NEUROTRANSMITTER SUBSTRATES OF ETHANOL-STIMULATED ACTIVITY: INVOLVEMENT OF GABA AND GLUTAMATE SYSTEMS

by

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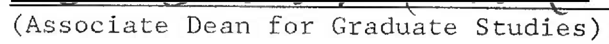

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ABSTRACT

The mechanisms underlying alcohol addiction are currently unknown; however, evidence suggests the existence of heritable factors which increase an individual's risk of developing this multifactorial disease. The evidence for genetic factors in alcoholism has led to a search for associated trait markers, which may aid detection of at-risk individuals and, if involved in the actions of alcohol, may further aid understanding of the mechanisms of alcohol addiction. One such trait marker is psychomotor stimulation by alcohol (ethanol), which has been demonstrated to parallel self-reports of euphoria in human subjects. Psychomotor stimulation in rodents is observed as increases in locomotor activity, and has been postulated to be a model of alcohol's euphoric effects. In addition, some theories of addiction have postulated that the neural pathways which mediate reward and reinforcement are homologous with the pathways which mediate locomotor stimulation, and that stimulation of these pathways leads to addictive behavior. Thus, understanding the mechanisms by which ethanol (EtOH) induces locomotor activation may lead to an understanding of the euphoric, and possibly addictive characteristics of the drug.

FAST and SLOW mice were bred, in replicate, for differential sensitivity to the locomotor stimulant effects of EtOH. C57BL/6J (B6) and DBA/2J (D2) inbred strains also differ in sensitivity to EtOH's activating effects due to genetic differences that arose arbitrarily at the time these strains were generated. FAST and D2 mice are more sensitive to the locomotor stimulant effects of EtOH compared to SLOW

and B6 mice, which are relatively resistant to EtOH's activating effects. These genotypes provide two unique genetic animal models with which to study mechanisms underlying locomotor responses to EtOH. Previous data demonstrated the involvement of dopamine (Shen, Crabbe and Phillips, 1995) and $GABA_B$ receptors (Shen, Harland and Phillips, 1995) in modulating expression of EtOH-stimulated activity in FAST mice. In the current work, the $GABA_B$ agonist, baclofen, dose-dependently decreased (0.625 mg/kg) and blocked (1.25 mg/kg) EtOH-stimulated activity (1.5 g/kg) in D2 mice, and may have enhanced locomotor depression in B6 mice (2.5 mg/kg). Receptor specificity of the inhibition of EtOH-stimulated activity by baclofen was demonstrated in EtOH-treated FAST and D2 mice by reversal of baclofen's effects by the $GABA_B$ antagonist, CGP-35348 (50 and 100 mg/kg). Taken together, these results provided additional support for the role of $GABA_B$ receptors in modulating expression of locomotor stimulation by EtOH.

Since a $GABA_B$ agonist decreased locomotor activation in FAST and D2 mice, it was hypothesized that enhanced $GABA_B$ activity was responsible for insensitivity to the locomotor stimulant effects of EtOH, and that blockade of $GABA_B$ activity would allow expression of EtOH-stimulation in SLOW and B6 mice. However, administration of the $GABA_B$ antagonist, CGP-35348 did not produce the predicted effect in SLOW (100 mg/kg) or B6 (100 - 200 mg/kg) mice. These data suggest that insensitivity to the locomotor stimulant effects of EtOH is unrelated to $GABA_B$ receptor function. Alternatively, $GABA_B$ function could have a minor or indirect role, so that alterations of other receptor subtypes or neurotransmitter systems are also necessary to induce stimulation in

these genotypes.

The mesoaccumbens-pallidal circuit comprises dopaminergic projections from ventral tegmental area (VTA) to nucleus accumbens (N Acc), and GABAergic projections from N Acc to ventral pallidum/substantia innominata (VP/SI). Glutamatergic projections to N Acc modulate the activity within this circuit, which has been demonstrated to mediate spontaneous locomotor activity, as well as novelty- and psychostimulant-induced locomotor activation. In addition, a GABAergic feedback projection from N Acc to VTA is thought to inhibit DA neuron activity via $GABA_B$ receptors. The neural circuitry underlying EtOH-stimulated activity has not been established; however, previous studies on individual systems have supported the involvement of dopamine, GABA, and glutamate. Thus, it is possible that mesoaccumbens-pallidal connections may also mediate EtOH-induced locomotor activation. The results that a $GABA_B$ agonist and several DA antagonists each decreased locomotor activation by EtOH are consistent with $GABA_B$ modulation of DA activity, and it was hypothesized that inhibition of EtOH activation by baclofen was due to inhibition of DA activity via $GABA_B$ receptors. It was predicted that DA agonists would reverse baclofen's inhibition of EtOH-stimulated activity in FAST and D2 mice. However, systemic injection of apomorphine (mixed agonist), SKF-38393 (D_1 agonist), quinpirole (D_2 agonist), or methamphetamine (increases synaptic DA) did not produce the predicted effects. These data suggest that the neural circuitry mediating locomotor activation by EtOH does not include DA/$GABA_B$ connections. Alternatively, it may be that microinjection of drugs directly into specific brain areas, rather than

systemic drug injection, would provide a better method for testing this neuroanatomically derived hypothesis.

