BEHAVIORAL ASSESSMENT OF COMMON DETERMINANTS OF DRUG AND ETHANOL-INDUCED SENSITIZATION AND THE ROLE OF ETHANOL-INDUCED SENSITIZATION IN VOLUNTARY ETHANOL DRINKING AND ETHANOL-INDUCED CONDITIONED TASTE AVERSION

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ABSTRACT

Repeated administration of low ethanol (EtOH) doses may lead to a progressive increase in the motor stimulant effects of EtOH, or behavioral sensitization. The lasting nature of EtOH sensitization points to neural adaptations in drug sensitivity, which may effectively maintain EtOH taking behavior leading to EtOH abuse. This dissertation aimed at investigating the nature of these neural adaptations through behavioral approaches.

First, it was assessed whether cross-sensitization existed between the stimulant effects of EtOH and those of morphine, cocaine, and methamphetamine. Since morphine, cocaine, and methamphetamine have specific neural targets, an effect of EtOH on those neural targets would be revealed through the presence of cross-sensitization. Genetically heterogeneous mice received repeated EtOH injections to induce EtOH sensitization, and 24 hours later, mice were challenged with single administrations of one of the other three drugs. Cross-sensitization would have been revealed if EtOH-sensitized mice had shown enhanced locomotor responses to challenge drug injections relative to their saline-treated counterparts. There was no evidence for cross-sensitization, as EtOH-sensitized mice had either similar, or a tendency for blunted, locomotor responses to challenge drug administrations.

Second, the influence of EtOH sensitization on sensitivity to EtOH reinforcement, as measured by EtOH two-bottle preference drinking, was examined in three mouse genotypes. It was additionally assessed whether voluntary EtOH drinking would induce EtOH sensitization. A change in EtOH consumption following EtOH sensitization would imply common neural substrates in the mediation of EtOH reinforcement and sensitization processes. It was found that the alcohol-preferring C57BL/6J inbred strain consumed enough EtOH to become sensitized, and that upon further repeated EtOH

injections, EtOH sensitization was enhanced and subsequently led to increased EtOH consumption.

Third, the influence of EtOH sensitization on sensitivity to EtOH aversion, as assessed using the conditioned taste aversion paradigm, was examined in genetically heterogeneous mice. As with EtOH reinforcement, a change in EtOH-induced taste aversion following EtOH sensitization would imply common neural substrates in the mediation of EtOH aversion and sensitization processes. The results showed that sensitized mice had much attenuated magnitude of taste aversion relative to their nonsensitized controls at an intermediate EtOH taste-conditioning dose. Overall, the data suggest that different neural mechanisms mediate sensitization to the effects of EtOH and those of morphine, cocaine, and methamphetamine, although this conclusion is tentative, particularly since the reciprocal conditions of repeated morphine, cocaine, or methamphetamine treatment followed by challenge EtOH injections have not been determined. The data also suggest that EtOH sensitization, EtOH reinforcement, and EtOH aversion processes may be mediated by common neural substrates. In addition, the fact that EtOH sensitization led to increased EtOH drinking and reduced EtOH aversion may implicate EtOH sensitization as an important phenomenon in the etiology of EtOH abuse.

OVERALL INTRODUCTION

Single or repeated exposures to a drug induce neural adaptations that may be manifest behaviorally as sensitization to the locomotor stimulant effects of the drug (Kuczenski and Segal, 1988; Pierce and Kalivas, 1997). Sensitization may develop to the effects of different classes of drugs of abuse including the psychostimulants cocaine and amphetamine (Segal and Mandell, 1974; Post and Rose, 1976; Shuster et al., 1977; Paulson et al., 1991; Hirabayashi et al., 1991), the opioid morphine (Babbini et al., 1975; Shuster et al., 1975; Volpicelli et al., 1999), the general central nervous system depressant ethanol (Masur and Boerngen, 1980; Masur et al., 1986; Phillips et al., 1994b; Phillips et al., 1995; Phillips et al., 1996), and general stimulants such as nicotine (Ksir et al., 1985; Blomqvist et al., 1996; Booze et al., 1999), and even caffeine (Meliska et al., 1990; Varani et al., 1999). This all-encompassing profile of drug sensitization implies that different drugs of abuse may exert some of their effects through common neural mechanisms. Behavioral sensitization has been shown to last for up to many months following termination of drug administration (Babbini et al., 1975; Shuster et al., 1977; Paulson et al., 1991; Lessov and Phillips, 1998; Volpicelli et al., 1999). This implies long-lasting neuroadaptations that may contribute to drug relapse following periods of abstinence (Sato et al., 1983; Robinson, 1993; Bartlett et al., 1997; Self, 1998; Spanagel and Weiss, 1999) and that may subserve proclivity toward drug abuse (Wise and Bozarth, 1987; Robinson and Berridge, 1993; Schenk and Davidson, 1998).

Because of the proposed role of drug sensitization in drug addiction (Wise and Bozarth, 1987; Robinson and Berridge, 1993), there has been great interest in elucidating the neural mechanisms mediating sensitization. The following paragraphs briefly review

the literature aimed toward this goal and focus on the role of the mesolimbic dopaminergic system. In the first half, discussion will focus on psychostimulant and opiate reward and sensitization, while the second half will focus on ethanol (EtOH), the main topic of this dissertation.

Drug Sensitization and Drug Reinforcement

One reason why drugs become abused is because of the feelings of pleasure or euphoria associated with their administration (Schulteis and Koob, 1996; Bigelow et al., 1998; Di Chiara, 1999). In animal models, drug reward has been most often inferred by measuring how much drug an animal will self-administer (Samson et al., 1988; Samson et al., 1998; Stafford et al., 1998). In operant terms, a drug is said to be positively reinforcing when an animal preferentially presses a lever associated with drug administration and when it is willing to press the lever at increasing frequency to receive the drug. Voluntary preference drinking behavior and the conditioned place preference paradigm offer two additional behavioral indices of drug reward (Cunningham et al., 1992; McBride and Li, 1998; Tzschentke, 1998). For the purposes of this introduction, "drug reinforcement" and "drug reward" will be interchangeably used, although it is understood that they may represent related, but different, phenomena.

There is interest in determining whether neuroadaptations that underlie drug sensitization may be associated with drug reinforcement processes. Investigations have been conducted both at the behavioral and neurochemical levels. Repeated administration of amphetamine resulted in faster and greater acquisition of amphetamine-reinforced operant behavior (Mendrek et al., 1998; Pierre and Vezina, 1998), and pre-

treatment with the dopamine D_1 receptor antagonist SCH-23390 blocked both the amphetamine-induced locomotor stimulation and the enhanced amphetamine self-administration (Pierre and Vezina, 1998). These findings implicate the D_1 receptor in the mediation of amphetamine reinforcement and suggest an association between amphetamine sensitization and reinforcement processes. Similarly, amphetamine and nicotine pre-treatment lead to faster acquisition and higher levels of cocaine-reinforced operant self-administration (Horger et al., 1992; Schenk et al., 1993; Valadez and Schenk, 1994), implying sensitization to the reinforcing effects of cocaine and common neural substrates in the effects of amphetamine and cocaine, and those of amphetamine and nicotine. In one study (Schenk et al., 1993), the amphetamine-induced increase in cocaine self-administration was blocked by pre-treatment with the NMDA antagonist MK-801. The glutamate and dopamine systems interact with one another via a glutamatergic input to the nucleus accumbens (NAcc) and ventral tegmental area (VTA) from the prefrontal cortex, and via dopaminergic VTA projections to the prefrontal cortex (Pierce and Kalivas, 1997; Kalivas and Nakamura, 1999). As with amphetamine, repeated cocaine administration leads to an enhancement of cocaine-reinforced operant self-administration (Horger et al., 1990), and to an increase in responding on a lever previously associated with reward (water reward in water-deprived animals) for intra-NAcc amphetamine infusions (Taylor and Horger, 1999). These data indicate that cocaine-induced NAcc adaptations may mediate both cocaine and amphetamine reward.

Studies employing the conditioned place preference paradigm have shown that repeated administration of cocaine, amphetamine, and morphine enhanced the magnitude of subsequent place preference conditioning for each drug as well as across drugs (Lett,

1989; Gaiardi et al., 1991; Shippenberg and Heidbreder, 1995). Using this behavioral paradigm, co-administration of the dopamine D_1 receptor antagonist SCH-23390 during repeated cocaine treatment blocked subsequent cocaine-induced place conditioning (Shippenberg and Heidbreder, 1995), indicating an involvement of D_1 receptors in the mediation of conditioned cocaine reward, as was also shown for amphetamine reward (Pierre and Vezina, 1998). Morphine and nicotine pre-exposure also resulted in faster acquisition of morphine-induced conditioned place preference (Shippenberg et al., 1996) indicating an association between a sensitizing regimen of morphine exposure and morphine reward, in addition to implicating nicotinic receptors in the mediation of morphine reward. Finally, in an animal model of drug craving, priming injections of cocaine, amphetamine, and heroin reinstated heroin-seeking behavior in previously heroin-experienced rats, but only cocaine and amphetamine, and not heroin, reinstated cocaine-seeking behavior in previously cocaine-experienced rats (De Vries et al., 1998). It was determined that drug-seeking behavior was reinstated only in the cases where sensitization or cross-sensitization between the effects of the priming drug and the pre-treatment drug were present (De Vries et al., 1998).

There seems to be considerable evidence to suggest that prior drug exposure may enhance or sensitize reinforcing drug properties. However, in most studies, it was generally assumed that drug pre-exposure resulted in neuroadaptations underlying drug sensitization, in that the behavioral manifestations of sensitization were rarely assessed.

Drug Reinforcement and Dopamine

Obviously, elucidating the mechanisms of drug reinforcement is of paramount importance to the understanding of drug abuse. Much research aimed at this goal seems to agree and converge on the mesolimbic dopaminergic system as one common neural substrate mediating drug reward (Newlin, 1994; Berridge and Robinson, 1998; Self, 1998; McBride et al., 1999). Systemic administrations of morphine, cocaine, amphetamine, and nicotine resulted in increased extracellular dopamine concentrations in rat NAcc and caudate (Di Chiara and Imperato, 1988), indicating that enhanced dopaminergic activity may mediate some of the effects of these drugs. Reinstatement of non-reinforced responding in previously cocaine-trained rats was achieved with priming injections of cocaine, of the dopamine reuptake inhibitor GBR-12909, and of the dopamine D_2 receptor agonist quinpirole (De Vries et al., 1999). In contrast, the dopamine D_1 receptor agonist SKF-82958 failed to reinstate cocaine-seeking behavior (De Vries et al., 1999). These results indicate that the dopamine transporter and the dopamine D_2 but not the D_1 receptor may mediate cocaine reward. In the same study, heroin-seeking behavior was reinstated by heroin and by GBR-12909, however the D_1 and D_2 agonists had no effect (De Vries et al., 1999) implicating the dopamine transporter, but not the D_1 or D_2 receptors in mediation of opiate reward. Interestingly, dopamine D_2 receptor knockout mice failed to develop morphine-induced conditioned place preference relative to their wild type counterparts implicating the D_2 receptor in mediation of opiate reward (Maldonado et al., 1997), in contrast to the conclusions of the previous study (De Vries et al., 1999). Thus, depending on the behavioral paradigm and procedural manipulation, opposite conclusions may be reached regarding the involvement

of a given receptor system in the mediation of drug reward. Further complications arise from studies showing that the dopamine D_1 and D_2 receptor agonists SKF-38393 and quinpirole, respectively, together but not individually, were self-administered into the shell but not the core subregion of the NAcc (Ikemoto et al., 1997a). These results indicate that adaptations at both D_1 and D_2 receptors are necessary to induce reward and refine regional specificity of drug site of action.

Investigation of brain areas that support drug reward has shown that morphine was self-administered directly into the VTA and NAcc, however microinjections of morphine into the VTA, but not into the NAcc, supported morphine-induced conditioned place preference (Olmstead and Franklin, 1997; McBride et al., 1999). Animals also self-administered cocaine and amphetamine directly into the NAcc, however while microinjections of amphetamine into the NAcc supported amphetamine-induced place preference, intra-NAcc cocaine microinjection did not support cocaine-induced place preference (McBride et al., 1999). It seems that both the VTA and NAcc mediate psychostimulant and opiate reward, but these areas may be differentially involved in the expression of drug reward as assessed in separate behavioral paradigms.

The effects of morphine in the VTA are likely mediated by way of μ opioid receptor inhibition of GABA interneurons (Kalivas and Nakamura, 1999; McBride et al., 1999; Spanagel and Weiss, 1999). Activation of μ opioid receptors inhibits tonic GABA activity, leading to disinhibition of dopamine neurons (Kalivas and Nakamura, 1999; Spanagel and Weiss, 1999). Support for this mechanism of action comes from studies showing that rats self-administered $GABA_A$ antagonists into the VTA (specifically into the anterior VTA, but not the posterior VTA or regions adjacent to the VTA, Ikemoto et

al., 1997c) implicating the resultant increase in extracellular dopamine in the mediation of drug reward. Imaging studies of human cocaine and opiate addicts also implicate the NAcc region in mediation of drug effects and drug salience (Breiter and Rosen, 1999; Sell et al., 1999).

At this level and point of investigation, it is difficult to argue against some dopaminergic mediation of drug reinforcement processes. However, the fact that dopamine transporter knockout mice, although hyperactive and insensitive to the locomotor stimulant effects of cocaine and amphetamine (Giros et al., 1996), did acquire significant cocaine-induced conditioned place preference (Sora et al., 1998) implicates additional systems, perhaps serotonergic (Ritz et al., 1987), in the mediation of drug reward and the need for detailed investigation. There is redundancy in the dopamine system in the mediation of drug effects and the design of potential therapeutic agents should take such redundancy into consideration (Sora et al., 1998). Since there are many components of the mesolimbic dopaminergic system including different subregions, different receptors and associated second messenger systems, and reuptake mechanisms, in addition to other modulatory neurotransmitter systems such as GABA and glutamate, much detail relating dopamine and drug reinforcement processes remains to be elucidated and discovered.

Drug Sensitization and Dopamine

Because of the proposed association between drug sensitization and drug reinforcement (Stewart et al., 1984; Wise and Bozarth, 1987; Robinson and Berridge, 1993), a large body of literature has been devoted to the elucidation of the neural adaptations associated

with the development and expression of drug sensitization. Interestingly, and perhaps not coincidentally, drug sensitization, like drug reinforcement, is also associated with enhanced dopaminergic activity (Robinson, 1993; Kalivas and Nakamura, 1999). Sensitization to the stimulant effects of a single amphetamine administration was associated with enhanced electrically evoked dopamine release from the NAcc, with both the behavioral expression of sensitization and the increase of dopamine release having greater magnitudes at 3 weeks, relative to 3 days, following amphetamine pre-exposure (Vanderschuren et al., 1999a). Similarly, repeated systemic cocaine administration, which resulted in behavioral sensitization, also enhanced extracellular dopamine in the NAcc beyond the increase induced by acute cocaine administration assessed 1 week following the final cocaine injection (Steketee, 1998). Such results suggest a sensitization of the dopamine response that is temporally parallel to the presence of behavioral drug sensitization.

Efforts to identify brain regions involved in psychostimulant sensitization have shown that repeated intra-VTA amphetamine administration resulted in sensitization to the effects of systemic and intra-NAcc amphetamine challenge and in cross-sensitization to intra-NAcc cocaine challenge (Cador et al., 1995; Bjijou et al., 1996). On the other hand, repeated intra-NAcc amphetamine administration did not result in sensitization to systemic or intra-NAcc amphetamine challenge (Cador et al., 1995). These data indicate that neuroadaptations in both the VTA and NAcc occur as a result of amphetamine administration, however these two regions may subserve different aspects, such as development versus expression, of psychostimulant sensitization (Cador et al., 1995; Li et al., 1997). Thus, whereas the VTA may be essential for the development and expression

of amphetamine sensitization, the NAcc may only underlie the expression of amphetamine sensitization (Vezina and Stewart, 1990; Cador et al., 1995). Investigation of the brain areas subserving morphine sensitization has shown that challenge morphine administered 15 days following a regimen of repeated systemic morphine injections resulted in an increase in extracellular dopamine concentrations in the caudate and in the core subregion of the NAcc, and a decrease in extracellular dopamine in the shell subregion of the NAcc (Cadoni and Di Chiara, 1999). In the same study, challenge amphetamine and cocaine administrations did not result in behavioral sensitization or in a change in extracellular dopamine levels, indicating an association between enhanced extracellular dopamine levels and behavioral sensitization (Cadoni and Di Chiara, 1999). Cross-sensitization studies have shown that repeated systemic morphine resulted in sensitization to challenge intra-NAcc amphetamine and cocaine (Cunningham et al., 1997). Intra-NAcc morphine also resulted in sensitization to a challenge intra-NAcc amphetamine administration (Cunningham et al., 1997). These results suggest morphine-induced adaptations in the NAcc that are similar to the neural targets of cocaine and amphetamine. Conversely, repeated intra-VTA amphetamine administration resulted in sensitization to the effects of systemic and intra-VTA morphine; intra-NAcc amphetamine did not result in sensitization to systemic morphine (Cador et al., 1995; Bjijou et al., 1996). These results suggest amphetamine-induced adaptations in the VTA are common to the sites of action of morphine, but do not support the idea of a common neural target in the NAcc. A morphological study of amphetamine and cocaine sensitized rats also showed that both groups had greater number of spines and greater branching in medium spiny neurons of the shell subregion of the NAcc (Robinson and Kolb, 1999).

Dopamine receptors have also been associated with psychostimulant sensitization. Amphetamine-sensitized rats showed attenuated locomotor responses to the dopamine D_2/D_3 receptor agonist, bromocriptine, and to the D_2 agonist, quinpirole, in addition to decreased dopamine D_2 receptor binding in the ventral striatum assessed 8 to 10 days after the final amphetamine administration (Chen et al., 1999). These results suggest alterations in dopamine receptor sensitivity as a function of repeated drug administration. The dopamine D_1 receptor antagonist SCH-23390, when administered to the VTA prior to repeated systemic cocaine injections, had no effect on the development of behavioral sensitization to cocaine, however it did block the cocaine-induced increase in extracellular dopamine levels in the NAcc (Steketee, 1998). These results indicate that VTA dopamine D_1 receptors may not be necessary for the development of behavioral sensitization to the effects of cocaine, but they may be necessary for cocaine-induced dopamine release in the NAcc. In order to better understand the roles that the dopamine D_1 and D_2 receptors in the VTA and NAcc might play in cocaine sensitization, these receptors were stimulated through repeated administration of their respective agonists SKF-38393 and quinpirole, separately and together, in essence mimicking the effects of repeated cocaine administration (Henry et al., 1998). It was shown that SKF-38393 administration induced dopamine D_1 receptor supersensitivity in the NAcc, while quinpirole administration induced dopamine D_2 receptor subsensitivity in the VTA and NAcc (Henry et al., 1998). The different treatment groups were tested for locomotor cross-sensitization to a challenge cocaine administration 1 day, 1 week, and 1 month after the final agonist administration. Interestingly, long term cross-sensitization was observed only in animals that had received both agonists together, suggesting that cocaine

sensitization is mediated through alterations in sensitivities of both D_1 and D_2 receptors in the VTA and NAcc (Henry et al., 1998).

Substrates of morphine sensitization appear to be similar to those of psychostimulant sensitization. Significant cross-sensitization between the effects of morphine and those of cocaine or amphetamine, for example, implies some dopaminergic mediation of morphine sensitization since cocaine and amphetamine are known to directly affect dopaminergic function (Ritz et al., 1987; Giros et al., 1996; Wu and Gu, 1999). In addition, morphine pre-treatment led to an enhanced response to the dopamine reuptake inhibitor GBR-12909 (Vanderschuren et al., 1999b), implying dopaminergic reuptake mechanisms that are known sites of cocaine and amphetamine action (Baldo and Kelley, 1991; Giros et al., 1996) in the mediation of the effects of morphine as well.

Repeated administration of selective dopamine agonists may also result in sensitization to their locomotor stimulant effects. Repeated systemic administration of the D_1/D_2 receptor agonist, apomorphine, led to the development of sensitization in both rats (Mattingly and Rowlett, 1989; Rowlett et al., 1991) and mice (Bedingfield et al., 1996; Battisti et al., 1999). Apomorphine-induced sensitization has also been associated with an increase in dopamine synthesis in the striatum (Rowlett et al., 1993). Repeated stimulation of D_1 and D_2 receptors via administration of their respective agonists, SKF-38392 and quinpirole, did not result in behavioral sensitization, however both pre-treatments cross-sensitized to a challenge apomorphine administration (Mattingly et al., 1993). Repeated administration of the dopamine reuptake inhibitor GBR-12909 also resulted in sensitization to its locomotor stimulant effects (Kelley and Lang, 1989). In addition, repeated administration of the NMDA receptor antagonist, MK-801, which has

been shown to block the development of drug sensitization (Kim et al., 1996; Broadbent and Weitemier, 1999) and to block sensitization to drug reward (Schenk et al., 1993), may also result in sensitization to its stimulant effects (Carey et al., 1995; Jessa et al., 1996; Itzhak and Martin, 1999). Taken together, these data further implicate components of the dopaminergic and associated systems in the mechanisms underlying mediation of behavioral sensitization.

The dose and temporal characteristics of drug administration can affect whether or not behavioral sensitization is observed (Kuribara, 1996; Vanderschuren et al., 1997b). One study took this into consideration and showed that behavioral sensitization developed following different amphetamine pre-treatment regimens, however there was no evidence for enhanced release of dopamine measured by *in vivo* microdialysis in the caudate-putamen or the NAcc for any of the drug regimens used (Kuczenski et al., 1997). Such results may reflect the role that other neurotransmitter systems may play in the mediation of drug sensitization. For example, amphetamine administration enhanced acetylcholine release from the NAcc, caudate, and medial prefrontal cortex (Vanderschuren et al., 1999a). Intra-VTA administration of a cholinergic agonist and antagonist both decreased alcohol-drinking behavior (Katner et al., 1997). NMDA and AMPA receptor antagonists disrupted aspects (development or expression) of psychostimulant sensitization (Ohmori et al., 1994; Li et al., 1997). Repeated systemic cocaine administration resulted in sensitization to the stimulant effects of cocaine, in enhancement of glutamate, in addition to dopamine, release in the NAcc (Reid and Berger, 1996), and in an increase in glutamate receptor subunit levels in the VTA (Churchill et al., 1999). Lesions of the pre-frontal cortex, from which glutamatergic

neurons project to the NAcc, blocked behavioral sensitization to cocaine and cocaine-induced VTA and NAcc neuroadaptations (Pierce et al., 1998; Li et al., 1999). The NMDA antagonist, MK-801 blocked the development of morphine sensitization at some doses (Jeziorski et al., 1994), although this effect has not always been shown (Vanderschuren et al., 1997a). The selective serotonin reuptake inhibitor, fluoxetine, blocked sensitization to the stimulant effects of morphine (Sills and Fletcher, 1997) implicating serotonin in the mediation of opiate sensitization. Finally, $GABA_A$ and benzodiazepine antagonists decreased EtOH drinking behavior and were self administered into the VTA (Ikemoto et al., 1997c; June et al., 1998a; June et al., 1998b; Nowak et al., 1998). It is important to keep in mind that the systems implicated in mediation of drug sensitization could be exerting their effects via interactions with the dopamine system (Kalivas and Nakamura, 1999).

Ethanol Reinforcement and Dopamine

The dopamine hypothesis of drug reinforcement applies to ethanol (EtOH) reinforcement as well. Systemic acute administration of a range of EtOH doses enhanced extracellular dopamine levels in the NAcc and striatum (Di Chiara and Imperato, 1988). Microinjection of the dopamine D_2 receptor agonist, quinpirole, into the NAcc tended to enhance EtOH-reinforced operant responding, whereas microinjection of the D_2 receptor antagonist, raclopride, significantly decreased EtOH-reinforced responding (Samson et al., 1992). In the same study, microinjection of amphetamine into the NAcc also significantly increased EtOH-reinforced operant responding (Samson et al., 1992). These data suggest that the D_2 receptor and dopamine reuptake mechanisms in the NAcc likely

mediate some of the reinforcing properties of EtOH. Activation or blockade of D_2 autoreceptor function affects synaptic dopamine availability with activation leading to a decrease and blockade leading to an increase. If an increase in dopamine is in fact experienced as rewarding, it can be seen how a decrease in dopamine might be behaviorally manifest as an increase in EtOH self-administration, whereas an increase in dopamine would be manifest as a decrease in EtOH self-administration. Bilateral lesions of the NAcc attenuated acquisition of voluntary EtOH drinking behavior but had no effect on established EtOH drinking behavior (Ikemoto et al., 1997b). These results indicate that NAcc dopamine may be necessary for the initial experience of drug reward but that subsequent drug reinforcement and maintenance of drug taking behavior recruits and involves other brain areas or neurotransmitter systems.

Many studies have examined and compared dopaminergic functioning between lines of rats genetically selected for high or low alcohol preference drinking. Because EtOH drinking behavior is used to index EtOH reinforcement, and assuming that dopamine mediates reinforcing drug properties, it has been rationalized that differences in dopaminergic activity should be observed between the selected lines. Systemic EtOH administration has consistently resulted in an increase in extracellular dopamine levels in the NAcc in genetically heterogeneous rats and in selectively bred alcohol preferring and alcohol avoiding lines (Di Chiara and Imperato, 1988; Yoshimoto et al., 1992; Blomqvist et al., 1993; Kiianmaa et al., 1995; Nurmi et al., 1996). However, no differences were found in the magnitude of EtOH-induced increase in extracellular dopamine concentrations between alcohol preferring HAD and AA and alcohol avoiding LAD and ANA lines of rats (Yoshimoto et al., 1992; Kiianmaa et al., 1995; Nurmi et al., 1996).

Such results indicate that the genes underlying differential voluntary EtOH consumption may differ from those involved in the acute effects of EtOH on dopamine release. In contrast, when access to EtOH was voluntary, alcohol preferring P rats self-administered EtOH directly into the VTA, whereas alcohol non-preferring NP rats did not (Gatto et al., 1994), implying some involvement of neural pathways in this brain region in the differential EtOH self-administration of these lines. In addition, operant oral self-administration of EtOH resulted in greater extracellular dopamine levels in the NAcc of P rats relative to the genetically heterogeneous Wistar rats (Weiss et al., 1993). These results may indicate differences in dopamine response based on whether the drug is passively or actively acquired (Nurmi et al., 1996).

The DBA/2J and C57BL/6J inbred mouse strains differ remarkably in their degree of voluntary EtOH consumption, with the DBA/2J mice showing alcohol avoidance and C57BL/6J mice showing alcohol preference (Belknap et al., 1993; Phillips et al., 1994a; Rodriguez et al., 1995). Efforts to identify neural substrates of this difference have shown that naïve C57BL/6J mice have greater dopamine D_1 and D_2 receptor mRNA and expressed protein in the striatum relative to naïve DBA/2J mice (Ng et al., 1994). The authors postulated that a lower basal dopaminergic activity of the C57BL/6J mice has led to compensatory changes in dopamine receptor levels and may be the reason for the proclivity of these mice toward alcohol drinking. Accordingly, the same group of investigators showed that systemic pre-treatment of C57BL/6J mice with the dopamine D_1 or D_2 receptor agonists SKF-38393 and bromocriptine, respectively, drastically decreased voluntary EtOH consumption and preference (Ng and George, 1994). These data indicate that activation of the dopamine system through stimulation of D_1 and D_2

receptors is reinforcing and leads to a decrease in the motivation for EtOH consumption (Ng and George, 1994).

Dopamine neuron activity is modulated by tonically active GABA interneurons in the VTA, GABA projections from the NAcc to the VTA and glutamatergic projections from the prefrontal cortex to the mesolimbic dopamine system (Kalivas and Nakamura, 1999; Spanagel and Weiss, 1999). Presumably, stimulation of GABA interneurons will inhibit VTA dopamine neurons resulting in lowered dopaminergic output in the NAcc, while inhibition of GABA neurons will disinhibit dopamine neurons resulting in enhanced dopaminergic output in the NAcc. If dopamine function mediates drug reinforcement, it is reasonable to suppose that activation of the dopamine system, which might result in an increase in extracellular dopamine in the VTA and NAcc, would be reinforcing; conversely, inhibition of dopamine functioning, which might result in decreased extracellular dopamine in the VTA and Nacc, could be aversive. Behaviorally, then, dopamine activation might be manifest as a decrease in drug intake, whereas dopamine inhibition might be manifest as an increase in drug intake. Consistent with these notions are data showing that microinjection of the $GABA_A$ antagonist picrotoxin into the anterior VTA dose-dependently and reversibly suppressed EtOH drinking in alcohol preferring P rats (Nowak et al., 1998). Systemic benzodiazepine antagonist administration also decreased voluntary EtOH drinking (June et al., 1998a) and EtOH-reinforced responding (June et al., 1998b) in alcohol preferring P rats. This is interesting, since benzodiazepine antagonists have been shown to have no effect on GABA-stimulated Cl^- flux, whereas $GABA_A$ antagonists block Cl^- flux (Feldman, 1997; Torben Neelands, personal communication). Similarly, however, administration of the opiate

antagonist, methylnaloxonium, into the NAcc decreased operant self-administration of EtOH in genetically heterogeneous rats (Heyser et al., 1999). Conversely, the benzodiazepine midazolam, which would be expected to inhibit dopamine neurons, enhanced alcohol drinking and hedonic (forward and lateral tongue extensions and paw licks) responses to EtOH (Söderpalm and Hansen, 1998).

Overall, it appears that alterations in VTA and NAcc activation may be one mechanism that mediates the reinforcing effects of EtOH.

Ethanol-Induced Sensitization and Dopamine

Repeated administration of stimulant doses of EtOH or long term EtOH drinking may be associated with the development of behavioral sensitization and seems to also involve adaptations in the mesolimbic dopamine system. Rats kept on an EtOH diet for 12 weeks had enhanced tyrosine hydroxylase activity in the VTA and enhanced cAMP-dependent protein kinase activity in the NAcc, both measures of neuronal activation (Ortiz et al., 1995). There was no evidence for enhanced neuronal activity in either brain area of rats kept on the EtOH diet for 1 or 6 weeks, nor in isocalorically pair-fed EtOH-naïve controls (Ortiz et al., 1995). However, in another study, voluntary EtOH drinking for about 4 weeks resulted in an increase in electrically evoked dopamine from the NAcc, but not from the caudate-putamen, although EtOH experienced rats did show dopamine D_1 receptor supersensitivity in the caudate-putamen relative to their EtOH naïve counterparts, as assessed 3 weeks following termination of EtOH drinking (Nestby et al., 1999). Repeated EtOH administration also induced enhanced electrically evoked dopamine release from the NAcc, with a similar dopamine increase seen from the

caudate-putamen, assessed 3 weeks following the final EtOH administration (Nestby et al., 1997). Inconsistencies in the brain areas where neuroadaptations were seen may arise from procedural differences of self- versus experimenter-administered EtOH. For example, rats that self-administered EtOH under operant conditions did not show an increase in extracellular NAcc dopamine, but their yoked controls, that received EtOH intragastrically and non-contingently, did (Nurmi et al., 1996).

Cross-sensitization studies represent another, albeit more indirect way, of assessing neural mechanisms mediating EtOH-induced sensitization. For example, rats exposed for 3 weeks to voluntary EtOH drinking showed greater amphetamine-stimulated locomotor activity tested 3 weeks following termination of EtOH drinking (Fahlke et al., 1995). In addition, mice kept on an EtOH diet for 3 weeks showed sensitization to the locomotor stimulant effects of a challenge amphetamine administration and to potentiation of sensitization to repeated amphetamine and cocaine administration (Manley and Little, 1997). Since cocaine and amphetamine directly affect dopamine activity by way of their action on dopamine transporters (Wu and Gu, 1999), enhanced sensitivity to their effects in previously EtOH exposed rats implies that adaptations induced by EtOH are similar to the neural targets of cocaine and amphetamine. Repeated systemic EtOH administrations also led to an enhanced response to a challenge cocaine administration (Itzhak and Martin, 1999) implying some similarity in the neural targets of cocaine and neuroadaptations induced by EtOH exposure. In fact, it was also shown that EtOH treatment was associated with increased dopamine transporter binding in the striatum (Itzhak and Martin, 1999).

Investigation of dopamine receptors in the mediation of EtOH-induced sensitization has shown that administration of the general dopamine antagonist, haloperidol, had no effect on the development of EtOH-induced sensitization in DBA/2J inbred mice, even though it blocked acute EtOH stimulation (Broadbent et al., 1995). One study using genetically heterogeneous mice showed that EtOH-sensitized mice had increased dopamine D_2 receptor binding in the caudate relative to saline-treated controls and relative to EtOH-treated, but not behaviorally sensitized counterparts(Souza-Formigoni et al., 1999). There were no differences in receptor binding in the NAcc between the treatment groups (Souza-Formigoni et al., 1999). These data imply some dissociation between the general effects of EtOH administration and the actual development of behavioral sensitization to repeated EtOH administration. In addition, there is evidence to suggest the involvement of dopamine receptors, and specifically the D_2 receptors, in the mediation of EtOH-induced sensitization.

