	--	--	--	-3.69	4.68

Data presented are the number of mice (n), selection intensity (*i*, in std. dev.), selection differential (S), cumulative selection differential (Cum. S), response to selection in each generation (R/Gen), and cumulative response to selection (R) for parental generation (P) and each line at each generation of artificial selection. First and 2nd litters were used to calculate statistics in generations S_3 and S_4 (with the exception that only 1st litters of HRT-2 and LRT-2 mice were tested in S_4). Breeding was not continued in replicate 2 after S_4, so there was no selection differential for the S_4 generation.

Means for body weight, baseline performance on day 1, and initial sensitivity to ethanol for mice in replicate 1 are shown in Table 2. In the later generations of selection, HRT-1 mice tended to weigh less than LRT-1 mice. They also performed better at baseline in S_3 and S_4. In each generation of selection LRT-1 mice were more sensitive to ethanol, as evidenced by a shorter latency to fall after ethanol. Even with these differences in body weight and baseline ability on day 1, there were no significant correlations between body weight and baseline performance in S_3 or S_4. When both lines were analyzed together, these correlations tended to be negative, but were nonsignificant (Pearson's r's < -.15, $p>.10$). In neither S_3 or S_4 did tolerance scores correlate with baseline scores (r's = .01, $p>.84$); but in S_4, body weight was negatively correlated with tolerance score ($r = -.32$, $p<.001$). When HRT-1 and LRT-1 were analyzed separately, body weight did not correlate with tolerance scores in HRT-1 mice ($r = -.24$, $p=.13$), but it did in LRT-1 mice ($r = -.26$, $p<.02$).

To see whether differences in rapid tolerance development were due to differences in motor learning, we examined the acquisition of rotarod performance before exposure to ethanol. In each generation of selection, motor learning was examined by comparing acquisition of ARR performance in HRT-1 and LRT-1 mice. This was done by two-way ANOVA (Line X Trial). Differences in the rate of acquisition were evidenced by a significant Line X Trial interaction. Only in S_3 was this interaction significant [$F(9,1161)=3.07$, $p<.01$], suggesting that HRT-1 mice acquired the task faster than LRT-1 mice (data not shown). In S_4, however, this interaction was no longer present [$F=0.9$, $p=.53$]. As S_4 was the generation where the lines showed the greatest difference

Table 2.

Selection Generation	Body Wt (g) HRT-1	Body Wt (g) LRT-1	Day 1 Baseline (sec) HRT-1	Day 1 Baseline (sec) LRT-1	Day 1 Post-EtOH (sec)* HRT-1	Day 1 Post-EtOH (sec)* LRT-1
S_1	22.0 ± 0.5	22.9 ± 0.7	38.2 ± 2.1	33.0 ± 2.7	27.3 ± 2.2	**18.5 ± 2.2**
S_2	22.3 ± 0.5	22.1 ± 0.6	42.8 ± 1.8	42.9 ± 2.7	33.6 ± 2.4	**26.0 ± 1.9**
S_3	21.8 ± 0.4	**24.0 ± 0.5**	50.4 ± 2.1	**43.1 ± 2.0**	21.1 ± 1.8	**14.2 ± 1.0**
S_4	21.3 ± 0.4	**23.0 ± 0.4**	48.9 ± 2.4	**41.7 ± 1.6**	16.6 ± 1.9	**11.1 ± 1.0**

Body weight and day 1 performance in HRT-1 and LRT-1 mice. Values represent means ± SEM for each generation of selection. *The ethanol dose was increased from 2.5 g/kg to 2.75 g/kg in selection generation 3. Bold = significantly different from HRT-1 for that measure ($p<.05$).

in rapid tolerance development, it is unlikely that differences in tolerance development are strictly due to differences in the lines' abilities to acquire the task.

Mice from S_5 were examined for differences in ethanol pharmacokinetics. Table 3 presents data for blood ethanol concentration (BEC) collected 30 min after 2.75 g/kg ethanol in HRT-1 and LRT-1 mice. Two-way ANOVA (Line X Day) for BEC revealed no significant differences of Line or Day [F's<2.7, p's > .15], suggesting no pharmacokinetic differences between the lines.

Chronic Tolerance. HRT-1 and LRT-1 mice were tested to see if they also differed in the development of chronic tolerance to ethanol-induced ataxia. Figure 5 shows performance of HRT-1 and LRT-1 mice over the 5 days of testing on the ARR. Three-way ANOVA (Line X Dose X Day) of the post-ethanol latency to fall showed significant effects of Line [$F(1,75)=38.18$, $p<.001$], Dose [$F(3,75)=40.17$, $p<.001$], and Day [$F(4,300)=46.77$, $p<.001$]. HRT-1 mice performed better than LRT-1 mice overall. Additionally, mice given lower doses of ethanol performed better than those given higher doses. Mice also improved their performance across days of testing. There was also a significant Day X Line interaction [$F(4,300)=10.98$, $p<.001$] with HRT-1 showing greater improvement across days than LRT-1 mice, demonstrating greater chronic tolerance development in HRT-1 mice. A significant Day X Dose interaction [$F(12,300)=6.02$, $p<.001$] showed that mice given lower doses of ethanol tended to develop more tolerance than those given higher doses.

Analysis of baseline data across days (Line X Dose X Day) revealed only a main effect of Day [$F(4,300)=19.80$, $p<.001$], demonstrating improvement in pre-ethanol

Table 3.

Line	Day 1 BEC (mg/ml)	Day 2 BEC (mg/ml)
HRT-1	2.60 ± 0.09	2.66 ± 0.10
LRT-1	2.83 ± 0.08	2.78 ± 0.10

Blood ethanol concentrations (BEC) collected 30 min after 2.75 g/kg ethanol on 2 consecutive days. Values represent means ± SEM for 12-13 mice/line. No significant differences were found between the lines on either day.

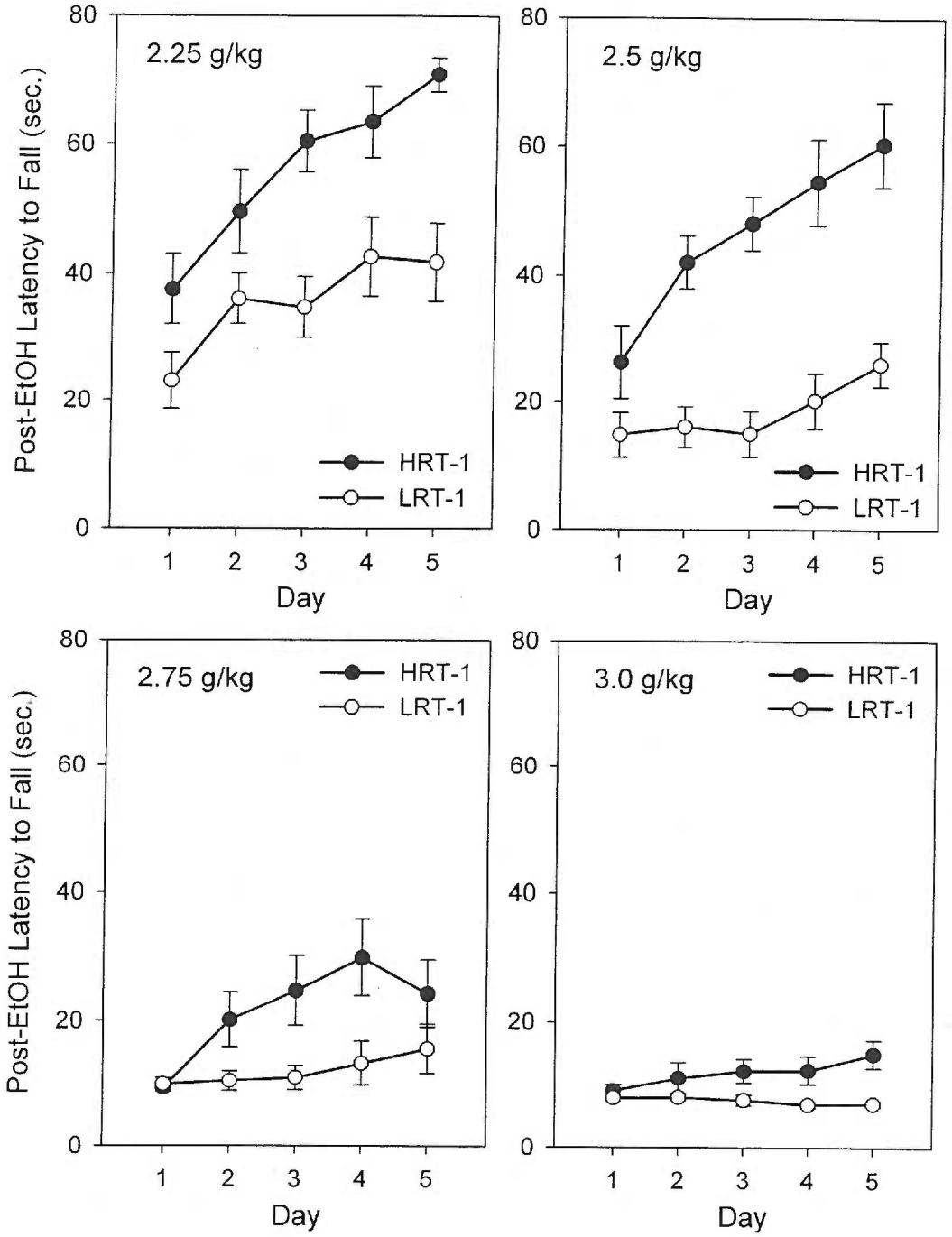


Figure 5. Post-ethanol latency to fall over 5 days of treatment in HRT-1 and LRT-1 mice. Values represent means and SEM for mice from S_5 (n = 9-12/line/dose).

performance in all mice, regardless of line or dose group (Fig 6). To make sure that tolerance development was specific to post-ethanol performance, and not simply to improvements in overall motor ability, post-ethanol scores were corrected for baseline performance. For each mouse, the baseline latency to fall was subtracted from the post-ethanol latency to fall. These data are shown in Figure 7. Three-way ANOVA (Line X Dose X Day) of the change from baseline scores revealed main effects of Line [$F(1,75)=37.78$, $p<.001$], showing that HRT-1 mice performed better than LRT-1 mice. Further there was a significant main effect of Dose [$F(3,75)=37.22$, $p<.001$]. Mice given lower doses performed better than those given higher doses. There was no significant effect of Day [$F=.97$, $p=.42$]; however, there were significant Line X Day [$F(4,300)=7.23$, $p<.001$] and Dose X Day [$F(12,300)=3.46$, $p<.001$] interactions, demonstrating that HRT-1 mice, and mice given lower doses performed better across days. These results suggest that HRT-1 mice developed greater chronic tolerance to ethanol than LRT-1 mice even when post-ethanol performance was corrected for changes in baseline ability across days.

Discussion

The results from these experiments demonstrate a role for genetics in the development of rapid tolerance to the ataxic effects of ethanol as measured by the accelerating rotarod. There was a significant difference among inbred strains in their ability to develop rapid tolerance (Fig 2). Results from the short-term artificial selection

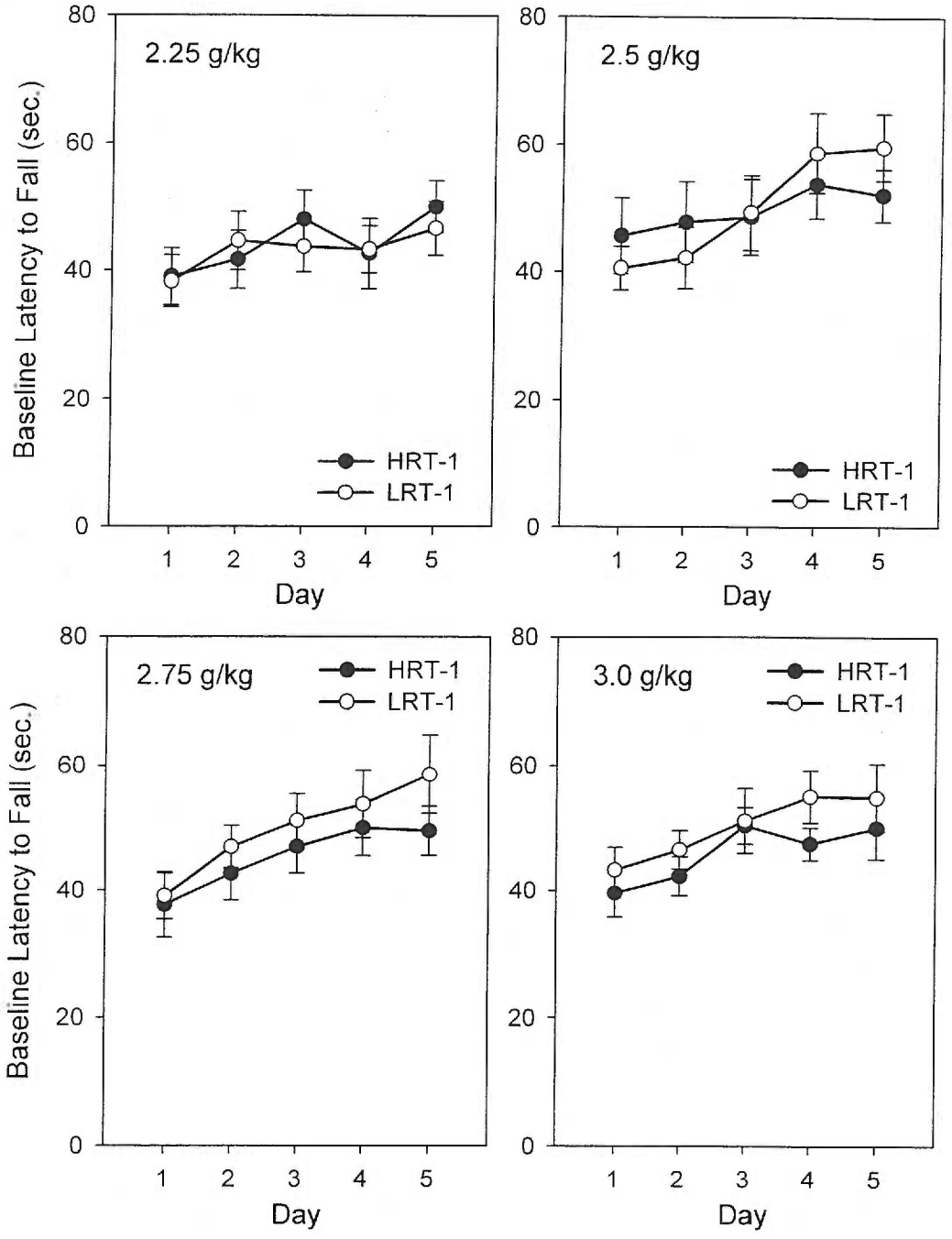


Figure 6. Pre-ethanol baseline performance across days in HRT-1 and LRT-1 mice. Values represent mean ± SEM latency to fall for 9-12 mice/dose/line.