Recent electrophysiological, biochemical, and behavioral data suggest interactions of EtOH with the NMDA glutamate receptor. In addition, EtOH was found to have an inhibitory effect on glutamate-stimulated Ca^{2+} influx in microsacs derived from brains of SLOW, but not FAST mice. It was hypothesized that the NMDA receptor is an important factor in determination of sensitivity to EtOH's stimulant effects. It was further hypothesized that NMDA receptors were differentially altered between FAST and SLOW mice during selection, and were also different between B6 and D2 mice. Locomotor responses to the noncompetitive NMDA receptor antagonist, MK-801, were assessed in naive- and EtOH-treated mice. MK-801 had dose-dependent biphasic effects on locomotor activity in all genotypes. Peak stimulation occurred at 0.2 mg/kg, followed by decreased stimulation at 0.5 mg/kg MK-801. FAST mice were more sensitive to the locomotor activating effects of MK-801 than SLOW mice, supporting the hypothesis that selection had differentially altered NMDA receptor function between the lines. There was no difference between B6 and D2 inbred strains in response to MK-801, suggesting that the difference in sensitivity to EtOH's stimulant effects between these two strains was not due to differences in NMDA receptor function, at least with respect to the MK-801 site. When given in conjunction with various doses of EtOH (0.5, 1.0, 1.5 g/kg in B6 and D2 mice; 0.5, 1.0, 2.0 g/kg in FAST and SLOW mice), low doses of MK-801 (0.05, 0.1 mg/kg) increased activity of mice given low doses of EtOH. The highest MK-801 dose tested (0.2 mg/kg) decreased the locomotor stimulant actions of higher

EtOH doses in FAST and D2 mice, sometimes to the level of saline controls, but did not cause locomotor depression. In SLOW and B6 mice, MK-801 given prior to EtOH resulted in slight decreases in EtOH-activity, suggesting a potentiation of sensitivity to the locomotor depressant actions of EtOH. This effect was most evident in mice given 1 g/kg EtOH and the highest dose of MK-801 (0.2 mg/kg). Taken together, these data indicate that MK-801 produced a leftward shift in the EtOH dose-response curve of FAST, D2, SLOW, and B6 mice.

In summary, possible neurochemical substrates of locomotor stimulation by EtOH were examined in two sets of genetic animal models: the FAST and SLOW selected lines, and B6 and D2 inbred strains. The data presented here provide evidence that $GABA_B$ receptors and NMDA receptors modulate the expression of activity after EtOH administration in FAST and SLOW selected lines, and B6 and D2 inbred strains, but do not provide support for an interaction between $GABA_B$ and dopamine systems. Future studies should 1) continue characterization of genetic differences between FAST and SLOW mice, and 2) continue to identify and characterize other neurotransmitter systems and receptor subtypes which mediate sensitivity to EtOH's stimulant effects. Furthermore, although pursuit of neuroanatomical hypotheses in mice awaits refinement of microinjection methods, future studies should also strive to identify the neural circuitry mediating EtOH-stimulated activity, as part of the continuing effort to understand EtOH's actions in the central nervous system.

INTRODUCTION

Alcohol abuse and alcohol dependence are prevalent concerns in the United States today. According to recent surveys (see Dufour, 1995; National Institute on Alcohol Abuse and Alcoholism, 1993), *per capita* alcohol consumption is 2.31 gallons of pure alcohol per year. Liver cirrhosis, a result of chronic alcohol consumption, was the 11th leading cause of death in the U.S. in 1991. Since 1979, alcohol-related traffic fatalities have been responsible for nearly 45% of all traffic deaths. The consequences of alcohol consumption are costly, both financially and in terms of human lives, and these consequences are not limited to those who abuse or are dependent upon alcohol. People who are able to drink socially without engaging in abusive or addictive behaviors are likely to become involved in alcohol-related accidents, and abstainers are often victims of such accidents. Though the full range of consequences of excessive alcohol consumption has not been discussed here, it is clear from these examples that research is needed to determine how to treat alcoholism, or possibly prevent it from occurring.

The mechanisms underlying alcohol addiction are not completely understood. However, a great deal of evidence from twin studies, adoption studies, and family studies suggests that genetic factors are important aspects of this multifactorial disease (see Goodwin, 1985; Devor and Cloninger, 1989; Schuckit, 1987). The evidence for a genetic component in the development of alcoholism has led to the search for an associated marker. This marker could be a biological trait that is directly involved in the actions of alcohol, or could be a closely linked gene product (Devor and Cloninger, 1989). One approach to

finding a marker for alcoholism has been the testing of biological sons of alcoholics, considered to be at high risk for development of alcoholism, and comparing them to low risk individuals (sons of nonalcoholics). It has been found that sons of alcoholic fathers, who had not yet developed alcoholism at the time of testing, showed lower sensitivity to the effects of alcohol compared to sons of nonalcoholic fathers. For example, as part of a prospective study being carried out in Denmark, Pollock et al. (1986) reported that biological sons of alcoholic men, defined as a high risk population, reported feeling less intoxicated and experienced less intense somatic symptoms (e.g. dizziness, nausea, headaches, palpitations, or excessive warmth) after ingestion of 0.5 g/kg alcohol compared to controls. Similar findings were observed in a study from the U.S. Sons of alcoholic fathers reported lower subjective feelings of "drug effect" and displayed less bodysway (static ataxia) after ingestion of 0.75 ml/kg alcohol, compared to subjects with negative family history (Schuckit, 1988). Hormonal changes in response to acute alcohol were also lower in individuals with positive family history of alcoholism; serum prolactin levels and cortisol levels were elevated in both groups, but the elevation was significantly lower in sons of alcoholic fathers. In both sets of studies (Danish population and U.S. population), differences between high and low risk subjects could not be attributed to differences in alcohol metabolism. The authors of both studies suggested that decreased sensitivity to the intoxicating and somatic effects of alcohol may be a risk factor for heavy alcohol consumption and later development of alcohol abuse or alcohol dependence. This suggestion was supported

by the results of a follow-up study in the U.S. population (Schuckit, 1994). All subjects, regardless of family history, were evaluated and divided on the basis of whether they had developed alcoholism in the approximately 10 years since their initial evaluation. It was found that subjective reports of intoxication, as well as body sway scores, had been lower in men who later developed alcoholism. In addition, a greater percentage of family history positive men developed alcoholism than men with no family history of alcoholism.