In addition to dopamine, GABA and glutamate have also been implicated in the mediation of sensitization to the stimulant effects of EtOH. The $GABA_B$ receptor agonist, baclofen, blocked both the development of EtOH-induced sensitization and acute EtOH stimulation, whereas the $GABA_A$ agonist, THIP, blocked only the acute EtOH stimulant response, and had no effect on EtOH-induced sensitization (Broadbent and Harless, 1999). The same group of investigators also showed that the NMDA receptor antagonist, MK-801, blocked both the acute and sensitized EtOH responses (Broadbent and Weitemier, 1999).

Summary

Drug administration seems to affect dopaminergic function through increases in extracellular dopamine, decreases in reuptake, and alterations in dopamine receptor sensitivity in the VTA, NAcc, or caudate-putamen. These changes seem to be associated both with the rewarding properties of drugs of abuse and with the development of sensitization to the locomotor stimulant effects of drugs of abuse, implicating at least one common neural substrate in the multiple effects of drugs of abuse. Elucidating commonalities of drug action may increase our understanding of the phenomenon of drug abuse in general (Newlin, 1994).

Inconsistencies in the implication of dopaminergic mediation of drug sensitization and reward (Maldonado et al., 1997; De Vries et al., 1999) may arise from the employment of many different drug administration regimens and drug doses used to induce drug sensitization, the different behavioral paradigms used to assess drug reward, the different procedures used to assess drug-induced neuroadaptation, and strain differences in rodent animal models. For instance, drugs have been administered once or on multiple occasions, daily or intermittently, systemically (intraperitoneally or subcutaneously) or microinjected directly into a brain area of interest, and their effects have been assessed at different times following termination of drug administration. Drug reinforcement has been measured in operant self-administration paradigms, in yoked control situations, in conditioned place preference paradigms and in voluntary or forced EtOH drinking situations. Neuroadaptation has been assessed via *in vivo* microdialysis, electrically evoked neurotransmitter release in brain slices, *in vitro* electrophysiological recordings, receptor binding assays, and site-specific injections of receptor agonists or

antagonists. In addition, rats and mice, as well as specific genetic models of each species have been employed. It is important to note that this plethora of experimental design differences can be applied to the rest of the neurotransmitter systems (GABA, glutamate, serotonin) implicated in the mediation of drug effects. Amidst this diversity of methodologies, the general consistency implicating dopaminergic mediation of drug action is quite remarkable.

It should also be noted that there are data showing drug self-administration behavior that is not accompanied by detectable alterations of dopaminergic function (Nurmi et al., 1996; Kuczenski et al., 1997), in addition to data showing enhanced dopaminergic activity in anticipation, rather than acquisition, of drug reward (Weiss et al., 1993; Hollerman and Schultz, 1998). It has recently been suggested that dopamine may act as a signal of the availability of drug reward and may participate in motivational processes that result in drug acquisition, but not in the specific processes of drug reward (Nurmi et al., 1996; Berridge and Robinson, 1998; Kalivas and Nakamura, 1999; Spanagel and Weiss, 1999; Stefanski et al., 1999).

Dissertation Goals

Sensitization to the stimulant effects of EtOH has been much less studied than psychostimulant-induced sensitization and investigation of a possible association between EtOH-induced sensitization and EtOH reinforcement is sorely lacking. Therefore, in this dissertation project, behavioral sensitization to the effects of EtOH is the central focus and topic of investigation. From a purely behavioral perspective, the experiments aimed at elucidating (1) whether some of the mechanisms mediating EtOH-induced sensitization

also mediate sensitization to the effects of morphine, cocaine, or methamphetamine and (2) whether neuroadaptations manifested as behavioral sensitization to the stimulant effects of EtOH also underlie EtOH reinforcement and aversion processes. Presence of cross-sensitization would imply similarity in mechanisms underlying drug sensitization processes. There are only three other studies that have specifically assessed cross-sensitization between the stimulant effects of EtOH and those of morphine, cocaine, or amphetamine (Wise et al., 1996; Nestby et al., 1997; Itzhak and Martin, 1999) using a systemic injection paradigm similar to the one used in the present experiments. Two of these studies were performed in mice and were inconsistent, one showing significant reciprocal EtOH-cocaine cross-sensitization (Itzhak and Martin, 1999) and the other showing no cross-sensitization to a challenge cocaine administration following repeated EtOH injections (Wise et al., 1996). Repeated EtOH administration in rats did not result in the development of EtOH-induced sensitization, although there was significant cross-sensitization to a challenge morphine, but not amphetamine, administration (Nestby et al., 1997). Rats may not be appropriate models for experiments examining EtOH-induced sensitization, as they generally do not show stimulant responses to EtOH (Masur et al., 1986). The present cross-sensitization experiments entailed a more detailed investigation of EtOH-drug cross-sensitization using genetically heterogeneous mice that have been shown to develop lasting and robust EtOH-induced sensitization (Lessov and Phillips, 1998). Repeated EtOH exposures were followed by challenge administrations of one of several doses of morphine, cocaine, or methamphetamine administered 24 hours after the final EtOH injection.

The second aim was addressed by determining whether repeated EtOH administration altered subsequent voluntary EtOH drinking or EtOH-induced conditioned taste aversion. Alterations in these two EtOH-induced behaviors as a consequence of repeated EtOH exposure would imply that neuroadaptations that may underlie EtOH-induced sensitization may be similar to those that underlie EtOH reinforcement and aversion processes. There are no studies that have examined the effect of repeated EtOH administration on EtOH drinking behavior and that have concurrently assessed the possible development of sensitization to the effects of EtOH. Sparse data indicate that continuous morphine, amphetamine, or nicotine exposure increased subsequent voluntary EtOH consumption (Potthoff and Ellison, 1982; Potthoff et al., 1983; Hubbell et al., 1988) while repeated cocaine administration decreased voluntary EtOH consumption (Uemura et al., 1998). These studies suggest that prior drug exposure alters subsequent alcohol drinking likely through some alterations in brain reinforcement mechanisms. There are also no studies that have repeatedly administered EtOH, measured behavoral sensitization to the effects of EtOH, and determined EtOH-induced conditioned taste aversion. Repeated EtOH administration has been shown to attenuate subsequent EtOH-induced conditioned taste aversion (Risinger and Cunningham, 1995), however no assessments of the effect of EtOH pre-exposure on the development of sensitization were made. An association between EtOH-induced sensitization and EtOH reinforcement would be strengthened if it could additionally be shown that voluntary EtOH consumption could induce sensitization to the activating effects of an acute EtOH injection. There is one EtOH-specific report showing that the alcohol-preferring C57BL/6J mice had greater activity response to EtOH administration following a period

of voluntary EtOH drinking, relative to pre-drinking EtOH-induced activity levels (Nocjar and Middaugh, 1997). Regarding other drugs, intravenous cocaine and operant heroin self-administration sensitized rats to the effects of challenge administrations of cocaine and heroin, respectively (Phillips and Di Ciano, 1996; De Vries et al., 1998; Marinelli et al., 1998). The present experiments were specifically designed to include an assessment of the development of EtOH-induced sensitization subsequent to voluntary EtOH consumption.

Given the sparseness of studies examining EtOH-induced sensitization in general, and the lack of studies examining the effect of EtOH-induced sensitization on other EtOH-related behaviors, in particular, the proposed experiments will begin to elucidate the role and potential importance of neuroadaptations manifested as behavioral sensitization to the stimulant effects of EtOH in the etiology of alcohol abuse.

Assessment of Cross-Sensitization Between the Locomotor Stimulant Effects of Ethanol and Those of Morphine, Cocaine and Methamphetamine

INTRODUCTION

Repeated administration of psychostimulants (Segal and Mandell, 1974; Post and Rose, 1976; Shuster et al., 1977; Hirabayashi et al., 1991; Paulson et al., 1991), opiates (Babbini et al., 1975; Shuster et al., 1975; Volpicelli et al., 1999), and ethanol (Masur and Boerngen, 1980; Masur et al., 1986; Phillips et al., 1994b; Phillips et al., 1995; Phillips et al., 1996) may lead to an increase in their locomotor stimulant effects, or behavioral sensitization. Because stimulant drug effects are thought to represent positive or euphoric drug properties, enhancement of these properties through a process such as sensitization is of great interest as one way of elucidating pathways of drug reward and reinforcement.

The mechanisms of psychostimulant sensitization have been widely studied. It appears that the ventral tegmental area (VTA) is important for initiation and expression of sensitization to the effects of amphetamine with ensuing increases in dopaminergic output in the nucleus accumbens (NAcc) and striatum (Vezina and Stewart, 1990; Bjijou et al., 1996; Cador et al., 1995). At least one study, however, shows no change in dopamine outflow in the NAcc or striatum as a function of amphetamine sensitization (Kuczenski et al., 1997). Cross-sensitization studies employing pharmacological agents support the involvement of dopamine reuptake mechanisms (Baldo and Kelley, 1991; Elmer et al., 1996), dopamine receptor systems (Henry et al., 1998; Steketee, 1998; Chen et al., 1999; Hondo et al., 1999; Vanderschuren et al., 1999d), and the glutamate NMDA

receptor in sensitization to the effects of amphetamine and cocaine (Ohmori et al., 1994; Churchill et al., 1999; Lu and Wolf, 1999; O'Neill and Sanger, 1999). Morphine-induced sensitization also appears to be mediated by dopaminergic (Cadoni and Di Chiara, 1999), perhaps glutamatergic (Iijima et al., 1996; Vanderschuren et al., 1997a; Carlezon et al., 1999), as well as serotonergic (Sills and Fletcher, 1997) mechanisms.

Since drugs of abuse likely share some common factors that mediate their abuse potential, it is important to determine overlapping mechanisms of drug action as a way to elucidate the phenomenon of drug addiction in general. Cross-sensitization studies provide a measure for assessing common behavioral and neurochemical factors associated with drug sensitization. It is no surprise that cross-sensitization between the stimulant effects of cocaine and those of the amphetamines has been consistently shown (Hirabayashi et al., 1991; Schenk et al., 1991; Cador et al., 1995; Bonate et al., 1997; Vanderschuren et al., 1999b) since these two drugs directly affect dopaminergic activity (Ritz et al., 1987; Giros et al., 1996). Cross-sensitization between the effects of morphine and those of cocaine and amphetamine has also been shown (Vezina et al., 1989; Vezina and Stewart, 1990; Cador et al., 1995; Bjijou et al., 1996; Vanderschuren et al., 1997b; Vanderschuren et al., 1999b; Vanderschuren et al., 1999c;), although much less consistently (Cadoni and Di Chiara, 1999), and not always reciprocally with treatment of one drug leading to enhanced response to a second drug and vice versa (Shuster et al., 1975; Vanderschuren et al., 1999c).

In the present experiments, it was of interest to determine whether cross-sensitization exists between the stimulant effects of EtOH and those of morphine, cocaine, and methamphetamine. Since morphine, cocaine, and methamphetamine have

specific neural targets (Ritz et al., 1987; Giros et al., 1996; Matthes et al., 1996; Kieffer, 1999; Wu and Gu, 1999), the present cross-sensitization experiments could both provide or eliminate potential targets for further investigation of mechanisms mediating EtOH-induced sensitization. Studies investigating neuroadaptations resulting from repeated EtOH administration have shown activation and changes in several neurotransmitter systems. For instance, repeated EtOH administration in rats resulted in an enhancement of dopamine and acetylcholine release in the NAcc and caudate-putamen (Nestby et al., 1997). Similar changes were observed following repeated administration of morphine, cocaine, and amphetamine indicating common neuroadaptations resulting from administration of EtOH and other drugs of abuse (Nestby et al., 1997). Behaviorally, the EtOH treated rats did not show sensitization to the effects of EtOH, and showed cross-sensitization to morphine, but not amphetamine, challenge injections (Nestby et al., 1997). In one study, mice that were repeatedly administered EtOH were separated into sensitized and non-sensitized groups (Souza-Formigoni et al., 1999). It was shown that the sensitized group had increased dopamine D_2 receptor binding in the caudate-putamen relative to both the non-sensitized group and to the saline control group (Souza-Formigoni et al., 1999). There were no group differences in D_2 receptor binding in the NAcc (Souza-Formigoni et al., 1999). Another study also performed in mice, showed evidence for sensitization and reciprocal cross-sensitization between the effects of EtOH and those of cocaine (Itzhak and Martin, 1999). In addition, both EtOH and cocaine treatment were associated with an increase in dopamine transporter binding in the striatum relative to saline treated controls (Itzhak and Martin, 1999).

Thus, it appears that neuroadaptations mostly related to the dopaminergic system occur in the NAcc and the striatum in response to repeated EtOH administration. Some of these adaptations may be common to the effects of several drugs of abuse. The presence of reciprocal cross-sensitization between EtOH and cocaine (Itzhak and Martin, 1999), for instance, implicates common dopaminergic mediation of sensitization to the effects of these drugs. The presence of cross-sensitization between EtOH and morphine (Nestby et al., 1997) likely indicates both GABAergic and dopaminergic mediation of sensitization as morphine acts via μ opioid receptors located on GABA interneurons, which modulate activity of dopamine neurons in the VTA and in the NAcc (Kalivas and Nakamura, 1999).

Pharmacological assessment of GABA involvement in EtOH-induced sensitization has shown that administration of the $GABA_A$ agonist, THIP, prior to repeated EtOH injections had no effect on the development of EtOH-induced sensitization; administration of the $GABA_B$ agonist, baclofen, on the other hand, blocked the development of EtOH-induced sensitization (Broadbent and Harless, 1999). One other study and experiments in our laboratory have not been able to show an effect of baclofen on EtOH-induced sensitization (Chester and Cunningham, 1999; unpublished observations). In addition, administration of the general dopamine antagonist, haloperidol, also failed to block the development of EtOH-induced sensitization (Broadbent et al., 1995). It is clear that while investigation of the neuroadaptations underlying EtOH-induced sensitization is under way, the studies are few, and much more information is necessary to allow for reasonable conclusions to be reached.

The present experiments initiated a behavioral investigation into the mechanisms mediating EtOH-induced sensitization. Mice were repeatedly administered EtOH followed by acute challenge administrations of one of four doses of morphine, cocaine, or methamphetamine. The reciprocal conditions, that of repeatedly treating with morphine, cocaine, or methamphetamine, followed by acute challenge EtOH administrations, are planned as future studies. Presence of cross-sensitization would imply some commonality in mechanisms mediating sensitization to the effects of EtOH and those of the other three drugs tested.

METHODS

Subjects

Female mice from two replicate lines of genetically heterogeneous populations were used because females have been previously used in EtOH-induced sensitization studies in this laboratory and because they have been shown to develop more robust sensitization than males (Robinson, 1984; Cailhol and Mormède, 1999). These lines have been maintained as control populations for selective breeding experiments (Crabbe et al., 1985) and are termed WSC1 and WSC2. Mice were kept on a 12:12 hour light:dark cycle (lights on at 0600 hr), with water and food available *ad libidum* except during behavioral testing. Animals ranged in age from 51 to 98 days old at the beginning of the experiments. There were 10-12 animals per treatment group. Studies were performed in accordance with the Institutional Animal Care and Use Committee and National Institutes of Health guidelines for the care and use of laboratory animals.

Drugs

Morphine sulfate (RBI), cocaine hydrochloride (Sigma), and methamphetamine hydrochloride (Sigma) were dissolved in saline (0.9% NaCl) for injection volumes of 10 ml/kg. Ethanol (EtOH, 200 proof, AAPER and Pharmco) was diluted in saline for a 20% v/v solution. All injections were intraperitoneal (i.p.).

Apparatus

Mice were contained in clear acrylic plastic boxes (40 x 40 x 30 cm) and tested in automated locomotor activity monitors (Accuscan, Columbus, OH) housed in sound attenuating chambers. Lights were mounted near the ceiling at the center of the rear wall of each chamber and fans were mounted in the rear right top corner of each chamber wall. During testing lights and fans were on inside the chambers; fans provided ventilation and masking noise. Eight infrared beams were mounted 2 cm above the test chamber floor outside an acrylic plastic test box on two perpendicular panels. Eight detectors were mounted on the opposing panels. As a mouse moved about the chamber floor, the infrared beams were interrupted and each interruption was automatically recorded as an activity count. Data were automatically translated to horizontal distance traveled (cm). The activity monitors automatically record several behavioral measures, including stereotopy such as rearing and repetitive head movements. Horizontal distance traveled was the chosen measure of activity because the stereotopy measure has been found unreliable (Tamara Phillips, personal communication).

Procedure

Three experiments were conducted, each assessing the presence of cross-sensitization between the stimulant effects of EtOH and those of morphine (Exp. 1), cocaine (Exp. 2) or methamphetamine (Exp. 3). For all experiments, mice were randomized into EtOH or saline treatment groups. Twenty-four hours following the final EtOH or saline treatment, each group received a challenge administration of one of four doses of morphine, cocaine, or methamphetamine. Twenty-four hours following the challenge injections, all mice were tested following saline administration to assess whether the experimental procedures and drug administration altered baseline activity.

Experiment 1: EtOH-Morphine Cross-Sensitization

EtOH-induced sensitization: The EtOH-induced sensitization protocol and challenge drug administrations are shown in Table 1. Littermates were randomly assigned to a Saline Control, EtOH Sensitized, EtOH Naïve, or Untreated treatment group. On activity test days, mice were weighed, injected with either saline or EtOH and immediately placed in the center of an activity monitor. Data were collected for 10 min in two 5-min epochs. On Days 1 and 2, all mice were tested following saline injections. Day 1 served as an apparatus habituation test day. Day 2 served as the activity baseline test day. On Day 3, the EtOH Sensitized group was tested following 2 g/kg EtOH, while the rest of the treatment groups were tested following saline injections. For the subsequent consecutive 10 days comprising the daily treatment period, mice were injected daily with either saline (Saline Control and EtOH Naïve groups), 2.5 g/kg EtOH (EtOH Sensitized group), or received no injections (Untreated group). Following injections, animals were returned to

their home cages; no activity testing took place during this period. On Day 14, Saline Control, EtOH Sensitized, and Untreated groups were tested following 2 g/kg EtOH, while the EtOH Naïve group was still tested following saline injections.

Treatment Groups: The Saline Control group received saline throughout the experiment, except for an acute EtOH administration on the last EtOH test day. The EtOH Sensitized group received EtOH administration throughout the experiment after 2 days of saline baseline testing. Comparison of the activity response to EtOH between the EtOH Sensitized and Saline Control groups on the last EtOH test day served as a measure of *between-group* sensitization. The EtOH response of the EtOH Sensitized group on Day 14 relative to its acute EtOH response on Day 3 served as a measure of *within-group* sensitization. The EtOH Naïve group received saline throughout the experiment and served as a control for the possible effects of a single EtOH administration (Saline Control) on subsequent response to the challenge drug administrations. The Untreated group did not receive daily injections during the 10-day treatment phase, and served as a control for the possible effects of handling and injection stress on the development of EtOH-induced sensitization (Roberts et al., 1995) by comparing its response to the Saline Control and EtOH Sensitized groups.

Cross-Sensitization Test: On Day 15, 24 hours following the last EtOH or saline administration, mice from each Saline Control, EtOH Sensitized, EtOH Naïve and Untreated treatment group were pseudo-randomized (see below) into four morphine dose subgroups (n=10-11 per subgroup) that received challenge administrations of either 0, 5, 10 or 20 mg/kg morphine immediately prior to 60-min activity tests. On Day 16, all mice were tested for 60 min following saline injections. This test day was included in order to

assess the possible effects (e.g. conditioning) of EtOH treatment and/or morphine administration on baseline activity.

Pseudo-randomization: Because a genetically heterogeneous population of mice was used, there was variability in the degree of acute stimulant and sensitized responses to EtOH across individual mice. Therefore, individuals from each of the four treatment groups were pseudo-randomized into the four morphine dose groups based on their magnitude of EtOH-induced sensitization (EtOH Sensitized group; Day 14 Sensitized – Day 3 acute scores) or their magnitude of acute stimulant response to EtOH (Saline Control and Untreated groups; Day 14 acute – Day 2 baseline scores). For the sake of consistency, the EtOH Naïve group was also pseudo-randomized on the basis of its Day 14 saline – Day 2 baseline scores. This procedure ensured that each morphine dose group included an equal number of high and low EtOH responders.

Blood Ethanol Concentrations (BECs): On Day 14, immediately upon termination of the 10-min testing period, the EtOH-injected mice were removed from the activity monitors and were lightly restrained in a clear plastic cylinder allowing for the tail to protrude freely. Tail tips were snipped, 20 µl of blood was collected in borosilicate glass micro pipets (Fisherbrand, PA), and was immediately expelled into 1.7 ml snaplock microcentrifuge tubes containing 50µl of 5% $ZnSO_4$. Tubes were kept on ice for the duration of the experimental day. Subsequently, 50µl of $Ba(OH)_2$ and 300 µl of MilliQ water were added to each tube. Tube contents were thoroughly mixed and spun at 12,000 rpm for 5 min (Beckman Microfuge). The supernatant was collected, placed in 2 ml clear crimp vials (Hewlett Packard) and analyzed by a gas chromatograph (Hewlett Packard) with a flame ionization detector.

Experiments 2 and 3: EtOH-Cocaine and EtOH-Methamphetamine Cross-Sensitization

These experiments followed the exact same procedures as Exp.1 except that an Untreated group was not included. Exp. 1 showed no significant difference in the response to EtOH between the Saline Control and Untreated groups indicating that handling and injection stress, by themselves, did not significantly contribute to EtOH-induced sensitization, thus eliminating the need for a stress control group for these experiments. In Exp. 2, there were 10-11 mice per group; in Exp. 3, all groups consisted of 12 mice.

Statistical Analyses

Locomotor activity data were analyzed with two and three-way ANOVAs, with horizontal distance traveled (cm) and stereotypy counts (for cocaine and methamphetamine only) as the dependent variables, and Group (Saline Control, EtOH Sensitized, EtOH Naïve, and Untreated), Dose (0, 5, 10, and 20 mg/kg morphine, 0, 5, 10, and 20 mg/kg cocaine, or 0, 0,5, 1, and 2 mg/kg methamphetamine), and Test Day as the independent variables. Simple main effects analyses, one-way ANOVAs and Newman-Keuls Posthoc multiple comparisons tests were performed as appropriate when significant interactions from the overall ANOVAs were observed. BECs, age and body weight data were analyzed with one or two-way ANOVAs as appropriate.

RESULTS

Experiment 1: EtOH-Morphine Cross-Sensitization

Sensitization to the Stimulant Effects of EtOH

Data from the EtOH-induced sensitization phase are shown in Figure 1. The treatment groups showed a different pattern of response across test days due to group differences on Days 3 and 14 (Group x Test Day interaction effect, $F_{9,471}=71.2$, $p<0.001$). The EtOH Sensitized group (black circles) showed a significant acute EtOH stimulant response on Day 3. This group further showed an enhanced EtOH response on Day 14 relative to both the acute EtOH response of the Saline Control group (white circles) on that day, and their own acute EtOH response on Day 3 (all $p<0.001$), indicating the presence of between- and within-group sensitization to the effects of EtOH, respectively. Activity responses of the Saline Control and Untreated (black triangles) groups completely overlapped indicating no significant contribution of stress due to daily handling and injection on EtOH response.

EtOH-Morphine Cross-Sensitization

Activity data for the total 60-min morphine challenge activity test (Days 15) across morphine doses are presented in Figure 2A. If cross-sensitization were present, the EtOH Sensitized groups (black bars) would have shown enhanced responses relative to the Saline Control groups (white bars) at least at some of the morphine doses used. There was an overall morphine-induced dose-dependent increase in activity (main effect of Dose, $F_{3,147}=5.2$, $p<0.01$), but no evidence for cross-sensitization between the locomotor stimulant effects of EtOH and those of morphine. Because the greatest

stimulant response to morphine occurred at the end of the 60-min test session, the last 10-min time bin was separately analyzed (data not shown) to determine whether cross-sensitization might be seen at this time point. The pattern of response across EtOH treatment and morphine doses was similar to that shown in Figure 2A, and the overall morphine-induced dose-dependent activation persisted (main effects of Dose, $F_{3,147}=11.2$, $p<0.001$), but significant effects of EtOH treatment were not detected.

Baseline Activity

An effect of EtOH or morphine (e.g. conditioning) on baseline activity could be assessed by analyzing the 0 mg/kg dose groups on the morphine challenge day (Figure 2A) and all sets of groups on the subsequent saline challenge test day (Day 16, Figure 2B). Both analyses showed no effect of EtOH treatment or morphine administration on saline-induced baseline activity.

Blood Ethanol Concentrations (BECs)

The EtOH Naïve groups were not included in this analysis since their locomotor activity was never assessed following EtOH administration. The EtOH Sensitized group had significantly higher BECs (one-way ANOVA, main effect of Group, $F_{1,117}=8.2$, $p<0.001$) than either of their Saline Control or Untreated counterparts (both $p<0.01$), which did not differ from one another. Saline Control, EtOH Sensitized and Untreated group BEC means were 1.08 ± 0.06, 1.37 ± 0.06, and 1.09 ± 0.06 mg/ml, respectively.

Age and Body Weight

There were no significant age differences between the treatment groups. The means for the Saline Control, EtOH Sensitized, EtOH Naïve, and Untreated groups collapsed on morphine dose were 76.7 ± 1.63, 76.6 ± 1.59, 81.3 ± 2.01, and 77.2 ± 1.57 days, respectively. Body weight was analyzed using a two-way Group x Dose ANOVA for each of the 16 days of the experiment. Generally, body weights varied between 22-26 g and there were no significant differences between the different treatment groups.

Experiment 2: EtOH-Cocaine Cross-Sensitization

Sensitization to the Stimulant Effects of EtOH

Activity data from the sensitization portion of this experiment are presented in Figure 3. The Saline Control, EtOH Sensitized, and EtOH Naïve treatment groups showed different patterns of response across test days (Group x Test Day interaction effect, $F_{6,363}=107.4$, $p<0.001$). The EtOH Sensitized group (black circles) showed significant EtOH-induced stimulation on Day 3, and a subsequent enhanced EtOH response on Day 14 relative to both their acute response on Day 3 and to the acute EtOH stimulant response of the Saline Control group (white circles) on Day 14, indicating significant within- and between-group sensitization, respectively (both $p<0.001$).

EtOH-Cocaine Cross-Sensitization

Data from the 60 min activity tests on the cocaine challenge test day (Days 15) are presented in Figure 4, Panel A. There was an overall dose-dependent increase in activity across cocaine doses (main effect of Dose, $F_{3,114}=28.4$, $p<0.001$). Since sensitization to

the effects of EtOH was tested for 10 min, and since the cocaine stimulant response was most robust in the first 10 min of the 60-min activity test, the first 10-min time bin was separately analyzed. The data (not shown) looked largely similar to those in Figure 4A and showed an overall dose-dependent cocaine activation (main effect of Dose, $F_{3,114}=22$, $p<0.001$). It can be seen in Figure 4A that the EtOH Sensitized groups (black bars) in the 5 and 20 mg/kg cocaine sets of groups had lower activity levels than the Saline Control (white bars) and EtOH Naïve (hatched bars) groups, although no significant differences were detected. Since the EtOH Sensitized groups did not show enhanced activity relative to the Saline Control groups, it may be concluded that there was no evidence for cross-sensitization between the locomotor stimulant effects of EtOH and those of cocaine. In fact, prior EtOH exposure may have blunted the response to cocaine. There were no differences in activity between the Saline Control and the EtOH Naïve groups across cocaine doses indicating no effect of the single EtOH injection administered to the Saline Control groups 24 hours prior on subsequent response to cocaine.

Baseline Activity

Data from the total 60-min saline challenge activity test (Day 16) for each of the previously cocaine-administered sets of groups are presented in Figure 4, Panel B. An overall Group x Dose ANOVA revealed a significant effect of EtOH treatment (main effect of Group $F_{2,114}=3.4$, $p<0.05$) likely due to the persistently slightly blunted saline responses of the EtOH Sensitized groups previously treated with 5 and 20 mg/kg cocaine. However, separate one-way ANOVAs for each cocaine dose set of groups revealed no significant group differences. Analysis of the first 10-min bin (data not shown) of the 60

min test period still revealed the overall blunted response of the EtOH Sensitized groups relative to the Saline Control and EtOH Naïve groups (main effect of Group, $F_{2,114}=3.2$, $p<0.05$). Separate analyses for each cocaine dose set of groups revealed no significant group differences. Albeit not strong, these data suggest a cocaine-induced conditioned suppression of activity in the EtOH Sensitized groups. There was no effect of EtOH treatment on baseline activity, as no group differences were detected for the saline treated groups on both the cocaine- and saline-challenge days (Figure 4A and B).

Blood Ethanol Concentrations (BECs)

There were no BEC differences between the EtOH Sensitized and the Saline Control groups. The mean BEC values were 1.12 ± 0.06 and 1.03 ± 0.05 mg/ml, respectively.

Age and Body Weight

Treatment group age means ranged around 71-83 days at the beginning of the experiment. No significant group differences were detected. Body weight was analyzed using a two-way Group x Dose ANOVA for each day of the experiment. Body weight averages ranged between 22-35 grams and there were no differences between the treatment groups.

Experiment 3: EtOH-Methamphetamine Cross-Sensitization

Sensitization to the Stimulant Effects of EtOH

Activity data for the EtOH-induced sensitization phase are shown in Figure 5. The treatment groups had differential patterns of response across test days due to significant

group differences on Days 3 and 14 (Group x Test Day interaction effect, $F_{6,417}$=141.2, $p<0.001$). The EtOH Sensitized group (black circles) had an enhanced response to EtOH on Day 14 relative to the acute EtOH stimulant response of the Saline Control group on that day and relative to their own acute EtOH response on Day 3 (both $p<0.001$). These differences indicated the presence of significant between- and within-group sensitization, respectively.

EtOH-Methamphetamine Cross-Sensitization

Data from the 60 min activity test on the methamphetamine challenge test day (Days 15) are presented in Figure 6, Panel A. Methamphetamine administration induced a dose-dependent increase in locomotor activity regardless of EtOH treatment (main effect of Dose, $F_{3,130}$=33.7, $p<0.001$). There was no evidence for cross-sensitization between the effects of EtOH and those of methamphetamine since the EtOH Sensitized groups (black bars) did not show higher responses than the Saline Control groups (white bars) at any methamphetamine dose used. A single EtOH administration also did not appear to affect subsequent response to methamphetamine as no significant differences between the Saline Control and EtOH Naïve (hatched bars) groups were found. For the 0 mg/kg methamphetamine set of groups, there was a trend toward significant group differences with greater activity of the Saline Control group relative to the EtOH Sensitized and EtOH Naïve groups (Figure 6A, $F_{2,33}$=3.2, p=0.052) indicating possible conditioned activation to EtOH. The most robust stimulant effect of methamphetamine was seen from about 20 min to 40 min following methamphetamine injection (time course data not shown). Therefore, this 20 min time period was binned into two 10-min periods that

were separately analyzed. At both time points, there was an overall dose-dependent activation (main effects of Dose, $Fs_{3,130}$=27.7 and 29.7, both $p<0.001$, data not shown). In the 20-30 min time bin, as with the 60-min data, for the 0 mg/kg methamphetamine set of groups, the Saline Control group had significantly greater activity than both EtOH Sensitized and EtOH Naïve groups which did not differ from each other (both $p<0.05$) suggesting possible conditioned activation to EtOH. Visual inspection of Figure 6A showed that as with cocaine, there was a tendency toward a blunted methamphetamine response of the EtOH Sensitized groups relative to both the Saline Control and EtOH Naïve groups.

Baseline Response

Activity data for the saline challenge test day (Day 16) are shown in Figure 6, Panel B. There was an overall blunted response of the EtOH Sensitized groups and an overall greater activity levels for the groups administered 0.5 mg/kg methamphetamine on the previous test day relative to the other sets of groups (main effects of Group and Dose, $F_{2,130}$=7.3 and $F_{6,130}$=3.2, respectively, both $p<0.05$). These effects indicate some conditioning to the effects of methamphetamine administered 24 hours previously. Separate analyses for each set of methamphetamine dose groups did not reveal the source of the main effects. Similar results were obtained in the 20-30 min and 30-40 min time bins (main effects of Group and Dose, $Fs_{2,130}$=7.9 and 4.8, respectively, for the Group effect; $Fs_{3,130}$=3.8 and 4.6, respectively, for the Dose effect; all $p<0.05$, data not shown). Separate analyses for each set of methamphetamine dose subgroups for the 20-30 min and 30-40 min time bines showed that, in the 20-30 min time bin, for the set of groups

previously administered 0.5 and 2 mg/kg methamphetamine, the EtOH Naïve group had nearly greater response relative to the EtOH Sensitized group (main effects of Group, $F_{2,32}$=3.3, p=0.0501, and $F_{2,33}$=3.2, p=0.053, for each dose, respectively), while the EtOH Sensitized and Saline Control groups did not differ from one another. This may indicate a conditioned activation to 0.5 and 2 mg/kg methamphetamine that was blocked by acute or repeated EtOH administration.