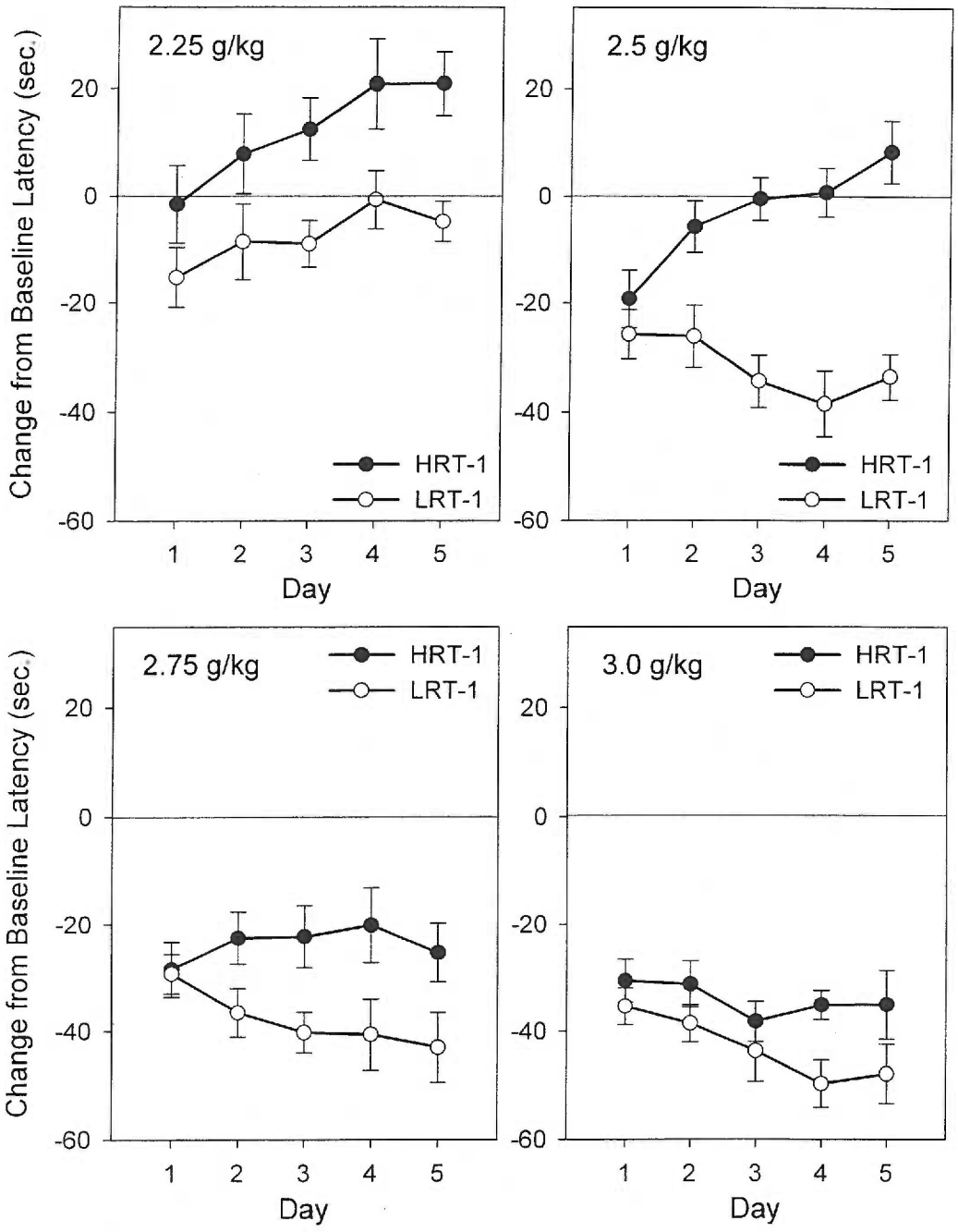


Figure 7. Change from baseline latency to fall over 5 days of ethanol treatment in HRT-1 and LRT-1 mice. Negative scores represent decreased performance compared to baseline, while positive scores represent increases in performance compared to baseline. Values represent means and SEM for mice from S_5 (n = 9-12/line/dose).

experiment support the results from the inbred strains, as one of the replicates showed a significant divergence between mice selected for high and low degrees of rapid tolerance development (Fig 4). HRT-1 and LRT-1 mice differed in a test of chronic tolerance, further suggesting a mechanistic link between the development of rapid and chronic tolerance.

Response to selection was observed in only one of the two sets of replicate lines. One of the main purposes of using replicate lines is to provide evidence that the response to selection is not the result of random genetic drift in the relatively small breeding population used in selection studies. With small breeding populations, inbreeding is an unavoidable occurrence, causing the segregation (or fixation) of genes that may or may not be relevant to the selection phenotype (Crabbe, 1999). Therefore, observed differences between one set of selected lines could be due solely to a random drift in gene frequencies within the breeding population, or specifically due to the selection pressure applied. Having a concurrently (or subsequently) selected replicate line that shows a similar response to selection provides evidence that the results are not strictly due to the random drift of alleles. Replicate lines are also useful for looking at correlated responses to selection. Seeing a correlated response to selection in both sets of replicate lines provides the strongest evidence that the two traits are sharing similar genetic control.

Even though replicate 2 mice showed no difference in tolerance development after 4 generations of selective breeding, there is still evidence that the response we saw in replicate 1 resulted from selection pressure. HRT-1 mice showed a very stable increase in tolerance development over the course of selection (Fig 4), a typical response for a trait controlled by many genes, each with a relatively small effect. We believe that

the lack of a response in replicate 2 may be a reflection of the starting population of mice and/or the number of breeding pairs used for each line. There are a number of options for the starting population of mice for selection studies (e.g. F2 of two inbred strains, outbred stock, genetically defined heterogeneous stock). We used heterogeneous mice derived from a systematic cross of 8 different inbred strains, mice which had been crossed for over 40 generations. While providing a great degree of genetic diversity (potentially 8 different alleles at each locus—1 from each contributing strain) as well as substantial reductions in linkage disequilibrium, this population has the potential to possess high or low gene frequencies at relevant loci for rapid tolerance development. This scenario opens the door for potential founder effects (Crabbe, 1999). If, when the original breeding pairs were selected, the gene frequencies for the trait-relevant loci were low and unfavorable, one would expect a rather slow response to selection. This is exactly what was seen in replicate 1 (Fig 4A). With low gene frequencies it is also possible that some relevant alleles were not included in either the initial breeding pairs, or in the first few generations of selection. If important alleles were lost, it may have been very difficult to observe a significant divergence in rapid tolerance, given the overall phenotypic variance of the trait (Crabbe, 1999). It is possible that this was the situation in replicate 2 (Fig 4B). Figure 4 shows evidence of a founder effect in the S_1 generation, where mice in both lines showed a lower degree of tolerance development compared to the mean of the starting population. The alternative explanation, an environmental effect, is less likely, because only 3 of the 4 lines showed lower scores than the starting population (for evidence of clear environmental effects in replicated selections, see (Crabbe et al., 1985).

A different choice for the starting population could have helped to minimize this potential founder effect. Using an F2 population derived from 2 inbred strains provides, by definition, a population with gene frequencies of .5 at all segregating gene loci (Falconer & Mackay, 1996). This provides an optimal starting population for short-term selected lines, since this gene frequency is conducive to a quick response to selection (Falconer & Mackay, 1996). We decided to use the 8-way cross of mice because of their genetic diversity and because they were readily available to us.

Kalant et al. (1971) proposed that the degree of tolerance development would be positively correlated with the initial disturbance produced by ethanol. That is, the greater the initial disturbance, the greater the tolerance development. Our data do not support this. In replicate 1 mice, HRT and LRT mice differed in their sensitivity to ethanol with HRTs being less sensitive than LRTs (Table 2), even though HRTs developed more tolerance in S_3 and S_4. One explanation for this discrepancy is that LRT-1 mice may have been too intoxicated by the increased selection dose to demonstrate any tolerance on day 2. LRT-1 mice showed as much tolerance as HRT-1 mice in S_1 and S_2, when the selection dose was 2.5 g/kg. When the dose was increased to 2.75 g/kg in S_3, they developed much less tolerance. This suggests perhaps an "inverted-U" dose-response curve for tolerance development, where tolerance increases as a function of dose to a point, at which higher doses actually inhibit tolerance development. For the HRTs and LRTs though, the difference in tolerance development cannot be attributed strictly to the degree of initial impairment. Figure 5 shows the performance in the chronic tolerance study. After 2.5 g/kg ethanol, HRT-1 and LRT-1 mice showed similar ethanol sensitivity. Yet, HRT mice developed much more rapid and chronic tolerance than LRTs

at this dose. Further, at the lowest dose tested (2.25 g/kg), LRT mice were not maximally impaired, yet developed very little rapid or chronic tolerance when post-ethanol performance was corrected for baseline changes across days (Fig. 7). These data suggest that initial sensitivity may be an important stimulus for tolerance development, but it is not the sole determinant of tolerance development.

The results of the chronic tolerance study support previous assertions that rapid and chronic tolerance share many common characteristics (Khanna et al., 1991b; Khanna et al., 1992c; Khanna et al., 1994b; Wu et al., 1993). HRT-1 mice developed greater chronic tolerance than LRT-1 mice at all doses of ethanol tested (Figs. 5 and 7), suggesting pleiotropic action of genes affecting both forms of tolerance development. Therefore, rapid tolerance may be a useful model for studies of mechanisms involved in chronic tolerance development. It is unclear from these studies what role acute functional tolerance (AFT) may have had in the expression of rapid tolerance in HRT and LRT mice. Since HRT and LRT mice were tested 30 min after ethanol administration, it is possible that HRT mice show rapid tolerance due to a greater development of AFT within that 30 min pre-treatment time. This could also explain the apparent difference in initial sensitivity seen between HRT-1 and LRT-1 mice (Table 2) If HRT-1 mice developed greater AFT, they would likely show less sensitivity to ethanol when tested 30 min later. Testing HRT and LRT mice for AFT would help answer this question. There is no clear link in the literature between AFT and rapid tolerance. Khanna et al. (1992b) had originally suggested that the two forms of tolerance are mediated by separate mechanisms. However, when they later changed their assessment of AFT, they were able to see a similar pharmacological blockade of AFT and rapid tolerance (Khanna et al.,

2002). Mice selected for High (HAFT) and Low (LAFT) AFT (Erwin & Deitrich, 1996) were tested to see whether the mice differed in chronic tolerance (Rustay et al., 2001). These data suggested that AFT and chronic tolerance may not share similar genetic influences. In these studies, however, the HAFT and LAFT mice were not tested for chronic tolerance on tasks which showed them to differ in AFT, so no clear conclusions can be drawn in this regard. Nonetheless, these mice, along with HRT and LRT mice, provide useful models for helping to determine the relationship between the different forms of tolerance.

The development of HRT and LRT mice also provide a helpful model for the study of the biological mechanisms involved in tolerance development. Theoretically, the significant response to selection is the result of changes in the biological systems that mediate rapid tolerance. There are numerous reports suggesting a role of the NMDA (Karcz-Kubicha & Liljequist, 1995; Khanna et al., 1992a, 1993a; Khanna et al., 1995a; Khanna et al., 1997), GABA (Barbosa & Morato, 2000, 2001; Zaleski et al., 2001), and serotonin systems (Khanna et al., 2002; Lê et al., 1981b), as well as their interactions (Barbosa & Morato, 2001; Khanna et al., 1994a), in tolerance development. Testing HRT and LRT mice with different pharmacological agents will help to determine the role of these systems in rapid tolerance.

Chapter 2. Pharmacology of rapid tolerance

Introduction

Tolerance can be defined as a decrease in responsiveness to ethanol after previous or repeated administrations. Chronic tolerance is defined as that which develops over days to weeks of ethanol exposure, and is thought to reflect the adaptations that occur in alcoholics. In fact, tolerance to alcohol is an important criterion for a diagnosis of alcoholism. Therefore, gaining information on the mechanisms behind tolerance development may lead to a better understanding of the disease, and the possibility of better treatment and prevention strategies.

Rapid tolerance has been described in rodents as tolerance which develops within 8-24 hours of an initial ethanol exposure (Bitrán & Kalant, 1991; Crabbe et al., 1979). Many pharmacological studies have provided convincing evidence that rapid and chronic tolerance share many characteristics, and suggest that rapid tolerance may represent the initial stages of chronic tolerance development. Using a behavioral genetic approach, we have recently selectively bred mice to show either high or low levels of rapid tolerance to ethanol's ataxic effects (Rustay & Crabbe, submitted). In this selection experiment, mice were first trained on the accelerating rotarod (ARR), as we have shown a significant learning component to performance on the apparatus (Rustay et al., 2003b). Twenty-four hrs after training, mice were tested on 2 consecutive days, each of which consisted of baseline trials followed by 2.75 g/kg ethanol and testing on the ARR 30 min later. Rapid tolerance was defined as the increase in post-ethanol performance on day 2 compared to day 1. After 4 generations of selection, the High Rapid Tolerance (HRT) and Low Rapid

Tolerance (LRT) lines demonstrated a 20 sec and 9 sec improvement, respectively, in post-ethanol performance on day 2. HRT and LRT mice also showed significantly different development of chronic tolerance to ethanol's ataxic effects on the accelerating rotarod.

Numerous reports have been completed investigating the neurotransmitter systems involved in tolerance to ethanol. These include serotonin (Frankel et al., 1975; Khanna et al., 1994a; Lê et al., 1979a; Lê et al., 1981b), GABA (Barbosa & Morato, 2000, 2001; Zaleski et al., 2001), vasopressin (Hoffman & Tabakoff, 1989), and glutamate (Khanna et al., 1992a; Szabo et al., 1994; Wu et al., 1993). In particular, it seems as though the N-methyl-d-aspartate (NMDA) type glutamate receptor plays a significant role in rapid tolerance to ethanol's ataxic and hypothermic effects. Non-competitive NMDA antagonists dizocilpine (MK-801) and ketamine have been shown to block rapid tolerance (Khanna et al., 1992a; Khanna et al., 1997; Khanna et al., 1991c), while D-cycloserine (DCS), an agonist at the glycine site on the NMDA receptor, has been shown to augment rapid tolerance development (Khanna et al., 1995a). In addition, NMDA receptor drugs have been shown to reverse the effects on tolerance development that other neurotransmitter system modulators elicit. In rats, rapid tolerance to ethanol's incoordinating effects on the tilt-plane task was shown to be potentiated by the ingestion of a tryptophan-rich diet, effectively boosting brain serotonin levels. This potentiation was blocked by the administration of NMDA antagonists (Khanna et al., 1994a). Similarly, when rapid tolerance to ethanol in mice was enhanced by the administration of pregnenolone sulfate, a positive modulator of the $GABA_A$ receptor, this effect was inhibited by the administration of MK-801 (MK) (Barbosa & Morato, 2001). These

results suggest that while many systems may interact to influence the development of rapid tolerance, the NMDA receptor system may be particularly important in mediating its development.