Some studies have found results opposite to those described above. In a study by Nagoshi and Wilson (1987), family history positive subjects reported *greater* sensitivity to alcohol's intoxicating effects. When tested at equivalent blood alcohol levels (60 mg/dl), male subjects with positive family history were more impaired in two behavioral measures of alcohol effects, hand steadiness and bead stringing, compared to subjects with no family history of problem drinking (Vogel-Sprott and Chipperfield, 1987). Subjective reports of alcohol effects did not differ between family history groups; however, blood alcohol levels were not equivalent when these subjective ratings were given. O'Malley and Maisto (1985) also reported that subjects with positive family history were more impaired by alcohol on a timed motor task, but reported lower levels of intoxication, behavioral impairment, anesthesia and central stimulation than family history negative subjects. Thus, there is some disagreement within the literature with regard to sensitivity to alcohol's effects in family history positive and family history negative individuals. In addition, the data reported by O'Malley and Maisto (1985) suggest that relative sensitivity may

depend on the alcohol effect being measured, since family history positive subjects were more sensitive to the motoric effects of alcohol, but perceived themselves to be less intoxicated than family history negative subjects.

Although the results of these studies appear contradictory, one implication of these approaches is that measures of acute sensitivity to alcohol may provide important information with regard to risk of developing alcoholism. Furthermore, since markers for the development of alcoholism may be biological traits involved in the pathophysiology of alcoholism, an understanding of the mechanisms by which alcohol produces these acute effects may provide greater understanding of the mechanisms by which alcoholism develops.

Alcohol-Induced Locomotor Stimulation

Clinical Studies

Psychomotor stimulation is produced by acute administration or ingestion of alcohol (ethanol; EtOH), and may serve as a trait marker for increased risk or predisposition for development of alcoholism. In keeping with this possibility, Newlin and Thomson (1991) found that sons of alcoholics showed greater motor activity compared to sons of nonalcoholics. Furthermore, psychomotor stimulation may be a biologically relevant marker, in that it may reflect processes which contribute to the development of alcoholism. Clinical studies have produced evidence suggesting that moderate alcohol doses can elicit stimulant, along with euphoric effects in human subjects. Subjects who had ingested alcohol reported feeling happier, more elated (measures of

euphoria), and simultaneously more talkative (taken to be a measure of stimulation), than when sober (Ahlenius et al., 1973; Ekman et al., 1963, Ekman et al., 1964). In a more recent study by Lukas and Mendelson (1988), subjects who drank 0.695 g/kg alcohol reported euphoric episodes beginning within 10 minutes after ingestion, and continuing for an additional 40 minutes. The increased incidence of subjective reports of euphoria (75% of subjects who ingested alcohol compared to 0% in a placebo control group), closely paralleled increases in plasma alcohol level, EEG alpha activity, and plasma ACTH levels, suggesting that the behavioral and physiological effects of intoxication occurred at low blood EtOH levels during the ascending limb of the blood EtOH curve. In a similar study, subjects who ingested alcohol rated themselves to be more elated, friendly, and "vigorous" during the ascending phase of the blood EtOH curve, and further described themselves as more angry, depressed, and fatigued as blood alcohol levels declined (Babor et al., 1983).

Animal Studies

In animals, psychomotor stimulation is expressed as increased locomotor activity. Effects of EtOH on locomotor activity in animals were documented as early as 1960 by Read, Cutting, and Furst. "Gross activity" was measured on an activity table, an apparatus which consisted of a light plastic container supported by a spring lever. Movement of a mouse placed inside the container closed a microswitch, which activated an electrical counter. An EtOH volume of 5 ml/kg (EtOH concentration was not reported), injected subcutaneously into mice,

produced marked stimulation with rapid onset, followed by a typical depressant phase. Low doses of EtOH (1.0 and 2.0 g/kg) produced increases in open-field activity when administered to gerbils (Järbe and Ohlin, 1977). Similar dose-dependent EtOH effects on locomotor activity were documented in albino rats by Buckalew and Cartwright (1968). Low doses of EtOH (30% EtOH in saline) up to 1.2 g/kg were observed to produce increases in exploratory activity in a Skinner box, while doses ≥ 1.8 g/kg caused decreases in exploration. However, locomotor stimulation in rats appears to be less easily demonstrated compared to other rodents, as some studies report only decreases in activity (e.g. Cunningham, Niehus and Noble, 1993; Frye and Breese, 1981; Masur, DeSouza and Zwicker, 1986). The reasons underlying differential responses of mice and rats have not been elucidated.