Blood Ethanol Concentrations (BECs)

EtOH Sensitized mice had significantly higher BECs than Saline Control mice (1.52 ± 0.06 and 1.26 ± 0.06 mg/ml, respectively, $p<0.01$). Such a result could be troublesome because it allows for sensitization to the effects of EtOH to be interpreted as a function of EtOH pharmacokinetics rather than as a function of altered neural sensitivity. On the other hand, such a result could be explained as statistical artifact rather than true difference because of the high sample size (47-48 per group, collapsed on methamphetamine dose) and low standard errors.

Age and Body Weight

The average age for each treatment group ranged between 68-71 days. No significant differences in age were detected. Body weight was analyzed using two-way Group x Dose ANOVAs for each experimental day. The average body weight for each treatment group ranged around 22-25 g and no group differences were detected.

DISCUSSION

The present experiments investigated whether cross-sensitization exists between the stimulant effect of EtOH and those of morphine, cocaine, and methampethamine in a daily drug administration paradigm using genetically heterogeneous female mice. The results showed no evidence for cross-sensitization between EtOH and any of the drugs tested. This conclusion is tentative, however, since only half of the relevant experiments have been performed, that is, a regimen of repeated EtOH administration was followed by challenge injections of morphine, cocaine, and methamphetamine. A full investigation would additionally require repeated administrations of morphine, cocaine, and methamphetamine, followed by challenge injections of EtOH. Repeated EtOH administration may lead to slight alterations in sensitivity at a number of neural targets. Such small effects may not be detected by subsequent administration of drugs such as the opiates and psychostimulants that have specific neural targets (Ritz et al., 1987; Giros et al., 1996; Kieffer, 1999). Conversely, repeated administration of the opioids or psychostimulants might lead to larger alterations in sensitivity of their neural targets, which may be more readily detected by subsequent EtOH administration. This is one reason why reciprocal cross-sensitization designs may be important and informative.

Interestingly, a consistent trend toward a blunted response to some of the doses of morphine, cocaine, and methamphetamine was seen in the EtOH sensitized groups. This was not an artifact of the longer testing period during the drug challenge days, as the blunted response was also seen in the first 10 min of the 60 min tests. It could be a manifestation of cross-tolerance, since it is possible that during the EtOH sensitizing regimen, tolerance to some effect of EtOH developed. It has also been observed that up

to 5 days following EtOH withdrawal, dopaminergic activity in the ventral tegmental area (VTA) and striatum was reduced (Rossetti et al., 1992; Diana et al., 1996; Bailey et al., 1998). It is possible that in the present experiments, EtOH sensitized mice had a general reduction of dopaminergic function at the time of cross-sensitization testing and this reduction was behaviorally manifested as a blunted response to challenge drug administration. The fact that the EtOH Sensitized groups showed slightly different responses than both control groups may still imply that overlapping mechanisms mediate the effects of repeated EtOH administration and the effects of morphine, cocaine, and methamphetamine. Clearly, a single EtOH administration was not sufficient to induce changes in response to the three other drugs as no differences were detected between Saline Control and EtOH Naïve groups.

Even though manifestation of cross-sensitization between some drugs may be context-dependent (Bonate et al., 1997), the experimental procedure used in the present experiments provided little opportunity for conditioning to occur. No consistent effects were detected to suggest a role of conditioning on the magnitude of EtOH-induced sensitization.

In two of the three experiments, mice repeatedly treated with EtOH had significantly higher blood ethanol concentrations 10 min following EtOH injection, than their acutely EtOH treated counterparts. This may be troublesome, as sensitization to the effects of EtOH may be attributed to EtOH pharmacokinetics, rather than EtOH pharmacodynamics. However, the mean group differences were in the order of 0.3 mg/ml EtOH that is unlikely to underlie the robust behavioral differences. In addition, the statistical analyses may have resulted in significant differences because of the high

sample size (around 45 mice per group), and the small standard error associated with the measurements. We also previously determined that the sensitization paradigm used in the present experiments does not alter EtOH pharmacokinetic parameters regardless of whether tail or intraorbital blood samples were collected (Lessov and Phillips, 1998). Intraorbital blood samples are more representative of brain EtOH concentration and 10 min following EtOH injection, intraorbital blood EtOH concentrations are about twice as high as concentrations determined through tail blood sampling. Importantly, the development of EtOH-induced sensitization in the present experiments was most likely due to altered neural sensitivity, rather than altered EtOH kinetics.

To our knowledge, there are three other studies that examined cross-sensitization between EtOH and other drugs through injection paradigms similar to the one used here. Five daily injections of EtOH or cocaine induced sensitization and reciprocal cross-sensitization to the stimulant effects of each drug in male Swiss Webster mice (Itzhak and Martin, 1999). In female Swiss Webster mice, however, there was no evidence for the development of sensitization to the effects of EtOH following 10 daily EtOH administrations, nor cross-sensitization between the effects of EtOH and those of cocaine (Wise et al., 1996). The latter study (Wise et al., 1996) used lower doses of both EtOH and cocaine and cross-sensitization was assessed 24 hours following drug treatment, whereas the former study (Itzhak and Martin, 1999) assessed both sensitization and cross-sensitization after 10 days of drug withdrawal. The current experiments used the same 2 g/kg dose of EtOH and included the cocaine dose used in one of these studies (Itzhak and Martin, 1999), however no withdrawal time following the sensitizing EtOH regimen was imposed. Thus, our results agree with one study (Wise et al., 1996), however it is

possible that this study and the experiments described here would have resulted in significant cross-sensitization if a drug withdrawal period had been imposed. In another study, rats, rather than mice, were injected with EtOH for 15 consecutive days, and challenged with morphine and amphetamine 3 weeks following termination of EtOH administration (Nestby et al., 1997). The EtOH treated rats did not develop sensitization to the effects of EtOH, and showed enhanced responses only to challenge morphine, but not amphetamine injections (Nestby et al., 1997). Under circumstances when sensitization to the effects of one of the drugs used is not shown, it is difficult to make conclusions about cross-sensitization phenomena *per se*. Additionally, rats are generally much more sensitive to the depressant, rather than stimulant effects of EtOH, even at low EtOH doses, making assessment of EtOH-induced sensitization difficult (Masur et al., 1986).

Other studies using EtOH liquid diets or voluntary EtOH consumption as EtOH administration regimens hint at cross-sensitization between EtOH and other drugs of abuse. For instance, mice that had undergone three weeks of EtOH liquid diet consumption showed enhanced responses to amphetamine challenge injections (Manley and Little, 1997). Importantly, and relevant to the discussion above, this enhanced response was first seen 6 days or 2 months, but not 24 hours, following cessation of EtOH availability (Manley and Little, 1997). EtOH treated mice also showed enhanced responses to cocaine challenge injections, but only after 10 or 16 daily cocaine injections that followed the liquid EtOHdiet (Manley and Little, 1997). Three weeks of voluntary EtOH drinking in high-preferring Wistar rats resulted in greater amphetamine-induced activity 4 weeks later relative to a low-preferring group (Fahlke et al., 1995). The same

investigators also showed that 5 weeks of prior amphetamine treatment enhanced voluntary EtOH consumption 3 months later (Fahlke et al., 1994) indicating some similarity in the neuroadaptations induced by EtOH drinking and amphetamine treatment.

Overlapping mechanisms of drug action can also be assessed through genetic studies by determining whether similar genes affect different behaviors. In two separate studies using panels of BXD recombinant inbred strains of mice to assess sensitization to the effects of EtOH and cocaine, no genetic relationship between the two phenotypes was found implying separate sets of genes in the mediation of each behavior (Phillips et al., 1995; Phillips et al., 1998c). One could therefore predict that no cross-sensitization would be observed between the effects of EtOH and cocaine. It has also been shown that the genetically selected alcohol preferring AA rats developed sensitization to the effects of lower doses of morphine and cocaine relative to alcohol non-preferring ANA and Wistar rats (Honkanen et al., 1999). This implies that genes that mediate avidity for EtOH may also mediate cocaine and morphine sensitization. Unfortunately, neither the AA, nor the ANA rats developed sensitization to the effects of EtOH (Honkanen et al., 1999), making it difficult to conclude that genes that mediate sensitization to EtOH also mediate sensitization to morphine and cocaine. As already mentioned, however, rats may be poor models for the study of EtOH-induced sensitization, since they tend to mostly show behavioral depression in response to EtOH.

To our knowledge, the experiments described here represent only the third study that has specifically examined cross-sensitization between EtOH and other drugs using mice. The two other studies were discussed above (Wise et al., 1996; Itzhak and Martin, 1999), and the results from all three studies thus far point to evidence both for and against

the presence of cross-sensitization between EtOH and cocaine, and no evidence for cross-sensitization between the locomotor stimulant effects of EtOH and those of morphine and the amphetamines. Clearly many more studies varying drug dose, withdrawal period, and including reciprocal cross-sensitization conditions are necessary for a reasonable conclusion to be reached. We would recommend that such experiments be performed in mice as this animal model more readily shows locomotor activation to stimulant EtOH doses than the rat model (Masur et al., 1986).

It is possible that neurochemical correlates to drug treatment are more consistent across drugs than actual behavioral cross-sensitization between the effects of the same drugs (Nestby et al., 1997). For instance, acute administration of EtOH, morphine, cocaine, and amphetamine resulted in elevated extracellular dopamine concentrations in rat nucleus accumbens (NAcc) and caudate (Di Chiara and Imperato, 1988). Repeated administration of EtOH, morphine, cocaine, and amphetamine also resulted in an increase in the electrically evoked release of dopamine and acetylcholine from rat NAcc slices that lasted for 3 weeks following cessation of drug administration (Nestby et al., 1997). These results show evidence not only for lasting neuroadaptations as a function of repeated drug administration, but also indicate that there may be common neuroadaptations between different classes of drugs of abuse. Behavioral cross-sensitization between EtOH and cocaine was associated with an increase in dopamine transporter binding sites in mouse striatum (Itzhak and Martin, 1999). Another study showed that following repeated EtOH administration, only those mice that developed significant behavioral EtOH-induced sensitization had increased dopamine D_2 receptor binding in the NAcc, whereas EtOH treated mice that failed to develop EtOH-induced

sensitization and saline treated controls showed no changes (Souza-Formigoni et al., 1999). Thus, there is some evidence to suggest that repeated EtOH administration results in neuroadaptation in the dopaminergic system and these neuroadaptations may underlie both behavioral EtOH-induced sensitization and sensitization tot other drugs of abuse.

SUMMARY AND CONCLUSIONS

The current studies showed no evidence for cross-sensitization between the effects of EtOH and those of morphine, cocaine, and methamphetamine tested 24 hours following repeated EtOH treatment. This conclusion is tentative, however, as the presence of cross-sensitization in the reciprocal situation of repeated morphine, cocaine, and methamphetamine treatment followed by EtOH challenge injections has yet to be determined. Based on the results in these studies, it appears that different neural mechanisms may mediate the sensitizing effects of EtOH and those of the three other drugs tested. However, other studies suggest neuroadaptations of the dopaminergic system as a common mechanism underlying the development of drug sensitization. In addition, different results might have been obtained if a period of EtOH withdrawal prior to challenge drug administrations had been imposed. Future studies will address this possibility.

Table 1. Cross-Sensitization Procedure. Activity testing and EtOH injection procedure used to induce sensitization to the effects of EtOH and to assess cross-sensitization to the effects of morphine (**M**), cocaine (**C**), and methamphetamine (**METH**).

Treatment Groups	**Habituation** Day 1	**Baseline 1** Day 2	**Acute EtOH** Day 3	**Daily Treatment** Days 4-13	**Sensitization** Day 14	**Cross Sensitization** Day 15	**Baseline 2** Day 16
Saline Control	Saline	Saline	Saline	Saline	EtOH 2g/kg	**M/C/METH**	Saline
EtOH Sensitized	Saline	Saline	EtOH 2g/kg	EtOH 2.5g/kg	EtOH 2g/kg	**M/C/METH**	Saline
EtOH Naïve	Saline	Saline	Saline	Saline	Saline	**M/C/METH**	Saline
Untreated	Saline	Saline	Saline	None	EtOH 2g/kg	**M**	Saline

Locomotor activity was tested for 10 min on Days 1, 2, 3, and 14, and for 60 min on Days 15 and 16. Activity data were collected in 5-min epochs. No activity testing took place during the Daily Treatment phase. All injections were intraperitoneal (i.p.). M=morphine administered at 0, 5, 10, or 20 mg/kg; C=cocaine administered at 0, 5, 10, or 20 mg/kg; METH=methamphetamine administered at 0, 0.5, 1, or 2 mg/kg. The *Untreated* group was included only in the **M**orphine experiment.

Habituation and **Baseline 1**: On Days 1 and 2, all treatment groups were tested following saline injections. **Acute EtOH**: On Day 3, the *EtOH Sensitized* group was tested following 2 g/kg EtOH, while the rest of the treatment groups were tested following saline. **Daily Treatment**: During Days 4-13, the *EtOH Sensitized* group received 10 daily injections of 2.5 g/kg EtOH; *Saline Control* and *EtOH Naïve* groups received daily saline injections, while the *Untreated* group (included for one out of four experiments) received no injections. **Sensitization**: On Day 14, *Saline Control*, *EtOH Sensitized*, and *Untreated* groups were tested following 2 g/kg EtOH, while the *EtOH Naïve* group was tested following saline injection. **Cross-Sensitization**: On Day 15, each of the *Saline Control*, *EtOH Sensitized* and *EtOH Naïve* groups was injected with one of four doses of morphine (**M**), cocaine (**C**), or methamphetamine (**METH**). The Untreated group was injected with one of four doses of morphine (**M**) only. **Baseline 2**: On Day 16, all mice were tested following saline injections.

Figure 1. Total 10-min locomotor activity of saline-treated (Saline Control, *white circles*; EtOH Naïve, *white triangles*; and Untreated, *black triangles*) and EtOH-treated (EtOH Sensitized, *black circles*) mice across activity test days for the EtOH-morphine cross-sensitization experiment. On Days 1 and 2, all mice received saline injections. On Day 3, the EtOH Sensitized group received 2 g/kg EtOH, while the saline-treated groups received saline. On Day 14, Saline Control, EtOH Sensitized, and Untreated groups received 2 g/kg EtOH, while the EtOH Naïve group received saline. n=39-42 per group. ** Significant enhancement of EtOH response in the EtOH Sensitized group relative to the Saline Control and Untreated groups on Day 14, and relative to their own acute EtOH response on Day 3. both $p<0.001$

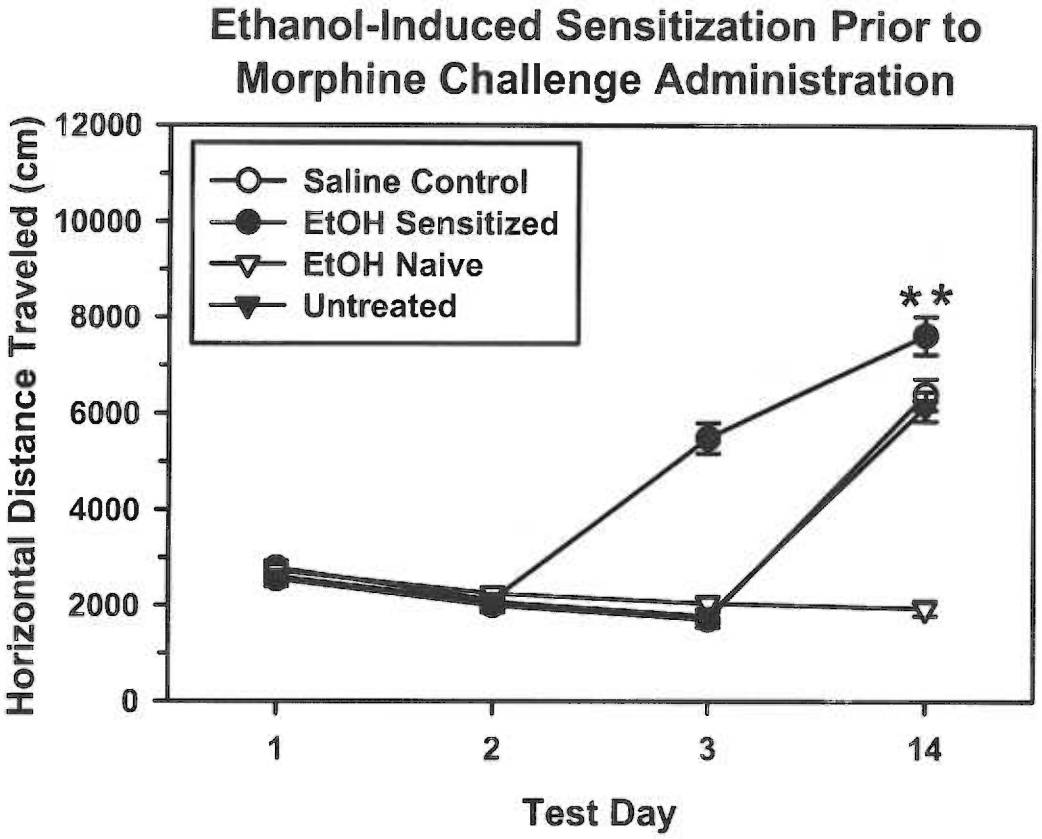

Ethanol-Induced Sensitization Prior to Morphine Challenge Administration
Horizontal Distance Traveled (cm)
12000
10000
8000
6000
4000
2000
0
Saline Control
EtOH Sensitized
EtOH Naive
Untreated
**
1
2
3
14
Test Day

Figure 2. Horizontal distance traveled in 60 min following challenge injections of 0, 5, 10, and 20 mg/kg morphine (*Panel A*) or saline (*Panel B*) on two consecutive test days, respectively. Even though *Panel B* represents responses to saline administration, treatment groups are graphed according to the morphine dose they received on the previous test day (*Panel A*). The bars represent differential treatment during the EtOH-induced sensitization period when groups received either repeated saline (Saline Control, EtOH Naïve, and Untreated groups; *white, diagonal,* and *hatched bars*, respectively) or repeated EtOH (EtOH Sensitized group, *black bars*) administration. There were no significant differences between treatment groups in response to morphine (*Panel A*) or saline (*Panel B*). n=10-11 per group

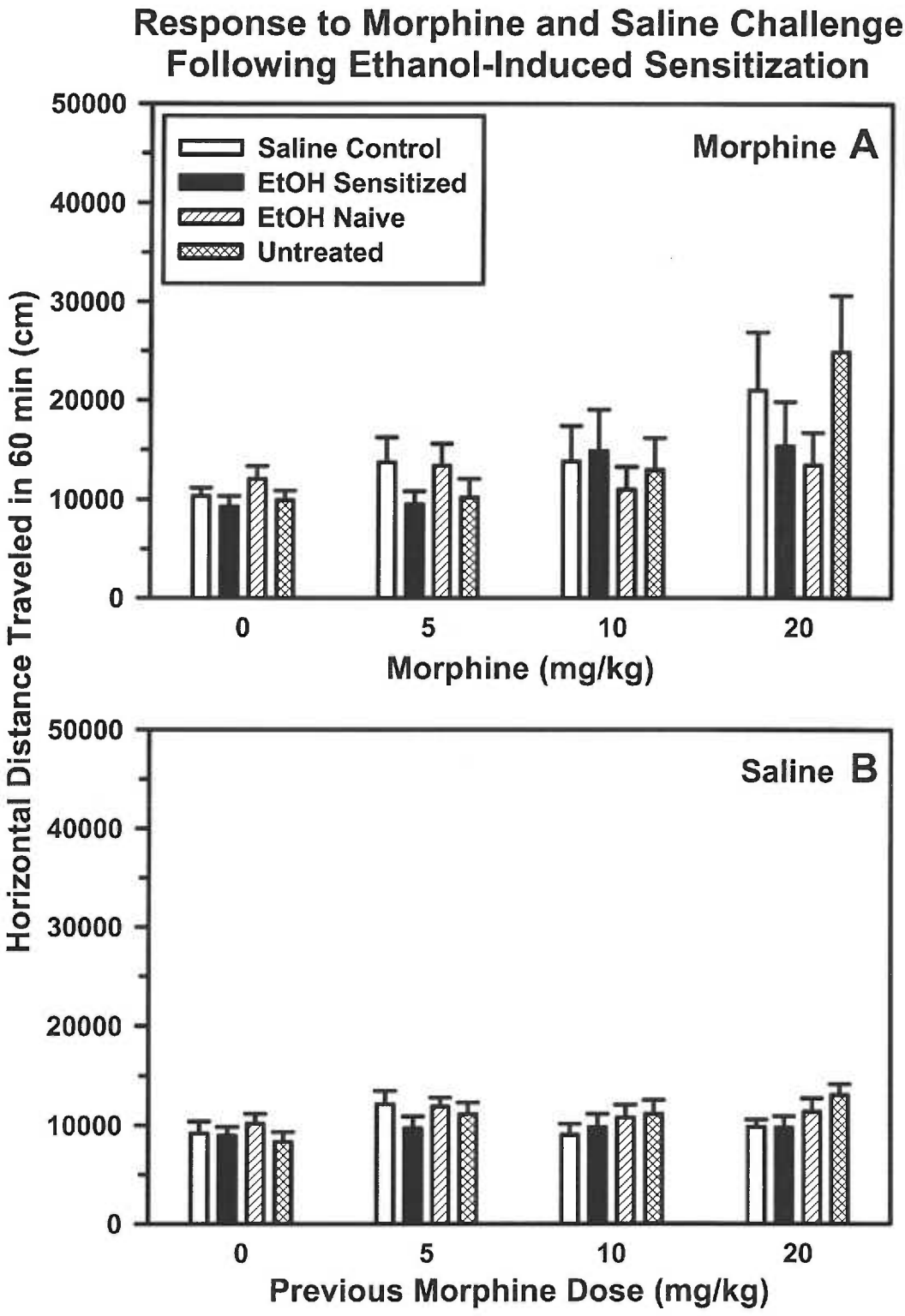

Response to Morphine and Saline Challenge
Following Ethanol-Induced Sensitization
Horizontal Distance Traveled in 60 min (cm)
50000
40000
30000
20000
10000
0
Saline Control
EtOH Sensitized
EtOH Naive
Untreated
Morphine A
0
5
10
20
Morphine (mg/kg)
Saline B
Previous Morphine Dose (mg/kg)

Figure 3. Total 10-min locomotor activity of saline-treated (Saline Control, *white circles* and EtOH Naïve, *white triangles*) and EtOH-treated (EtOH Sensitized, *black circles*) mice across activity test days for the EtOH-cocaine cross-sensitization experiment. On Days 1 and 2, all mice received saline injections. On Day 3, the EtOH Sensitized group received 2 g/kg EtOH, while the saline-treated groups received saline. On Day 14, Saline Control, EtOH Sensitized, and Untreated groups received 2 g/kg EtOH, while the EtOH Naïve group received saline. n=40-42 per group

** Significant enhancement of EtOH response in the EtOH Sensitized group relative to the Saline Control group on Day 14, and relative to their own acute EtOH response on Day 3. both $p<0.001$

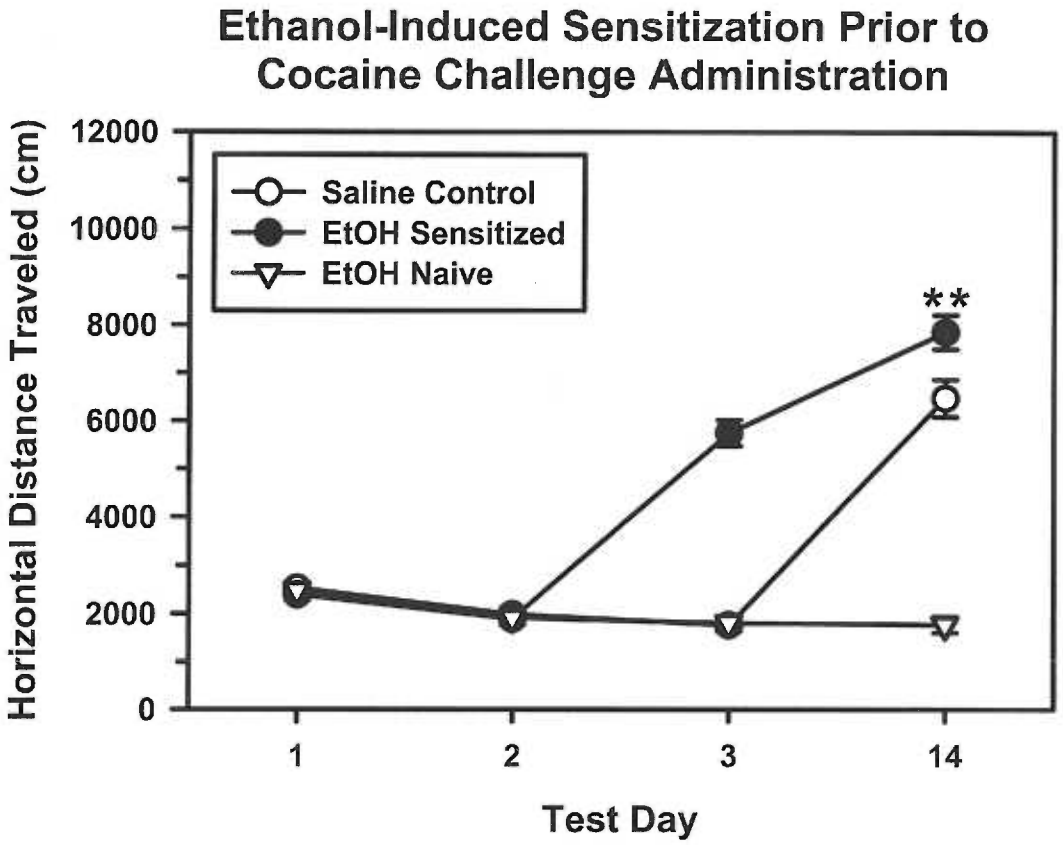

Ethanol-Induced Sensitization Prior to Cocaine Challenge Administration
Horizontal Distance Traveled (cm)
12000
10000
8000
6000
4000
2000
0
Saline Control
EtOH Sensitized
EtOH Naive
**
1
2
3
14
Test Day

Figure 4. Horizontal distance traveled in 60 min following challenge injections of 0, 5, 10, and 20 mg/kg cocaine (*Panel A*) or saline (*Panel B*) on two consecutive test days, respectively. Even though *Panel B* represents responses to saline administration, treatment groups are graphed according to the cocaine dose they received on the previous test day (*Panel A*). The bars represent differential treatment during the EtOH-induced sensitization period when groups received either repeated saline (Saline Control, EtOH Naïve, and Untreated groups; *white, diagonal,* and *hatched bars*, respectively) or repeated EtOH (EtOH Sensitized group, *black bars*) administration. There were no significant differences between treatment groups in response to cocaine (*Panel A*) or saline (*Panel B*). n=10-11 per group

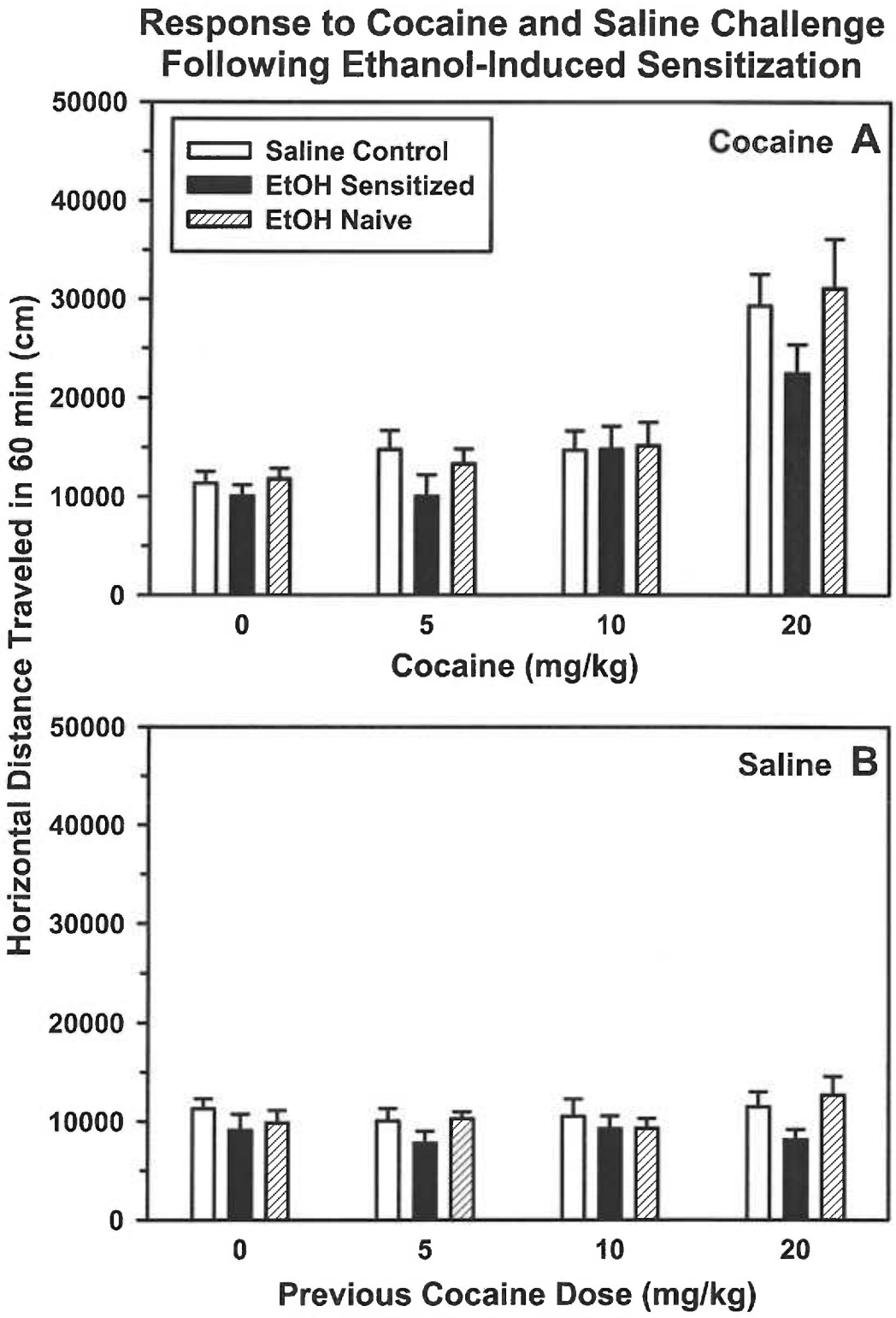

Response to Cocaine and Saline Challenge
Following Ethanol-Induced Sensitization
Saline Control
EtOH Sensitized
EtOH Naive
Cocaine A
Saline B
Horizontal Distance Traveled in 60 min (cm)
50000
40000
30000
20000
10000
0
0
5
10
20
Cocaine (mg/kg)
Previous Cocaine Dose (mg/kg)

Figure 5. Total 10-min locomotor activity of saline-treated (Saline Control, *white circles* and EtOH Naïve, *white triangles*) and EtOH-treated (EtOH Sensitized, *black circles*) mice across activity test days in the EtOH-methamphetamine cross-sensitization experiment. On Days 1 and 2, all mice received saline injections. On Day 3, the EtOH Sensitized group received 2 g/kg EtOH, while the saline-treated groups received saline. On Day 14, Saline Control, EtOH Sensitized, and Untreated groups received 2 g/kg EtOH, while the EtOH Naïve group received saline. n=46-48 per group

** Significant enhancement of EtOH response in the EtOH Sensitized group relative to the Saline Control group on Day 14, and relative to their own acute EtOH response on Day 3. both $p<0.001$