The current set of experiments was aimed at determining the role of NMDA receptors in the development of tolerance to ethanol's ataxic effects using HRT and LRT mice. The significant difference in rapid tolerance expressed in these lines should reflect changes in the biological systems mediating tolerance development (Crabbe, 1999). Therefore, we expect that HRT mice, which develop at least twice as much rapid tolerance as LRT mice (Rustay & Crabbe, submitted), should be more sensitive to drugs that modulate rapid tolerance than LRT mice. HRT and LRT mice were tested with MK and DCS to look for the ability of these drugs to block or enhance rapid tolerance, respectively. Tolerance development has been shown to be influenced by intoxicated practice (a learning component) (Bitrán & Kalant, 1991; Khanna et al., 1997; LeBlanc et al., 1973; LeBlanc et al., 1976), and MK has been shown to block learning in numerous tasks (Chiamulera et al., 1990; Heale & Harley, 1990; McLamb et al., 1990). Since we have shown that there is a learning component to ARR performance (Rustay et al., 2003b), we also examined the effects of MK on acquisition of ARR performance. Based on the results of MK's effects on motor learning, we also examined the effects of the NMDA receptor drugs on tolerance development in mice that were forced to learn the task under the influence of ethanol.

Materials and Methods

Mice. Male and female HRT and/or LRT mice from selected generation 5 were used in experiments 1-3, 5 and 6. Mice were between the ages of 49-115 days old at the start of testing and were housed 1-6 per polycarbonate cage on Carefresh® paper bedding. All HRT and LRT mice were housed in the Department of Comparative Medicine vivarium at Oregon Health & Science University. Due to a shortage of HRT and LRT mice, male and female genetically heterogeneous WSC-2 mice (Crabbe et al., 1983) were used to examine the role of MK-801 on motor learning (Experiment 4). These mice were housed 3-5 per cage on Bed-o-cob® bedding. WSC-2 mice were housed in the Veterinary Medical Unit at the Portland Department of Veterans Affairs Medical Center. All mice were kept on a 12 hr light cycle (lights on at 0600 hrs) in a colony room with controlled humidity and a temperature of 20-22 ^{o}C. All procedures were approved by the Institutional Animal Care and Use Committee at the respective institution in accordance with NIH guidelines.

Apparatus. The AccuRotor Rota Rod (Accuscan Instruments, Columbus, OH) was modified to have a 63 cm fall height above sawdust bedding. The rod was divided into 4 running lanes each 10 cm wide, separated by white acrylic disks. The 6.35 cm diameter dowel was covered with 320 grit wet/dry sandpaper to provide a uniform surface and to reduce slipping (Rustay et al., 2003b). For all experiments, the rod had a continuous acceleration rate of 20 rpm/min.

Drugs. (+)-MK-801 hydrogen maleate and D-cycloserine were purchased from Sigma-Aldrich (St. Louis, MO). These drugs were dissolved in 0.9% saline and injected intraperitoneally (i.p.) with an injection volume of .01 ml/g body weight. Ethanol (200 proof, Pharmco, Brookfield, CT) was dissolved in saline to a final concentration of 20% ethanol (v/v). Ethanol injections were given i.p. in a volume adjusted according to body weight. For all studies, 0.9% saline was used for vehicle injections.

Blood Ethanol Concentration (BEC). Following experiments 2 and 3 (see below), BECs were measured to determine whether treatment with MK or DCS significantly changed the pharmacokinetics of ethanol. For BEC determination, mice were gently restrained while 20 µl of blood was taken from the periorbital sinus. Blood samples were added to centrifuge tubes containing 50 µl $ZnSO_4$. Fifty µl $Ba(OH)_2$ and 300 µl dH_2O were added and samples were centrifuged at 12,000 rpm for 5 min. The supernatant was removed and analyzed by gas chromatography (Model 6890N, Agilent Technologies, Palo Alto, CA).

A brief overview of each experiment is given in Table 4. Specific methods are given in the procedure section under each experiment's subheading.

Table 4. Summary of Experiments

Expt	Target	ARR Training	Drug Treatments Day 1	Drug Treatments Day 2	Mice (n/group)
1	Sensitivity, RT	10 Trials	MK	MK	HRT/LRT (8-9)
2	EtOH RT, EtOH CT	10 Trials	MK, EtOH	EtOH	HRT (17-19)
3	EtOH RT	10 Trials	DCS, EtOH	EtOH	HRT/LRT (15-16)
4	Motor Learning	20 Trials (over 2 days)	MK	none	WSC-2 (11-13)
5	EtOH RT	None	MK, EtOH	EtOH	HRT (12-27)
6	EtOH RT	None	DCS, EtOH	EtOH	LRT (13-14)

"ARR Training" refers to pre-treatment acquisition trials in experiments 1-3. For experiment 4, it refers to total number of trials over the 2 days of testing. CT, chronic tolerance; DCS, D-cycloserine; EtOH, ethanol; MK, MK-801; RT, rapid tolerance

Experiment 1: Sensitivity and rapid tolerance to the ataxic effects of MK-801

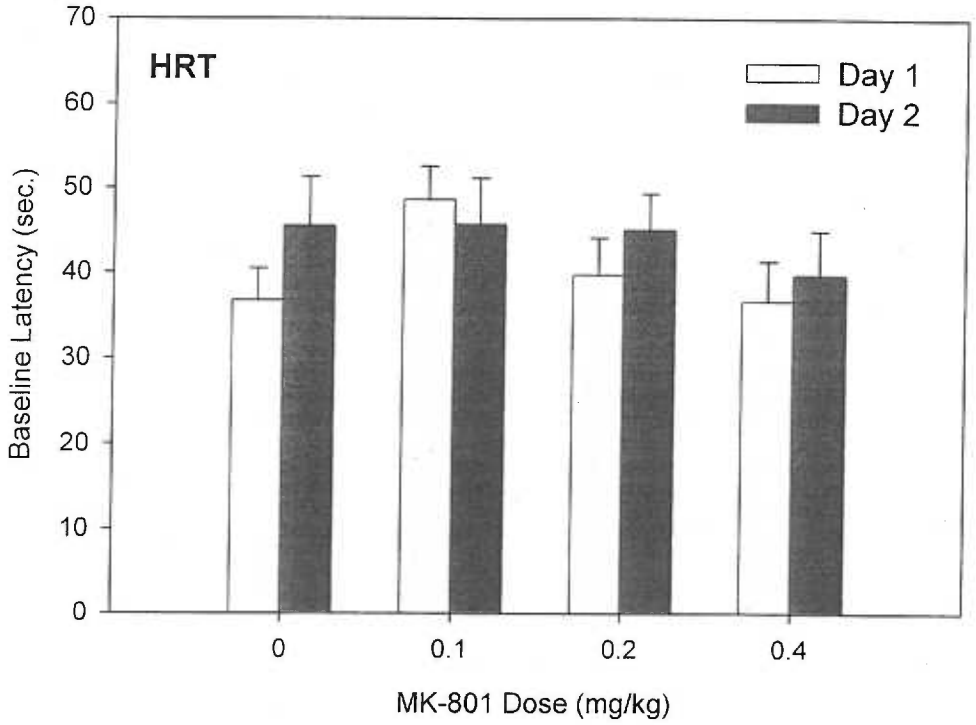
As presented in the introduction, numerous reports have provided evidence that rapid tolerance is mediated, at least in part, through the NMDA receptor system. Artificial selection is a powerful tool to investigate the mechanisms underlying rapid tolerance. As lines diverge in response to selection pressure, it is assumed that the systems mediating the response are changing in parallel. Once a significant difference in the selection response is present, testing the lines for correlated responses to selection can provide information on the processes that share common mechanisms (Crabbe, 1999). HRT and LRT mice were tested for their sensitivity to the ataxic effects of acutely and repeatedly administered MK. It was predicted that the artificial selection for high and low rapid tolerance had changed the sensitivity to NMDA receptor drugs. Given that increased NMDA receptor function increases rapid tolerance in unselected rodents (Khanna et al., 1993a), I predicted that HRT mice would be more sensitive to the ataxic effects of MK than would LRT mice. Mice were tested for rapid tolerance development to MK's ataxic effects to examine whether selection for rapid tolerance to ethanol's effects confer increased or decreased propensity to develop tolerance to another ataxia-producing agent. It was predicted that HRT mice would develop greater rapid tolerance to MK than would LRT mice, suggesting similar mechanisms for tolerance development to ethanol and MK.

Procedure. Naïve HRT and LRT mice were moved into the testing room, weighed, and allowed to sit undisturbed for 30 min. For training, mice were each given 10 consecutive trials on the ARR with a 30 second inter-trial interval. For each trial, 4 mice were placed

on the rod while it was stationary. The rod was then turned on, and the time it took each mouse to fall from the rod was recorded as the measure of motor coordination. After 10 trials, the mice were put back in their home cage and returned to the colony room. The following day, mice were given 3 baseline trials on the ARR immediately before being injected i.p. with either saline or 0.1, 0.2 or 0.4 mg/kg MK. Sixty min later, mice were given 3 more tests. For each mouse, the average baseline latency to fall (average of trials 2 and 3 before injection) was subtracted from the post-MK latency to fall (average of trials 2 and 3 post-injection) to generate an index of MK-induced ataxia. Therefore, negative values represent impaired performance while positive values represent enhanced performance compared to baseline. The following day, mice were treated in an identical manner to look for the development of rapid tolerance to MK.

Results. Differences in baseline ability on the ARR were examined by two-way ANOVA (Line X Dose) for baseline latency to fall on the first day of drug treatment. There were no significant differences between lines or dose groups in basal ability on test day 1 [F's<1.78, p's>.10, see Fig. 8]. The ability of MK to produce ataxia was assessed by two-way ANOVA (Line X Dose) on the change from baseline latency 60 min after drug treatment on day 1. This analysis revealed a significant effect of Dose [$F(1,57)=13.96$, $p<.001$] with the 0.4 mg/kg dose having produced significant performance deficits (Fig. 9). Mice from both HRT and LRT lines were affected similarly by MK, as there was no significant Line effect [$F=3.00$, $p=.08$]; however, HRT mice tended to be less sensitive than LRT mice. There was also no significant Line X Dose interaction [$F=1.32$, $p=.28$].

Figure 8. Baseline performance in HRT and LRT mice in Experiment 1. Bars represent means and SEM for performance prior to treatment with saline or MK. There were no significant differences between lines or treatment groups.


HRT
Day 1
Day 2
Baseline Latency (sec.)
70
60
50
40
30
20
10
0
0
0.1
0.2
0.4
MK-801 Dose (mg/kg)

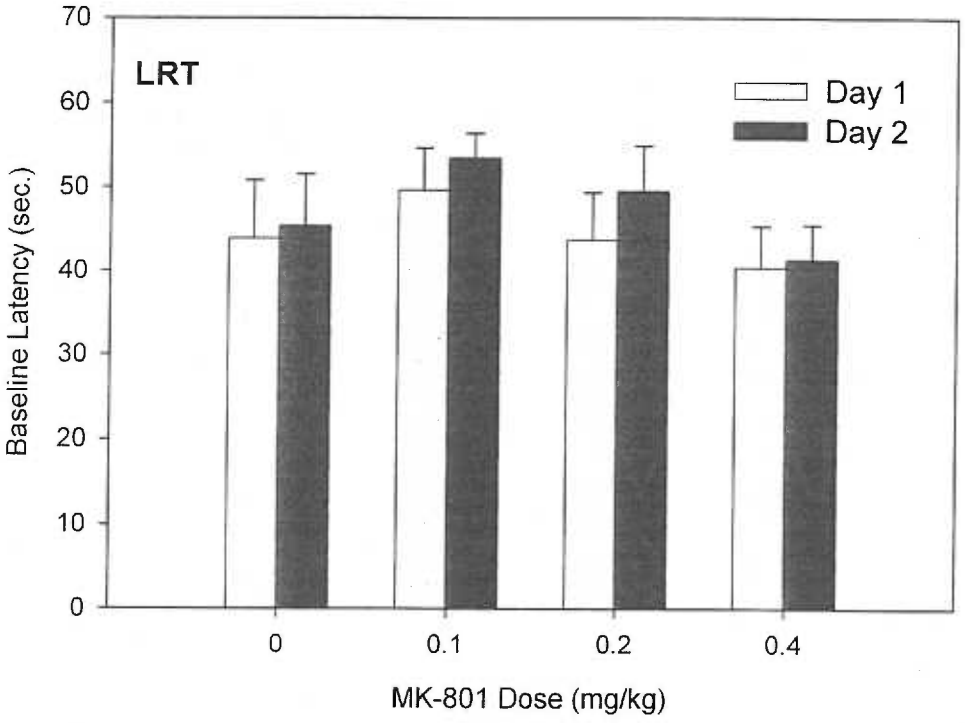
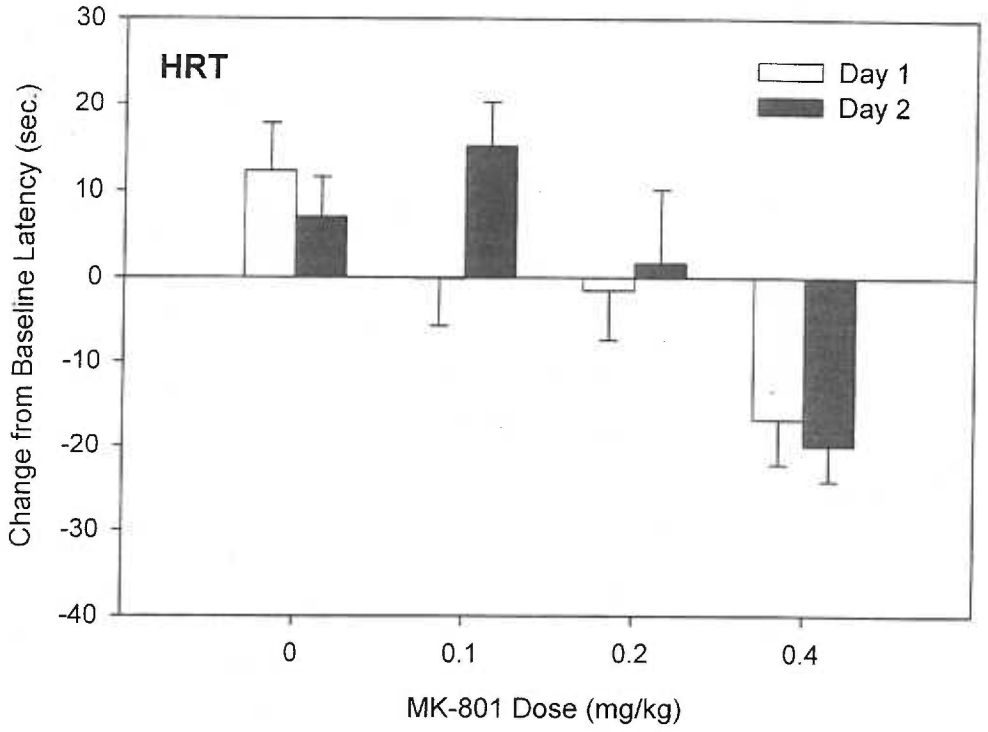
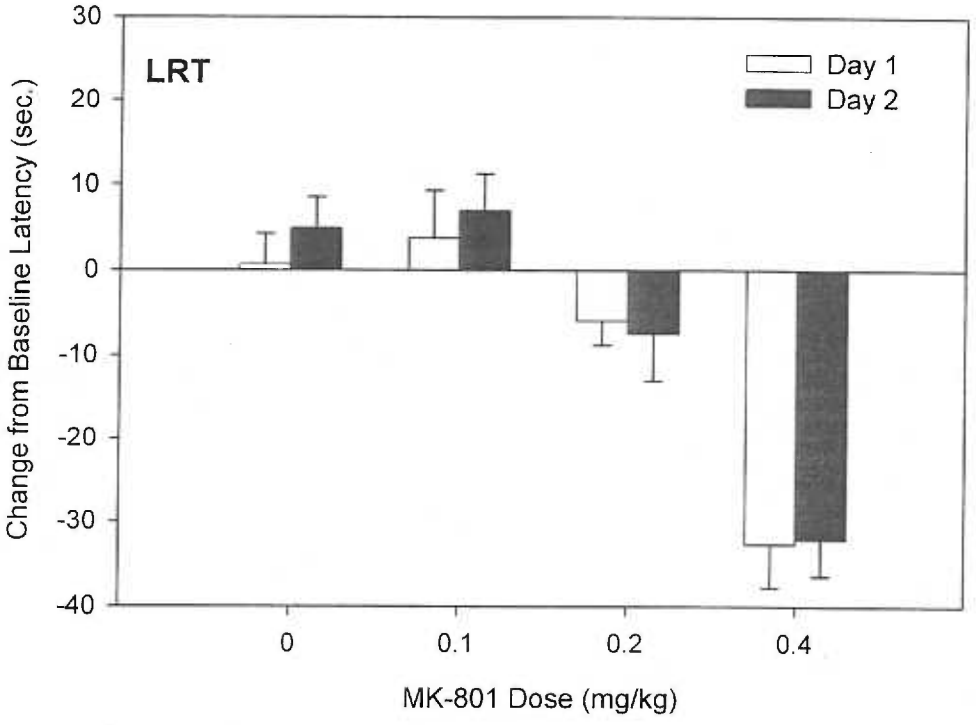