Overall, these studies are typical examples of the biphasic actions of EtOH (Pohorecky, 1977; Dudek, Phillips and Hahn, 1991). Whereas high doses of EtOH have sedative effects, the stimulant properties of EtOH are evident at low doses or at early time points after exposure to higher doses of EtOH. Interestingly, there is some evidence that EtOH also produces biphasic hedonic effects in DBA/2 inbred mice as measured by conditioned place preference (Risinger and Cunningham, 1992). Mice placed in a conditioned place preference apparatus immediately after EtOH injection showed preference for the environment in which they had been exposed to EtOH. In contrast, mice placed into the apparatus 30 minutes after EtOH injection showed a conditioned aversion to the drug-paired environment. These data suggested that the rewarding effects of EtOH occurred early after

injection, presumably during the time that blood ethanol concentrations (BECs) were increasing, and that aversive effects of EtOH occurred during peak BEC, or when BECs were declining. A similar conclusion was reached after investigation of EtOH effects on brain stimulation reward in rats (Lewis and June, 1990). When measured 0 - 20 minutes after EtOH injection (0.25, 0.5 g/kg, i.p.), response rates were significantly increased and reward thresholds were significantly decreased, suggesting that EtOH was reinforcing at these doses. However, at 30 - 50 minutes after injection, response rates and reward thresholds were unchanged. BECs determined in an independent group of rats were found to increase immediately after injection and reach peak levels by 20 minutes after administration. Taken together, these data suggested that the reinforcing effects of EtOH occurred when BECs were increasing.

Genetic influences may also be important in conferring sensitivity to the stimulant effects of EtOH in mice, as supported by variability in locomotor responses to EtOH among inbred strains (Crabbe, 1986; Crabbe et al., 1994; Dudek, Phillips and Hahn, 1991; Phillips et al., 1995), and by the development of selectively bred mouse lines which differ in locomotor responses to EtOH (Crabbe et al., 1987; Phillips et al., 1991; Shen et al., 1995).

Significance of Locomotor Activation

The parallel biphasic effects of EtOH on activity and hedonia in both rodents and humans have led investigators to postulate that 1) in humans, stimulation by EtOH may reflect euphoria or rewarding effects; and 2) EtOH-stimulated activity in animals can serve as a model of

alcohol's euphoric effects in humans (Pohorecky, 1977). Thus, psychomotor activation by EtOH may be a biologically relevant trait marker in human genetic studies, reflecting processes (euphoria or reward) that may contribute to the development of alcoholism. The notion that locomotor stimulation may reflect activation of mechanisms underlying reward or reinforcement has been applied to all drugs of abuse in a psychomotor stimulant theory of addiction. Most recently formalized by Wise and Bozarth in 1987, the theory proposes that the locomotor stimulant properties of all drugs of abuse and the mechanisms of reward or reinforcement are homologous, and therefore mediated by a common neural substrate, the mesolimbic dopamine projection. According to this theory, investigation of the mechanisms underlying locomotor stimulation will lead to an understanding of the mechanisms by which drugs become addictive. Alternative views, as stated by Koob and colleagues (Koob, 1992; Koob and Bloom, 1988), suggest that the mesolimbic system is an important component of reward, but is not the primary mediator of reward for all drugs of abuse.

Genetic Animal Models

The use of genetic animal models has gained a popularity in alcohol research that is nearly unparalleled in any other field of study. The evidence that alcoholism is influenced by genetic factors, and the difficulty in elucidating one specific site of EtOH's actions, have contributed to this popularity. In turn, genetic animal models have supported a role of genetic factors in mediating many of EtOH's effects, and have suggested multiple sites of EtOH actions. Rodents,

especially mice, have been favored as genetic models. Mice have enjoyed a long history in genetics research, and many inbred strains of mice currently exist. On a more practical side, mice require relatively little space, have large litters, require very little time between successive generations of litters, and are amenable to controlled matings. More importantly, nearly all human genes have homologs in the mouse genome, and there is a great deal of linkage homology (synteny) between mouse and human chromosomes. Thus, genetic factors found to influence complex alcohol traits in mice can then be screened as possible candidate genes for related alcohol traits in humans (Copeland et al., 1993; Silver, 1995, p. 266). Two genetic models of interest here are inbred strains and selectively bred lines of mice.

Inbred Strains of Mice

All mice within an inbred strain are genetically identical (isogenic) and homozygous at all gene loci. By definition, a strain is considered to be inbred after at least 20 consecutive brother-sister matings (F20), and when its origins can be traced to a single ancestral breeding pair either at F20 or a subsequent generation. After 20 generations of brother-sister matings, approximately 98.7% of the genome should be homozygously fixed. For any given trait, phenotypic variance (V_P) within a population is, in its simplest conceptualization, due to a combination of genotypic variance (V_G) and environmental variation (V_E): $V_P = V_G + V_E$. Since members of an inbred strain are genetically identical, differences between individuals of a strain for a given phenotype are assumed to be due to environmental factors. Conversely,

when environmental conditions are held constant, differences between inbred strains for a given phenotype are due to genetic factors. One advantage of inbred strains is that, because of the genetic identity within each strain, data collected from laboratories in various locations and at various times can be directly compared.

Many of the inbred mouse strains in use today were derived from strains originally developed for mouse fancy, a hobby which began in China and Japan, and eventually spread to England and the United States (Silver, 1995, p. 26). Mice were bred and named for special or rare coat colors, such as white English sable or creamy buff, or for interesting behaviors, such as those observed in "waltzing" mice, a motor behavior in which mice appear to be twirling and dancing. In the United States, Abbie Lathrop bred many of these pet strains for American mouse fanciers in the early 1900's and was the main supplier of mice to William E. Castle and his colleagues at the Bussey Institution for Applied Biology of Harvard University, whose initial experiments on the inheritance of coat color and cancer susceptibility laid the groundwork for the applicability of Mendel's laws to mammalian species (Morse, 1978).