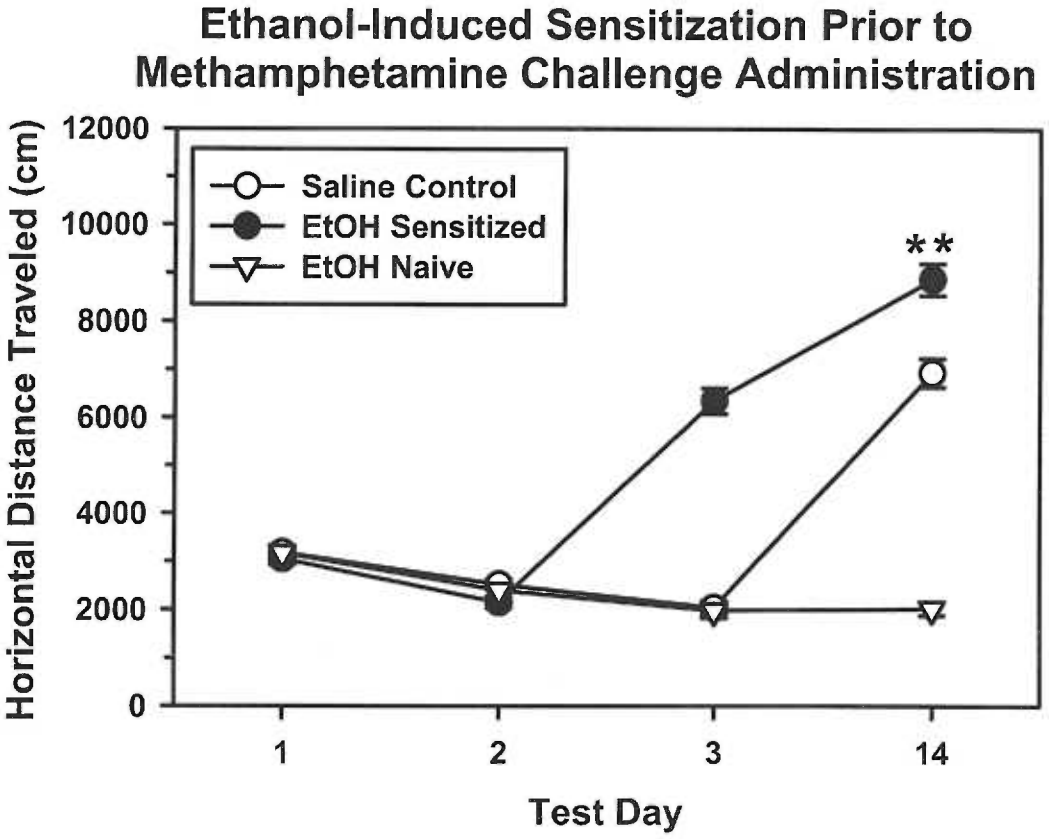
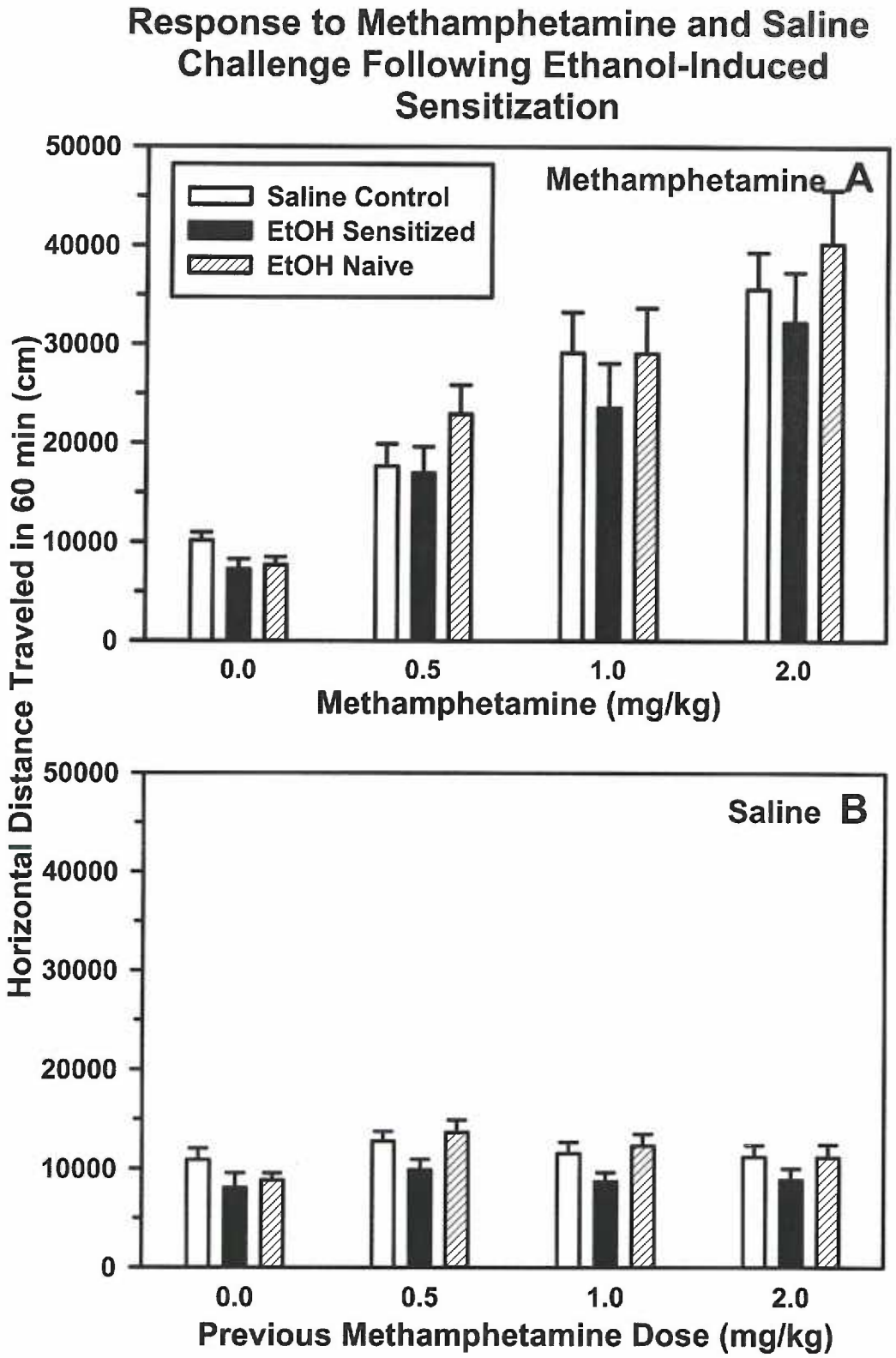

Ethanol-Induced Sensitization Prior to Methamphetamine Challenge Administration
Horizontal Distance Traveled (cm)
12000
10000
8000
6000
4000
2000
0
Saline Control
EtOH Sensitized
EtOH Naive
**
1
2
3
14
Test Day

Figure 6. Horizontal distance traveled in 60 min following challenge injections of 0, 0.5, 1, and 2 mg/kg methamphetamine (*Panel A*) or saline (*Panel B*) on two consecutive test days, respectively. Even though *Panel B* represents responses to saline administration, treatment groups are graphed according to the methamphetamine dose they received on the previous test day (*Panel A*). The bars represent differential treatment during the EtOH-induced sensitization period when groups received either repeated saline (Saline Control, EtOH Naïve, and Untreated groups; *white, diagonal,* and *hatched bars*, respectively) or repeated EtOH (EtOH Sensitized group, *black bars*) administration. There were no significant differences between treatment groups in response to methamphetamine (*Panel A*) or saline (*Panel B*). n=12 per group


Response to Methamphetamine and Saline Challenge Following Ethanol-Induced Sensitization
Saline Control
EtOH Sensitized
EtOH Naive
Methamphetamine A
Horizontal Distance Traveled in 60 min (cm)
50000
40000
30000
20000
10000
0
0.0
0.5
1.0
2.0
Methamphetamine (mg/kg)
Saline B
Previous Methamphetamine Dose (mg/kg)

Voluntary Ethanol Drinking in Mice Sensitized to the Locomotor Stimulant Effects of Ethanol

INTRODUCTION

Repeated administration of a low dose of ethanol (EtOH) may lead to an increase in its locomotor stimulant effect (Masur and Boerngen, 1980; Masur et al., 1986; Phillips et al., 1995), or behavioral sensitization. EtOH-induced sensitization is long lasting (Lessov and Phillips, 1998) and may be accompanied by neuroadaptations that influence EtOH abuse and addiction. Investigation of the mechanisms mediating sensitization to the stimulant effects of cocaine and amphetamine has led to theories implicating sensitization in drug craving and drug reward (Wise and Bozarth, 1987; Robinson and Berridge, 1993). Since EtOH-induced sensitization has been much less studied, there has been little discussion of its contribution to EtOH abuse. It has been suggested that behavioral sensitization might underlie uncontrolled drinking behavior (Hunt and Lands, 1992).

The present series of experiments were initiated in order to determine whether sensitization to the effects of EtOH would alter EtOH reinforcement as indicated by a change in subsequent voluntary EtOH consumption, and whether voluntary EtOH drinking itself would induce sensitization. Several lines of evidence suggest that administration of drugs of abuse in a manner consistent with the development of sensitization to the effects of the drugs enhances the reinforcing drug properties. Drug reinforcement had been assessed through operant self-administration and conditioned place preference paradigms (Samson et al., 1998; Tzschentke, 1998). For example, faster acquisition and greater magnitude of cocaine or amphetamine operant self-administration

was observed in rats pretreated with cocaine, amphetamine, or nicotine (Horger et al., 1990; Horger et al., 1992; Schenk et al., 1993; Valadez and Schenk, 1994; Mendrek et al., 1998; Pierre and Vezina, 1998; Taylor and Horger, 1999). Sensitization to the effects of the pretreatment drugs was confirmed in some of these studies (Horger et al., 1990; Horger et al., 1992; Mendrek et al., 1998; Taylor and Horger, 1999). Similarly, amphetamine, cocaine, and morphine pretreatment enhanced subsequent drug-induced acquisition and magnitude of conditioned place preference, both in a same-drug and across-drug assessment (Lett, 1989; Shippenberg and Heidbreder, 1995; Shippenberg et al., 1996). Neither of the conditioned place preference studies showed evidence for sensitization to the effects of the pretreatment drugs, but the drugs were administered in a regimen often shown to induce sensitization. In a model of drug-seeking behavior, it was shown that priming injections of heroin, cocaine, and amphetamine reinstated heroin and cocaine self-administration, but only in a manner that paralleled the expression of sensitization to heroin and amphetamine (De Vries et al., 1998). EtOH consumption or preference, the index of EtOH reinforcement used in the present studies (McBride and Li, 1998), was enhanced in rats previously treated with amphetamine or sensitized to the effects of nicotine (Fahlke et al., 1994; Blomqvist et al., 1996). Even though sensitization due to drug pretreatment was not always determined, it is notable that sensitization was present in each instance that it was assessed.

A stronger argument regarding the potential contribution of drug sensitization to the process of drug addiction could be advanced if sensitization is shown to develop as a function of drug self-administration, rather than experimenter-administered drug injections. Toward this end, rats trained to nose-poke in a hole associated with heroin

infusions showed a progressive increase in their locomotor activity, assessed during self-administration sessions (Marinelli et al., 1998), and showed enhanced locomotor response to a challenge heroin injection administered 3 weeks following cessation of self-administration (De Vries et al., 1998). Rats trained in an operant task to self-administer cocaine infusions, showed an enhanced locomotor response to a challenge intravenous cocaine administration 3 days after the final self-administration session relative to pre-self-administration levels (Phillips and Di Ciano, 1996). The alcohol preferring C57BL/6J mouse inbred strain showed enhanced activity to a challenge EtOH injection following a period of voluntary EtOH consumption relative to pre-drinking activity levels (Nocjar and Middaugh, 1997). Similarly, immediately following a 15-min voluntary EtOH access period selectively bred Sardinian alcohol preferring rats had significantly higher spontaneous locomotor activity relative to water consuming controls (Colombo et al., 1998). Examination of alcohol related phenotypes in genetic animal models suggests that genes involved in mediating avidity for EtOH may also mediate some of the locomotor stimulant properties of EtOH implying similarity of mechanisms underlying stimulant drug effects and drug reinforcement.

In the present series of experiments, mice from a genetically heterogeneous population, and from the C57BL/6J (B6) and DBA/2J (D2) inbred strains were taken through a process that included (1) an initial 2-bottle EtOH preference drinking paradigm, in which half of the animals were offered EtOH vs. water and half were maintained on water only (EtOH naïve), (2) a subsequent sensitization protocol during which mice from each of the previously EtOH naïve or EtOH experienced groups were repeatedly injected with saline or EtOH, and (3) a final EtOH preference drinking

procedure during which all mice had access to EtOH. By placing the sensitization procedure in the middle of two EtOH drinking periods, both development of sensitization subsequent to EtOH drinking and changes in EtOH drinking subsequent to sensitization could be assessed in a between- and within-group design. The animal models were chosen on the basis of their differential avidity for EtOH and their differential sensitivity to the sensitizing effects of EtOH. The genetically heterogeneous mice have shown robust sensitization to the effects of EtOH and are moderate drinkers (Lessov and Phillips, 1998; unpublished observations), the B6 mice are avid drinkers and have shown insensitivity to the sensitizing effects of EtOH, while the D2 mice are alcohol-avoiders and have shown robust sensitization to the effects of EtOH (Belknap et al., 1993; Phillips et al., 1994a; Phillips et al., 1994b; Phillips et al., 1995; Rodriguez et al., 1995; Phillips et al., 1996). We hypothesized that sensitization to the effects of EtOH would alter subsequent EtOH drinking and that, at least in the alcohol-preferring B6 strain, EtOH drinking would induce sensitization.

METHODS

Subjects

Female mice from three genotypes were used. They included two replicate lines of a genetically heterogeneous population, WSC1 and WSC2, and the C57BL/6J (B6) and DBA/2J (D2) inbred strains. Female mice were chosen to facilitate comparison with prior data from this laboratory and because female rodents have been characterized as more susceptible to drug sensitization (Robinson, 1984; Cailhol and Mormède, 1999). The heterogeneous lines have been maintained as controls for selective breeding

experiments (Crabbe et al., 1985). They were born and raised at the VA Animal Care Facility in Portland, Oregon. The B6 and D2 mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) at least two weeks prior to utilization in experiments. Animals were kept on reverse 12:12 hour light: dark cycle (lights OFF at 9 am [Exp. 1 and 3] or 10 am [Exp. 2 and 4]) with food and water available ad libidum, except during behavioral testing. They were acclimated to this lighting schedule for a minimum of 2 weeks prior to study initiation. Animals ranged in age from 58 to 97 days old at the beginning of the experiments. Experiments were performed in accordance with the Institutional Animal Care and Use Committee and National Institutes of Health guidelines for the care and use of laboratory animals.

Drugs

For injections, ethanol (EtOH, 200 proof, AAPER or Pharmco) was diluted in saline (0.9%NaCl) for a final solution of 20% v/v. Saline was used for control injections. All injections were intraperitoneal (i.p.). As a drinking fluid, EtOH was mixed with tap water for final solution concentrations of 3%, 6% or 10% v/v.

Apparatus

Mice were tested in automated locomotor activity monitors (40 x 40 x 30 cm, Accuscan, Columbus, OH) housed in sound attenuating chambers. Lights and fans were mounted on the rear middle wall and right upper corner of each chamber, respectively. During testing, fans were on inside the chambers, providing ventilation and masking noise. Lights were on for one out of four experiments. Eight infrared beams were mounted 2

cm above the test chamber floor outside an acrylic plastic test box on two perpendicular panels. Eight detectors were mounted on opposing panels. As a mouse moved about the chamber floor, the infrared beams were interrupted and each interruption was automatically recorded as an activity count. Data were automatically translated to horizontal distance traveled (cm) and this was the chosen measure of activity.

General Procedure

Four experiments were conducted. In Exp. 1 and 2, the genetically heterogeneous population of mice was used; in Exp. 3, the B6 and D2 inbred strains were used; in Exp. 4, only the B6 strain was used. Each experiment consisted of at least 3 phases: (1) Pre-Sensitization Drinking during which half of the animals were offered water (EtOH-naive) and half were offered a choice between EtOH and water (EtOH-experienced), (2) EtOH-induced sensitization during which mice from each of the EtOH-naïve and EtOH-experienced groups were repeatedly injected with either saline (Saline Control) or EtOH (EtOH Sensitized) and were tested for subsequent locomotor response, and (3) Post-Sensitization Drinking during which all mice were given free access to water and EtOH as drinking fluids. A fourth phase of Post-Drinking Activity was assessed in Exp. 1, 2, and 3. It consisted of consecutive EtOH and saline activity test days, during which all animals were tested following 2.0 g/kg EtOH or saline injections, respectively. The purpose of the EtOH and saline test days was to determine whether sensitization to the effects of EtOH was still present in EtOH Sensitized groups and whether the experimental procedures affected baseline locomotor activity, respectively.

The next few paragraphs describe each phase in detail, followed by a discussion regarding the particular procedural peculiarities of each experiment (section Experiment-Specific Procedures).

Pre-Sensitization Drinking: Mice were single housed and for the first 4 days, water was presented in two 25 ml calibrated tubes adapted with drinking spouts. For subsequent two or three 4-day periods (depending on the experiment), half of the animals had one of their water drinking tubes replaced with a tube containing an EtOH solution (EtOH-experienced), while the other half continued to receive only water (EtOH-naive). The EtOH solution was sometimes offered in ascending concentrations of 3%, 6%, and 10% EtOH, depending on the experiment, with each concentration available for a 4-day period. Water and EtOH solution consumption volumes were recorded immediately before lights off (9 or 10 am, depending on the experiment) and at 3 and 6 hours into the dark cycle (the 3-hour reading was omitted for Exp. 4). Data collection was binned into 3 time periods: the first 3-hour, the second 3-hour, and the remaining 18-hour periods. For data presentation and analyses, consumption volumes were expressed as g/kg EtOH and EtOH preference ratios (ml EtOH /ml EtOH + ml water). Drinking tube positions were rotated every 48 hours to control for side preferences. Averages were calculated based on data collected on Days 2 and 4 of every 4-day access period as the most stable estimate of preference and consumption (Phillips et al., 1994a). Water and EtOH tubes were replaced with clean ones every 8 and 4 days, respectively. Cages were changed and mice were weighed every 4 days.

Final volume consumption was recorded in the morning of Day 13 or 17, depending on the experiment. Drinking tubes were removed and replaced with standard

water bottles. Each of the EtOH-experienced and EtOH-naïve groups was pseudo-randomized into saline (Saline Control) and EtOH (EtOH Sensitized) treatment groups in preparation for the next EtOH-induced sensitization phase. Because of water and EtOH consumption variability across individual mice, the pseudo-randomization procedure ensured that an equal number of low and high consumers would be represented in each of the Saline Control and EtOH Sensitized groups. The variable we chose for the basis of the pseudo-randomization was the average consumption of 6% EtOH (10% EtOH for Exp. 4) in g/kg for the first 6 hours of the dark cycle.

EtOH-induced sensitization: This phase was initiated 24 hours following removal of water and EtOH drinking tubes. Volume consumption was not recorded. A summary of the EtOH-induced sensitization protocol is shown in Table 2. On activity test days, animals were moved to the testing room immediately before lights off and allowed to acclimate to the testing environment for at least 90 min prior to experiment initiation. Activity testing took place in the dark (lights off in the testing room and in the activity monitors), except for Exp. 1 during which testing was conducted with lights on in the testing room and inside the activity monitors (differences explained below). On Days 1 and 2, all mice were injected (i.p.) with saline and immediately placed in the center of an activity monitor. Data were collected for 10 min in two 5-min epochs. Day 1 served as an habituation day to the testing apparatus. Day 2 served as a baseline activity test day. On Day 3, Saline Control and EtOH Sensitized groups were tested following saline or 2 g/kg EtOH injections, respectively. Day 3 served as a measure of the acute stimulant response to EtOH of EtOH Sensitized groups. For the subsequent 10 consecutive days (Day 4-13), Saline Control and EtOH Sensitized mice received saline or 2.5 g/kg EtOH

injections, respectively, in their home cages. This treatment phase was conducted in the light, before lights off, to ease animal handling and injection for the experimenter. No activity testing took place. On the final activity test day, Day 14, Saline Control and EtOH Sensitized mice were tested following saline or 2 g/kg EtOH injections, respectively. This day served as a measure of the sensitized response to EtOH of EtOH Sensitized mice.

Post-Sensitization Drinking was initiated 24 hours following the EtOH-induced sensitization activity test day. This phase was conducted in exactly the same way as Pre-Sensitization Drinking with the exception that all animals, instead of half of the animals, were offered a choice between water and EtOH.

For Exp. 1, 2, and 3, Post-Drinking Activity was initiated 24 hours following removal of the EtOH drinking tubes. Two water drinking tubes continued to be available, but consumption volumes were not recorded. On the first of two activity test days, all mice were tested for 10 min following 2 g/kg EtOH injections. For Saline Control groups, this was an acute EtOH injection. On the second test day 24 hours later, all mice were tested following saline injections.

Treatment Groups

To summarize, there were 4 treatment groups represented in each of the four experiments: Naïve-Saline Control, Naïve-EtOH Sensitized, EtOH-Saline Control and EtOH-EtOH Sensitized. The first designation, Naïve or EtOH, indicates whether or not EtOH was available for consumption during Pre-Sensitization Drinking, while the second designation, Saline Control or EtOH Sensitized, indicates treatment during the EtOH-

induced sensitization period. There was a possibility that stress due to daily handling and injections could have sensitized Naïve- or EtOH-Saline Control groups (Roberts et al 1995) and potentially could have altered subsequent drinking behavior. In Exp. 2 and 3, therefore, an additional control group was included that was offered only water during Pre-Sensitization Drinking and was injected with saline on activity test days during EtOH-induced sensitization in the same manner as the Naïve-Saline Control group, but did not receive injections during the daily treatment phase (Days 4-13). It was designated as a Naïve-Untreated group.

Experiment-Specific Procedures

Experiments 1 and 2

Genetically heterogeneous female mice were used in these experiments and all four treatment phases described above were conducted. Each treatment group consisted of 11-12 mice for Exp. 1 and 10-14 mice for Exp. 2. During Pre- and Post-Sensitization Drinking, animals were offered a choice between water and each of 6% and 10% EtOH solutions. There were three procedural differences between these two experiments: (1) lights went off at 9 am in Exp. 1, and 10 am in Exp. 2, (2) activity testing during EtOH-induced sensitization was conducted in the light, between 6 and 9 am in Exp. 1, whereas testing took place in the dark, starting around noon, in Exp. 2, and (3) a Naïve-No Treatment group was added in Exp. 2. The first change occurred due to the daylight savings time change. The second change was made in order to avoid testing animals so near the end of their light cycle when all prior sensitization experiments had been initiated at least 2 hours following light onset. The third change was made in order to

control for the potential effects of stress on sensitization to the effects of EtOH and on subsequent drinking behavior.

Experiments 3 and 4

Exp. 3 served as a pilot study to assess the effects of repeated EtOH administration on subsequent EtOH drinking behavior in two differentially EtOH sensitive inbred strains. Treatment group size in Exp. 3 was 6-7, while Exp. 4 had 12 mice represented in each group. All four phases described in General Procedures were conducted. Female mice from the C57BL/6J (B6) and DBA/2J (D2) inbred strain were used in this experiment. EtOH drinking solutions were available in ascending order of 3%, 6%, and 10% EtOH, in consecutive 4-day periods. Consumption volumes were recorded before lights off (around 9 am), and 3 and 6 hours into the dark cycle. A Naïve-No Treatment group was also included.

In order to simplify and replicate the most significant findings of Exp. 3, Exp. 4 was designed using only B6 mice, a single EtOH concentration (10%), and consumption volume readings were recorded before lights off and 6 hours into the dark cycle. A Naïve-No Treatment group was not included and Post-Drinking Activity was also eliminated. Otherwise, procedures were as described for Exp. 3.

Statistical Analyses

Behavioral sensitization data were initially analyzed with a three-way Group (Saline Control vs. EtOH Sensitized) x Drinking Experience (EtOH-naïve vs. EtOH-experienced) x Test Day (Days 1, 2, 3, 14, and both post-drinking test days) ANOVA. In the absence

of an effect of Drinking Experience, the data were collapsed on this factor and re-analyzed with a two-way Group x Test Day ANOVA. Significant interaction effects were followed up with Simple Main Effects analyses and Newman-Keuls posthoc multiple comparisons tests. Based on a priori decisions, within-group sensitization to the effects of EtOH was evaluated for the EtOH Sensitized groups using one-way repeated measures ANOVAs followed by Newman-Keuls posthoc tests.

Post-sensitization drinking data were analyzed using between-subjects Drinking Experience x Group x EtOH Concentration ANOVAs. Pre- vs. post-sensitization drinking measures could only be analyzed for the EtOH-experienced groups using within-subject Group x EtOH Concentration x Time (pre- vs. post-sensitization) ANOVAs. Significant interaction effects were followed by relevant two-way ANOVAs with Simple Main Effects analyses and Newman-Keuls posthoc multiple comparisons tests. Additionally, post-sensitization drinking data were analyzed using separate two-way Group x EtOH concentration ANOVAs for each of the EtOH-naive and EtOH-experienced conditions. These analyses were justified based on a priori decisions to separate the data and to complement the already separate within-subject analyses for the pre-sensitization EtOH-experienced groups. The dependent variables included EtOH consumption expressed in g/kg, EtOH preference expressed as a ratio of the amount (ml) of EtOH consumed relative to the total amount (ml) of fluid (EtOH + water) consumed, and total fluid (ml, EtOH + water) intake. Data from Day 2 and 4 of each 4-day EtOH access period were averaged across each EtOH concentration and each time point.

RESULTS

Experiment 1: Genetically Heterogeneous Mice

Sensitization to the Stimulant Effects of EtOH

Locomotor activity data collected during the EtOH-induced sensitization (Days 1-3 and 14) and during the post-drinking EtOH and saline test days are presented in Figure 7. The behavioral pattern of response differed across test days as a result of differential EtOH treatment (significant Group x Test Day interaction, $F_{5,210}=69.6$, $p<0.001$). There was no effect of prior drinking experience (Naïve-EtOH Sensitized vs. EtOH-EtOH Sensitized groups; Figure 7, black circles vs. black triangles) on activity response to EtOH pointing to a lack of sensitization as a function of EtOH consumption. Therefore, data were collapsed across the Drinking Experience factor and re-analyzed using a two-way Group (Saline Control vs. EtOH Sensitized) x Test Day ANOVA. There was a significant interaction effect ($F_{5,220}=70.8$, $p<0.001$) indicating a significant EtOH stimulant response of the EtOH Sensitized groups on Days 3 and 14 (both $p<0.001$). There was significant within-group sensitization as mice repeatedly treated with EtOH (Naïve-EtOH Sensitized and EtOH-EtOH Sensitized groups) collectively showed enhanced EtOH responses on Day 14 relative to their acute EtOH responses on Day 3 (one-way ANOVA for EtOH Sensitized groups collapsed on Drinking Experience, $p<0.05$). EtOH activity assessed on the EtOH post-drinking test day remained higher than the acute EtOH response on Day 3 and did not differ from the sensitized EtOH response on Day 14 ($p<0.05$) indicating that sensitization lasted through the 8-day post-sensitization drinking period (denoted as a break on the x-axis of Figure 7).

Within-group sensitization for each of the EtOH-naïve and EtOH-experienced EtOH Sensitized groups was also separately assessed because of a priori decisions and because the drinking data were also separately analyzed. With the reduced sample size, there was no evidence for significant within-group sensitization in either the Naïve-EtOH Sensitized or EtOH-EtOH Sensitized groups. It is possible that the unusual time of testing (6-9 am) immediately prior to onset of the animals' dark cycle may have precluded the expression of EtOH-induced sensitization in this study, even though the mice might have in fact been sensitized. It may be concluded that this experiment provided weak evidence for the presence of EtOH-induced sensitization. However, this did not preclude analyses of the effects of prior EtOH drinking on locomotor sensitivity and the effects of repeated EtOH exposure on subsequent EtOH drinking.

All animals were tested following EtOH injections on the EtOH post-drinking test day. There was no evidence of between-group sensitization as would have been revealed if the EtOH Sensitized groups had shown greater EtOH-induced activity relative to the acute EtOH response of the Saline Control groups. There were also no group differences on the subsequent saline post-drinking test day indicating that the experimental procedures had no effect on baseline activity.

Ethanol Preference Drinking

After examination and analysis of drinking measures for 5 time periods – the first 3-hour, second 3-hour, total first 6-hour, the remaining 18-hour, and the total 24-hour periods – the 24-hour period was chosen as the most representative and informative. Hence, data

presented and discussed only pertain to 24-hour drinking and are presented in Figure 8, Panels A, B, and C.

The three-way ANOVA applied to post-sensitization drinking data revealed no overall group differences in EtOH consumption (Figure 8A), and greater overall 6% EtOH preference in EtOH-naïve mice (Figure 8B, white and black bars) relative to 6% EtOH preference of EtOH-experienced mice (Figure 8B, diagonal and hatched bars) and relative to their own 10% EtOH preference (significant Drinking Experience x EtOH Concentration interaction, $F_{1,42}=6.4$, $p<0.05$). Analyses of the EtOH-naïve groups (Group x EtOH Concentration ANOVAs) showed an overall trend toward increased EtOH consumption by the Naïve-EtOH Sensitized group (black bars) relative to their Naïve-Saline Control (white bars) counterparts regardless of EtOH concentration (Figure 8A; trend for main effect of Group, $F_{1,21}=4.2$, $p=0.055$). Analyses of the EtOH-experienced groups (Group x EtOH Concentration ANOVAs) showed an overall greater consumption of 10% relative to 6% EtOH solution regardless of EtOH treatment (Figure 8A, main effect of EtOH Concentration, $F_{1,21}=10.1$, $p<0.05$). Inspection of Figure 8C appears to show little, if any, group differences. However, analyses did reveal an overall greater fluid intake during the time when 10% relative to 6% EtOH was offered (main effect of EtOH Concentration, $F_{1,42}=19.8$, $p<0.01$), greater fluid intake during the time of 10% EtOH availability by the Naïve-Saline Control group relative to the Naïve-EtOH Sensitized group (Group x EtOH Concentration interaction, $F_{1,21}=4.4$, $p<0.05$), and greater 10% EtOH and water consumption of the EtOH-experienced groups relative to their 6% EtOH and water consumption levels ($F1,21=6.3$, $p<0.05$). Overall, it seemed that the EtOH-naïve mice consumed more and showed higher preference for the lower

6% EtOH concentration, whereas the EtOH-experienced mice consumed more of the 10% EtOH solution. There was also a tendency for greater EtOH consumption in the Naïve-EtOH Sensitized group relative to their Naïve-Saline Control counterparts. There was no effect of repeated EtOH administration on subsequent drinking behavior of EtOH-experienced mice.

Within-subject analyses showed that following sensitization, EtOH-experienced mice consumed more EtOH (g/kg), showed greater EtOH preference and greater total fluid intake relative to their pre-sensitization levels (main effects of Time, $Fs_{1,21}$=5.9, 6.4 and 5.2, respectively, all $p<0.05$). There were no significant effects of repeated EtOH administration on subsequent EtOH consumption or preference.

Age and Body Weight

The average ages of the different treatment groups ranged around 72-73 days. There were no significant group differences. Body weight was analyzed using two-way Group x Drinking Experience ANOVAs for each day of each phase that body weight was assessed. No differences between treatment groups were detected and the average group weight ranged around 21-23 g.

Experiment 2: Genetically Heterogeneous Mice

Sensitization to the Stimulant Effects of EtOH

Locomotor activity data collected during the EtOH-induced sensitization phase (Days 1-3 and 14) and on the final post-drinking EtOH and saline test days are presented in Figure 9. Overall, as in Exp. 1, the EtOH Sensitized groups (black symbols) had higher

locomotor activity than the Saline Control groups (white symbols) and the pattern of activity across test days differed as a function of repeated EtOH treatment (three-way Group x Drinking Experience x Test Day ANOVA, significant Group x Test Day interaction effect $F_{5,215}=64.1$, $p<0.001$). There was no effect of pre-sensitization drinking (Naïve-EtOH Sensitized vs. EtOH-EtOH Sensitized groups) on activity response to EtOH. Therefore, data were collapsed on Drinking Experience and re-analyzed. The resulting two-way Group x Test Day ANOVA revealed a significant interaction effect ($F_{5,225}=63.9$, $p<0.001$) indicating that, overall, the EtOH Sensitized groups had higher activity than the Saline Control groups on Days 3 and14, and on the EtOH post-drinking test day (all $p<0.01$). The group difference at this latter test day provided evidence for significant between-group sensitization to the effects of EtOH since the EtOH Sensitized groups showed a higher response to EtOH relative to the acute EtOH stimulant response of the Saline Control groups.

Within-group sensitization was assessed separately for each EtOH Sensitized group because of a priori decisions to do so and because the drinking data were also separated into the EtOH-naïve and EtOH-experienced conditions. Each of the previously EtOH-naïve or EtOH-experienced EtOH Sensitized groups (Figure 9, black circles and black triangles, respectively), showed significant within-group sensitization to the effects of EtOH evidenced by their increased activity responses to EtOH on Day 14 relative to their acute EtOH responses on Day 3 (both $p<0.01$) and sensitization persisted through the 8 days of post-sensitization drinking (denoted as a break on the x axis, all $p<0.01$). In addition, there was significant between-group sensitization on the post-drinking EtOH test day for each set of EtOH-naïve (Naïve-Saline Control vs. Naïve-EtOH Sensitized)

and EtOH-experienced (EtOH-Saline Control vs. EtOH-EtOH Sensitized) groups ($p<0.01$ and $p<0.05$, respectively).

A separate analysis was performed to evaluate potential differences between the Naïve-Saline Control group and the Naïve-Untreated group. There were no significant differences in activity between these treatment groups across activity test days (Group x Test Day ANOVA) indicating no effect of daily handling and injection stress on activity.