LRT
Day 1
Day 2
Baseline Latency (sec.)
70
60
50
40
30
20
10
0
0
0.1
0.2
0.4
MK-801 Dose (mg/kg)

Figure 9. Sensitivity and lack of rapid tolerance to the ataxic effects of MK-801. Ataxia was indexed as the change from baseline latency to fall. Positive values represent improved performance, while negative values represent impaired performance compared to baseline. On day 1, there were no differences between lines in their sensitivity to MK. Further, neither line developed significant rapid tolerance to the ataxic effects of MK.


HRT
Day 1
Day 2
Change from Baseline Latency (sec.)
30
20
10
0
-10
-20
-30
-40
0
0.1
0.2
0.4
MK-801 Dose (mg/kg)


LRT
Day 1
Day 2
Change from Baseline Latency (sec.)
30
20
10
0
-10
-20
-30
-40
0
0.1
0.2
0.4
MK-801 Dose (mg/kg)

Rapid tolerance was examined by examining the change from baseline latency on days 1 and 2 using three-way ANOVA (Line X Dose X Day). Rapid tolerance would be demonstrated as a smaller change from baseline on day 2 compared to that on day 1. This revealed a main effect of Line [$F(1,57)=5.37$, $p<.03$] and Dose [$F(3,57)=23.82$, $p<.001$], showing that HRT mice were less affected by MK overall, and that only the highest MK dose resulted in significant ataxia. There was no effect of Day [$F=1.46$, $p=.23$], suggesting that there was no significant rapid tolerance to MK. Even in the 0.4 mg/kg group, which showed ataxia, there was no rapid tolerance development in either the HRT or LRT line.

Experiment 2: Effect of MK-801 on rapid and chronic tolerance to ethanol in previously trained HRT mice

Although there was no significant difference in sensitivity to MK in experiment 1, HRT mice were tested to see whether rapid or chronic tolerance could be blocked by MK, in accordance with much of the literature. This would provide evidence that even if the selected lines did not differ in NMDA receptor function, the NMDA receptor does still play a role in tolerance to ethanol's ataxic effects in HRT mice. Only HRT mice were tested, as LRT mice did not show significant tolerance development in a previous dose response study (Rustay & Crabbe, submitted).

Procedure. Naïve HRT mice (n=17-19/dose) were given the standard training protocol of 10 consecutive trials on the ARR at 20 rpm/min. The following day (test day 1), mice were given 3 baseline trials, followed immediately by injection with saline or 0.05, 0.1 or

0.2 mg/kg MK and placed into individual holding cages. Thirty min later, mice were injected with either saline or 2.5 g/kg EtOH. Thirty min after EtOH (60 min after MK), mice were given 3 more trials on the ARR. After testing, mice were returned to their home cage and to the colony room. On test day 2, mice were tested for rapid tolerance. They were tested in an identical manner, except that all mice received saline immediately after baseline trials, and 2.5 g/kg EtOH 30 min prior to post-EtOH testing. Test days 3 and 4 were procedurally identical to test day 1, and test day 5 was identical to test day 2. Immediately following day 5 testing, blood samples were taken for a measure of BEC.

Results. Analysis of baseline performance across days was completed by two-way ANOVA (Treatment X Day). This analysis revealed no effect of Treatment [$F=0.76$, $p=.55$], suggesting that all groups were matched for baseline ARR ability. A significant effect of Day [$F(4,336)=15.08$, $p<.001$] showed that all groups were increasing their baseline performance across the days of testing (Fig 10). MK's effects on sensitivity to EtOH were examined on test day 1 and are shown in Fig 11. One-way ANOVA of the change from baseline performance revealed a significant effect of treatment group [$F(4,85)=28.68$, $p<.001$]. Tukey's post-hoc test revealed that mice treated with saline performed better than all other groups ($p<.05$), demonstrating EtOH's impairing effects. Further, groups treated with any dose of MK along with ethanol were significantly more impaired than the group treated with EtOH alone ($p's<.05$). This demonstrated the efficacy of MK to potentiate the ataxic effects of EtOH.

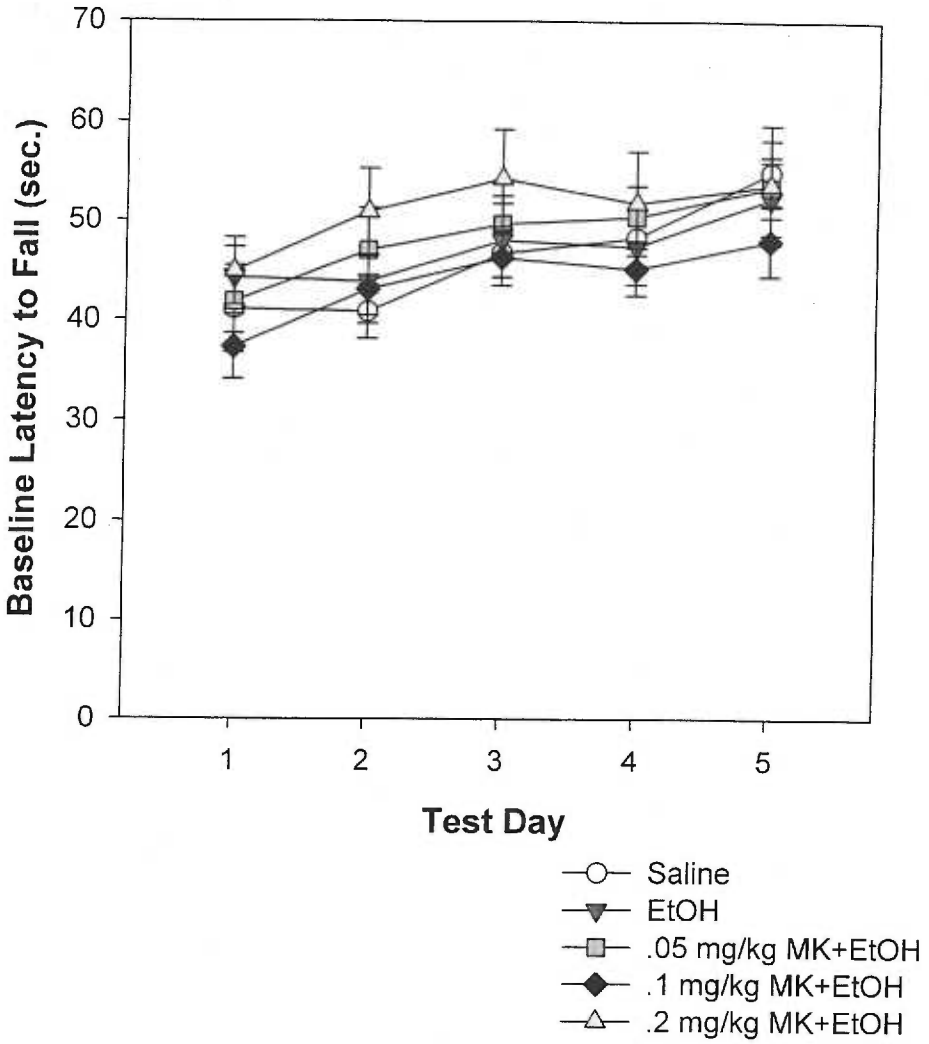


Figure 10. Baseline latency to fall (sec) across 5 days of testing on the ARR in HRT mice. Values represent the mean and SEM for performance immediately prior to treatments noted. Mice in all groups showed significant improvement in pre-drug performance across days of testing ($p<.001$).

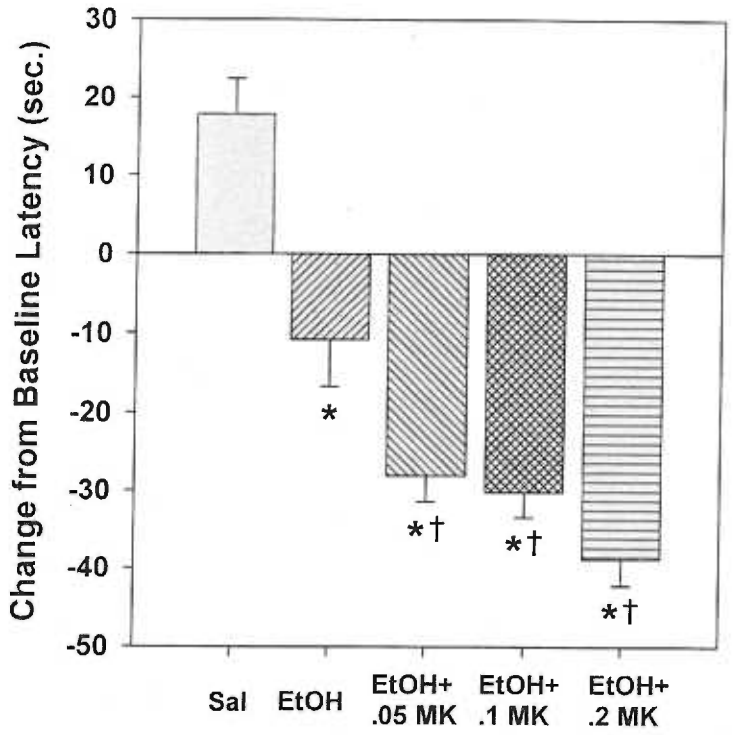


Figure 11. MK-801 potentiates sensitivity to ethanol on the ARR in HRT mice. Administration of 2.5 g/kg EtOH on day 1 produced a significant impairment of performance (*, $p<.05$ compared to Sal). Administration of any dose of MK with EtOH produced greater impairment than EtOH alone (†, $p<.05$ compared to EtOH).

Rapid tolerance was assessed by between-group comparisons on day 2 (Fig 12A). On this day, all groups received ethanol without MK. Analysis of the change from baseline performance resulted in no significant differences among groups [$F=1.52$, $p=.20$], indicating that there was no significant between-groups tolerance development on day 2. Chronic tolerance was assessed by between-group comparisons on test day 5 (Fig 12B). Again, all groups were challenged with ethanol alone on this day. On day 5, there was a significant difference among treatment groups in the change from baseline performance [$F(4,84)=2.54$, $p<.05$]. Tukey's post-hoc testing revealed the only difference was between the chronically ethanol treated and the saline control groups ($p<.05$), demonstrating significant between-groups chronic tolerance development. Groups treated with MK did not differ from the chronic ethanol group, suggesting that MK treatment did not block chronic tolerance in trained HRT mice. The MK-treated groups also did not differ from the saline treated group, however, indicating that treatment with MK did slightly attenuate tolerance development.

BECs were analyzed to determine whether MK treatment had altered ethanol pharmacokinetics. Values are shown in Table 5. Blood samples were taken immediately after testing on day 5. ANOVA revealed no differences among treatment groups [$F=1.15$, $p=.35$]; however, groups treated with daily with ethanol tended to have lower BECs than the saline control group.