C57BL/6 and DBA/2 Inbred Strains of Mice

Of the many inbred strains developed by colleagues and students of William Castle, the C57BL/6 (B6) and DBA/2 (D2) strains have been regarded to be standards in EtOH research because they differ in many EtOH-related responses, including preference drinking, conditioned taste aversion, ataxia, tolerance to hypothermic and ataxic effects of EtOH,

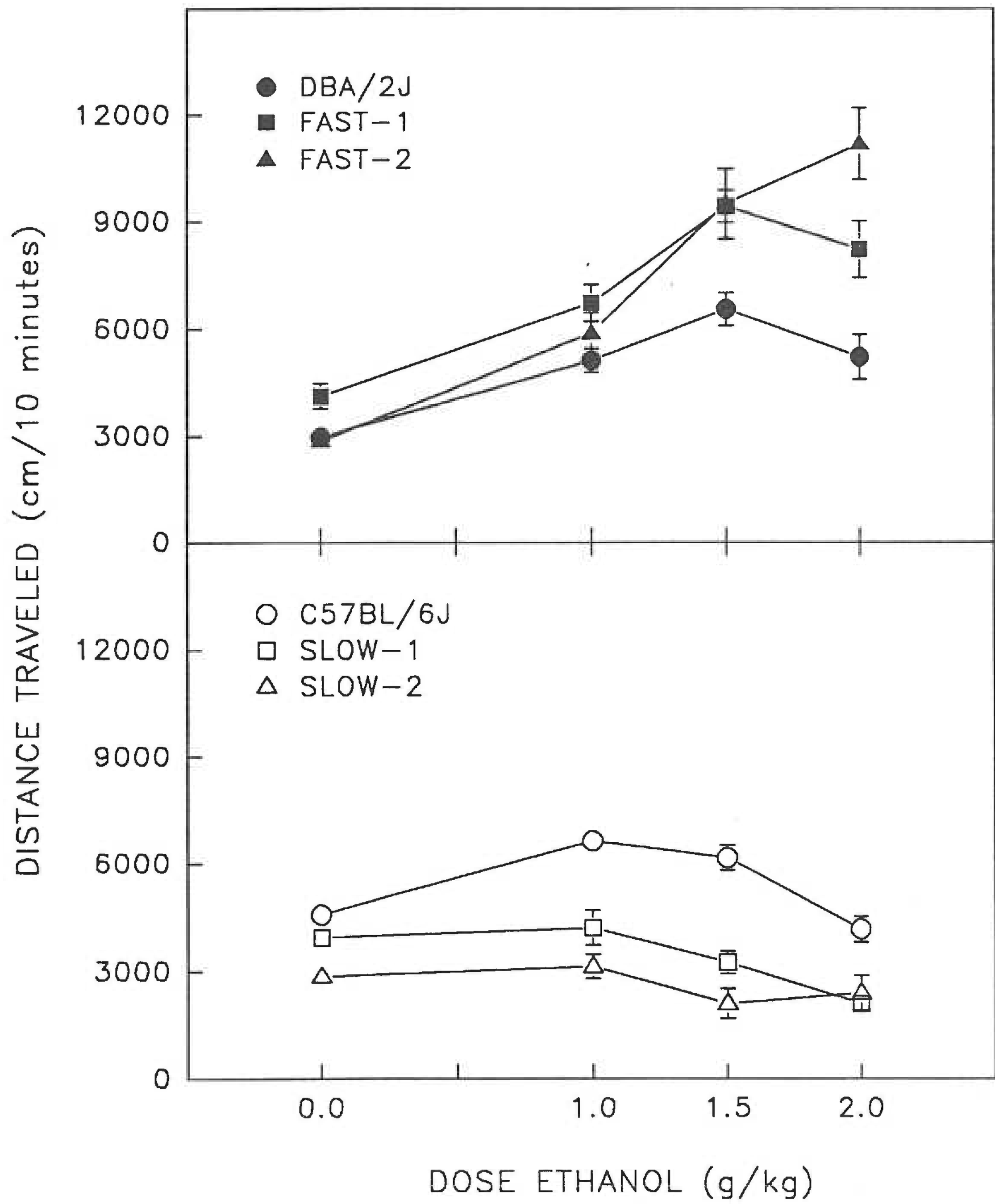
withdrawal, and sensitivity to prenatal effects of EtOH (Phillips and Crabbe, 1991). Both C57 and DBA strains were originally developed by Clarence Little, a student of Castle's who eventually founded Jackson Laboratory. Breeding of the DBA strain began in 1909, thus making it the oldest of inbred mouse strains. New substrains, including the D2 substrain, were established at Jackson Laboratory around 1930. Development of C57 mice began in 1921 using mice from Miss Abbie Lathrop's stock, and B6 was one of several substrains derived from this original inbreeding program (Festing, 1989). B6 and D2 mice differ markedly in locomotor responses to EtOH, as demonstrated by a recent study presented in Figure 1 (E. Shen, unpublished results). This figure shows that D2 mice were significantly stimulated by all doses of EtOH tested (1.0, 1.5, and 2.0 g/kg), with peak stimulation occurring at 1.5 g/kg. In B6 mice, a slight locomotor activation was observed after administration of 1.0 and 1.5 g/kg EtOH, but decreased to saline levels after 2.0 g/kg. These results in B6 and D2 mice are consistent with published characterizations of locomotor behavior in these strains in response to EtOH (Crabbe et al., 1982; Dudek and Phillips, 1990).