Ethanol Preference Drinking

EtOH consumption, preference, and total volume intake for the 24-hour period are presented in Figure 10, Panels A, B, and C, respectively. Three-way ANOVAs for post-sensitization data revealed an overall greater consumption of 10 % vs. 6% EtOH regardless of EtOH treatment or drinking experience (Figure 10A; main effect of EtOH Concentration, $F_{1,43}=14.7$, $p<0.001$). There were no other differences for EtOH preference or total volume intake measures (Figure 10B and 10C, respectively). Because of a priori decisions, separate Group x EtOH Concentration ANOVAs were performed for each of the EtOH-naïve and EtOH-experienced conditions. There were no effects of EtOH treatment or EtOH concentration on drinking measures for the EtOH-naïve mice (Figure 10, all panels, white and black bars). Thus, the trend for increased EtOH consumption (g/kg) by the EtOH Sensitized group found in Exp. 1 was not replicated. EtOH-experienced mice showed overall greater consumption of 10% vs. 6% EtOH paralleling their greater total fluid intake during the time of 10%EtOH availability, regardless of EtOH treatment (main effects of EtOH Concentration, $Fs_{1,25}=15.7$ and 6.8, both $p<0.05$). No group differences in EtOH preference were detected.

Within-subject analyses performed for the EtOH-experienced mice revealed that during pre-sensitization drinking, the EtOH-Saline Control group showed greater total 10% and water fluid intake than total 6% EtOH and water intake levels (Figure 10C, diagonal bars; Group x EtOH Concentration interaction effect, $F_{1,25}=10.0$, $p<0.01$). The significance of this effect is difficult to interpret, since no pre-sensitization group differences were expected to occur.

There were no significant differences in post-sensitization EtOH drinking between the Naïve-Saline Control and Naïve-Untreated groups indicating no effect of a single EtOH administration on subsequent drinking behavior. Data for the Naïve-Untreated group are not shown.

Age and Body Weight

The average age across treatment groups ranged around 72-75 days. There were no group differences with respect to age. Body weight was analyzed with two-way Group x Drinking Experience ANOVAs for each day that body weights were measured. The average body weights ranged around 22-25 g throughout the experiment and no significant group differences were detected.

Experiment 3: Pilot, DBA/2J and C57BL/6J Mice

Sensitization to the Stimulant Effects of EtOH

Locomotor activity data during EtOH-induced sensitization and during the post-drinking EtOH and saline test days for both C57BL/6J (B6) and DBA/2J (D2) mice are presented in Figure 11, Panels A and B, respectively. For each strain, three-way Group x Drinking

Experience x Test Day ANOVAs were conducted. Whereas there were significant Group x Test Day interaction effects for both B6 and D2 strains ($F_{5,110}$=15.1 and $F_{5,115}$=71.5, both $p<0.001$), pointing to a significant effect of EtOH treatment across test days, only data for the B6 mice also resulted in a significant Drinking Experience x Test Day interaction ($F_{5,110}$=4.1, $p<0.01$) indicating an effect of pre-sensitization drinking on subsequent locomotor response to EtOH injections. Prior drinking experience for B6 mice induced higher and more sustained sensitization (Figure 11A) as the EtOH-EtOH Sensitized group (black triangles) showed greater EtOH-induced stimulation than the Naïve-EtOH Sensitized group (black circles) on Day 14 and on the post-drinking EtOH test day (Drinking Experience x Test Day ANOVA for the EtOH Sensitized groups, both $p<0.05$). There was also a trend toward sensitization as a function of drinking experience as the EtOH-EtOH Sensitized groups showed a nearly significantly greater acute EtOH response on Day 3 relative to the Naïve-EtOH Sensitized group ($p=0.063$). This trend was supported by separate Group x Test Day ANOVAs for each of the EtOH-naïve and EtOH-experienced conditions, which showed that the EtOH-EtOH Sensitized group (Figure 11A, black triangles) had a significant stimulant EtOH response on Day 3 relative to its EtOH-Saline Control group (white triangles, $p<0.01$), whereas no group differences were detected between the Naïve-EtOH Sensitized and Naïve-Saline Control groups on that day (black and white circles, respectively).

Surprisingly, both B6 and D2 strains developed significant within-group sensitization to the effects of EtOH as indicated by the higher responses of EtOH Sensitized groups on Day 14 relative to their acute EtOH responses on Day 3 (one-way ANOVAs, all $p<0.05$; separate analyses for B6 and D2 Naïve-EtOH Sensitized and

EtOH-EtOH Sensitized groups). The EtOH-experienced B6 EtOH Sensitized group (Figure 11A, black triangles) also had increased activity on the post-drinking EtOH test day relative to their acute EtOH response on Day 3 ($p<0.01$), indicating that within-group sensitization lasted through the 12-day post-sensitization drinking phase.

In addition, between-group sensitization assessed on the post-drinking EtOH test day showed that for B6 mice, the Naïve- and EtOH-EtOH Sensitized groups had higher EtOH responses than their respective Naïve- and EtOH-Saline Control groups (Figure 11A; separate Group x Test Day ANOVAs for EtOH-naïve and EtOH-experienced conditions; both $p<0.05$). There were no group differences on the following saline test day indicating no effect of the experimental procedure on B6 baseline activity. For D2 mice, a Group x Time ANOVA collapsed on Drinking Experience revealed no group differences on the EtOH or saline post-drinking test days indicating no between-group sensitization, no effect of experimental procedure on baseline activity, and no sustained sensitization following the post-sensitization drinking.

There were no significant differences in activity between Naïve-Saline Control and Naïve-Untreated groups for either the B6 or D2 mice, indicating no effect of daily animal handling and stress on locomotor activity.

EtOH Preference Drinking: C57BL/6J Mice

EtOH consumption, preference, and total fluid intake (ml) data for the B6 mice are presented in Figure 12, Panels A, B, and C, respectively. B6 mice showed a dose-dependent increase in EtOH consumption, with the greatest consumption at the highest EtOH concentration (Figure 12A). Analyses of post-sensitization data showed a trend

toward differential response as a function of EtOH treatment regardless of prior drinking experience (near significant Group x EtOH Concentration interaction effect, $F_{2,44}$=3.1, p=0.053). The same interaction effect was highly significant for the 6-hour time period ($F_{2,44}$=5.3, p<0.01, data not shown). Follow up analyses indicated that the EtOH sensitized groups (black and hatched bars) consumed more 10% EtOH relative to the Saline Control groups (white and diagonal bars, p<0.01), while consumption levels at the lower EtOH concentrations were comparable. Prior drinking experience differentially affected preference for the different EtOH concentrations (Drinking Experience x EtOH Concentration interaction effect, $F_{2,44}$=4.1, p<0.05). As can be seen in Figure 6B, EtOH-naïve groups (white and black bars) showed less preference for 3% EtOH relative to EtOH-experienced groups (diagonal and hatched bars), but increased their preference to the level of the EtOH-experienced groups at the higher EtOH concentrations. There was an overall increase in total fluid intake with increasing EtOH concentrations regardless of EtOH treatment or pre-sensitization drinking experience (Figure 12C; main effect of EtOH Concentration, $F_{2,44}$=8.4, p<0.01).

For the EtOH-experienced groups (diagonal and hatched bars), within-subject analyses revealed that following sensitization, mice showed an overall lower 10% EtOH consumption relative to pre-sensitization levels, whereas consumption of the lower EtOH concentrations was comparable before and after sensitization (Figure 12A, significant Time x EtOH Concentration interaction effect, $F_{2,20}$=3.6, p<0.05). Additionally, total fluid intake during the time when 10% EtOH was offered was higher following sensitization relative to pre-sensitization levels, while total fluid intake for the lower EtOH concentrations was comparable across time (Figure 12C; significant Time x EtOH

Concentration interaction effect, $F_{2,20}$=9.7, $p<0.01$). There was a slight, but overall significantly enhanced EtOH preference with increasing EtOH concentrations before and after sensitization (Figure 12B; main effect of EtOH Concentration, $F_{2,20}$=3.9, $p<0.05$).

Ethanol Preference Drinking: DBA/2J Mice

Data for EtOH consumption, preference, and total EtOH and water fluid intake for D2 mice are presented in Figure 13, Panels A, B, and C, respectively. It is somewhat misleading to apply the word "preference" to the drinking behavior of D2 mice, when it should be more appropriately labeled as "aversion". Therefore, data will be discussed in terms of more or less aversion, rather than lower or greater preference, respectively. Pre-sensitization drinking differentially affected post-sensitization EtOH consumption and aversion at the three EtOH concentrations (significant Drinking Experience x EtOH Concentration interactions effects, $Fs_{2,46}$=6.0 and 14.9, respectively, both $p<0.01$). The EtOH-naïve groups (Figure 13A and 13B, white and black bars) consumed more and showed less aversion for 3% EtOH relative to EtOH-experienced groups (diagonal and hatched bars, both $p<0.01$), but EtOH consumption decreased and EtOH aversion increased to the levels of the EtOH-experienced groups at higher EtOH concentrations. On the other hand, the EtOH-experienced groups showed an increase in EtOH consumption with increasing EtOH concentration (Figure 13A, diagonal and hatched bars, $p<0.05$), but showed no change in EtOH aversion across EtOH concentrations (Figure 7B). There was no effect of EtOH treatment or EtOH drinking experience on total fluid intake as overall intake increased across EtOH concentrations (Figure 13C; main effect of EtOH concentration, $F_{2,46}$=7.6, $p<0.01$).

There was no effect of EtOH-induced sensitization when pre- vs. post-sensitization drinking measures were compared for the EtOH-experienced D2 mice (Figure 13, diagonal and hatched bars). Overall, animals consumed greater quantities of 6% and 10% EtOH relative to 3% EtOH (Figure 13A) and showed overall less EtOH aversion during the pre-sensitization relative to the post-sensitization period (Figure 13B; main effects of EtOH Concentration and Time, respectively, $F_{2,20}=4.2$, $p<0.05$ and $F_{1,10}=4.9$, $p=0.051$, respectively). The groups also showed greater total fluid intake when 10% EtOH was available during the post-sensitization period relative to their pre-sensitization levels, while fluid intake before and after sensitization was comparable at the lower EtOH concentrations (Figure 13C; significant Time x EtOH Concentration interaction effect, $F_{2,20}=4.7$, $p<0.05$).

There were no differences in EtOH drinking measures between the Naïve-Saline Control and Naïve-Untreated groups for either the B6 or D2 strains indicating no effects of stress due to daily handling and injections on drinking behavior (data not shown).

Age and Body Weight

All mice were age-matched at 74 days at the beginning of the experiment. Body weights were analyzed with Group x Drinking Experience ANOVAs for each day that body weights were measured. The analyses resulted in some significant main effects of Group or Drinking Experience for both B6 and D2 mice. However, the differences were on the average about 1.5 g or so, likely not enough to contribute to a meaningful change in behavior. Significant differences were likely detected because of the very small standard errors associated with body weight measures in this experiment.

Experiment 4: C57BL/6J Mice

Sensitization to the Stimulant Effects of EtOH

The prior Exp. 3 resulted in unexpected and never before shown EtOH injection-induced sensitization to the stimulant effects of EtOH in B6 mice. In fact, B6 mice had been previously shown to be resistant to EtOH-induced sensitization (Cunningham et al., 1992; Phillips et al., 1994b; Phillips et al., 1995; Phillips et al., 1996). These results, combined with the avidity of this inbred strain for 10% EtOH solution, necessitated replication and extension of the sample size. The current experiment, therefore, aimed at (1) replicating the significant sensitization to the effects of EtOH in B6 mice, and (2) assessing whether EtOH drinking experience induced sensitization to the effects of EtOH in a larger sample size of B6 mice.

Activity data are presented in Figure 14. Overall, EtOH-experienced mice (black and white triangles) had higher activity than EtOH-naïve mice (black and white circles) and locomotor response differed across days as a function of repeated EtOH administration (three-way Group x Drinking Experience x Test Day ANOVA, main effect of Drinking Experience and significant Group x Test Day interaction effects, $F_{1,44}=9.1$, $p<0.01$ and $F_{3,132}=73.3$, $p<0.001$, respectively). Follow up two-way Group x Test Day ANOVAs for each set of EtOH-naïve and EtOH-experienced groups revealed significant differences between EtOH-Sensitized and Saline Control groups on Days 3 and 14, indicating significant EtOH-induced stimulant responses in the EtOH Sensitized groups (all $p<0.01$). Because of a priori decisions, the presence of within-group sensitization to the effects of EtOH was assessed using one-way ANOVAs for each of the Naïve-EtOH Sensitized and EtOH-EtOH Sensitized groups. Both groups developed

sensitization to the effects of EtOH as evidenced by their enhanced EtOH responses on Day 14 relative to their acute EtOH responses on Day 3 (both $p<0.001$). The same analyses also showed that the EtOH-experienced EtOH Sensitized group (black triangles) had a significant acute stimulant EtOH response on Day 3 relative to baseline Day 2 activity ($p<0.02$), whereas the EtOH-naïve EtOH Sensitized group (black circles) did not. This indicated that prior EtOH drinking experience sensitized B6 mice to the stimulant effects of EtOH. However, both Naïve- and EtOH-EtOH Sensitized groups showed significant acute EtOH responses on Day 3 relative to their respective Naïve- and EtOH-Saline Control counterparts (Group x Test Day ANOVAs for each set of Naïve- vs. EtOH-experienced groups, both $p<0.01$). It is possible that the significant difference between the Naïve-EtOH Sensitized (black circles) and Naïve-Saline Control (white circles) groups on Day 3 was due to the continued habituation of the Naïve-Saline Control group (Day 3 vs. Day 2 saline response, $p<0.02$), rather than to a significant EtOH stimulant response of the Naïve-EtOH Sensitized group. The EtOH-Saline Control group showed similar activity levels on Days 2 and 3. The potential effect of prior drinking experience on subsequent EtOH response was directly compared between the Naïve- and EtOH-EtOH Sensitized groups (Drinking Experience x Time ANOVA). The analysis resulted in main effects of Drinking Experience and Time ($F_{1,22}=8.3$ and $F_{3,66}=70.8$, respectively, both $p<0.01$), but no interaction effect, indicating that EtOH drinking experience contributed to an overall enhanced, but not differential, locomotor response to EtOH across time.

EtOH Preference Drinking

EtOH consumption, preference, and total fluid intake data are presented in Figure 15, Panels A, B, and C, respectively. Following sensitization, the EtOH-experienced EtOH Sensitized group (hatched bar) consumed significantly more EtOH relative to their EtOH-experienced Saline Control counterparts (diagonal bar, Figure 15A; Group x Drinking Experience interaction effect, $F_{1,44}=5.3$, $p<0.05$). This could be due to the generally lower EtOH consumption levels of the EtOH-Saline Control group (diagonal bar), which also significantly differed from EtOH consumption levels of the Naïve-Saline Control group (white bar, $p<0.05$).

There was no effect of sensitization to the effects of EtOH on EtOH preference (Figure 15B). EtOH-naïve groups (white and black bars) showed overall greater EtOH preference than EtOH-experienced groups (diagonal and hatched bars; main effect of Drinking Experience, $Fs_{1,44}= 5.2$, $p<0.05$).

Within-group analyses (Group x Time ANOVAs) could only be conducted for the EtOH-experienced EtOH Sensitized and Saline Control groups. The EtOH-EtOH Sensitized group had significantly greater EtOH consumption following sensitization relative to their pre-sensitization levels (Group x Time interaction effect, $F_{1,22}=5.2$, $p<0.05$) indicating that sensitization contributed to increased drinking behavior. No group differences in EtOH preference or total fluid intake were detected.

Age and Body Weight

All mice were age matched at 66 days at the beginning of this experiment. There were no group differences in body weight during the pre-sensitization drinking. There were some

significant group differences in body weight during the EtOH-induced sensitization phase and post-sensitization drinking. However, the actual weight differences varied between 1.0 and 1.5 g, differences we believe were unlikely to have affected behavior. The significant differences are likely due to the very small error associated with body weight measurements, and are not reported.

DISCUSSION

The main results from these experiments included (1) a trend toward sensitization to the effects of EtOH following voluntary EtOH drinking in the alcohol preferring C57BL/6J (B6) mouse strain, (2) a significant increase in EtOH consumption and preference in EtOH-experienced B6 mice following a sensitizing EtOH regimen, and (3) a tendency for increased EtOH consumption in EtOH-naïve genetically heterogeneous mice following a sensitizing regimen of EtOH administration. The surprising results were that the B6 mice developed robust sensitization to the stimulant effects of EtOH as a function of repeated EtOH administrations, and that the magnitude of sensitization in D2 mice was smaller than expected. To our knowledge, this is the first demonstration of sensitization in B6 mice. Previously, EtOH was administered to B6 mice every other day (Phillips et al., 1995; Phillips et al., 1996) or daily (Phillips et al., 1994b). In these studies, no significant sensitization to the effects of EtOH was seen in B6 mice. In one study, EtOH was administered daily for 10 days as in the present experiments, however, mice were tested every third day, and both the treatment and testing EtOH dose was 2 g/kg (Phillips et al., 1994b). These procedural differences may have been enough to account for the absence of sensitization to the effects of EtOH in B6 mice in previous studies and its presence in

two of the current experiments. B6 sensitization was also subsequently replicated in a separate series of experiments in this laboratory (unpublished data). Other studies have also failed to show sensitization to the effects of EtOH in B6 mice following repeated EtOH injections (Cunningham et al., 1992; Cunningham, 1995).

We replicated previous results (Nocjar and Middaugh, 1997), showing that EtOH consumption induced sensitization to acute EtOH injections in B6 mice. The B6 mice likely consumed sufficient amounts of EtOH to induce sensitization. The ability to show the presence of sensitization as a function of EtOH self-administration has implications both for human alcohol consumption and for the phenomenon of sensitization as a likely contributor to the maintenance of alcohol taking behavior. This effect was likely dose-dependent, as genetically heterogeneous mice, which actually consumed moderate amounts of about 4-6 g/kg EtOH per day, and D2 mice, which consumed only about 1 g/kg EtOH per day, may not have reached the necessary EtOH amounts to induce sensitization. Perhaps restricted or forced EtOH access (EtOH solution being the only fluid provided) might lead to sensitization in these two strains. Alternatively, factors that motivate differential avidity for alcohol may also underlie differential central nervous system sensitivity to the effects of alcohol. In such case it is possible that some strains or some individuals may be incapable of developing sensitization to the effects of EtOH.

Repeated administration of EtOH in a paradigm that induced sensitization to the effects of EtOH contributed to greater EtOH consumption in B6 and genetically heterogeneous mice. The inability of sensitization to alter subsequent EtOH drinking in D2 mice may have been due to both their innate low EtOH consumption and preference and to their low magnitude of sensitization in the current study. D2 mice have been

shown to develop significant sensitization to the effects of EtOH (Cunningham et al., 1992; Cunningham, 1995; Phillips et al., 1994b; Phillips et al., 1996), however in one study (Phillips et al., 1995) they failed to do so. The high sensitivity of D2 mice to the acute stimulant effects of EtOH in the current study may have contributed to a ceiling stimulant effect making sensitization less robust. A lower dose of EtOH might reveal a greater degree of sensitization to the effects of EtOH in these mice. The data from the D2 mice are not conclusive, however, and might change with a higher sample size. The study including D2 mice served as a pilot and based on the data, a more precise study was designed (Exp. 4) using only B6 mice.

Whereas it was the EtOH-experienced sensitized B6 mice that consumed more EtOH relative to their non-sensitized controls, it was the EtOH-naïve sensitized genetically heterogeneous mice that consumed more EtOH than their non-sensitized controls. The source of this difference is unclear but it could be a function of the differential EtOH avidity of the B6 and genetically heterogeneous strains. For example, the combination of factors that motivate the moderate EtOH consumption of the genetically heterogeneous mice (e.g., taste, pharmacologic EtOH effects, and sensitivity to either or both) might preclude an effect of experimenter-induced EtOH behavioral sensitization on subsequent drinking behavior. Unfortunately, in the genetically heterogeneous mice, the trend toward higher EtOH consumption following repeated EtOH administration seen in Exp.1 was not replicated in Exp. 2 (compare Figures 7 and 9). In addition, sensitization to the effects of EtOH was less robust in Exp. 1 than in Exp. 2, speculatively because in Exp. 1, locomotor activity was tested during the last 3 hours of the light cycle, whereas in Exp. 2 mice were tested beginning two hours after dark

onset. We have always tested animals beginning two hours after light onset, a time more comparable to testing in Exp. 2, than in Exp. 1. The mice in Exp. 1 were likely sensitized, but the unusual time of testing could have precluded our detection of sensitization.

EtOH-naïve D2 and genetically heterogeneous mice showed higher post-sensitization EtOH consumption relative to their EtOH-experienced counterparts regardless of treatment during the EtOH-induced sensitization phase. Stress due to daily handling and injections during EtOH-induced sensitization may have contributed to enhanced EtOH consumption in EtOH-naïve animals (Cappell and Herman, 1972; Bond, 1978; Powers and Kutash, 1985; Volpicelli et al., 1990), whereas prior EtOH exposure in EtOH-experienced animals may have altered their sensitivity to the stress of daily handling and injections, keeping their subsequent EtOH consumption at lower levels. Alternatively, EtOH-experienced animals may have developed a taste aversion to EtOH manifest as reduced post-sensitization drinking levels relative to EtOH-naïve groups (Pizzi and Cook, 1996).

Investigation of the association between drug sensitization and drug reinforcement is warranted considering some neuroanatomical and neurochemical factors that the two behaviors share. The mesolimbic dopaminergic system has been implicated as one common pathway that mediates drug-induced locomotion, drug reward and drug relapse (Di Chiara and Imperato, 1988; Bozarth, 1989; Robinson, 1993; Phillips and Shen, 1996; Self, 1998; De Vries et al., 1999; Di Chiara, 1999; McBride et al., 1999; Vanderschuren et al., 1999b). Changes in dopaminergic transmission and receptor systems have been implicated both in sensitization to the effects of EtOH and

reinforcement from EtOH (Diana et al., 1993; Samson and Hodge, 1993; Ng and George, 1994; Ortiz et al., 1995; Nestby et al., 1997). Specifically, increases in dopamine D_2 receptor binding in the caudate-putamen were associated with sensitization to the effects of EtOH (Souza-Formigoni et al., 1999), while the lack of D_2 receptors was associated with decreased sensitivity to the depressant effects of EtOH and decreased EtOH preference drinking behavior (Phillips et al., 1998b). A region on mouse chromosome 9 that contains the gene for the D_2 receptor has been significantly associated with variability in alcohol consumption (Phillips et al., 1998a). These studies are examples of convergent evidence for a role of a specific neural receptor in EtOH locomotion and reinforcement.

Sensitization to the effects of drugs of abuse is not unique to animal models. Sensitization to the stimulant effects of EtOH (increase in response rate for money reinforcement) and amphetamine (increased motor activity, speech, eye blink rate, subjective mood elevation) has been recorded in humans as well (Rumbold and White, 1987; Strakowski et al., 1996; Strakowski, Sax, 1998). There was no evidence for sensitization to the effects of cocaine as a function of two cocaine administrations in cocaine users (Rothman et al., 1994). However, sons of alcoholics tend to develop chronic sensitization to some autonomic arousal measures such as finger pulse amplitude, skin conductance and general motor activity, whereas sons of nonalcoholics tend to develop chronic tolerance to these measures (Newlin and Thomson, 1990; Newlin and Thomson, 1991; Newlin and Thomson, 1999). EtOH-induced elevations in mood and feelings of euphoria tend to be reported during the rising limb of the blood alcohol concentration curve (Babor et al., 1983; Lukas et al., 1986; Newlin and Thomson, 1990).

Because sensitization tends to develop to the initial, stimulant EtOH effect observed as blood alcohol levels rise, it might represent a model that captures the enhancement of the positive or euphoric EtOH effects with repeated EtOH exposure. If, for the animal or human, the sense of euphoria is enhanced over time, it is easy to predict that drug-taking behavior will be maintained. Indeed, drug sensitization has been implicated as a contributor to drug craving (Robinson and Berridge 1993). In addition, because behavioral sensitization tends to last for up to months following cessation of drug administration (Babbini et al., 1975; Shuster et al., 1977; Paulson et al., 1991; Lessov and Phillips, 1998; Volpicelli et al., 1999), it has also been implicated as a contributor to relapse in drug-taking behavior following periods of abstinence (Sato et al., 1983; Robinson, 1993; Bartlett et al., 1997).

SUMMARY AND CONCLUSIONS

This series of experiments investigated whether sensitization to the effects of EtOH could be induced by voluntary EtOH drinking and whether it could alter subsequent EtOH drinking behavior in genetically heterogeneous mice and in the C57BL/6J (B6) and DBA/2J (D2) inbred strains. The key results were that voluntary EtOH drinking induced sensitization to a challenge EtOH injection in the B6 mice and that sensitized B6 mice showed enhanced EtOH consumption relative to their non-sensitized controls. B6 mice also developed robust sensitization as a result of repeated EtOH administration, which was a surprising result and to our knowledge, a first demonstration. Results in the genetically heterogeneous mice were inconsistent. In D2 mice, the robust acute stimulant

EtOH response, the low magnitude of sensitization and the low drinking levels may have precluded detection of significant effects.

The fact that sensitization can develop as a function of EtOH self-administration and that it can subsequently contribute to greater EtOH self-administration has powerful implications for the role of sensitization in the mediation of EtOH reinforcement, particularly in an animal model genetically predisposed to voluntary EtOH drinking. It is possible that sensitive individuals, through EtOH consumption, become sensitized to the euphoric or positive aspects of EtOH and that sensitization maintains further EtOH consumption potentially leading to EtOH abuse and addiction.

Table 2. Procedure for Ethanol-Induced Sensitization. Locomotor activity and EtOH injection procedure used to induce sensitization to the stimulant effects of EtOH.

	Habituation Day 1	**Baseline** Day 2	**Acute EtOH** Day 3	**Daily Treatment** Days 4-13	**Sensitization** Day 14
Saline Control	Saline	Saline	Saline	Saline	Saline
Test	Yes	Yes	Yes	No	Yes
EtOH Sensitized	Saline	Saline	EtOH 2g/kg	EtOH 2.5g/kg	EtOH 2g/kg
Test	Yes	Yes	Yes	No	Yes
Untreated	Saline	Saline	Saline	None	Saline
Test	Yes	Yes	Yes	No	Yes

Locomotor activity was tested on Days 1, 2, 3, and 14. Activity data were collected for 10 min in two 5-min epochs. All injections were intraperitoneal (i.p.).
Habituation and **Baseline**: On Days 1 and 2, all treatment groups were tested following saline injections. Day 1 served as a habituation test day to acclimate mice to the injection procedure and apparatus. Day 2 provided a measure for baseline activity. **Acute EtOH**: On Day 3, *Saline Control* and *Untreated* groups were tested following saline, while the *EtOH Sensitized* group was tested following 2 g/kg EtOH. Day 3 provided a measure for the acute response to EtOH of the *EtOH Sensitized* group. **Daily Treatment**: During Days 4-13, *Saline Control* and *EtOH Sensitized* groups received 10 daily saline or 2.5 g/kg EtOH injections, respectively, with no activity testing. The *Untreated* group received no injections. **Sensitization**: On Day 14, the *Saline Control* and *Untreated* groups were tested following saline, while the *EtOH Sensitized* group was tested following 2 g/kg EtOH. Day 14 provided a measure for the EtOH sensitized response of the *EtOH Sensitized* group.

Figure 7. Total 10-min locomotor activity of genetically heterogeneous mice across EtOH-induced sensitization activity test days 1, 2, 3, and 14 and post-drinking EtOH and saline (Sal) test days. White and black circles: groups with no prior EtOH drinking experience (Naïve) that received repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations, respectively. White and black triangles: groups with prior EtOH drinking experience (EtOH) that received repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations, respectively. On Days 1 and 2, all mice received saline injections. On Days 3 and 14, EtOH Sensitized groups received 2 g/kg EtOH, while Saline Control groups received saline. On post-drinking EtOH and Sal test days, all mice received 2 g/kg EtOH or saline injections, respectively. n=11-12 per group

* Significant within-group sensitization assessed as increased EtOH responses on Day 14 and on the post-drinking EtOH test day relative to the acute EtOH response on Day 3 (both $p<0.05$). Within-group sensitization was significant only when data for the Naïve- and EtOH-EtOH Sensitized groups were collapsed on prior drinking experience.

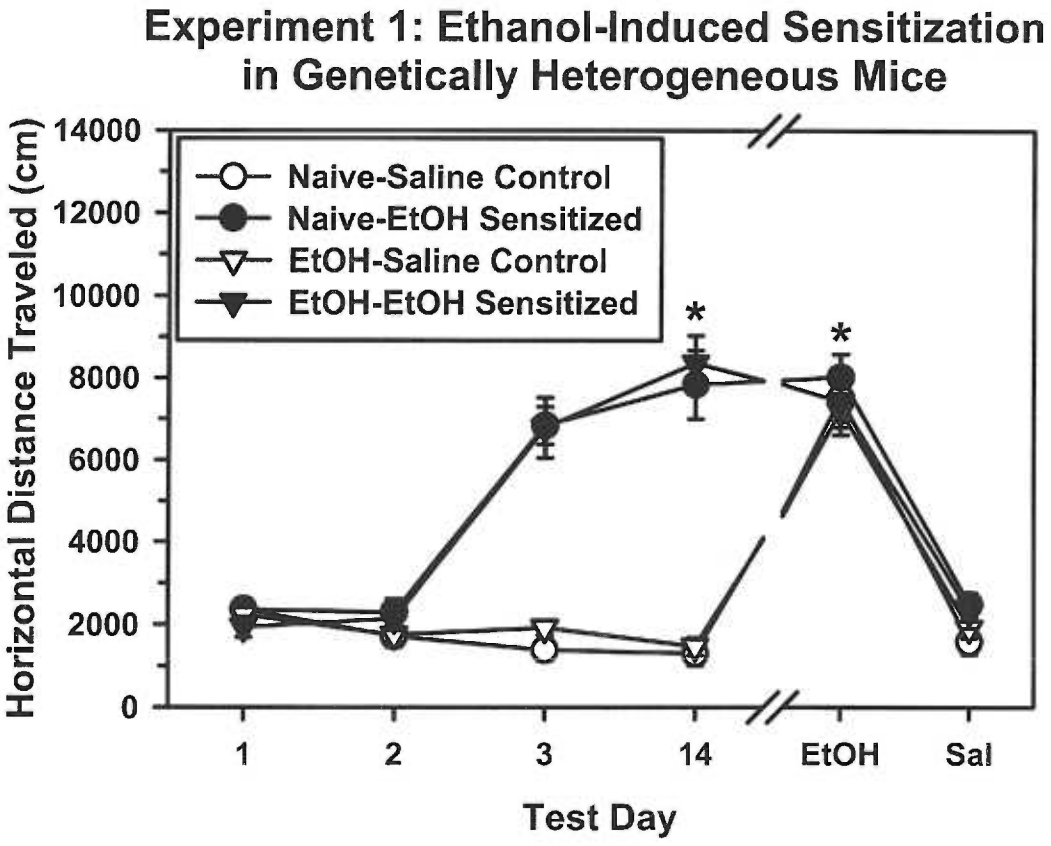

Experiment 1: Ethanol-Induced Sensitization
in Genetically Heterogeneous Mice
Horizontal Distance Traveled (cm)
14000
12000
10000
8000
6000
4000
2000
0
Naive-Saline Control
Naive-EtOH Sensitized
EtOH-Saline Control
EtOH-EtOH Sensitized
*
*
1
2
3
14
EtOH
Sal
Test Day

Figure 8. 24-hour EtOH consumption (g/kg, Panel A), EtOH preference (EtOH consumed/total fluid consumed, Panel B), and total fluid intake (ml, Panel C) measures for 6% and 10% EtOH solutions before (Pre-Sensitization) and after (Post-Sensitization) a sensitizing regimen of EtOH administration in genetically heterogeneous mice. White and black bars: groups with no prior Pre-Sensitization EtOH drinking experience (Naïve) and subsequently treated with repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations during the EtOH-induced sensitization period. Diagonal and hatched bars: groups with prior Pre-Sensitization EtOH drinking experience (EtOH) and subsequently treated with repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations during the EtOH-induced sensitization period. n=11-12 per group

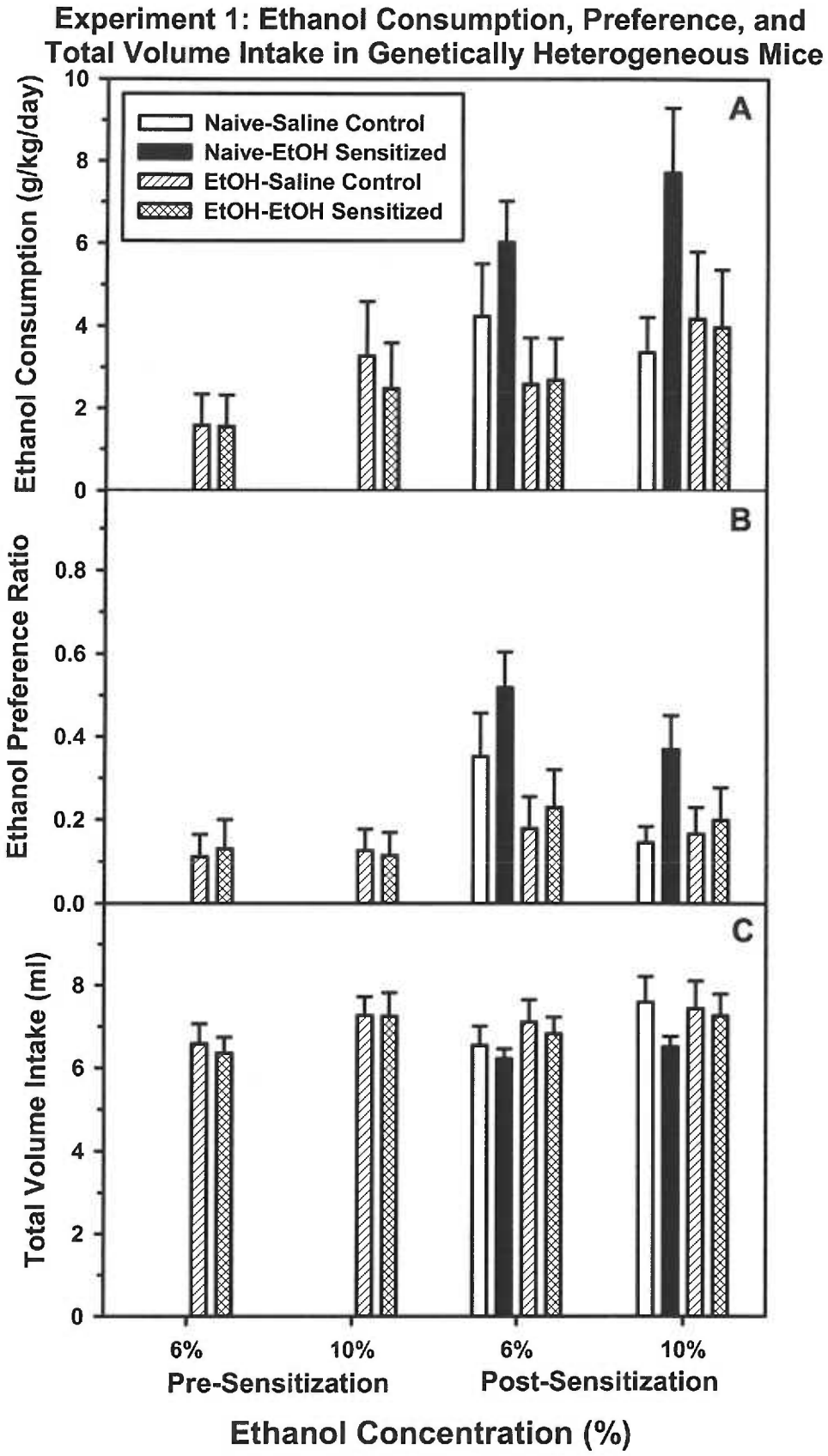

Experiment 1: Ethanol Consumption, Preference, and Total Volume Intake in Genetically Heterogeneous Mice
Naive-Saline Control
Naive-EtOH Sensitized
EtOH-Saline Control
EtOH-EtOH Sensitized
A
B
C
Ethanol Consumption (g/kg/day)
Ethanol Preference Ratio
Total Volume Intake (ml)
0
2
4
6
8
10
0.0
0.2
0.4
0.6
0.8
6%
10%
6%
10%
Pre-Sensitization
Post-Sensitization
Ethanol Concentration (%)

Figure 9. Total 10-min locomotor activity of genetically heterogeneous mice across EtOH-induced sensitization activity test days 1, 2, 3, and 14 and post-drinking EtOH and saline (Sal) test days. White and black circles: groups with no prior EtOH drinking experience (Naïve) that received repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations, respectively. White and black triangles: groups with prior EtOH drinking experience (EtOH) that received repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations, respectively. White square: group with no prior EtOH drinking experience (Naïve) that did not receive daily injections (Untreated) during Days 4-13 treatment phase. On Days 1 and 2, all mice received saline injections. On Days 3 and 14, EtOH Sensitized groups received 2 g/kg EtOH, while Saline Control and Untreated groups received saline. On post-drinking EtOH and Sal test days, all mice received 2 g/kg EtOH or saline injections, respectively. n=10-14 per group

* Significant within-group sensitization assessed as an increase in the EtOH response of each of the Naïve- and EtOH-EtOH Sensitized groups, relative to their acute EtOH responses on Day 3 (all $p<0.001$).