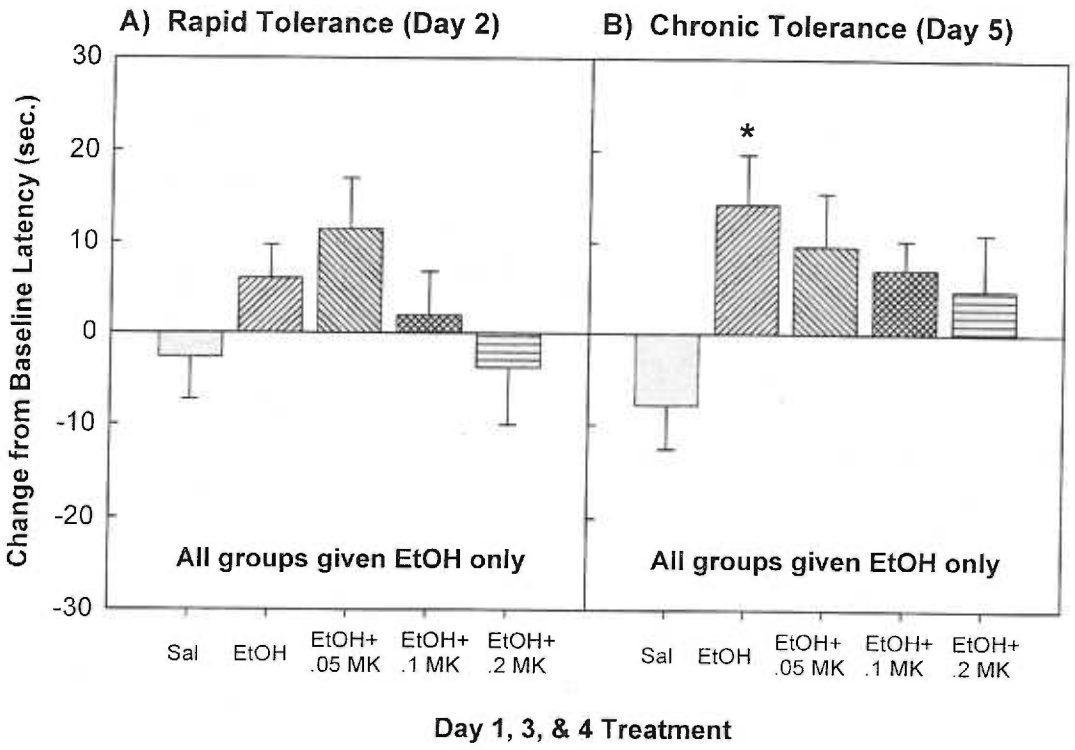


Figure 12. Effects of MK-801 on the development of rapid and chronic tolerance to EtOH in HRT mice. Data are expressed as a change from baseline latency for each day. (A) Rapid tolerance was assessed with between-groups comparisons on day 2, when all mice were treated only with 2.5 g/kg EtOH. No significant rapid tolerance was detected between Sal and EtOH groups. (B) Chronic tolerance was assessed with between-groups comparisons on day 5. Mice chronically treated with EtOH performed significantly better than Sal group. MK treatment did not significantly decrease chronic tolerance when compared to the EtOH group.

Table 5. Blood Ethanol Concentrations (BEC)

Experiment	Treatment Group	BEC (mg/ml) HRT	LRT
2	Sal	3.10 ± 0.09	--
	EtOH	2.95 ± 0.05	--
	EtOH + 0.05 MK	2.91 ± 0.10	--
	EtOH + 0.1 MK	2.93 ± 0.06	--
	EtOH + 0.2 MK	2.95 ± 0.05	--
3	EtOH	2.83 ± 0.06	2.76 ± 0.13
	EtOH + 10 DCS	2.79 ± 0.09	2.68 ± 0.13
	EtOH + 20 DCS	2.80 ± 0.10	2.73 ± 0.10
	EtOH + 40 DCS	2.83 ± 0.08	2.64 ± 0.05

Values represent means ± SEM for BECs immediately after testing on day 5 (Expt 2) or day 2 (Expt 3), when all groups had been given ethanol only (see Methods). There were no statistically significant differences among groups in either experiment. DCS, D-cycloserine; EtOH, ethanol; MK, MK-801; Sal, saline.

Experiment 3: Effect of D-cycloserine on rapid tolerance in previously trained HRT and LRT mice

To further examine whether modulation of NMDA receptor function could change rapid tolerance, HRT and LRT mice were tested to see whether D-cycloserine would enhance rapid tolerance development. Previous research has found that D-cycloserine pretreatment can enhance tolerance to ethanol's ataxic effects in rats (Khanna et al., 1993a); however, this effect has not been shown in mice.

Procedure. Naïve male and female HRT and LRT mice (n=14-15/group/line) were tested with D-cycloserine (DCS). Mice were tested in a similar manner as in experiment 2, except that HRT mice were treated with 2.75 g/kg and LRT mice were given 2.5 g/kg. Twenty-four hours after training, mice were given 3 baseline trials, followed by an i.p. injection of either saline or 10, 20 or 40 mg/kg DCS. Thirty min later, mice were injected with either 2.5 (LRT) or 2.75 (HRT) g/kg EtOH, and tested 3 times 30 min after EtOH. HRT and LRT mice were given different doses of EtOH because in previous studies, it appeared that LRT mice would be too impaired to develop tolerance if treated with the dose of ethanol used during selective breeding (2.75 g/kg, Rustay & Crabbe, submitted). The following day, all mice were given 3 baseline trials followed by an injection of saline and placed in individual holding cages for 30 min. All mice were then given EtOH (2.5 g/kg for LRT and 2.75 g/kg for HRT) and placed back in the holding cage. Thirty min after EtOH, mice were given 3 more tests on the ARR. Blood samples were taken immediately after testing on day 2. The effects of DCS on tolerance development were determined by analyzing day 2 change from baseline latency. Due to a shortage of mice,

rapid tolerance was assessed by within-group repeated measure analysis in ethanol-treated mice on days 1 and 2. Since the HRTs and LRTs were treated with different doses of EtOH, each line was analyzed separately.

Results. After training, both HRT and LRT mice were matched in baseline performance across treatment groups. One-way ANOVA within each line revealed no significant differences among the treatment groups in day 1 baseline performance [F's<0.55, p's>.65, see Fig 13]. Similarly, HRTs and LRTs treated with EtOH or EtOH + DCS did not differ in post-EtOH performance on Day 1 [F's<0.42, p's>.74], showing that DCS did not potentiate the ataxic effects of ethanol in either HRT or LRT mice (Fig. 14). Rapid tolerance was assessed by repeated measures analysis of the EtOH treated group in each line on day 1 and 2. Rapid tolerance was said to develop when the change from baseline was significantly less on day 2 compared to day 1 in the ethanol treated group. These analyses revealed a significant effect of Day in both the HRT [$F(1,13)=13.33$, $p<.01$] and LRT [$F(1,14)=10.14$, $p<.01$] mice, showing that both HRTs and LRTs developed significant rapid tolerance. On day 2, the effects of DCS were assessed by comparing performance among treatment groups within each line. In neither line did DCS treatment affect performance as none of the DCS treated groups differed from the EtOH treated group [F's<1.2, p's >.32]. BECs from HRT and LRT mice are shown in Table 5. There were no differences in BEC among treatment groups in either HRT [F=0.06, p=.98] or LRT [F=0.26, p=.86] mice after testing on day 2.

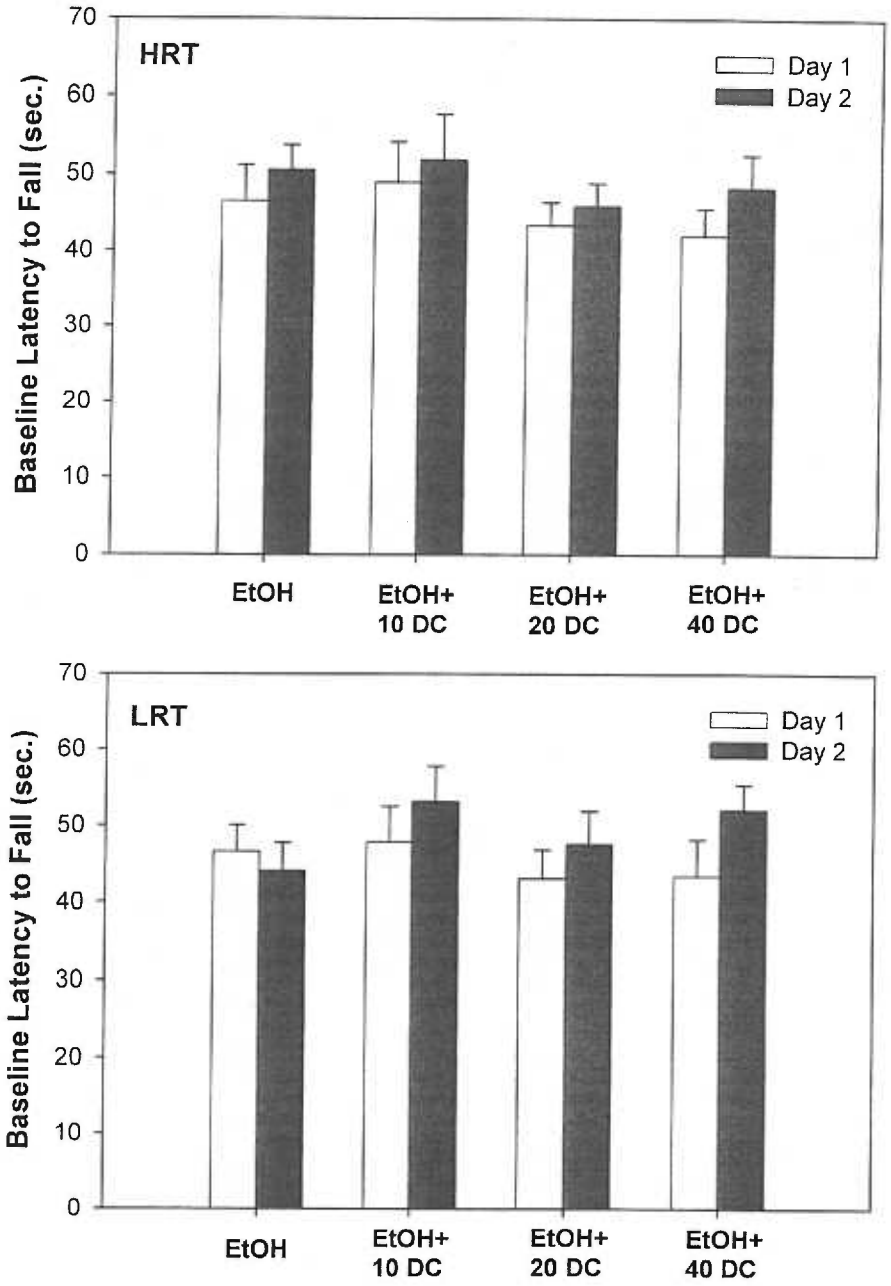


Figure 13. Baseline performance in HRT and LRT mice in Experiment 3. Bars represent means and SEM for performance just prior to treatment on days 1 and 2.

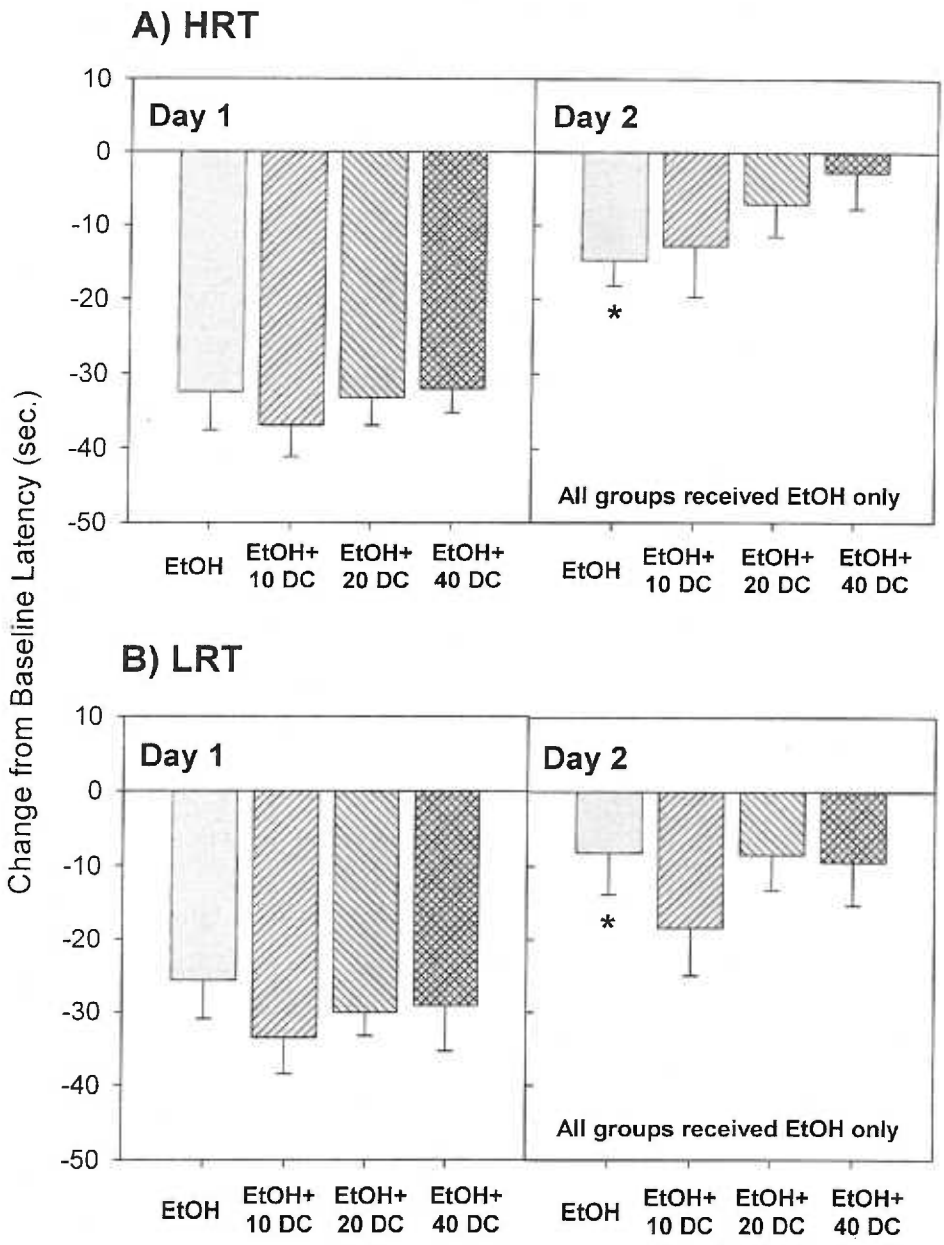


Figure 14. Effects of D-cycloserine on rapid tolerance in HRT (A) and LRT (B) mice. DCS treatment did not affect sensitivity to EtOH in either HRT or LRT mice (day 1). Both HRT and LRT mice developed significant rapid tolerance to EtOH (*, $p<.05$ compared to EtOH group on day 1). DCS treatment did not significantly enhance rapid tolerance in either HRT or LRT mice.

Experiment 4: Effect of MK-801 on motor learning on the ARR

Neither MK or DCS was shown to significantly affect tolerance development in HRT or LRT mice. As a result, the focus was shifted to the study of learning's role in the development of tolerance. The tolerance literature is full of reports on the effects of learning on tolerance development (see reviews by Siegel, 1989; Wolgin, 1989). Along with its importance in both cellular and animal models of learning, the NMDA receptor has been shown to be important in mediating the learned component of tolerance development (Khanna et al., 1997; Szabo et al., 1994). Given the learning component to performance on the ARR, MK was first tested for its ability to block the acquisition of ARR performance.