Selective Breeding

Selective breeding involves the systematic intermating of individuals to produce a population with a specifically desired phenotype or phenotypes. The method of selective breeding has been called "the most important application of quantitative genetics" (Falconer, 1989), and has been employed for many years in the fields of agriculture, horticulture, animal husbandry, and canine breeding.

Figure 1. Effects of EtOH on locomotor activity of FAST, SLOW, DBA/2J (D2) and C57BL/6J (B6) mice. Genotypes with high sensitivity to EtOH's stimulant effects (FAST-1, FAST-2, D2) are shown in the upper panel; genotypes with low sensitivity (SLOW-1, SLOW-2, B6) are shown in the lower panel. Mice were injected with saline or EtOH (20% v/v) and immediately tested for locomotor activity for 10 minutes. Mice were tested in activity chambers with no ambient light. D2, FAST-1, and FAST-2 mice were significantly stimulated by all doses of EtOH. B6 mice were significantly stimulated by 1.0 and 1.5, but not 2.0 g/kg EtOH. The activity of SLOW mice was not affected by EtOH, except 2.0 g/kg EtOH significantly decreased the activity of SLOW-1 mice. Significance level was $p < 0.05$ in all cases. S.E.M. larger than symbol size are shown; $n = 10$ per genotype per dose.

FIGURE 1



Bidirectional selective breeding has also gained popularity in alcohol addiction research (Crabbe, Belknap and Buck, 1994), and several sets of selected mouse and rat lines currently exist that differ with respect to a particular alcohol response. Production of sets of bidirectionally selected lines is accomplished by testing and scoring offspring for the phenotype under consideration. Subsequently, high-scoring animals are mated to each other and low-scoring animals are mated to each other. This process of testing offspring and mating extreme scorers is repeated with each generation, so that, given some genetic control of the phenotype, the phenotypic means of the lines diverge. Replication of this process using independent, but comparable, foundation populations results in two sets of lines, each set comprising one high and one low line. As described in greater detail below, replicated lines aid in the interpretation of data collected in selected lines.

Selective breeding theoretically alters allelic frequencies within a line so that all trait-relevant gene loci become homozygously fixed, while all trait-irrelevant genes continue to segregate independently. Selected lines thus provide unique genetic animal models for investigation of traits that may be genetically correlated with the selection phenotype, as well as identification and characterization of physiological mechanisms underlying the selection trait.

FAST and SLOW Selected Lines

A replicated bidirectional breeding program has produced lines of mice which differ in their locomotor responses to EtOH. Most recently characterized through the 35th generation of selection (S_{35}), FAST mice

are highly sensitive to the locomotor stimulant effects of low doses of EtOH, whereas SLOW mice are insensitive to EtOH's stimulant effects, and sometimes more sensitive to EtOH's locomotor depressant effects at these same doses (Shen et al., 1995).