** Significant between- and within-group sensitization assessed as an increase in EtOH responses of each of the Naïve- and EtOH-EtOH Sensitized groups relative to their respective Naïve- and EtOH-Saline Control counterparts on the post-drinking EtOH test day, and relative to their own acute EtOH response on Day 3. p values for within-group sensitization were as stated above; $p<0.001$ for between-group sensitization for the Naïve-EtOH Sensitized and Naïve-Saline Control groups; $p<0.05$ for between-group sensitization for the EtOH-EtOH Sensitized and EtOH-Saline Control groups.

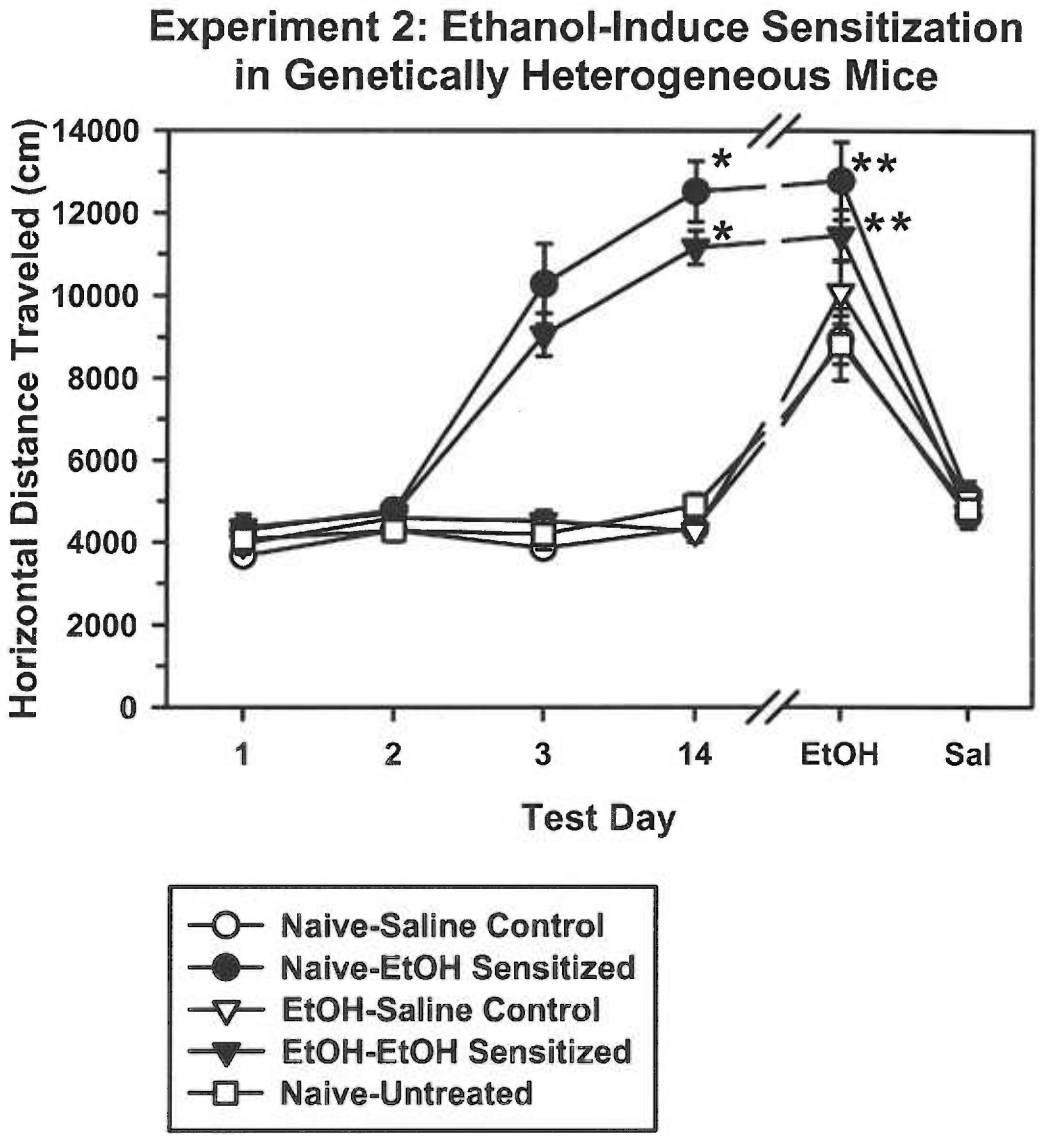

Experiment 2: Ethanol-Induce Sensitization
in Genetically Heterogeneous Mice
Horizontal Distance Traveled (cm)
14000
12000
10000
8000
6000
4000
2000
0
1
2
3
14
EtOH
Sal
Test Day
*
*
**
**
Naive-Saline Control
Naive-EtOH Sensitized
EtOH-Saline Control
EtOH-EtOH Sensitized
Naive-Untreated

Figure 10. 24-hour EtOH consumption (g/kg, Panel A), EtOH preference (EtOH consumed/total fluid consumed, Panel B), and total fluid intake (ml, Panel C) measures for 6% and 10% EtOH solutions before (Pre-Sensitization) and after (Post-Sensitization) a sensitizing regimen of EtOH administration in genetically heterogeneous mice. White and black bars: groups with no prior Pre-Sensitization EtOH drinking experience (Naïve) and subsequently treated with repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations during the EtOH-induced sensitization period. Diagonal and hatched bars: groups with prior Pre-Sensitization EtOH drinking experience (EtOH) and subsequently treated with repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations during the EtOH-induced sensitization period. n=10-14 per group

Experiment 2: Ethanol Consumption, Preference, and Total Volume Intake in Genetically Heterogeneous Mice

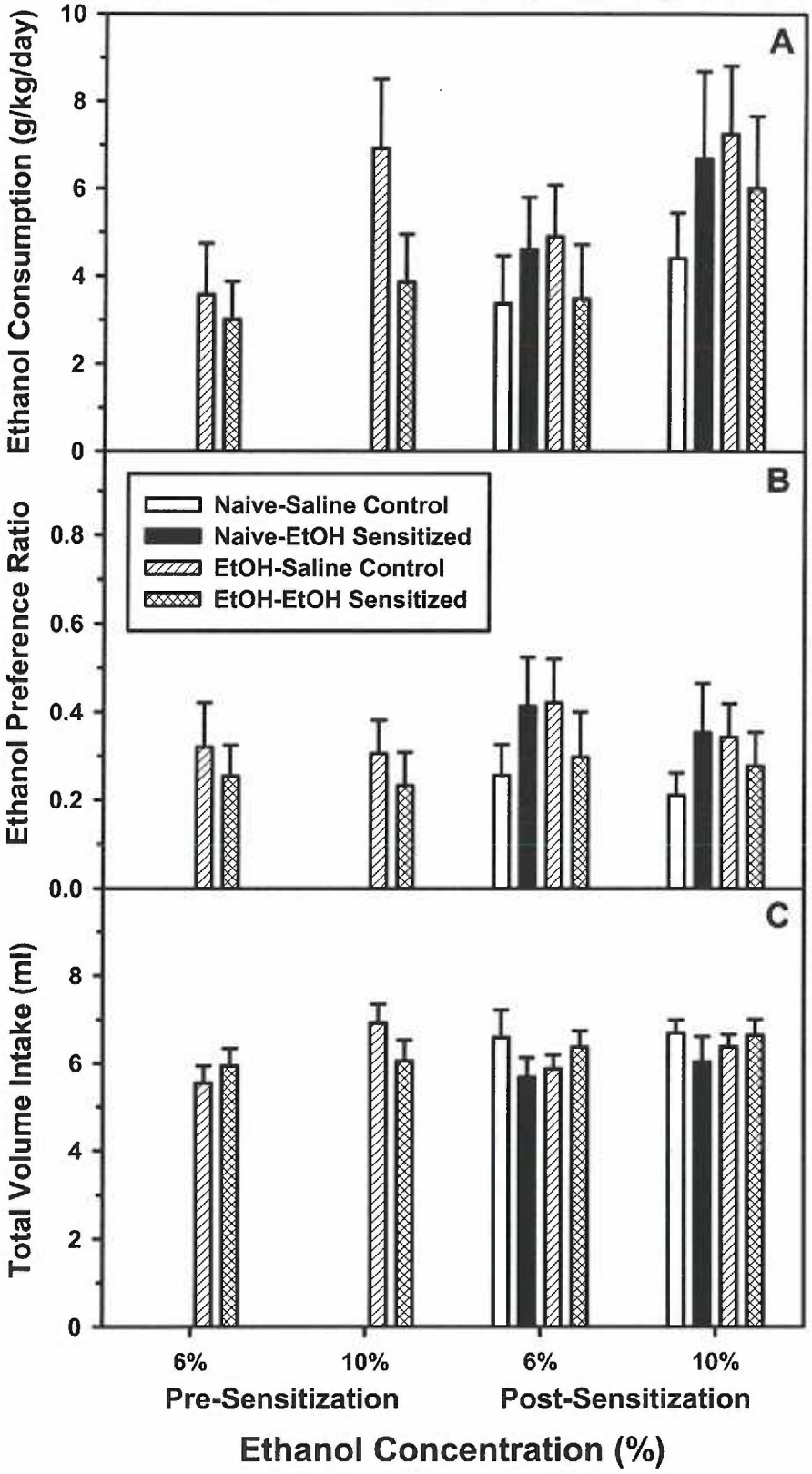


Figure 11. Total 10-min locomotor activity of C57BL/6J (Panel A) and DBA/2J (Panel B) inbred mice across EtOH-induced sensitization activity test days 1, 2, 3, and 14 and post-drinking EtOH and saline (Sal) test days. White and black circles: groups with no prior EtOH drinking experience (Naïve) that received repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations, respectively. White and black triangles: groups with prior EtOH drinking experience (EtOH) that received repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations, respectively. White square: group with no prior EtOH drinking experience (Naïve) that did not receive daily injections (Untreated) during Days 4-13 treatment phase. On Days 1 and 2, all mice received saline injections. On Days 3 and 14, EtOH Sensitized groups received 2 g/kg EtOH, while Saline Control and Untreated groups received saline. On post-drinking EtOH and Sal test days, all mice received 2 g/kg EtOH or saline injections, respectively. n=6-8 per group

* Significant within-group sensitization assessed as an increase in the EtOH response of each of the Naïve- and EtOH-EtOH Sensitized groups, relative to their acute EtOH responses on Day 3. $p<0.01$ for C57BL/6J mice; $p<0.05$ for DBA/2J mice

** Significant between- and within-group sensitization assessed as an increase in EtOH responses of the EtOH-EtOH Sensitized group relative to their EtOH-Saline Control counterparts on the post-drinking EtOH test day, and relative to their own acute EtOH response on Day 3. $p<0.01$ for both measures of sensitization

Significant between-group sensitization assessed as greater EtOH response of the Naïve-EtOH Sensitized group relative to their Naïve-Saline Control counterparts on the post-drinking EtOH test day. $p<0.05$

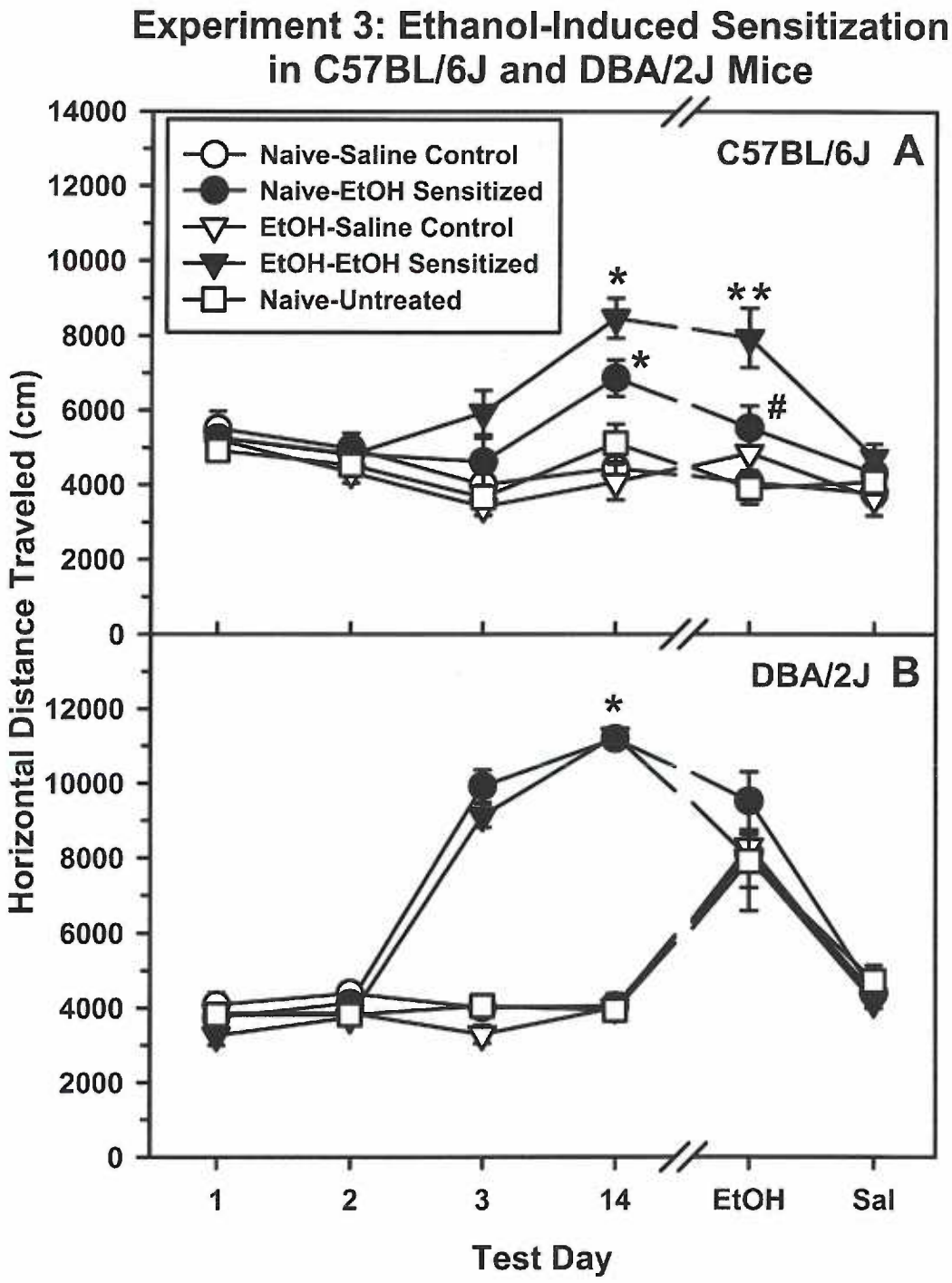

Experiment 3: Ethanol-Induced Sensitization
in C57BL/6J and DBA/2J Mice
Naive-Saline Control
Naive-EtOH Sensitized
EtOH-Saline Control
EtOH-EtOH Sensitized
Naive-Untreated
C57BL/6J A
DBA/2J B
Horizontal Distance Traveled (cm)
14000
12000
10000
8000
6000
4000
2000
0
*
**
*
#
1
2
3
14
EtOH
Sal
Test Day

Figure 12. 24-hour EtOH consumption (g/kg, Panel A), EtOH preference (EtOH consumed/total fluid consumed, Panel B), and total fluid intake (ml, Panel C) measures for 3%, 6%, and 10% EtOH solutions before (Pre-Sensitization) and after (Post-Sensitization) a sensitizing regimen of EtOH administration in C57BL/6J inbred mice. White and black bars: groups with no prior Pre-Sensitization EtOH drinking experience (Naïve) and subsequently treated with repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations during the EtOH-induced sensitization period. Diagonal and hatched bars: groups with prior Pre-Sensitization EtOH drinking experience (EtOH) and subsequently treated with repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations during the EtOH-induced sensitization period. n=6-7 per group

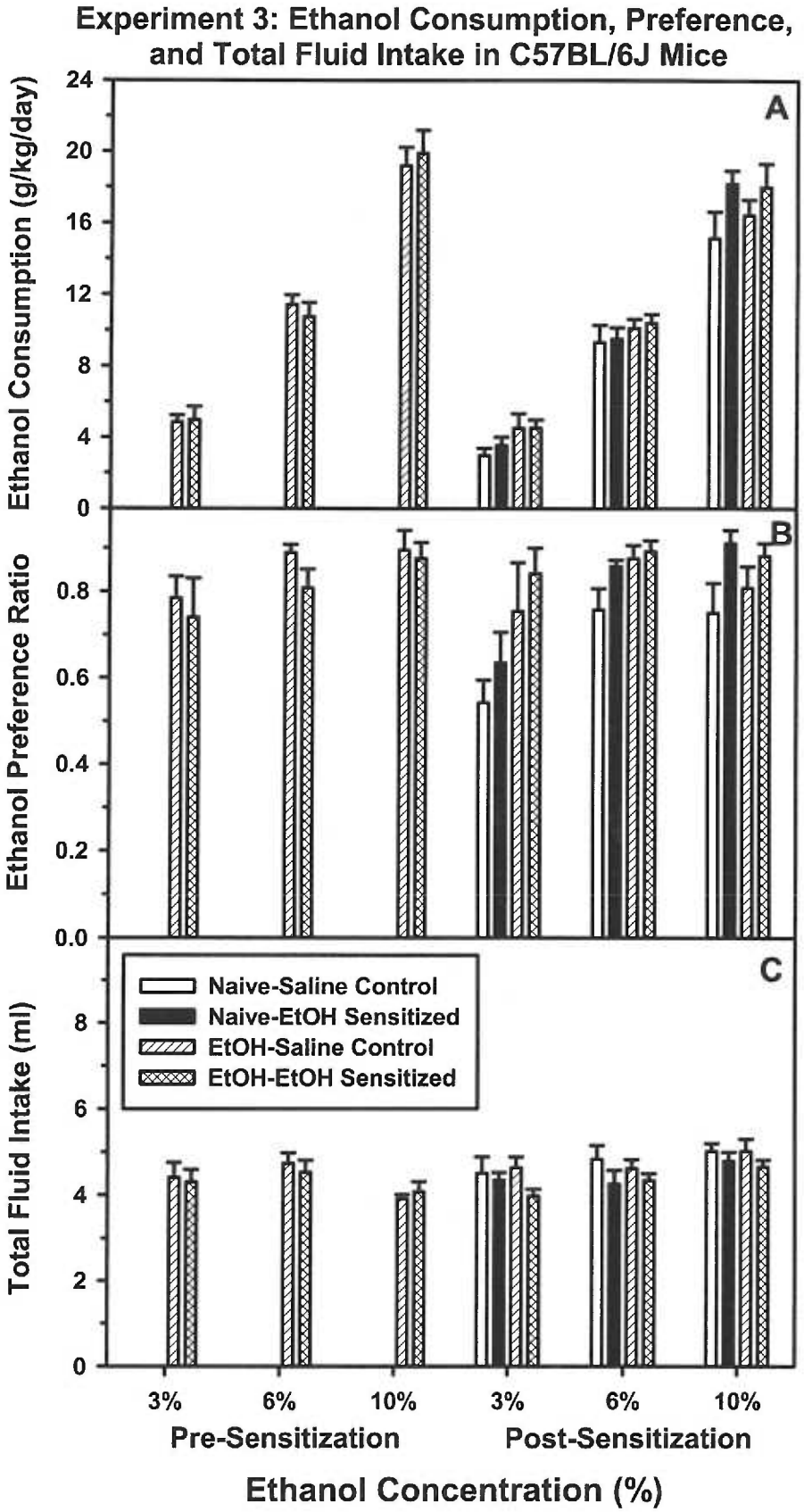

Experiment 3: Ethanol Consumption, Preference, and Total Fluid Intake in C57BL/6J Mice
Ethanol Consumption (g/kg/day)
Ethanol Preference Ratio
Total Fluid Intake (ml)
A
B
C
Naive-Saline Control
Naive-EtOH Sensitized
EtOH-Saline Control
EtOH-EtOH Sensitized
3%
6%
10%
3%
6%
10%
Pre-Sensitization
Post-Sensitization
Ethanol Concentration (%)

Figure 13. 24-hour EtOH consumption (g/kg, Panel A), EtOH preference (EtOH consumed/total fluid consumed, Panel B), and total fluid intake (ml, Panel C) measures for 3%, 6%, and 10% EtOH solutions before (Pre-Sensitization) and after (Post-Sensitization) a sensitizing regimen of EtOH administration in DBA/2J inbred mice. White and black bars: groups with no prior Pre-Sensitization EtOH drinking experience (Naïve) and subsequently treated with repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations during the EtOH-induced sensitization period. Diagonal and hatched bars: groups with prior Pre-Sensitization EtOH drinking experience (EtOH) and subsequently treated with repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations during the EtOH-induced sensitization period. n=6-8 per group

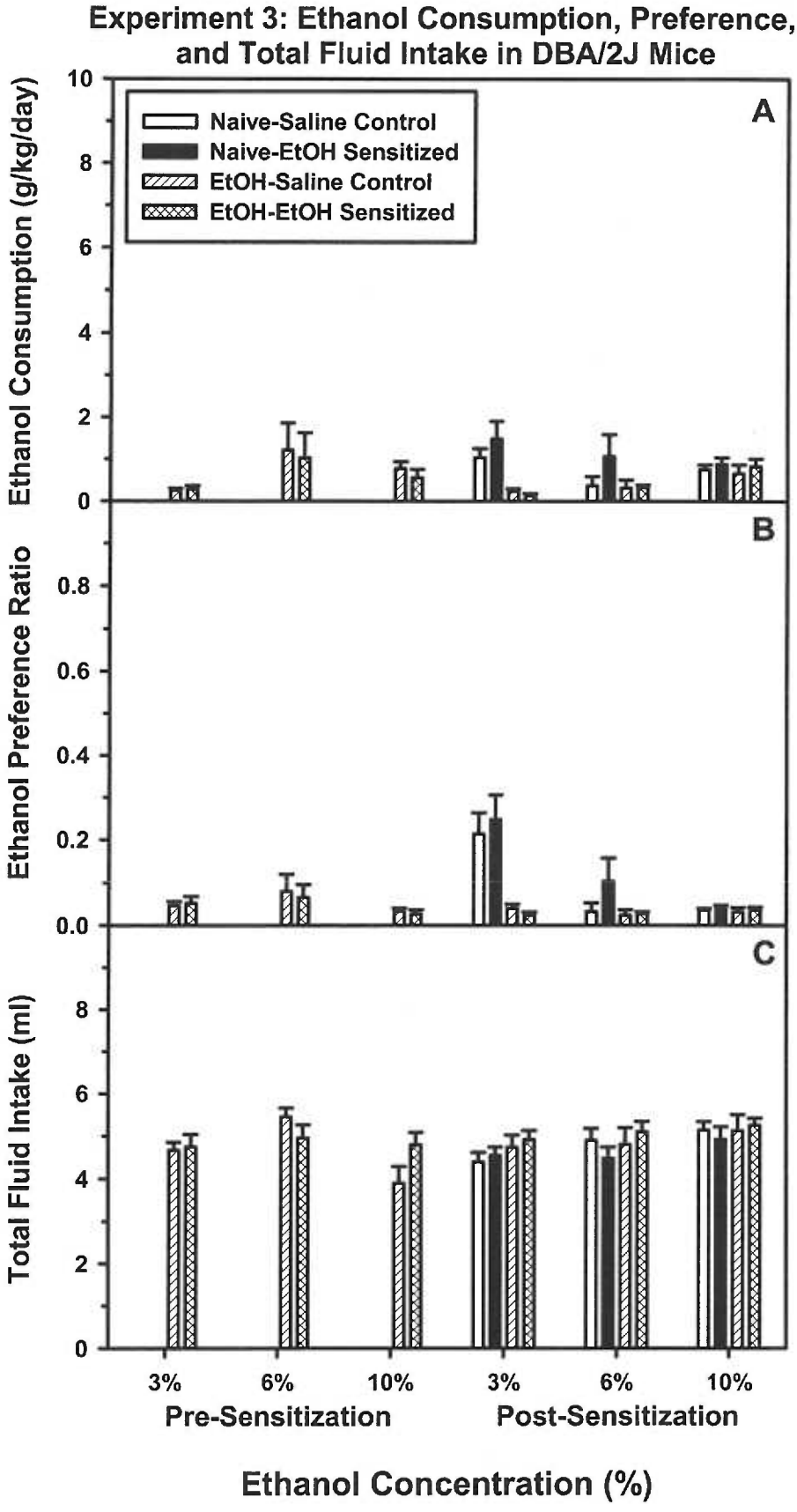

Experiment 3: Ethanol Consumption, Preference, and Total Fluid Intake in DBA/2J Mice
Naive-Saline Control
Naive-EtOH Sensitized
EtOH-Saline Control
EtOH-EtOH Sensitized
A
B
C
Ethanol Consumption (g/kg/day)
Ethanol Preference Ratio
Total Fluid Intake (ml)
0
2
4
6
8
10
0.0
0.2
0.4
0.6
0.8
3%
6%
10%
3%
6%
10%
Pre-Sensitization
Post-Sensitization
Ethanol Concentration (%)

Figure 14. Total 10-min locomotor activity of C57BL/6J inbred mice across EtOH-induced sensitization activity test days 1, 2, 3, and 14. White and black circles: groups with no prior EtOH drinking experience (Naïve) that received repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations, respectively. White and black triangles: groups with prior EtOH drinking experience (EtOH) that received repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations, respectively. On Days 1 and 2, all mice received saline injections. On Days 3 and 14, EtOH Sensitized groups received 2 g/kg EtOH, while Saline Control groups received saline. n=12 per group

* Significant within-group sensitization assessed as an increase in the EtOH response of each of the Naïve- and EtOH-EtOH Sensitized groups on Day 14, relative to their acute EtOH responses on Day 3. both $p<0.001$

@ Significantly greater EtOH response of the EtOH-EtOH Sensitized group on Days 3 and14 relative to the EtOH response of the Naïve-EtOH Sensitized group on those days. $p<0.01$ and 0.05 for Day 3 and Day 14, respectively