Procedure. Due to a shortage of HRT and LRT mice, male and female genetically heterogeneous WSC-2 mice were used in this study. These mice are the randomly bred control line used in the Withdrawal Seizure Prone and Withdrawal Seizure Resistant selected lines (Crabbe et al., 1983). Mice were divided into 6 groups (n=11-13/group). Four of the groups were treated with either saline or MK (0.05, 0.1 or 0.2 mg/kg i.p.). Thirty min after the treatment, mice were given 10 consecutive trials on the ARR to examine MK's effects on acquisition of rotarod performance. Because a pilot study showed that 0.2 mg/kg MK significantly decreased performance in this paradigm (data not shown), we included another group that was treated with saline, but during testing was removed from the rotarod at the same time the 0.2 mg/kg MK group fell. This equated these two groups for total practice on the rotarod on day 1. Another group was treated with saline but received no rotarod training on day 1. After testing, all mice were

returned to their home cage. The following day, mice in all groups were given 10 consecutive trials on the rotarod with no injection to look for differences in performance. As we have shown that WSC-2 mice typically reach a plateau in performance by the 5th trial on the acquisition day (Rustay et al., 2003b), we analyzed performance for only the first 5 trials on day 2.

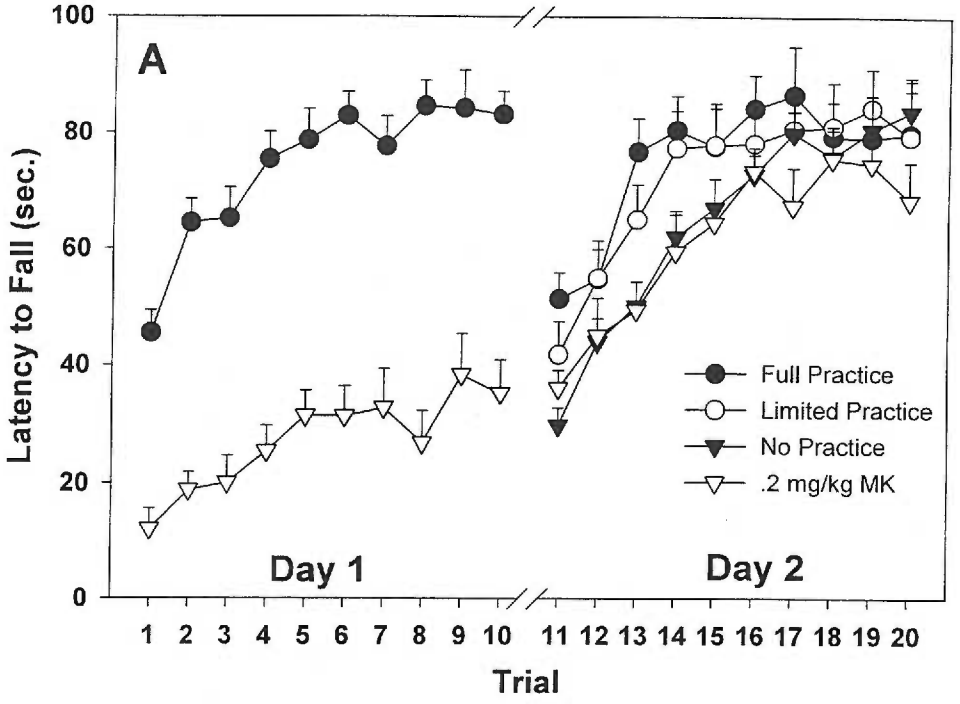
Results. Results for experiment 4 are shown in Figure 15. Day 1 performance was analyzed in the 4 groups that were given 10 full trials on day 1. This included the saline full practice group and the 3 MK treated groups. Repeated measures ANOVA for latency to fall over the 10 trials revealed a significant effect of Group [$F(3,43)=23.78$, $p<.001$] and Trial [$F(9,387)=29.17$, $p<.001$]. Mice given 0.2 mg/kg MK demonstrated significantly shorter latencies to fall across trials than all other groups (Fig 15A). The significant effect of Trial and lack of Group X Trial interaction [$F=1.21$, $p=.22$] suggests that all groups improved over trials, and that all groups improved at the same rate.

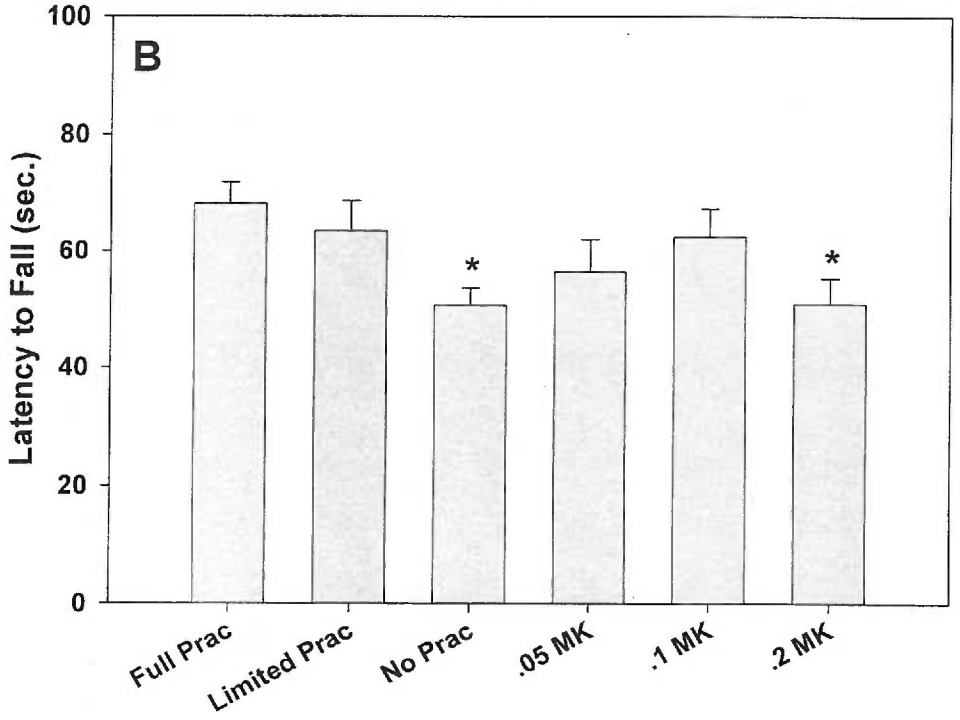
Day 2 performance was analyzed by one-way ANOVA for the average latency to fall over trials 1-5 and results are shown in Figure 15B. This analysis revealed a significant effect of Group [$F(5,67)=2.67$, $p<.04$]. Mice given 0.2 mg/kg MK performed similarly to those mice given no practice on day 1, suggesting that treatment with this dose of MK is sufficient to block the acquisition of ARR ability.

Experiment 5: Effects of MK-801 on rapid tolerance in untrained HRT mice

Since we were able to show a significant effect of MK on motor learning, we tested HRT mice to see whether MK would block rapid tolerance when mice were forced

Figure 15. Effect of MK-801 on acquisition of ARR performance. (A) Performance of saline and MK treated groups across trials on days 1 and 2. Mice treated with 0.2 mg/kg MK showed significantly lower performance under the influence of MK. Groups treated with 0.05 or 0.1 mg/kg MK did not differ from saline treated mice on day 1 (data from these groups omitted for clarity). Differences in performance on day 2 were restricted to trials 1-5. (B) Average latencies to fall on trials 1-5 on day 2 show that mice given 0.2 mg/kg MK and saline treated mice with no practice on day 1 performed significantly worse on day 2 compared to the saline full practice group (*, $p<.05$). Mice treated with saline but given limited practice on day 1 (equated with 0.2 mg/kg MK group, see Expt 4 methods) did not differ from the saline full practice group. The 2 lower doses of MK given on day 1 did not significantly decrease performance on day 2. Mice treated with saline and given either full practice or limited practice on day 1 did not differ on day 2, suggesting that total practice on day 1 did not affect day 2 performance. The two low doses of MK were not sufficient to block acquisition of ARR performance, as these groups did not differ from the full practice group.


A
Latency to Fall (sec.)
100
80
60
40
20
0
Day 1
Day 2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Trial
Full Practice
Limited Practice
No Practice
.2 mg/kg MK


B
Latency to Fall (sec.)
100
80
60
40
20
0
*
*
Full Prac
Limited Prac
No Prac
.05 MK
.1 MK
.2 MK

to learn the task under the influence of ethanol. We compared groups given intoxicated practice with those given ethanol with no intoxicated practice, as tolerance resulting from intoxicated practice has been shown to be more susceptible to blockade by MK treatment (Khanna et al., 1997). It was predicted that MK would inhibit rapid tolerance development in HRT mice given intoxicated practice, but not in groups tested for physiological tolerance (i.e., given ethanol, but no intoxicated practice on day 1).

Procedure. Naïve male and female HRT mice were assigned to one of two groups: intoxicated practice (P) or no intoxicated practice (NP). Mice in each group were further divided into 3 treatment conditions given either saline, EtOH, or EtOH + MK (n=12-27/group). Mice were not give pre-training on the ARR. On day 1, mice in all groups were moved into the testing room, weighed, and allowed to sit undisturbed for 30 min. Mice were then removed from the home cage, injected with either saline or MK (0.2 mg/kg), and placed in individual holding cages. Thirty min after the first injection, mice were given a second injection of either saline or 2.75 g/kg EtOH (20% v/v i.p.) and placed back in the holding cage for 30 min. Mice in the P group were then given 5 consecutive trials on the ARR and returned to the home cage. Mice in the NP group were returned to the home cage without rotarod testing.

The following day, mice were moved to the testing room and weighed. Mice in both the P and NP groups were given 3 baseline trials on the ARR immediately before injection with saline. All mice were placed in individual holding cages for 30 min before being injected with 2.75 g/kg EtOH. Thirty min after EtOH injection, all mice were given 5 trials on the ARR. Comparisons in performance were made between the three

treatment groups within the P and NP groups on day 2, when all mice were given 2.75 g/kg EtOH. Rapid tolerance was evidenced by increased performance in groups given their second injection of EtOH compared to those given their first.

Results. Mice in the intoxicated practice (P) group were analyzed for the effect of drug treatment on rotarod performance on day 1. One-way ANOVA for average performance on day 1 revealed a significant effect of Treatment [$F(2,74)=203.19$, $p<.001$], with mice given saline performing better than groups given EtOH or EtOH + MK ($p<.05$). Mice treated with EtOH or EtOH + MK did not differ in performance on day 1 ($p=.16$, Fig 16A).

Baseline performance on day 2 did not differ among treatment groups in either the P or NP groups (in each group [$F<3.01$, $p>.05$]), suggesting that day 1 treatment did not alter baseline performance on day 2 (Table 6). Post-EtOH performance was analyzed using one-way ANOVA for the change from baseline performance averaged over the 5 post-EtOH trials. In the P group, there was a significant effect of Treatment [$F(2,74)=9.86$, $p<.001$]. Tukey's post-hoc testing showed that mice treated with saline on day 1, were more impaired on day 2 than either the EtOH or the EtOH + MK groups (p's$<.02$). There was no difference in performance in the EtOH and EtOH + MK groups, suggesting that MK treatment on day 1 did not block tolerance development (Fig 16B).

A similar result was found in the NP group. ANOVA of day 2 change from baseline performance found a significant effect of Treatment [$F(2,34)=6.70$, $p<.01$]. Again, saline treated mice were more impaired than mice given either EtOH or EtOH + MK (p's$<.04$). This demonstrated the significant development of rapid tolerance in the

Figure 16. Effects of MK-801 in untrained HRT mice. Mice were divided into groups that received either intoxicated practice (P) or no intoxicated practice (NP) on the ARR on day 1. (A) Average latency to fall on day 1 shows that mice treated with saline performed significantly better than mice treated with 2.75 g/kg EtOH or MK+EtOH (*, $p<.05$ compared to P Sal group). (B) Mice in both P and NP groups showed significant between-groups tolerance, as mice previously treated with EtOH showed an attenuated change from baseline latency to fall compared to mice previously treated with saline, when challenged with 2.75 g/kg EtOH on day 2 (*, $p<.05$ compared to respective Sal group). MK treatment did not block the development of rapid tolerance in either the P or NP group.

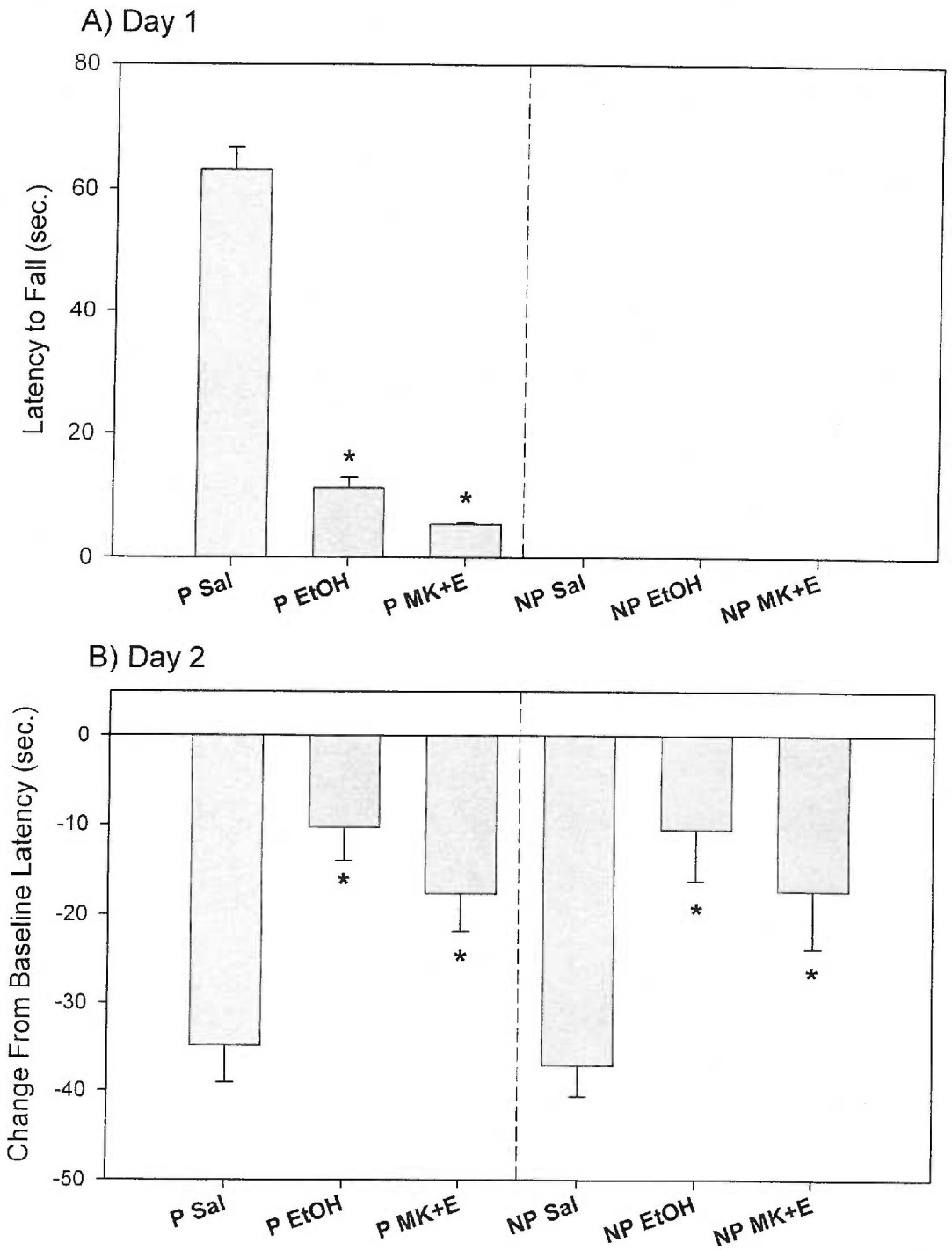

A) Day 1
Latency to Fall (sec.)
80
60
40
20
0
*
*
P Sal
P EtOH
P MK+E
NP Sal
NP EtOH
NP MK+E
B) Day 2
Change From Baseline Latency (sec.)
0
-10
-20
-30
-40
-50
*
*
*
*
P Sal
P EtOH
P MK+E
NP Sal
NP EtOH
NP MK+E