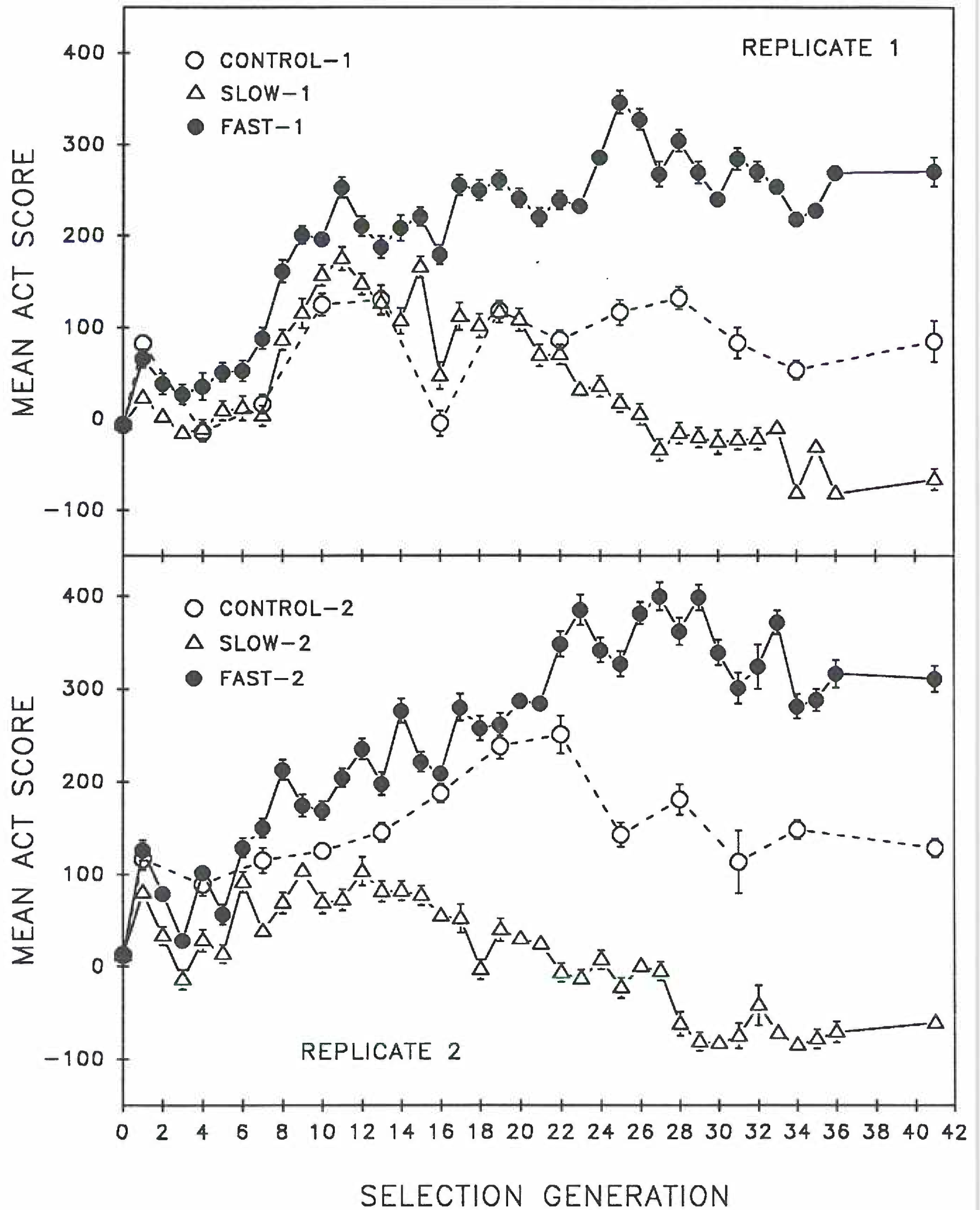
Selection protocol. Selective breeding of FAST and SLOW mice has been described in detail by Crabbe et al. (1987) and Phillips et al. (1991). Briefly, derivation of replicate sets of FAST and SLOW mice began with comparable, but genetically independent, populations of HS/Ibg mice, resulting in FAST-1, FAST-2, SLOW-1, and SLOW-2 lines. In addition, two control lines (CON-1 and CON-2) were randomly bred from this stock and maintained with the selected lines. HS/Ibg mice are a genetically heterogenous population created by an 8-way cross of inbred strains chosen for their widely divergent genetic backgrounds (McClearn, Wilson and Meredith, 1970). In each generation, locomotor activity of first-litter offspring was assessed for four minutes on each of two consecutive test days in Lehigh Valley circular open-field activity monitors (61 cm diameter). Locomotor activity is automatically measured by six sets of radially oriented photocell beams and receptors which transect the open-field into 36 squares, except at the rounded edges. Saline or EtOH (2.0 g/kg; 20% v/v) was injected i.p., 2 minutes prior to testing. For early selection generations (through S_6), mice were tested for locomotor activity under dim lighting conditions after saline injection on day 1, and 1.5 g/kg EtOH injection 24 hours later on day 2. Subsequently, the selection protocol was changed so that 2.0 g/kg EtOH was administered on day 1, saline was administered on day 2, and activity testing occurred under normal fluorescent lighting conditions.

In all generations, breeders were selected on the basis of ACT scores (EtOH activity - saline activity).

A within-family breeding scheme was used throughout selection. Within each replicated line, one male and one female from each of 9 families were chosen as parents and mated using a rotational breeding scheme, to produce the next generation. Individuals with the highest ACT scores were mated together to form the FAST lines, while individuals with the lowest (including negative) ACT scores were mated to produce the SLOW lines. Each of the lines was maintained as an independent breeding population, and the breeding scheme avoided brother-sister matings. Mice for the control lines were tested for activity responses to EtOH as described above, and individuals were randomly bred (i.e. without regard to their ACT scores), avoiding brother-sister matings. Selective breeding of FAST and SLOW mice continued for 36 generations, and was recently relaxed. Currently, each line is maintained as an independent breeding population using the same within-family breeding scheme, but no selection pressure is placed on the lines, and individuals are bred without regard to ACT scores.

Response to selection. Response to 36 generations of selection (S_{36}) for differential sensitivity to the locomotor stimulant effects of 2.0 g/kg EtOH, and the effects of 5 generations of relaxed selection ($S_{36}G_{41}$) are presented in Figure 2. Genetic analyses and characterization of FAST and SLOW mice have been described through S_{35} (Shen et al., 1995). A clear divergence between FAST and SLOW mice occurred in both replicates within the first generation of selection, suggesting a relatively large influence of a single gene on the

Figure 2. Response to 36 generations of selection for differential locomotor responses to low dose EtOH. Mean ACT scores of FAST-1, SLOW-1, and CON-1 mice (upper panel), and FAST-2, SLOW-2, and CON-2 mice (lower panel) are shown. For most selection generations, mice were tested under bright lighting conditions after injection of EtOH (2.0 g/kg) on day 1, and after saline injection 24 hours later on day 2. In generations 1 - 6, mice were tested under dim lighting conditions after saline on day 1 and EtOH (1.5 g/kg) on day 2. Mice were always selected on the basis of ACT scores (EtOH activity - saline activity). After S_{36}, mice were randomly mated within each line for 5 generations (relaxed selection), and tested again in $S_{36}G_{41}$. Mean ACT scores did not change after relaxed selection, suggesting that all trait-relevant loci have become homozygously fixed. Thus, FAST and SLOW lines are currently maintained without continued selection. S.E.M. larger than symbol size are shown. (Source of data: Shen et al., in press).