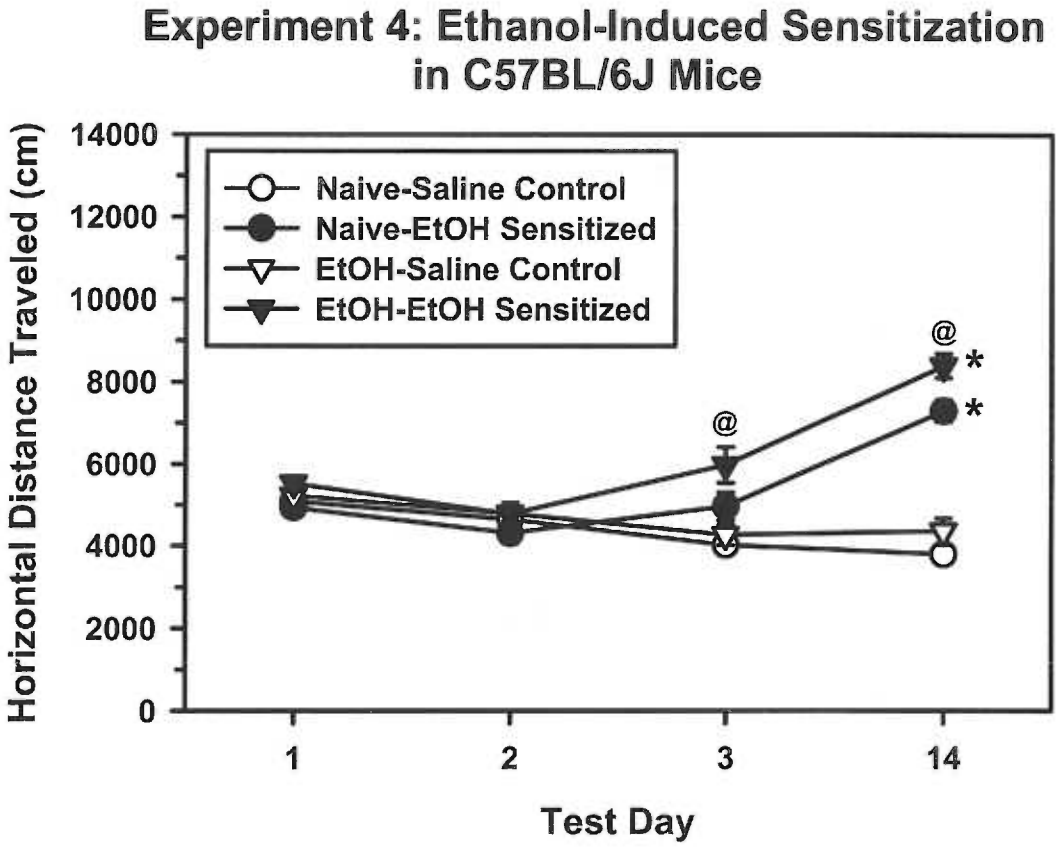
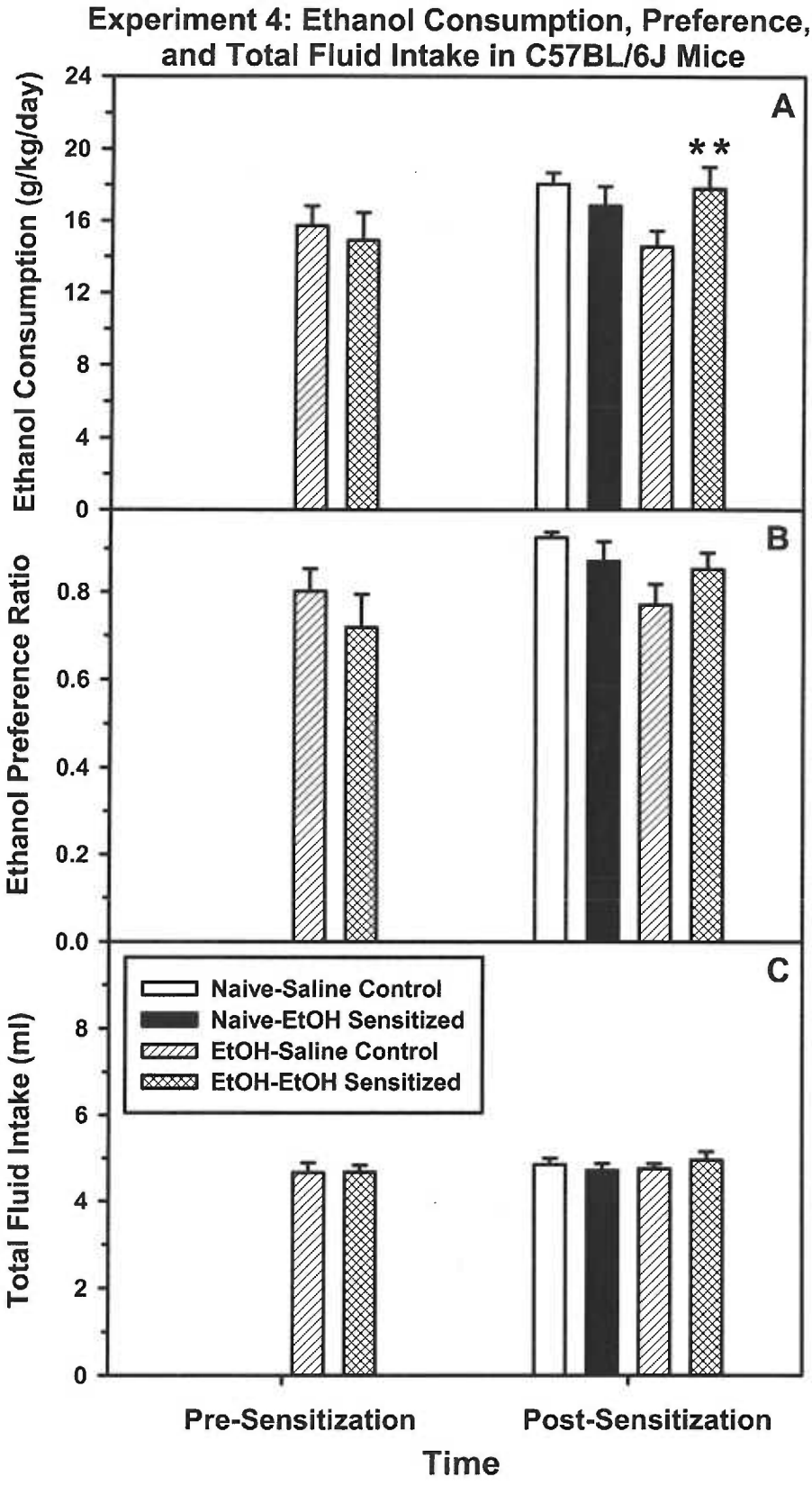

Experiment 4: Ethanol-Induced Sensitization in C57BL/6J Mice
Horizontal Distance Traveled (cm)
14000
12000
10000
8000
6000
4000
2000
0
Naive-Saline Control
Naive-EtOH Sensitized
EtOH-Saline Control
EtOH-EtOH Sensitized
@
@
*
*
1
2
3
14
Test Day

Figure 15. 24-hour EtOH consumption (g/kg, Panel A), EtOH preference (EtOH consumed/total fluid consumed, Panel B), and total fluid intake (ml, Panel C) measures for 10% EtOH solution before (Pre-Sensitization) and after (Post-Sensitization) a sensitizing regimen of EtOH administration in C57BL/6J inbred mice. White and black bars: groups with no prior Pre-Sensitization EtOH drinking experience (Naïve) and subsequently treated with repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations during the EtOH-induced sensitization period. Diagonal and hatched bars: groups with prior Pre-Sensitization EtOH drinking experience (EtOH) and subsequently treated with repeated saline (Saline Control) or EtOH (EtOH Sensitized) administrations during the EtOH-induced sensitization period. n=12 per group

** Significantly greater EtOH consumption of EtOH-EtOH Sensitized group relative to its EtOH-Saline Control counterpart and relative it its own pre-sensitization consumption levels. both $p<0.05$


Experiment 4: Ethanol Consumption, Preference, and Total Fluid Intake in C57BL/6J Mice
Ethanol Consumption (g/kg/day)
Ethanol Preference Ratio
Total Fluid Intake (ml)
A
B
C
**
Naive-Saline Control
Naive-EtOH Sensitized
EtOH-Saline Control
EtOH-EtOH Sensitized
Pre-Sensitization
Post-Sensitization
Time

Attenuation of Ethanol-Induced Conditioned Taste Aversion in Mice Sensitized to the Locomotor Stimulant Effects of Ethanol

INTRODUCTION

Sensitivity to both ethanol (EtOH) reward and aversion likely contribute to the ultimate probability of developing alcohol abuse (Tabakoff and Hoffman, 1988). When EtOH is repeatedly administered to mice, an enhancement of its acute locomotor stimulant effect may develop over time (Masur and Boerngen, 1980; Masur et al., 1986; Phillips et al., 1994b; Phillips et al., 1995; Phillips et al., 1996; Itzhak and Martin, 1999). This phenomenon, known as behavioral sensitization, has also been demonstrated with other drugs of abuse such as cocaine, amphetamine and morphine in both mice and rats (Segal and Mandell, 1974; Shuster et al., 1975; Post and Rose, 1976; Hirabayashi et al., 1991; Robinson, 1993; Badiani et al., 1997; Pierce and Kalivas, 1997; Cadoni and Di Chiara, 1999). It has been suggested that behavioral sensitization results from alterations in neural sensitivity that may enhance sensitivity to the positive or euphoric effects of a drug, thus maintaining drug use and potentially leading to drug abuse (Hunt and Lands, 1992; Robinson, 1993; Robinson and Berridge, 1993; Wise and Leeb, 1993; Bozarth, 1994). Likewise, sensitization could alter sensitivity to the negative or aversive effects of a drug. The current study investigated the effects of a sensitizing regimen of EtOH administration on EtOH-induced aversion.

EtOH-induced aversion can be measured using a Pavlovian taste conditioning procedure in which the presentation of a distinctive flavored solution (the Conditioned Stimulus or CS) is temporally paired with EtOH exposure (the Unconditioned Stimulus

or US). Typically, in this conditioned taste aversion (CTA) design, animals will avoid consumption of the flavor with repeated flavor-EtOH pairings (Risinger and Cunningham, 1995; Risinger and Cunningham, 1998). In addition to EtOH, taste aversion can be conditioned to a number of drugs of abuse including cocaine (Hunt et al., 1985), amphetamine (Poulos and Cappell, 1979; Rabin et al., 1987), morphine (Stewart and Eikelboom, 1978), and nicotine (Iwamoto and Williamson, 1984). Interestingly, exposure to any of these drugs prior to taste aversion conditioning generally results in attenuation of the magnitude of taste aversion. For example, repeated cocaine administration or a single pre-exposure to a high cocaine dose reduced subsequent cocaine-induced CTA (Riley and Simpson, 1999). Repeated morphine or nicotine administration also attenuated subsequent morphine- or nicotine-induced CTA, respectively (Stewart and Eikelboom, 1978; Iwamoto and Williamson, 1984; Martin et al., 1988; Gaiardi et al., 1991). Repeated EtOH injections in mice or voluntary 1-month EtOH consumption in alcohol-preferring P rats also attenuated subsequent EtOH-induced CTA (Stewart et al., 1991; Risinger and Cunningham, 1995). The reason behind such attenuation is not known but most common explanations of this phenomenon include non-associative development of tolerance to the aversive properties of the drugs or associative US pre-exposure effects, which posits that drug pre-exposure interferes with subsequent learning of the CS-US contingency. Unfortunately, few studies have specifically explored these possibilities. EtOH pre-exposure regimens that induced tolerance to the hypnotic effects of EtOH assessed as reduction in sleep time attenuated subsequent EtOH-induced CTA (Hunt and Rabin, 1988). Rats that developed tolerance to the discoordinating effects of EtOH as a result of intoxicated operant EtOH self-

administration showed an attenuation in subsequent EtOH-induced CTA, whereas their yoked counterparts that received passive EtOH administration did not (Gauvin and Holloway, 1992). A single EtOH pre-exposure considered to represent a non-tolerance inducing regimen also attenuated EtOH-induced CTA (Hunt and Rabin, 1988; Rabin et al., 1989), and in this instance, CTA attenuation may be attributed to US-pre-exposure effect. One study showed that groups of rats pre-exposed to morphine in different environmental conditions all showed equal attenuation of morphine-induced CTA, however tolerance to morphine analgesia was environment-specific, thus dissociating tolerance development form CTA attenuation (Stewart and Eikelboom, 1978). The authors concluded that different mechanisms likely mediate morphine tolerance and morphine-induced CTA attenuation.

To the best of our knowledge, there are no data showing the possible development of sensitization due to repeated drug administration prior to taste aversion conditioning. Sensitizing regimens of cocaine pre-exposure attenuated subsequent cocaine-induced CTA (Riley and Diamond, 1998; Riley and Simpson, 1999). The authors did not assess the behavioral consequences of cocaine pre-exposure even though they discussed the possible effects of sensitization (Riley and Diamond, 1998), and aimed to determine whether cocaine pre-exposure would sensitize acquisition of subsequent cocaine-induced CTA (Riley and Simpson, 1999). Therefore, it remains to be shown whether animals that develop behavioral sensitization during drug pre-exposure will show subsequent attenuation of drug-induced CTA.

In the present experiment, a sensitizing regimen of EtOH exposures was administered to mice. The presence of EtOH-induced sensitization was determined and

taste aversion conditioning was subsequently assessed. In order to evaluate the specificity of the sensitizing EtOH regimen on EtOH-induced CTA, in addition to EtOH, lithium chloride (LiCl)-induced CTA was also determined, as LiCl is an emetic agent often used to induce taste aversion. One study has shown significant acquisition of LiCl-induced CTA following a single exposure to a high EtOH dose, but significant attenuation of EtOH-induced CTA following a single exposure to LiCl (Rabin et al., 1989). These results indicate some degree of overlap and some specificity in the mechanisms that mediate taste aversion to EtOH and LiCl (Rabin et al., 1989).

In addition to being a measure of aversive drug properties, the CTA paradigm has been used as aversion training with animals (McKinzie et al., 1996; Thiele et al., 1996a) and human alcoholics (Baker and Cannon, 1979; Logue, 1985; Elkins, 1991) to decrease alcohol consumption and craving. An association between behavioral sensitization to the stimulant effects of EtOH and taste aversion might implicate sensitization, or at least repeated administration of EtOH, as a factor contributing to and altering the interoceptive properties of EtOH and perhaps altering the probability of alcohol abuse.

METHODS

Subjects

Female mice from two replicate lines of a genetically heterogeneous population were used. Since this study was conducted as an initial assessment of dose-response relationships between two behavioral measures, a readily available population of mice, which was also representative of mice in general, was the most appropriate animal model. These lines, WSC1 and WSC2, have been maintained as controls for selective breeding

experiments (Crabbe et al., 1985). Mice were born and raised at the VA Animal Care Facility in Portland, Oregon, and were kept on a 12:12 hour light:dark cycle (lights on at 6 am) with food available *ad libidum*, except during behavioral testing. Animals were maintained on restricted water access (see *Procedure* section below) and ranged in age from 56 to 85 days old at the beginning of the experiment. The experiment was performed in accordance with the Institutional Animal Care and Use Committee and National Institutes of Health guidelines for the care and use of laboratory animals.

Drugs

Ethanol (EtOH, 200 proof, Pharmco) was diluted in saline (0.9% NaCl) for a final solution of 20% v/v. Lithium Chloride (LiCl, Sigma) was dissolved in MilliQ water to create doses of 2 and 4mEq/kg when injected in a 10 ml/kg volume (848 mg or 1696 mg LiCl in 100 ml of MilliQ water, respectively). All injections were intraperitoneal (i.p.). Sodium chloride (NaCl, Sigma) for consumption was dissolved in tap water for a final concentration of 0.2M (23.2 g NaCl in 2000 ml of water).

Apparatus

Mice were contained in clear acrylic plastic boxes (40 x 40 x 30 cm) and tested in automated locomotor activity monitors (Accuscan, Columbus, OH) housed in sound attenuating chambers. Lights were mounted in the top center of the rear wall of each chamber; fans were located in the top right corner of each chamber. During testing, lights were on inside the chambers. Fans provided ventilation and masking noise. Eight infrared beams were mounted 2 cm above the chamber floor and were paired with

detectors on the opposing walls. As a mouse moved about the chamber floor, the infrared beams were interrupted and each interruption was automatically recorded as an activity count. Data were translated to horizontal distance traveled (cm) and this was the chosen measure of activity.

Procedure

This experiment consisted of four distinct phases: (1) habituation to single housing and drinking tubes, (2) habituation to water deprivation, (3) EtOH-induced sensitization and (4) EtOH- and LiCl-induced conditioned taste aversion (CTA).

1. Habituation to single housing and drinking tubes: 4 days

On Day 1, each mouse was weighed and single housed with food and water available *ad libidum*. For each mouse, water was presented in a single 25 ml calibrated tube adapted with a drinking spout. Volume consumption was recorded at 10 am daily for 4 days.

2. Habituation to water deprivation: 4 days

On the morning of Day 4, water consumption volumes were recorded at 10 am and again at noon, after which water tubes were removed. For the rest of this and the next phases of the experiment, water tubes were introduced for 2 hours daily starting at 9 am. Water levels were recorded at the beginning and end of water availability. Mice were weighed daily prior to the introduction of the water tubes.

3. EtOH-induced sensitization

The EtOH-induced sensitization protocol is shown in Table 3. Littermates were randomly assigned to Saline Control or EtOH Sensitized groups. Mice were weighed, injected with either saline or EtOH, and immediately placed in the center of an activity

monitor. Data were collected for 10 min in two 5 min epochs. For the first two test days, all mice were tested following saline administration. On the third day, mice were injected with either 2 g/kg EtOH (EtOH Sensitized group) or saline (Saline Control group). For the subsequent 10 consecutive days, EtOH Sensitized and Saline Control groups were injected with either 2.5 g/kg EtOH or saline, respectively, but no activity testing took place. On the final activity test day, all mice were tested following 2 g/kg EtOH administration. Activity testing and daily injections took place following the daily 2-hour water access period.

4. EtOH-induced conditioned taste aversion (CTA)

For all mice, six taste conditioning trials spaced 48 hours apart were conducted beginning 24 hours following the final EtOH-induced sensitization test day. A taste conditioning trial consisted of 1-hour access to a single drinking tube containing 0.2M NaCl solution, with volume levels recorded at the beginning and end of NaCl availability, immediately followed by injection of one of several unconditioned stimuli (USs). Excluding the first and last conditioning trials, mice from each of the Saline Control and EtOH Sensitized groups were injected with saline, one of three doses of EtOH (1, 2 or 4 g/kg), or one of two doses of LiCl (2 and 4 mEq/kg). There were 11-12 mice per treatment group. Since mice varied in the magnitude of their acute and sensitized EtOH responses, care was taken to assure that each of the saline-, EtOH- and LiCl-induced taste conditioning groups included an equal number of high and low locomotor responders. This pseudo-randomization was based on the difference scores for magnitude of the acute (Day 3 acute – Day 2 baseline scores) and sensitized (Day 14 sensitized – Day 3 acute scores) EtOH response for the Saline Control and EtOH Sensitized groups, respectively. Five

hours after conditioning trials, a water drinking tube was made available to each mouse for 30 min to prevent dehydration. Water volumes were recorded at the beginning and end of water availability. On days alternate to conditioning trials, mice received the usual 2-hour water access. Mice were weighed daily prior to NaCl or water availability.

Statistical Analyses

EtOH-induced sensitization and CTA data were analyzed with two- and three-way ANOVAs with horizontal distance traveled and consumption measures as the dependent variables, and Group (Saline Control vs. EtOH Sensitized), Dose (0, 1, 2 and 4 g/kg EtOH, 2 and 4 meq/kg LiCl), or Test Day/Conditioning Trial as the independent variables. Simple Main Effects and Neuman-Keuls Posthoc Multiple Comparisons analyses were performed where appropriate.

RESULTS

Sensitization to the Stimulant Effects of EtOH

Data from the EtOH-induced sensitization phase of this experiment are shown in Figure 16. Sets of Saline Control and EtOH Sensitized groups are separately presented as a function of the aversive unconditioned stimulus (US) used for subsequent taste aversion conditioning (Figure 16, Panels A-F). The presence of EtOH-induced sensitization was evaluated using both *between-* and *within-group* measures. Between-group sensitization entailed comparisons between the acute EtOH responses of the Saline Control groups relative to the final EtOH responses of the EtOH Sensitized groups on Day 14. Within-group sensitization entailed comparisons between the final (Day 14) and initial (Day 3)

EtOH responses of the EtOH Sensitized groups. For each panel, the Saline Control group showed a different behavioral pattern of response than the EtOH Sensitized group (Group x Test Day ANOVAs, significant interaction effects, all $p<0.001$). One reason was the significant acute EtOH stimulant responses of all EtOH Sensitized groups on Day 3 (all $p<0.001$) relative to the Saline Control group responses on that day. Another reason was the significant group differences on Day 14, indicating significant between-group sensitization. Even though this measure of sensitization was somewhat more variable than the within-group measure, Simple Main Effects analyses revealed significant between-group sensitization for sets of groups to be conditioned with 1 and 2 g/kg EtOH, and 2 mEq/kg LiCl as the USs (Figure 16B, C, and E, all $p<0.05$). There was a trend for significant between-group sensitization for groups to be conditioned with saline and 4 g/kg EtOH as the USs (Figure 16A and D, $p=0.055$ and 0.067, respectively). There was significant within-group sensitization for all EtOH Sensitized groups (all $p<0.01$). When the groups were collapsed across USs, increasing the sample size six-fold, both between- and within-group measures of sensitization were significant (Figure 16A inset, both $p<0.001$).

Conditioned Taste Aversion (CTA)

CTA data showing consumption of 0.2M NaCl (ml) across taste conditioning trials are presented in Figure 17. Sets of Saline Control and EtOH Sensitized groups conditioned to the different USs are separately presented across Panels A-F. The lower the amount of NaCl consumed the greater the degree of taste aversion.

Data in each panel were ultimately separately analyzed for optimum clarity and detection of group differences. However, three-way Group (Saline Control vs. EtOH Sensitized) x Dose (Saline, 1, 2, and 4 g/kg EtOH or Saline, 2 and 4 mEq/kg LiCl) x Conditioning Trial ANOVAs were initially performed for each set of EtOH and LiCl conditioned groups. There was a significant three-way interaction effect for the EtOH conditioned set of groups ($F_{15,425}$=1.9, $p<0.05$) indicating that the EtOH taste conditioning doses had a different effect on sensitized relative to non-sensitized mice across conditioning trials. The three-way interaction for the LiCl set of groups was not significant, but treatment groups as well as LiCl conditioning doses showed different degrees of CTA across conditioning trials (Group x Conditioning Trial and Dose x Conditioning Trial interaction effects, $F_{5,310}$=2.5 and $F_{10,310}$=25.2, $p<0.05$ and 0.001, respectively).

Data for each panel in Figure 17 were analyzed with two-way Group x Conditioning Trial ANOVAs. When presentation of the flavored NaCl solution was paired with saline administration (Figure 17A), taste aversion did not develop and there was no difference in NaCl consumption between the Saline Control and the EtOH Sensitized mice. There was in fact an overall increase in NaCl consumption over the course of conditioning trials (significant main effect of Conditioning Trial, $F_{5,105}$=11.8, $p<0.001$). The data with EtOH as the aversive US are shown in Figure 2B-D. When presentation of the flavored NaCl solution was paired with administration of 1 or 4 g/kg EtOH (Figure 17B and D), no differences between Saline Control and EtOH Sensitized groups were seen. These EtOH doses differed in the degree of taste aversion they produced, with no evidence for taste aversion induced by 1 g/kg EtOH, and robust taste

aversion induced by 4 g/kg EtOH. There was an overall increase in NaCl consumption in the 1 g/kg EtOH groups and an overall decrease in NaCl consumption in the 4 g/kg EtOH groups (significant main effects of Conditioning Trial, $F_{5,105}=7.1$ and $F_{5,105}=100.2$, respectively, both $p<0.001$). When presentation of NaCl solution was paired with administration of 2 g/kg EtOH (Figure 17C), the EtOH Sensitized mice showed significantly less taste aversion relative to Saline Control mice on all but the first two conditioning trials (significant Group x Conditioning Trial interaction effect $F_{5,110}=7.0$, $p<0.001$). Since the first flavor-US pairing was not until after the second conditioning trial, the third trial was essentially the first time that taste aversion could be assessed.

The data with LiCl as the aversive US are shown in Figure 17, Panels E and F. Groups for whom NaCl presentation was paired with the lower 2 mEq/kg LiCl (Figure 17E) showed significant acquisition of CTA, and overall, the EtOH Sensitized mice showed less taste aversion than the Saline Control mice (significant main effects of Conditioning Trial and Group, $F_{5,96}=12.7$ and $F_{1,19}=5.0$, $p<0.001$ and 0.05, respectively). The Group x Conditioning Trial interaction effect only approached significance ($F_{5,95}=2.1$, $p<0.07$), precluding further analyses on the effect of EtOH-induced sensitization on 2 mEq/kg LiCl-induced CTA. There was no effect of EtOH-induced sensitization on groups for whom NaCl presentation was paired with 4 mEq/kg LiCl (Figure 2F). Both EtOH Sensitized and Saline Control mice acquired similar degrees of CTA (significant main effect of Conditioning Trial, $F_{5,110}=66.6$, $p<0.001$).

Water Consumption

Water consumption data were analyzed with separate Group x Dose x Test Day ANOVAs for each distinct phase of this experiment. During the periods of habituation to single housing and habituation to water deprivation, all mice progressively increased their water consumption (main effects of Day, $F_{3,390}$=29.1 and $F_{2,260}$=208.0, both $p<0.001$). There were no water consumption differences between treatment groups during these phases, nor during the EtOH-induced sensitization phase. Since during the CTA, water was available for 0.5 hours on conditioning trial days, but for 2 hours on alternate days, separate three-way ANOVAs were performed for each set of those days. The analyses revealed a near significant three-way interaction for the conditioning trial days ($F_{20,520}$=1.6, p=0.054) likely due to the lower water consumption of both Saline Control and EtOH Sensitized 4 g/kg EtOH-treated groups on conditioning trial 2 (consumption = 0.23 ±.09 ml and 0.47 ± 0.13 ml for Saline Control and EtOH Sensitized groups, respectively), relative to the rest of the treatment groups and to the rest of the conditioning trials for which water consumption volumes ranged from 1.1 - 2.3 ml. Detailed follow up analyses were not statistically justified, however. All treatment groups consumed equivalent volumes of water on days alternate to taste conditioning trials.

Age and Body Weight

When mice were first placed on the limited 2-hour water access schedule, they lost on average 1.5-2.5 g of body weight (9-12%), after which their weights remained stable for the duration of the experiment. There were no differences among the treatment groups in

amount or percentage of weight loss. There were also no differences between the treatment groups in their weight during the EtOH-induced sensitization phase, nor during the CTA portion of this experiment. There were no significant differences in age between the treatment groups.

DISCUSSION

The aim of this study was to evaluate whether a sensitizing regimen of EtOH treatments would alter EtOH-induced conditioned taste aversion (CTA). The results showed that sensitized mice acquired less CTA than their non-sensitized counterparts at the 2 g/kg EtOH and 2 mEq/kg LiCl taste conditioning doses. Both sensitized and non-sensitized mice acquired similar degrees of CTA at the 4 g/kg EtOH and 4 mEq/kg LiCl taste conditioning doses. Neither sensitized, nor non-sensitized mice acquired CTA to 1 g/kg EtOH or saline. The attenuated taste aversion in sensitized mice at the medium EtOH and lower LiCl doses could be due to one or a combination of any one of three plausible explanations. It is possible that sensitization to the effects of EtOH, or at least the sensitizing regimen of EtOH administration, was responsible for CTA attenuation. However, at least two additional interpretations are possible.

Since tolerance was not specifically measured, its potential contribution to the attenuated CTA seen in some EtOH pre-exposed mice cannot be decisively excluded. There are, however, reasons to doubt tolerance as an explanation. For example, a tolerance-inducing EtOH regimen attenuated subsequent radiation-induced CTA at three different radiation doses (Hunt and Rabin, 1988). In the present study, if EtOH tolerance was a major contributor to the attenuation of EtOH- and LiCl-induced CTA in sensitized

mice, such attenuation, or at least delayed acquisition of CTA, should have been revealed at the higher EtOH and LiCl taste conditioning doses. On the other hand, it is possible that the high EtOH and LiCl taste conditioning doses overrode an existing tolerance effect. However, it was clear to the experimenter that during the CTA procedure, mice receiving 4 g/kg EtOH were developing tolerance to the sedative effects of EtOH as they became more vigilant over conditioning trials at 5 hours following EtOH injections. During taste aversion conditioning, both sensitized and non-sensitized mice were likely developing tolerance to the sedative effects of 4 g/kg EtOH to a similar degree. However, if sensitized mice had been rendered tolerant by prior EtOH exposure, they should have been immediately more tolerant, which should have been revealed as a difference in CTA magnitude between sensitized and non-sensitized mice at the 4 g/kg EtOH dose. Thus, we do not believe that tolerance played a major role in the attenuated EtOH- and LiCl-induced CTA seen in EtOH sensitized mice. At least one study showed dissociation between the mechanisms mediating tolerance to the analgesic effects of morphine and those mediating the attenuation of morphine-induced CTA as a function of repeated morphine pre-exposure (Stewart and Eikelboom, 1978). In studies of nicotine and amphetamine pre-exposure with subsequent attenuation of nicotine- and amphetamine-induced CTA, respectively, tolerance to the effects of the drugs was the common explanation, even though no data were presented to support such contentions (Cappell and Le Blanc, 1975; Iwamoto and Williamson, 1984).

Another possible explanation for our results is a US pre-exposure effect (Rabin et al., 1989). However, all non-sensitized groups in the present study received single EtOH administrations prior to taste aversion conditioning. If the US pre-exposure effect was a

robust phenomenon, the significant group differences at the 2 g/kg EtOH taste conditioning dose would have been obscured because the non-sensitized group should have also shown CTA attenuation. Not only did this group develop significant CTA both relative to its sensitized counterparts and relative to its non-sensitized control group for which NaCl was paired with saline injections (Figure 17C, and compare Panel C and Panel A Saline Control groups), but its level of CTA was similar to that of the non-sensitized group conditioned to 2 mEq/kg LiCl, which was expected to show significant LiCl-induced CTA. Any degree of CTA attenuation in the non-sensitized groups could have been revealed in comparison to CTA levels of EtOH-naïve control groups, but, unfortunately, such controls were not included. We cannot conclusively refute a contribution of US pre-exposure effects to the significant attenuation of EtOH- and LiCl-induced CTA in mice sensitized to the effects of EtOH. However, for the reasons given above, we believe that sensitization to the stimulant effect of EtOH, or at least the sensitizing regimen of EtOH administration, was primarily responsible for the diminution of subsequent EtOH- and LiCl-induced CTA.

An inherent problem with the sensitization procedure is the dissociation between the effects of the behavioral sensitization itself from those of the regimen of EtOH exposures that induce sensitization. It would be ideal to evaluate EtOH-induced CTA following a different number of EtOH pre-exposure events with concurrent measures of tolerance and sensitization to the effects of EtOH. In the absence of such systematic evaluation, distinguishing between the effects of repeated EtOH exposure, tolerance, or sensitization to the effects of EtOH on subsequent attenuation of EtOH-induced CTA is difficult. It is possible that tolerance to some effect of EtOH does indeed develop during

the EtOH-induced sensitization paradigm. In a genetic analysis, we found that those strains that developed the greatest sensitization were not the ones that developed the most EtOH tolerance (Phillips et al., 1996) implying different neural mechanisms and perhaps different time courses contributing to the development of these EtOH effects. A notable aspect of this study (Phillips et al., 1996) was that sensitization and tolerance to the effects of EtOH were seen in the same experimental paradigm. It has been shown that cocaine pre-exposure attenuated subsequent cocaine-induced CTA (Riley and Simpson, 1999). The authors advanced the concepts of adaptation, habituation and tolerance as possible explanations for CTA attenuation, even though the behavioral consequences of cocaine pre-exposure were not evaluated, in addition to the fact that cocaine was administered in a regimen that would have likely resulted in sensitization. However, in a prior publication, the same group (Riley and Diamond, 1998) did discuss the possible development of sensitization to the effects of cocaine during cocaine pre-exposure. Another study showed that repeated morphine pre-exposure induced conditioned place preference and attenuated morphine-induced CTA in separate groups of rats (Gaiardi et al., 1991). The authors concluded that morphine pre-exposure sensitized animals to the rewarding properties of morphine and the enhanced reinforcement interfered with the formation of a drug-induced CTA (Gaiardi et al., 1991). Indeed, repeated drug exposure has been shown to sensitize rats and mice to reinforcing drug properties (Lett, 1989; Horger et al., 1992; Mendrek et al., 1998; Taylor and Horger, 1999), and at times these effects have correlated with sensitization to the locomotor stimulant effects of these drugs (De Vries et al., 1998). It has even been suggested that it is because the reinforcing properties of a US override those of the flavor CS, that flavor consumption decreases

with flavor-drug pairings (Grigson, 1997). Therefore, a decrease in flavor consumption is due to the reinforcing, rather than the aversive drug properties (Grigson, 1997). Such suggestions, along with data here and elsewhere (Riley and Diamond, 1998; Riley and Simpson, 1999) implicating sensitization to the stimulant effects of drugs in the attenuation of aversive properties of these drugs, support the notion that sensitization to the effects of drugs may underlie drug craving and addiction (Wise and Bozarth, 1987; Robinson and Berridge, 1993).

SUMMARY AND CONCLUSIONS

Mice sensitized to the stimulant effects of EtOH showed attenuated EtOH- and LiCl-induced conditioned taste aversion relative to their non-sensitized counterparts. Data from the literature and theoretical considerations argue for the effects of sensitization as contributing factors, rather than the effects of tolerance or US pre-exposure effects. Future studies will determine whether this sensitizing regimen induces tolerance to the effects of EtOH. Lack of tolerance development will implicate sensitization to the effects of EtOH as a major player in the attenuation of the aversive stimulus properties of EtOH. It is reasonable and exciting to suppose that by virtue of decreasing EtOH aversion, sensitization to the stimulant effects of EtOH may contribute to the maintenance of alcohol drinking behavior which may in turn lead to alcohol abuse.

Table 3. Procedure for Ethanol-Induced Sensitization. Locomotor activity and EtOH injection procedure used to induce sensitization to the stimulant effects of EtOH.

	Habituation Day 1	**Baseline** Day 2	**Acute EtOH** Day 3	**Daily Treatment** Days 4-13	**Sensitization** Day 14
Saline Control	Saline	Saline	Saline	Saline	EtOH 2g/kg
Test	Yes	Yes	Yes	No	Yes
EtOH Sensitized	Saline	Saline	EtOH 2g/kg	EtOH 2.5g/kg	EtOH 2g/kg
Test	Yes	Yes	Yes	No	Yes

Locomotor activity was tested on Days 1, 2, 3, and 14. Activity data were collected for 10 min in two 5-min epochs. All injections were intraperitoneal (i.p.).
Habituation and **Baseline**: On Days 1 and 2, both *Saline Control* and *EtOH Sensitized* treatment groups were tested following saline injections. Day 1 served as a habituation test day to acclimate mice to the injection procedure and apparatus. Day 2 provided a measure for baseline activity. **Acute EtOH**: On Day 3, *Saline Control* and *EtOH Sensitized* groups were tested following saline or 2 g/kg EtOH, respectively. Day 3 provided a measure for the acute response to EtOH of the *EtOH Sensitized* group. **Daily Treatment**: During Days 4-13, *Saline Control* and *EtOH Sensitized* groups received 10 daily saline or 2.5 g/kg EtOH injections, respectively, with no activity testing. **Sensitization**: On Day 14, both *Saline Control* and *EtOH Sensitized* treatment groups were tested following 2 g/kg EtOH. Day 14 provided a measure for acute EtOH and EtOH sensitized responses for the *Saline Control* and *EtOH Sensitized* groups, respectively.