Table 6. Baseline performance in untrained HRT and LRT mice

Experiment	Line	Treatment Group	Baseline Latency (sec)
5	HRT	P Sal	51.7 ± 2.7
		P EtOH	43.7 ± 2.7
		P MK + EtOH	51.0 ± 2.4
		NP Sal	51.2 ± 3.6
		NP EtOH	52.7 ± 4.1
		NP MK + EtOH	54.3 ± 4.0
6	LRT	Sal	37.5 ± 2.5
		EtOH	41.6 ± 2.8
		10 DCS + EtOH	49.2 ± 3.6
		40 DCS + EtOH	43.7 ± 3.9

HRT and LRT mice were given no acquisition trials prior to the beginning of testing. On day 1, mice were either given practice (P) or no practice (NP) on the ARR 30 min after saline, ethanol, or MK + ethanol. On day 2, mice were given 5 baseline trials (data shown here) before treatment with ethanol alone. There were no significant differences in baseline performance among treatment groups in either HRT or LRT mice.

groups that were given EtOH on day 1. Mice in the EtOH and EtOH + MK groups again did not differ (p=.64), suggesting that MK treatment did not block rapid tolerance in mice that received no intoxicated practice.

Experiment 6: Effect of D-cycloserine on rapid tolerance in untrained LRT mice

In one additional experiment, we examined whether DCS treatment would enhance tolerance in LRT mice forced to learn the task under the influence of ethanol. As LRT mice have been shown to develop very little rapid tolerance with pre-training, we did not include the physiological tolerance (no intoxicated practice) group in this study. It was predicted that LRT mice treated with DCS would show greater tolerance than those treated with ethanol alone.

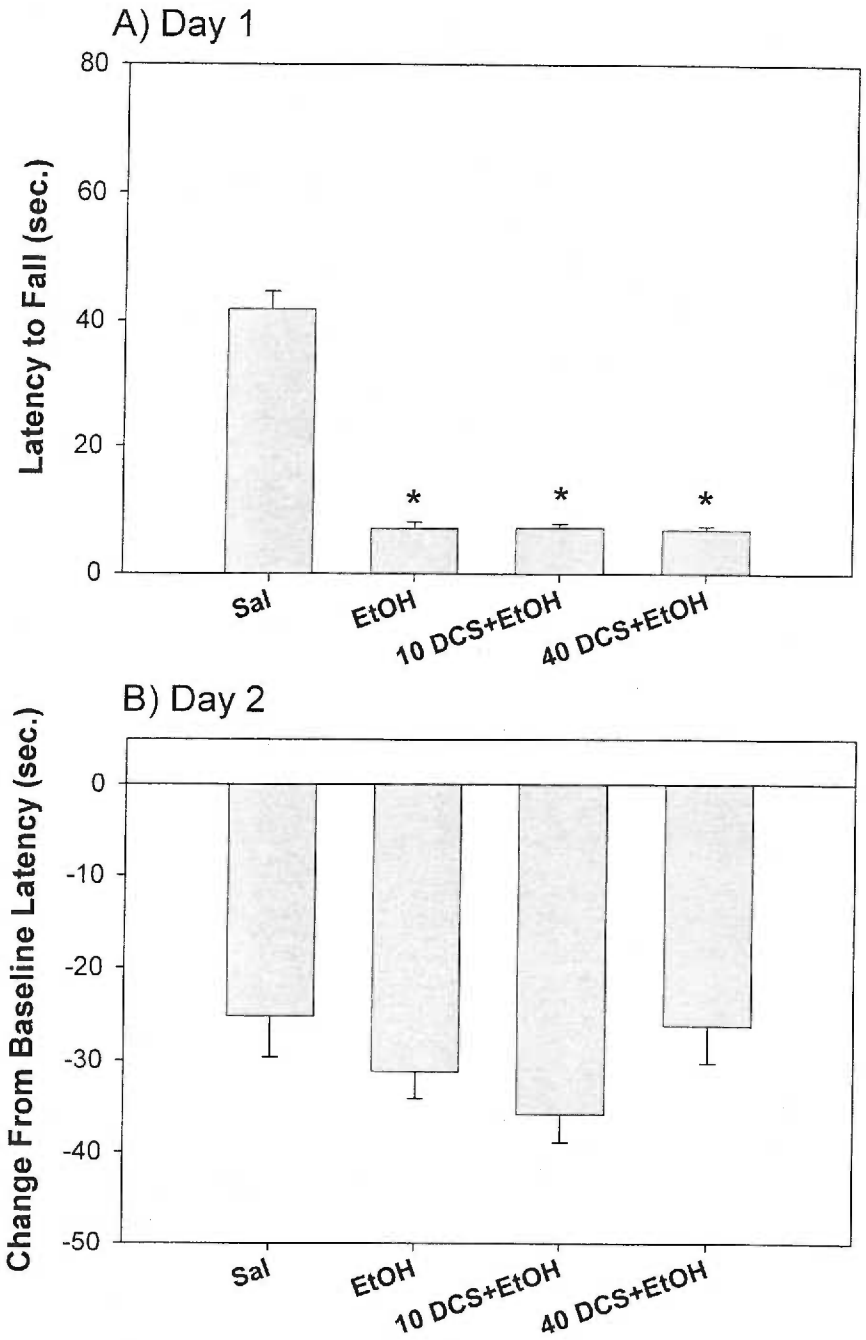
Procedure. Naïve LRT mice (n=13-14/group) were tested in this experiment. Procedurally, mice were treated similarly to those in the intoxicated practice group in Experiment 5. Mice were divided into 4 treatment groups that differed in day 1 treatment. On day 1, mice were given either saline or DCS (10 or 40 mg/kg) 30 min prior to treatment with saline or 2.5 g/kg EtOH. Thirty min after the last injection, mice were given 5 trials on the ARR before being returned to the home cage. The next day, mice were given 3 baseline trials before being injected with saline, followed 30 min by 2.5 g/kg EtOH. Thirty min after EtOH, all mice were given 5 trials more trials on the ARR. Rapid tolerance was assessed by comparing the performance among groups on day 2.

Results. Performance on day 1 was analyzed by one-way ANOVA on the average performance across the 5 ARR trials. This analysis revealed a significant effect of treatment [$F(3,51)=69.52$, $p<.001$], with saline treated mice performing better than all other groups (Fig 17A). Analysis of day 2 baseline performance found no significant differences between groups [$F=2.26$, $p=.09$], suggesting that day 1 treatment did not affect baseline performance on day 2 (Table 6). The change from baseline performance after ethanol on day 2 (Fig 17B) was also not different on day 2 [$F=1.97$, $p=.13$]. This provides evidence that there was no significant between-groups tolerance development, and that treatment with DCS did not significantly enhance the development of rapid tolerance in LRT mice.

Discussion

Overall, the results from the above experiments do not allow us to support a role for NMDA receptors in the development of rapid tolerance to ethanol in HRT and LRT mice. HRT and LRT mice did not differ in their sensitivity to the ataxic effects of MK. This suggests that for ethanol-induced ataxia, artificial selection did not differentially alter the function of NMDA receptors in the two lines. Neither line developed rapid tolerance to the ataxic effect of the high dose of MK, suggesting that tolerance to ethanol and MK do not share common underlying mechanisms in these mice. Further, in previously trained mice, neither MK or DCS significantly modulated the development of rapid tolerance to ethanol. These results suggest that blockade of the NMDA receptor is

Figure 17. Effects of D-cycloserine on rapid tolerance in untrained LRT mice. (A) Treatment with 2.5 g/kg EtOH significantly decreased average latency to fall on day 1 (*, $p<.05$ compared to Sal group). (B) No significant rapid tolerance developed in LRT mice on day 2, as the EtOH group did not differ from the Sal group in the change from baseline latency to fall. Further, mice treated with DCS did not differ from the EtOH group, suggesting that DCS treatment did not enhance rapid tolerance development in untrained LRT mice.


A) Day 1
Latency to Fall (sec.)
80
60
40
20
0
*
*
*
Sal
EtOH
10 DCS+EtOH
40 DCS+EtOH
B) Day 2
Change From Baseline Latency (sec.)
0
-10
-20
-30
-40
-50
Sal
EtOH
10 DCS+EtOH
40 DCS+EtOH

not sufficient to block tolerance to ethanol's ataxic effects in HRT mice, and that potentiation of NMDA receptor function is unable to increase tolerance in either HRT or LRT mice.

We were able to show in genetically heterogeneous mice that treatment with MK during acquisition trials on the ARR inhibited learning on the task. This inhibition was independent of the amount of practice the mice received on day 1. Saline-treated mice that were limited to the same amount of practice as the MK-treated mice on day 1 did not differ from the saline treated mice with full practice when compared on day 2. It was apparent that 0.2 mg/kg MK produced some ataxia on its own (Fig 15A). Mice given this dose of MK did appear to learn the task over the course of the 10 trials, as their rate of improvement was not statistically different from the other groups. However, with our testing paradigm, it takes 20-25 min to give WSC-2 mice 10 consecutive trials on the ARR. Because of this long test duration, some of the improvement seen in the high MK dose group could be the result of pharmacokinetic factors.

Given these effects of MK on ARR acquisition (motor learning), we thought that we might be able to see an effect of MK or DCS on rapid tolerance if mice were not extensively trained on the apparatus, therefore being forced to learn the task under the influence of ethanol. This would suggest a motor learning component to rapid tolerance development. Even under these conditions, the NMDA receptor drugs did not affect tolerance development in either HRT or LRT mice (Figs. 16 and 17). Taken together, these results may suggest that HRT mice develop tolerance in this paradigm independently of any effects of learning. Caution should be used in this interpretation, however, as we did not test for effects of MK on ARR acquisition in HRT or LRT mice.

That is, we do not know whether MK would block the acquisition of rotarod performance in these mice. The results also suggest that HRT and LRT mice may have been selected for rapid tolerance via an NMDA-independent mechanism. It was clear from the studies with MK that mice were still sensitive to the incoordinating effects of the drug, but there was no significant effect of MK on tolerance development in any study.

Many reports have provided evidence for a learning component to ethanol tolerance. LeBlanc et al. (1973) showed that tolerance to the motor impairing effects of ethanol on a moving belt task was increased in rats allowed to practice the task under the influence of ethanol. Similarly, Khanna and colleagues (Khanna et al., 1997) showed that intoxicated practice accentuates tolerance development on the tilt-plane task in rats. Interestingly, the Khanna et al. study showed that tolerance which developed as a result of intoxicated practice was more susceptible to NMDA receptor blockade than the "physiological tolerance" which developed in groups given ethanol without intoxicated practice. Similarly, Szabo and colleagues (Szabo et al., 1994) found that MK blocked tolerance to the impairing effects of ethanol on the rotarod in C57BL/6 mice, but only when ethanol was administered acutely before testing. In a concurrent study, they found that MK failed to block tolerance when ethanol was administered via liquid diet, and therefore was not environment-dependent. It should be noted that in the latter study, MK administration occurred only twice daily, making it unlikely that MK was active at all times that ethanol was active. Both of these latter studies provide evidence that any learning component to tolerance development may be more susceptible to blockade by NMDA receptor antagonists.

There are two pieces of evidence that support the possibility that HRT mice develop tolerance independent of learning processes, and are provided from the results of experiment 5. In this experiment, mice were not trained on the ARR. Hence, mice in the intoxicated practice group were forced to learn the task under the influence of ethanol, while mice in the no intoxicated practice group did not have any experience on the ARR on day 1. Figure 16 shows that even though they were given post-EtOH trials on day 1 (intoxicated practice), ethanol intoxication limited their exposure on the apparatus compared to the saline treated group. Even with little or no training, both groups developed significant rapid tolerance, suggesting that the ethanol experience alone was able to elicit rapid tolerance even in the absence of any (or very little) intoxicated practice. Perhaps if HRT mice develop tolerance independent of learning processes, it is not surprising that tolerance is not affected by NMDA receptor drugs in these mice.

There were a few results in the present set of experiments that suggest that NMDA receptors may still be playing a small role in ethanol tolerance in HRT and LRT mice. In experiment 2, HRT mice developed significant chronic tolerance. While MK treatment did not significantly decrease performance compared to the chronic EtOH group, there was a tendency for increasing doses of MK to decrease performance on the challenge day (Fig. 12B) to the point that MK-treated mice did not differ from saline-treated mice. In experiment 3, while HRT mice did not show a significant potentiation of tolerance by DCS, there was again a tendency for increasing doses of DCS to increase tolerance (Fig. 13). It is conceivable that the lack of a significant effect in these mice is due to a ceiling effect. In the same experiment, LRT mice given DCS showed marked improvement from day 1 to day 2; however, the fact that the control group also developed

tolerance makes this result hard to interpret. There were no significant differences among groups on day 2, so we cannot say that DCS potentiated tolerance in LRT mice in this study. This was the only study in which we saw significant rapid tolerance in LRT mice to any dose of ethanol (current studies and Rustay & Crabbe, submitted).