Figure 16. Locomotor activity of saline-treated (Saline Control, *white symbols*) and EtOH-treated (EtOH Sensitized, *black symbols*) mice across activity test days. Each panel represents independent sets of groups, which received subsequent taste aversion conditioning to saline (*Panel A*), 1 g/kg EtOH (*Panel B*), 2 g/kg EtOH (*Panel C*), 4 g/kg EtOH (*Panel D*), 2 mEq/kg LiCl (*Panel E*), or 4 mEq/kg LiCl (*Panel F*). On Days 1 and 2, all mice received saline injections. On Day 3, EtOH Sensitized groups received 2 g/kg EtOH, while Saline Control groups received saline. On Day 14, both Saline Control and EtOH Sensitized groups received 2 g/kg EtOH. The *inset* represents activity data averaged across all groups. n=11-12 per group

* Significant within-group sensitization assessed as increased activity of EtOH Sensitized groups on Day 14 relative to their acute EtOH responses on Day 3. all $p<0.01$

** Significant within- and between-group sensitization assessed as increased activity of the EtOH Sensitized groups on Day 14 relative to their acute EtOH responses on Day 3, and relative to the acute EtOH responses of the Saline Control groups on Day 14. all $p<0.01$ for within-group sensitization; all $p<0.05$ for between-group sensitization

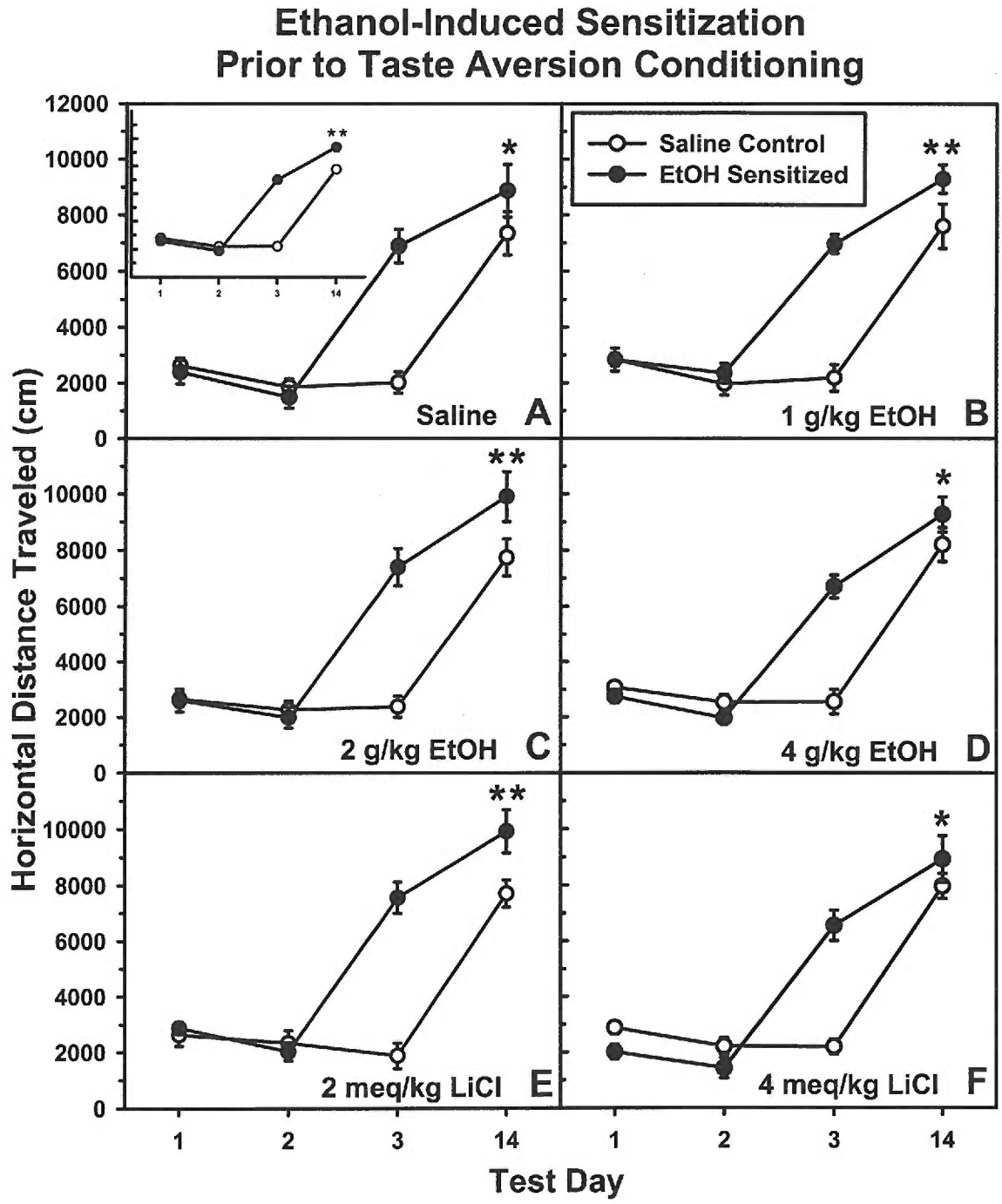
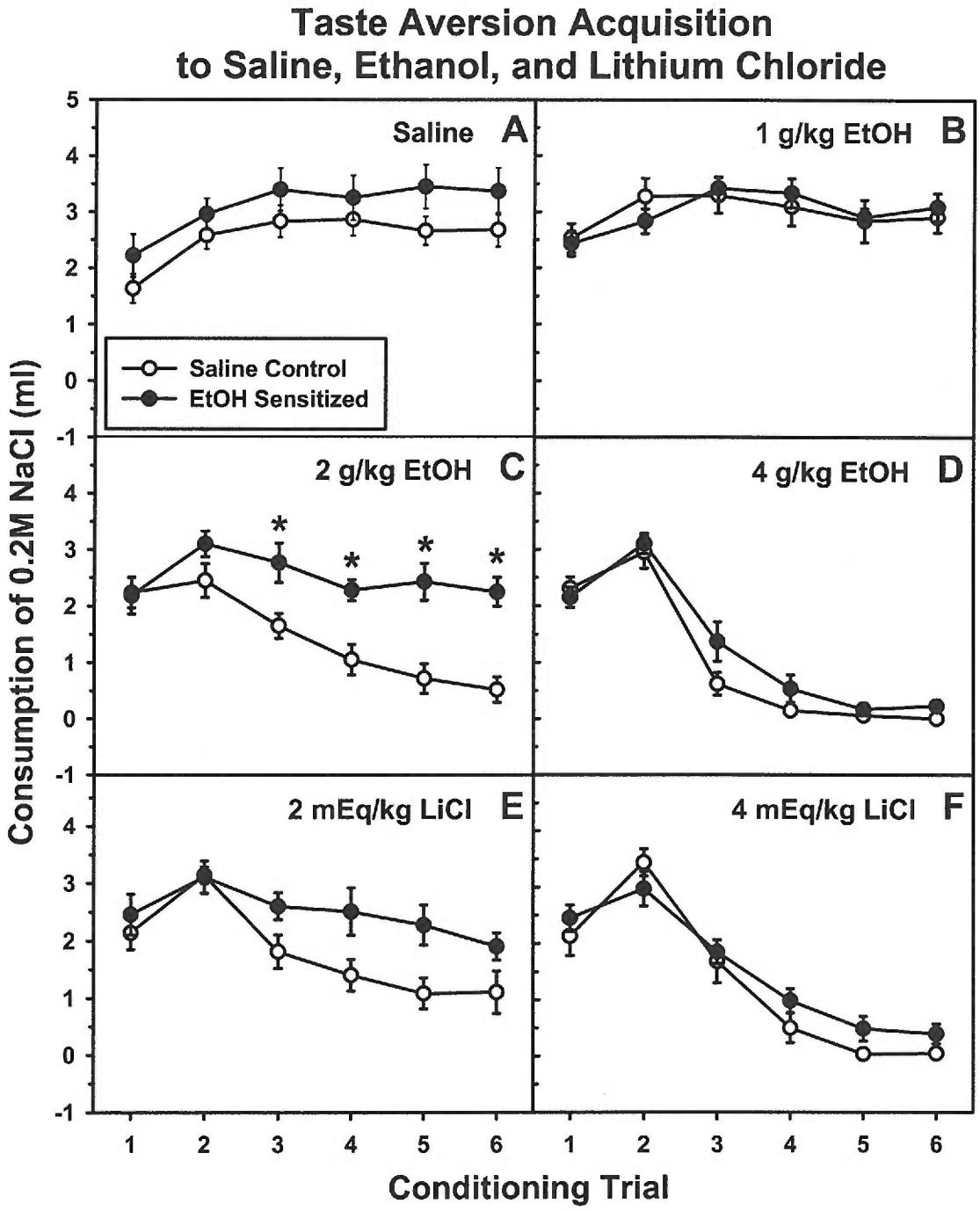

Ethanol-Induced Sensitization
Prior to Taste Aversion Conditioning
Horizontal Distance Traveled (cm)
Test Day
Saline Control
EtOH Sensitized
Saline A
1 g/kg EtOH B
2 g/kg EtOH C
4 g/kg EtOH D
2 meq/kg LiCl E
4 meq/kg LiCl F
0
2000
4000
6000
8000
10000
12000
1
2
3
14
*
**

Figure 17. Consumption of 0.2M NaCl by previously saline-treated (Saline Control, *white symbols*) and EtOH-treated (EtOH Sensitized, *black symbols*) groups across taste aversion conditioning trials. Decrease in NaCl consumption indicates the development of conditioned taste aversion. Each panel represents separate sets of groups conditioned to the aversive properties of saline (*Panel A*), 1g/kg EtOH (*Panel B*), 2 g/kg EtOH (*Panel C*), 4 g/kg EtOH (*Panel D*), 2 mEq/kg LiCl (*Panel E*), and 4 mEq/kg LiCl (*Panel F*). n=11-12 per group

* Significant difference in NaCl consumption between the Saline Control and EtOH Sensitized group for that conditioning trial. all $p<0.01$


Taste Aversion Acquisition
to Saline, Ethanol, and Lithium Chloride
Saline A
1 g/kg EtOH B
2 g/kg EtOH C
4 g/kg EtOH D
2 mEq/kg LiCl E
4 mEq/kg LiCl F
Saline Control
EtOH Sensitized
Consumption of 0.2M NaCl (ml)
Conditioning Trial
5
4
3
2
1
0
-1
1
2
3
4
5
6
*

OVERALL DISCUSSION

The central theme of this thesis was EtOH-induced sensitization: its role in EtOH reward and aversion, and common mechanisms with other abused drugs. The studies evaluated cross-sensitization between EtOH and morphine, cocaine, and methamphetamine, and examined the effect of a sensitizing regimen of EtOH exposures on subsequent EtOH oral self-administration and aversion.

Cross-Sensitization Between the Effects of Ethanol and Those of Morphine, Cocaine, and Methamphetamine

Complete assessment of drug cross-sensitization would require pre-treatment with one drug followed by a challenge administration of another drug, and vice versa. In the present experiments, repeated EtOH administration was followed by challenge drug administrations 24 hours later, but the reciprocal experiments were not conducted. Under these conditions, there was no evidence for cross-sensitization between the effects of EtOH and those of morphine, cocaine, or methamphetamine. To the best of our knowledge, there are only two other studies in mice that have specifically examined cross-sensitization between EtOH and other drugs. In one study, male Swiss Webster mice received 5 daily injections of EtOH or cocaine during which time no activity testing took place; sensitization and cross-sensitization to the stimulant effects of each drug were assessed 10 days later by EtOH and cocaine challenge injections (Itzhak and Martin, 1999). In the second study, female Swiss Webster mice received 10 daily injections of EtOH or cocaine and their locomotor responses were assessed daily following drug administration (Wise et al., 1996). There was no evidence for the development of either

EtOH or cocaine sensitization and no evidence for cross-sensitization assessed by EtOH or cocaine challenge injections administered 7 days after the final drug administration (Wise et al., 1996). There are a number of reasons for the contrasting results between these studies. First, in one study (Wise et al., 1996), the lower doses of both EtOH and cocaine that were used may have been inadequate to induce sensitization under the particular experimental conditions. Second, the number of repeated drug exposures in the study that showed significant sensitization (Itzhak and Martin, 1999) was half that of the other study (Wise et al., 1996). This is somewhat surprising, as one might have expected a greater number of drug exposures to be necessary for the induction of drug sensitization. Third, locomotor activity was assessed during drug pre-treatment in one study (Wise et al., 1996) but not in the other (Itzhak and Martin, 1999). Activity testing may have interfered with the development or expression of drug sensitization. However, this is unlikely, as conditioned activation that may have developed to the testing environment (Cunningham and Noble, 1992; Jodogne et al., 1994) would have either contributed to the development of sensitization or would have enhanced an existing sensitized response. Finally, the studies used different genders of mice, which may not have significantly contributed to the results, as the study that used female mice (Wise et al., 1996) failed to show sensitization, even though female animals have been shown to develop more robust sensitization than their male counterparts (Robinson, 1984; Cailhol and Mormède, 1999).

The present experiments used the same EtOH dose and included the cocaine dose used in one study (Itzhak and Martin, 1999), and the number of EtOH pre-exposures was similar to the other study (Wise et al., 1996). However, one major difference between

these two studies and the present experiments was that, here, cross-sensitization was assessed 24 hours after the final EtOH administration instead of 7 to 10 days later. Another difference is that care was taken to specifically assess both the acute and sensitized responses to EtOH in a between- and within-groups design. It is possible that cross-sensitization would have been evident if an EtOH withdrawal period had been imposed. In general, sensitization studies using drugs other than EtOH have shown exacerbated sensitization responses following a period of a few days to weeks of drug withdrawal. Even though EtOH-induced sensitization seems to be most robust 24 hours following the final EtOH administration and does not seem to become enhanced following a period of no EtOH exposure (Lessov and Phillips, 1998), sensitization to other drugs of abuse may require time for some other processes to occur.

EtOH does not have a specific neural target and is know to affect components of several neurotransmitter systems. Repeated EtOH administration may lead to alterations in sensitivity of some of those components. EtOH may have small effects on many systems so that in the conglomerate EtOH-induced sensitization is seen, but individual component changes may not support the expression of sensitization to drugs with more specific effects. Additionally, EtOH may simply act at different neurotransmitter systems than the other drugs tested and may have sensitized components of those systems to a greater degree than components of the dopaminergic system. For example, mice selectively bred for initial sensitivity (FAST) or insensitivity (SLOW) to the locomotor stimulant effects of EtOH, also differed in their sensitivity to the barbiturate, pentobarbital, the benzodiazepine, diazepam, and the NMDA receptor antagonist, MK-801 (Phillips et al., 1992; Shen and Phillips, 1998). However, FAST and SLOW mice

did not differ in their locomotor responses to morphine, cocaine, amphetamine, or to the dopamine antagonists SCH-23390, haloperidol, and raclopride (Phillips et al., 1992; Shen et al., 1995; Phillips and Shen, 1996). These results indicate that polymorphic genes alleles of the GABA and glutamate, but not the dopamine, systems may have differentially diverged as a result of selection for locomotor sensitivity to EtOH, and implicate the GABA and glutamate systems in the mediation of at least initial EtOH sensitivity. It is possible that GABA or glutamate, rather than dopamine may also mediate sensitization to the stimulant effects of EtOH to a greater degree. Since morphine, cocaine, and methamphetamine used in the present studies exert their effects at least partially via the dopaminergic system (Ritz et al., 1987; Giros et al., 1996; Wu and Gu, 1999), EtOH-induced neuroadaptations of the GABA and glutamate systems may not have been readily detected. Of course, the role of dopamine in mediation of the effects of EtOH cannot be discounted (Di Chiara and Imperato, 1988; Samson et al., 1992; Souza-Formigoni et al., 1999); however it is quite likely that dopaminergic alterations could occur as a consequence of GABAergic activation, and are therefore not the primary system involved in the actions of EtOH.

The EtOH-sensitized groups did not show cross-sensitization to the other drugs tested, however, they tended to show a blunted response to challenge drug administrations. At least one other study has shown a blunted response to challenge cocaine and amphetamine in repeatedly morphine-treated animals (Volpicelli et al., 1999). The authors attributed these results to a possible effect of morphine withdrawal (Volpicelli et al., 1999). It remains to be determined whether the EtOH-induced sensitization regimen and the EtOH doses used in the present experiments lead to

withdrawal symptoms. EtOH withdrawal is generally observed following dependence-inducing EtOH regimens (Crabbe et al., 1985), although a single high dose of EtOH can also induce acute withdrawal in mice during a specific time period (Metten et al., 1998; Metten and Crabbe, 1999). During EtOH withdrawal, reductions in dopamine concentration in the ventral striatum and reduced firing of VTA neurons have been shown (Rossetti et al., 1992; Diana et al., 1996; Bailey et al., 1998). These effects persisted for 3 to 5 days following cessation of EtOH treatment and following termination of behavioral withdrawal symptoms, which generally completely subside by 24 hours (Rossetti et al., 1992; Diana et al., 1996). In addition, the reduced firing of VTA neurons measured in slices prepared 24 hours following termination of repeated EtOH administration was not altered by application of amphetamine (Bailey et al., 1998). It is possible that in the present experiments, while sensitization to the effects of EtOH was being recorded for the first 10 min following EtOH administration, withdrawal-related neuroadaptations may have also been occurring at later time points. Therefore, if there was a general reduction of dopaminergic function at the time of cross-sensitization testing, it could have contributed to a blunted response in the EtOH-treated mice relative to their saline-treated counterparts.

It is also possible that the EtOH treatment regimen induced tolerance to some effect of EtOH. A similar EtOH administration regimen has been shown to induce tolerance to the ataxic effects of EtOH (Phillips et al., 1996). There are data indicating that tolerance may develop to the locomotor depressant effects of morphine (Schnur, 1985; Rauhala et al., 1995), and to the locomotor stimulant effects of cocaine (King et al., 1999) and amphetamine (Martin-Iverson and Iversen, 1989). Therefore, the tendency

toward a blunted response to drug challenge in the EtOH-sensitized groups may be a reflection of cross-tolerance between the effects of EtOH and those of morphine, cocaine, and methamphetamine.

Effects of Ethanol-Induced Sensitization on Voluntary Ethanol Consumption

The results showed that sensitized C57BL/6J (B6) mice drank more alcohol relative to their non-sensitized controls and relative to their pre-sensitization levels, indicating an enhancement of EtOH reward. In addition, this alcohol-preferring strain consumed enough EtOH to become sensitized as evidenced by the enhanced locomotor response to a subsequent EtOH injection. These results replicate the single previous report showing that B6 mice were sensitized as a consequence of voluntary EtOH drinking (Nocjar and Middaugh, 1997). There are few other instances showing that drug self-administration actually induced drug sensitization (Phillips and Di Ciano, 1996; De Vries et al., 1998; Marinelli et al., 1998). It is very important and relevant to continue investigation on the potential for the development of drug sensitization following drug self-administration, since such an experimental design most closely resembles the human condition, and might prove most informative as to the role of sensitization in the development of drug addiction.

The surprising and novel result of the present studies was that B6 mice developed significant sensitization to the effects of EtOH with repeated EtOH injections. This strain has been repeatedly shown to be resistant to the development of EtOH-induced sensitization using different EtOH injection paradigms (Cunningham et al., 1992; Cunningham, 1995; Phillips et al., 1994b; Phillips et al., 1995; Phillips et al., 1996). The

present results indicate that repeated EtOH administration induced neuroadaptations that may also underlie EtOH-induced sensitization and EtOH reinforcement. Considering that EtOH administration results in increased extracellular dopamine levles (Di Chiara and Imperato, 1988; Weiss et al., 1993) and that EtOH-induced sensitization may also be associated with enhanced extracellular dopamine levles (Nestby et al., 1997) as well as with changes in sensitivity of dopamine receptors (Souza-Formigoni et al., 1999), it could be the case that enhanced dopaminergic activity is responsible for the increased EtOH drinking of B6 mice. However, manipulations that increase extracellular dopamine concentrations, namely direct dopamine receptor activation and inhibition of GABA interneurons (Ng and George, 1994; Nowak et al., 1998), have been shown to result in a decrease of EtOH consumption. These results stand in contrast to the idea of increased dopamine mediating the increased EtOH drinking in the present studies. Essentially, it seems that both an increase and a decrease in voluntary EtOH drinking could be interpreted as rewarding depending on the underlying neurochemical correlates. Much more work on the mechanisms mediating EtOH-induced sensitization and EtOH reinforcement is necessary before meaningful conclusions about neural mediation of each or both can be made. The results obtained from the alcohol-preferring B6 mice strongly suggest an association between these two EtOH-induced behaviors.

Further investigation using B6 mice could be conducted by manipulating the presence or absence of EtOH-induced sensitization through employment of different EtOH injection paradigms. The effects of each paradigm on voluntary EtOH drinking could subsequently be assessed. If repeated EtOH administration induces neuroadaptations in the VTA or NAcc, areas also implicated in the mediation of EtOH

reinforcement, regardless of the behavioral expression of sensitization, both groups should show an equal increase in EtOH consumption. Such results would imply that neuroadaptations that mediate EtOH reinforcement are not necessarily the ones that mediate the behavioral expression of EtOH-induced sensitization, thus dissociating behavioral EtOH-induced sensitization from reinforcement processes and necessitating a different measure, perhaps a neurochemical measure of EtOH-induced sensitization. On the other hand, if behaviorally sensitized B6 mice consume more EtOH than similarly EtOH-treated but non-sensitized mice, it could be concluded that neuroadaptations that mediate behaviorally expressed EtOH-induced sensitization also mediate EtOH reinforcement, strengthening the proposed association between drug sensitization and the potential for drug abuse (Wise and Bozarth, 1987; Robinson and Berridge, 1993).

The genetically heterogeneous and DBA/2J (D2) mice showed little or no evidence, respectively, for an effect of the sensitizing regimen of EtOH administration on subsequent EtOH consumption. In one of the two experiments using genetically heterogeneous mice, there was a trend for greater EtOH consumption in the sensitized groups relative to their non-sensitized controls, however this trend was not replicated in the subsequent study. In addition, neither of these two strains showed evidence for sensitization as a function of prior EtOH drinking experience. However, it is likely that these mice, particularly the alcohol-avoiding D2 strain, did not consume sufficient quantities of EtOH to induce sensitization. The lower drinking levels should not have precluded an effect of sensitization on subsequent drinking, however, especially if sensitization contributes to an *increase* in EtOH consumption, as shown in the B6 mice. It could be that such effects are more easily manifest in an animal model genetically

predisposed to high alcohol consumption such as the B6 strain. Perhaps not coincidentally, sons of alcoholics, who are considered to be genetically at risk for developing alcoholism, tend to develop sensitization to autonomic physiologic measures following repeated alcohol administration, whereas sons of non-alcoholics tend to develop tolerance to the same measures (Newlin and Thomson, 1991; Newlin and Thomson, 1999).

Effects of Ethanol-Induced Sensitization on Ethanol- and Lithium Chloride-Induced Conditioned Taste Aversion

The conditioned taste aversion experiment showed that at the 2 g/kg EtOH and 2 mEq/kg LiCl taste conditioning doses, sensitized mice had lower magnitude of EtOH- and LiCl-induced taste aversion than their non-sensitized counterparts. These data indicate that the effect of the sensitizing EtOH regimen is not specific to EtOH-induced taste aversion, but also generalizes to taste aversion induced by emetic agents such as LiCl. This is not surprising considering that acquisition of conditioned taste aversion (CTA) seems to be mediated by several interconnected brain structures that carry taste and visceral information to the cortex. In general, taste information is carried along branches of the facial, glossopharyngeal, and vagus nerves; other branches of the glossopharyngeal and vagus nerves carry visceral information (Martin, 1996; Reilly, 1999). Taste and visceral information travels to the nucleus of the tractus solitarius (NTS) in the brain stem, which projects to the parabrachial nucleus (PBN) in the pons, which in turn projects to both the gustatory cortex (insular cortex) via the thalamus, and directly to the amygdala (Yamamoto et al., 1994; Reilly, 1999). Another brain stem structure, the area postrema

(AP), which responds to toxins and emetic stimuli such as LiCl for instance, also projects to the PBN (Stewart et al., 1988; Yamamoto et al., 1994). Essentially, the PBN is the site of integration of gustatory and visceral information (Yamamoto et al., 1994) and not surprisingly, lesions of the PBN completely disrupt acquisition of CTA (Yamamoto et al., 1995). Injection of large doses of EtOH and LiCl resulted in neuronal activation in the NTS, the AP and the PBN assessed through c-Fos like immunoreactivity, implying similarity in the mechanisms mediating taste aversion to these agents (Thiele et al., 1996b). However, at least one area, the AP, appears to have differential effects on CTA depending on the nature of the US, as AP lesions did not prevent acquisition of EtOH-induced CTA, but did block acquisition of methylscopolamine-induced CTA (Stewart et al., 1988). In addition, amphetamine microinjection into the AP induced CTA to amphetamine (Carr and White, 1986) implicating the AP in the mediation of amphetamine-induced CTA.

In the present experiment, we wished to differentiate whether the attenuation of EtOH- and LiCl-induced taste aversion was associated with EtOH-induced sensitization, rather than with the development of tolerance to some effect of EtOH, or to US (EtOH) pre-exposure effects. Tolerance-inducing EtOH pre-exposure regimens have been shown to attenuate both EtOH- and LiCl-induced taste aversion (Hunt and Rabin, 1988; Rabin et al., 1989). This could argue that tolerance to some effect of EtOH in the sensitized mice contributed to the decrease in taste aversion magnitude. However, if tolerance was a major contributor to taste aversion attenuation, the sensitized mice should have also shown attenuated taste aversion magnitude or perhaps slowed development of taste aversion to the higher 4 g/kg EtOH and 4 mEq/kg LiCl conditioning doses. It is possible

that an effect of tolerance could have been revealed at an EtOH taste-conditioning dose intermediate to the stimulant 2 g/kg and the sedative 4 g/kg EtOH doses. Possible effects of tolerance cannot be completely discounted in the current study.

US pre-exposure effects are revealed in instances where a single EtOH exposure may attenuate subsequent EtOH-, but *not* LiCl-induced taste aversion (Rabin et al., 1989). In the present experiment, all saline-treated control groups received a single EtOH administration on the final activity test day prior to taste aversion conditioning. If US pre-exposure explained taste aversion attenuation, the saline control groups conditioned to the aversive effects of 2 g/kg EtOH and 2 mEq/kg LiCl would have been expected to show the presence or absence of taste aversion attenuation, respectively. Not only did the saline control group conditioned to 2 g/kg EtOH show significant taste aversion, but its level of taste aversion was similar to that of the saline control group conditioned to 2 mEq/kg LiCl. Thus, the potential contribution of US pre-exposure effects cannot be completely refuted, but if existent, it is likely small. Therefore, sensitization to the effects of EtOH was likely a main contributor to subsequent attenuation of EtOH- and LiCl-induced taste aversion.

The CTA paradigm can also be considered a measure of drug reward, as drugs of abuse have both rewarding and aversive stimulus properties (Hunt and Amit, 1987) and drug administration induces neuroadaptations in the mechanisms mediating both of these stimulus properties (Carr and White, 1986). There may also exist some overlap in the mechanisms and neurotransmitter systems mediating drug reward and aversion. For example, in regard to the dopamine system that has repeatedly been implicated in drug reward, administration of both the dopamine D_1 and D_2 receptor agonists SKF-38392 and

quinpirole, respectively, induced development of CTA (Asin and Montana, 1989). In addition, both cocaine and amphetamine-induced CTAs were attenuated by pretreatment with dopamine receptor antagonists (Hunt et al., 1985; Lin et al., 1994). There are also data implicating glutamatergic involvement in CTA. Glutamate release was recorded in the amygdala following intraoral administration of a flavor previously paired with LiCl injection and this effect was partially blocked by intra-amygdala injection of the NMDA receptor antagonist, MK-801 (Tucci et al., 1998). On the other hand, systemic pre-treatment with MK-801 enhanced the magnitude of EtOH-induced CTA (Bienkowski et al., 1998). Even though these two studies show opposite effects of MK-801 on LiCl- or EtOH-induced CTA, they implicate the NMDA receptor, in particular, and the glutamate system, in general, in the mediation of formation of taste aversion. Overall, there appear to be similarities, at least at the level of neurotransmitter systems, in the mediation of drug reward and aversion, although the main brain areas implicated in drug reward appear to be different than those implicated in the formation of taste aversions.

Behavioral experiments have shown both an association and dissociation in the rewarding and aversive drug properties. For instance, rats that developed the strongest amphetamine- induced CTA were also the ones that developed the strongest amphetamine-induced conditioned place preference (Turenne et al., 1996). In the same study, this relationship did not hold for morphine as rats that developed both strong and weak morphine-induced CTAs developed similar levels of morphine-induced place preference (Turenne et al., 1996). The selectively bred alcohol-nonpreferring NP rats developed greater and more prolonged EtOH-induced CTA than their alcohol-preferring P counterparts (Froehlich et al., 1988). Conversely, selectively bred taste aversion-prone

(TAP) rats, in a voluntary EtOH drinking paradigm, consumed less EtOH and showed lower EtOH preference than their taste aversion-resistant (TAR) counterparts (Orr et al., 1997). These results indicate that high EtOH preference is associated with low sensitivity to EtOH aversion. This negative genetic relationship implies that common genetic architecture underlies EtOH drinking, a measure of EtOH reward, and EtOH aversion. Studies using genetic mouse models have shown no significant association between EtOH reward and aversion. That is, strains that developed the greatest EtOH-induced CTA were not the ones that showed the greatest EtOH-induced place conditioning or that consumed the most EtOH solution (Risinger and Cunningham, 1998). Such results imply that the genes that mediate drug reward may be different from those that mediate drug aversion (Risinger and Cunningham, 1998). Interestingly, the same study reported a tendency for a negative correlation between these EtOH aversion and reward-related behaviors, similar to the relationship shown in the selectively bred rat lines. Thus, either a negative or a positive relationship between EtOH reward and EtOH aversion could imply commonality in the mechanisms mediating the reinforcing and aversive properties of EtOH.

In the grand scheme of things, there is evidence for an association between drug sensitization and drug reward and between drug reward and drug aversion. The results from the experiments in this thesis lend support to the association of drug sensitization and drug reward assessed in an alcohol-preferring mouse model, and add the additional association between drug sensitization and drug aversion, which has previously been discussed and assumed, but not shown. Thus, there emerges a three-way reciprocal association between drug sensitization, drug reward, and drug aversion processes. We

believe that further research will support a role for the neuroadaptations underlying drug sensitization in the mediation of drug reward and aversion, thus implicating drug sensitization as an important behavioral correlate to the development of drug abuse.

OVERALL SUMMARY AND CONCLUSIONS

Cross-sensitization studies demonstrated that disparate neural mechanisms likely mediate sensitization to the effects of EtOH versus those of morphine, cocaine, and methamphetamine. However, given the importance of drug injection schedules and drug withdrawal periods for the demonstration of sensitization and cross-sensitization to the effects of some drugs, especially the psychostimulants, it is possible that cross-sensitization between EtOH and some of the drugs tested does exist, but was not detected under the present conditions. In addition, only half of the relevant experiments have been performed thus far. Therefore, the conclusion of differential mediation of sensitization phenomena is at present tentative.

The demonstration that mice sensitized to the stimulant effects of EtOH voluntarily consumed more EtOH than their non-sensitized counterparts, and that sensitization could be induced by prior voluntary EtOH consumption provided strong evidence for a role of EtOH-induced sensitization in the maintenance of EtOH self-administration. Generally, an increase in voluntary EtOH consumption has been interpreted as increased reinforcement from EtOH. Therefore, it could be further stated that EtOH-induced sensitization enhanced EtOH reinforcement, and that it is likely mediated by some of the same mechanisms underlying drug reinforcement processes (mesolimbic dopaminergic system). In addition, sensitized mice showed attenuation in

EtOH-induced conditioned taste aversion, indicating reduced sensitivity to the aversive stimulus properties of EtOH. It is reasonable and exciting to suppose that by enhancing the reinforcing while concurrently decreasing the aversive stimulus properties of EtOH, sensitization to the effects of EtOH may effectively support continued alcohol drinking, potentially leading to alcohol abuse.

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