It appears from these results that while the NMDA receptor may be playing a small role in tolerance development in HRT and LRT mice, there are clearly other mechanisms involved. It is possible that by selecting for high rapid tolerance, we have produced mice that develop tolerance though many different mechanisms. When we blocked the NMDA receptor, the other systems may have been able to compensate for its loss. This does not explain why we were not able to potentiate tolerance with the NMDA agonist, however. It would be useful to test other pharmacological agents to elucidate the mechanism(s) through which HRT and LRT mice display their differences in rapid tolerance development.

General Discussion

The results of the present set of experiments provide evidence that the development of rapid tolerance to the ataxic effects of ethanol is partially mediated by genetics. Inbred strains of mice were shown to differ significantly in rapid tolerance development, and from this result, I was able to estimate the genetic contribution to the trait (narrow-sense heritability; Falconer & Mackay, 1996). The estimation of narrow-sense heritability was rather low (.11) compared to the realized heritability calculations in some selective breeding studies (~.25) (Crabbe et al., 1987a; Erwin & Deitrich, 1996), but was comparable to another successful artificial selection study (McClearn & Kakihana, 1981); however, none of these studies focused on rapid tolerance. The results from the breeding study suggests that the narrow-sense heritability was relatively accurate, as the calculated heritability estimates in HRT-1 and LRT-1 mice were .25 and .06, respectively. The successful response to selection in replicate 1 supports the results of the inbred strain study. HRT-1 mice showed a steady increase in the amount of rapid tolerance which developed over the first 4 generations of selection, representative of a characteristic controlled by many genes, each with a relatively small effect on the trait (Crabbe, 1999).

AFT has been shown to have a significant genetic component. This was reported by studies using inbred strains of mice (Gallaher et al., 1996; Ponomarev & Crabbe, submitted). Artificial selection of the HAFT and LAFT mice solidified this claim (Erwin & Deitrich, 1996). The inbred strain study and artificial selection study presented here provide evidence that rapid tolerance is also under significant genetic control. Further, they provide the first genetic link between rapid and chronic tolerance to ethanol's ataxic

effects. Numerous reports have been published describing the pharmacological manipulation of rapid and chronic tolerance, with many showing a congruence between the two forms of tolerance (Barbosa & Morato, 2000, 2001; Khanna et al., 1991b; Khanna et al., 1994b). Similarly, drugs that have been shown to confer cross-tolerance to ethanol do so in both rapid and chronic tolerance paradigms (Khanna et al., 1992c). These data taken together, suggest that rapid tolerance and chronic tolerance share many physiological underpinnings, and that rapid tolerance may represent the initial stages of chronic tolerance development. Therefore, using a rapid tolerance paradigm may be a more efficient way to investigate the processes involved in the development of ethanol tolerance (Kalant, 1996).

A clear link, genetic or pharmacologic, has yet to be established between AFT and either rapid or chronic tolerance. Further research on this relationship is needed. The HRT and LRT lines provide a useful model for these studies, as testing HRT and LRT mice for AFT would help to establish a possible relationship between AFT and rapid tolerance. Similarly, HAFT and LAFT mice could be tested for rapid tolerance. In the few studies in which HAFT and LAFT mice have been investigated for chronic tolerance, there was no association seen between the two (Rustay et al., 2001). Interpretation of these results is somewhat complicated, though, in that the HAFT and LAFT mice were tested for AFT and chronic tolerance on different tasks (fixed-speed rotarod for AFT vs. grid test and hypothermia for chronic tolerance). Had they been tested for chronic tolerance with a task on which they were shown to differ in AFT, perhaps a clearer link would have been found.

The results from the current tolerance studies in HRTs and LRTs also allow some insight into the relationship between initial sensitivity and tolerance to the effects of ethanol. According to Kalant and colleagues (1971), tolerance should increase in parallel with the initial disturbance produced by ethanol. If this were true, HRT mice should have shown greater sensitivity to ethanol than LRT mice. However, Table 3 suggests the opposite; LRT mice tend to show greater initial sensitivity than do HRT mice at the selection dose of ethanol. Further, it appeared from Figure 6 that LRT mice were more sensitive to low doses of ethanol than were HRT mice on their initial exposure. This is the opposite of what would be expected by Kalant's postulate. As discussed in chapter 1, these data provide evidence that initial sensitivity is not the sole predictor of tolerance development.

There is a possible genetic explanation for this departure from Kalant's hypothesis. It is conceivable that there are multiple mechanisms for the propensity to develop high rapid or chronic tolerance. One or more of these mechanisms may result from increased sensitivity to the effects of ethanol, while others may not. It is entirely possible that the selection for HRT and LRT mice excluded those alleles responsible for increased sensitivity-dependent mechanisms for rapid tolerance, while selecting alleles for other, unknown mechanisms. This could result in lines of mice which differ in tolerance regardless of their sensitivity to ethanol. The results from the dose-response chronic tolerance study in HRTs and LRTs suggests that this may have been the case.

Many previous studies have shown a role for learning in the development of tolerance to ethanol's effects (see reviews by Siegel, 1989; Wolgin, 1989). This was an important consideration when the artificial selection experiment was initiated. I wanted

to be sure that I was selecting specifically for tolerance development, while controlling for differences in motor learning. One could imagine that a mouse that learned more quickly might also be more likely to show greater tolerance development in my testing paradigm. That is, a better learner might learn more quickly under the influence of ethanol, resulting in greater rapid tolerance. If this were the case, artificial selection could have ended with lines of mice that differed in basal rotarod acquisition and ability. Rotarod acquisition was analyzed in the mice selected from the parental population for the starting breeding pairs for selection. Mice selected for high and low tolerance in the starting population did not differ in rotarod acquisition. Subsequent analysis with each generation of selection revealed only one generation in which HRT mice acquired the task more quickly than LRT mice (see Chapter 1 results). This was not the same generation where they showed the greatest difference in tolerance development, suggesting that differential rapid tolerance between HRT and LRT mice did not arise from differences in motor learning.

Based on the results that NMDA receptor blockade inhibited ARR acquisition (Fig. 15), it may not be too surprising that the HRTs and LRTs did not differ in their sensitivity to the ataxic effects of MK. The fact that the lines did not differ in acquisition of ARR performance provides indirect evidence that they have similar NMDA receptor function in relation to rotarod performance. However, this conclusion must be met with caution, as we did not test the sensitivity of ARR acquisition to MK in HRT and LRT mice. But, based on the fact that the lines did not differ in ARR acquisition, and the results of the sensitivity to MK-induced ataxia study (Fig. 9), finding the lines different in their sensitivity to MK's blockade of motor learning seems unlikely.

The results of the current pharmacology studies were not in line with much of the literature on the neurotransmitter systems involved in rapid tolerance. Based on the review provided in the introduction, the NMDA receptor plays a critical role in the development of rapid tolerance to both the hypothermic and ataxic effects of ethanol. The role of the NMDA receptor has been shown in both rats and mice, and in several different tests of ataxia (tilt-plane, ARR, treadmill). However, I found no effect of a NMDA receptor agonist or antagonist on rapid tolerance development on the ARR. Initial attempts to block tolerance showed no effect of the NMDA drugs on tolerance development in mice that had been given previous training on the ARR, an identical testing design as the artificial selection protocol. Perhaps my inability to replicate the effects of NMDA receptor drugs on tolerance development with HRTs and LRTs, may have been the result of influences of motor learning. We were able to show in WSC-2 mice that the blockade of NMDA receptors resulted in the inhibition of learning on the ARR. It was thought that perhaps the NMDA receptor's effect on ARR performance was being exhausted by training mice prior to testing with ethanol, thereby minimizing the influence of the NMDA receptor when we tested for ethanol tolerance. To examine the role of learning on tolerance development in HRTs and LRTs, mice were tested for tolerance to ethanol without the extensive pre-training. Instead, mice were forced to learn to perform on the ARR while under the influence of ethanol. While HRT mice treated with ethanol did not show much improvement on the first day of ethanol testing (Fig 16), they showed significant tolerance development when tested after ethanol on day 2. Mice given MK with ethanol showed a similar response to those treated with ethanol alone on day 1 and on day 2, suggesting no blockade of tolerance by MK. A similar

response was seen in mice given no pre-training or intoxicated practice, further supporting that HRT mice do not need intoxicated practice or undrugged experience on the ARR in order to show MK-insensitive rapid tolerance development. It is clear from these studies that HRT mice must be developing tolerance through some non-NMDA-dependent mechanism(s).

This fact that HRT mice are able to develop such extensive rapid tolerance without any intoxicated practice is interesting. Several of the studies mentioned have investigated the learned component of tolerance compared to a "physiological" control group (Khanna et al., 1997; LeBlanc et al., 1973). While tolerance in the physiological tolerance groups was apparent in these studies, groups given intoxicated practice showed the greatest tolerance. In fact, another study by Wenger and colleagues (Wenger et al., 1980) showed that when the "physiological" group was given no intoxicated practice (instead of testing under ethanol every 4 days as in the LeBlanc et al. studies), rats showed no significant tolerance development. These results showed that learning to perform a given task under the influence of ethanol was a key component to the development of tolerance. HRT mice are not only able to develop tolerance without significant levels of intoxicated practice, but with only 1 prior exposure to ethanol.

It is interesting that NMDA receptor antagonists have shown an inhibitory effect on rapid tolerance in the past. Ethanol alone has an inhibitory effect on the function of hippocampal NMDA receptors using an *in vitro* slice preparation (Lovinger et al., 1990). Since acute ethanol and NMDA receptor antagonists have similar effects, it is unclear why drugs which inhibit NMDA receptor function have been shown to block ethanol tolerance development. Perhaps ethanol inhibition of the NMDA receptor results in a

change in the receptor conformation or function, or alters some down-stream signal cascade, which then decreases the effectiveness of subsequent ethanol exposures to inhibit the receptor (tolerance). Administering NMDA receptor antagonists before ethanol (when they are most effective at blocking tolerance) could then block the ability of ethanol to act on the receptor, disallowing any tolerance to develop through an NMDA receptor mechanism. Perhaps ethanol's actions at the NMDA receptor result in the recruitment of other neurotransmitter systems to produce the necessary changes for tolerance development, and in other mouse and rat populations, blockade of the NMDA receptor prevents these down-stream changes from occurring. It is possible that HRT mice have developed a way to bypass the input of the NMDA receptor in this process, thereby showing no effect of the NMDA receptor antagonists.

It is clear that an individual's ability to develop rapid tolerance is controlled in part by one's genetic make-up. Two separate genetic mouse models showed that rapid tolerance to ethanol's ataxic effects is under significant genetic control. While the NMDA-type glutamate receptor has been implicated in rapid tolerance development, the results from the current studies suggest that the NMDA receptor is not the sole mediator of rapid tolerance. Tolerance development was not altered by NMDA receptor drugs in either the HRT or the LRT selected lines. Further, tolerance was not found to be influenced by intoxicated practice.

Summary and Future Directions

It appears that the study of rapid tolerance provides a useful model for the study of chronic tolerance to ethanol. Similar results have been found in both rapid and chronic

tolerance paradigms using pharmacological approaches, and now with the successful artificial selection of HRT and LRT mice. These mice were shown to differ significantly in the degree of rapid tolerance the lines developed to ethanol's ataxic effects. HRT mice also developed greater chronic tolerance than did LRT mice. This was true for tolerance development to several doses of ethanol.

The current results also provide evidence that the NMDA receptor does not play a prominent role in the development of rapid tolerance in HRT and LRT mice. MK did not prevent rapid tolerance development in any of the paradigms tested. This suggests that HRT mice develop tolerance via an NMDA-independent mechanism. Additionally, LRT mice treated with D-cycloserine did not show increased tolerance development. Even though the present results do not support previous reports of a significant role for the NMDA receptor in ethanol tolerance, they do support the conclusion that there are other mechanisms that must play a role in the development of tolerance. HRT and LRT mice provide a useful model for examination of these other mechanisms. Future studies are aimed at examining the role of serotonin and GABA receptor drugs, as previous research has suggested that these systems may be involved in ethanol tolerance.

In the discussion of Chapter 2, it was suggested that the HRT mice might develop tolerance independent of any learning processes. This idea was brought forth due to the large amount of tolerance that was shown to develop in HRT mice given no training or intoxicated practice on the ARR ("physiological tolerance" group). This group developed as much tolerance as a group given intoxicated practice (Fig. 16). It would be interesting to test for an explicitly learned component, such as conditioned tolerance, using a paradigm similar to Mansfield and Cunningham (1980). By testing HRT and

LRT mice in familiar or novel environments, we could test for the development of conditioned tolerance, and whether this contributes to the expression of rapid tolerance in these mice. Although it did not appear that learning was contributing to the rapid tolerance seen in HRT mice, having experienced the ethanol administration procedure (even only once) may have been enough to elicit a conditioned response to counteract ethanol's effects on day 2. If this occurred, I would predict that HRT mice would show greater conditioned tolerance than LRT mice if tested in a conditioned tolerance paradigm.

As there is no clear link between rapid tolerance and AFT, HRT and LRT mice should be tested for the development of AFT. We have a protocol devised for testing AFT on the fixed-speed rotarod, which we have shown produces similar results to the stationary dowel in HAFT and LAFT mice (Rustay et al., 2001). Testing the HRTs and LRTs in this paradigm would provide needed data on the relationship between these forms of tolerance. I predict that HRT mice would show greater AFT than LRT mice, and propose that this is one mechanism by which the HRTs could develop greater rapid tolerance than LRTs (Littleton, 1980).

Also, the HRT and LRT mice provide a good model to examine the effects of tolerance development on ethanol drinking. Many reports have used selected lines of rodents selected for high and low ethanol intake to examine the relationship between ethanol drinking and tolerance development. It has been shown that rat lines which drink more ethanol also may develop greater tolerance to ethanol (Bell et al., 2001; Kurtz et al., 1996; Lê & Kiianmaa, 1988). HRT and LRT mice would allow us to as the opposite question; i.e., does tolerance induction lead to increased ethanol consumption? Erwin

and colleagues have already shown that naïve HAFT mice voluntarily drink more ethanol than LAFT mice, although both lines consumed low levels (Erwin et al., 2000). We have shown that HRT mice develop substantial tolerance to the ataxic effects of ethanol even when they are given no intoxicated practice on the test apparatus. If they concurrently develop tolerance to the subjective effects of ethanol, they might drink more ethanol in order to achieve the desired physiological effects of the drug. I predict that HRT mice would drink more ethanol than LRT mice after being treated with injections to induce ethanol tolerance.

To address the issue of whether increased tolerance to one of ethanol's effects confers increased tolerance to others, HRT and LRT mice could be tested for tolerance to another effect, such as ethanol-induced hypothermia. A positive correlation between these two traits would suggest some common tolerance mechanisms that are not specific to the specific test modality. Further, to test the specificity of tolerance development within the domain of ataxia, HRT and LRT mice could be tested for tolerance on another ataxia measure, such as the grid test or even a very similar task to the ARR, the fixed-speed rotarod.

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