ACTIVITY SELECTION
REPLICATE 1
CONTROL-1
SLOW-1
FAST-1
MEAN ACT SCORE
400
300
200
100
0
-100
REPLICATE 2
CONTROL-2
SLOW-2
FAST-2
MEAN ACT SCORE
400
300
200
100
0
-100
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
SELECTION GENERATION

selection phenotype. Because little additional divergence occurred thereafter, and in response to data showing that different environmental factors produced an even greater EtOH stimulant response in Swiss-Webster or FAST and SLOW mice (Crabbe et al., 1988), changes in the selection protocol were implemented after S_6. These changes included increases in EtOH dose and light intensity, and a change in the order of drug and saline injection, and resulted in consistent and gradual increases in the magnitude of divergence between FAST and SLOW mice, suggesting recruitment of several additional trait-relevant genes. The continued divergence was characterized by increased activation in FAST mice, and decreased activation followed by increased locomotor depression in SLOW mice in later generations. The locomotor activity of CON-1 mice resembled that of SLOW-1 mice in earlier generations, but mean ACT scores of recent generations have been intermediate, relative to FAST-1 and SLOW-1 mice. CON-2 mice more closely resembled FAST-2 mice in early generations and for a period of time between S_{16} - S_{22}; however, mean ACT scores of CON-2 mice have generally been intermediate to the selected lines during the course of selection. Little systematic increase (FAST mice) or decrease (SLOW mice) in response to selection has occurred since S_{28}, and it is likely that the limits of selection have been reached. Recent heritability estimates reported by Shen et al. (1995) support this suggestion. Heritability (h^2) reflects the proportion of phenotypic variance in a trait that can be attributed to genotypic variance, and can be derived in selective breeding programs by the ratio of total response to selection and cumulative selection pressure. Heritability calculated through S_{35} was 0.08 and 0.06 for

FAST-1 and FAST-2 mice, respectively, and 0.02 and 0.1 for SLOW-1 and SLOW-2 mice, respectively. These estimates in FAST mice are lower than those previously reported by Phillips et al. (1991), which suggests that additive genetic variance in FAST mice has decreased with successive selection generations. This is the expected result of homozygous fixation of trait-relevant loci. In contrast to FAST mice, h^2 increased in SLOW mice. This was due to a great deal of selection pressure for locomotor depression (selection for negative ACT scores) which initially resulted in decreased activation of SLOW mice and negative (ostensibly zero) or near zero heritabilities. In later generations however, selection for negative ACT scores finally resulted in increased sensitivity to locomotor depression around S_{28} in SLOW mice, which caused h^2 estimates to increase. However, as noted previously, little systematic divergence has occurred since S_{28}. In addition, after five generations of random mating ($S_{36}G_{41}$), the mean ACT score of each line was not different from the pre-relaxation score. These results suggest that homozygous fixation of all trait-relevant gene loci has occurred in each line.

As reported by Shen et al. (1995), analysis of baseline data collected during selection testing (saline day) showed that, except for FAST-1 mice, baseline activities of the lines had not been altered, whereas the responses to EtOH were significantly altered during the course of selection. These results suggest that selection has selectively altered EtOH responses largely independently of baseline activity, and provides some validation of the use of difference scores as the selection phenotype. The result that baseline activity of FAST-1

mice had increased slightly during selection was consistent with results from experiments in recent generations of FAST and SLOW mice, in which FAST-1 mice displayed higher baseline activities than all other lines. However, these changes were slight compared to the change in EtOH-stimulated behavior in FAST-1 mice.

Finally, several locomotor behavior characteristics in response to a range of EtOH doses (0.5 - 3.0 g/kg) were described by Shen et al. (1995). At 5-minutes post-injection, EtOH (1.0 - 3.0 g/kg) increased distance traveled by FAST mice in a dose-dependent biphasic manner with peak activation at 2.0 g/kg and decreasing activation at higher doses. Biphasicity of stimulant responses was less evident in FAST-1 mice compared to FAST-2 mice. In SLOW mice, distance traveled was lower than, but not significantly different from baseline activity. FAST and SLOW mice differed in distance traveled in response to every dose of EtOH tested. In addition, FAST mice spent more time in motion, ambulated at greater speeds, and moved farther per movement "bout" compared to SLOW mice.

Interpretation of Results with Selected Lines and Inbred Strains

Selectively bred lines and inbred strains provide powerful genetic tools for determination of physiological processes which mediate a particular phenotype of interest, as well as the extent to which different phenotypes share common genetic influences. When bidirectionally selected lines differ in a trait other than the selection phenotype, this is said to be a correlated response to selection which arises when the genes, or a subset of genes, which

mediate the selection phenotype also mediate the other trait. Because inappropriate analysis and interpretation of results can lead to spurious or misleading information, guidelines for interpretation of experiments using selectively bred and inbred animals have been suggested and discussed in detail (Crabbe et al., 1990). For selectively bred lines, one important caveat is that selective breeding programs necessarily use finite animal populations, inevitably leading to inbreeding over successive selection generations. Inbreeding may result in spurious genetic correlations since it effectively forces all gene loci to homozygosity, regardless of relevance to the selection phenotype. The use of replicated sets of lines reduces the incidence of finding spurious genetic correlations due to inbreeding because strong evidence for a true genetic correlation requires that the correlated responses occur in both replicates. The likelihood that chance fixation of alleles at gene loci unrelated to the selection phenotype would result in trait differences in the same direction in both replicates is very low. A situation in which a correlated response occurs in one replicate, but not the other, may arise for two distinct reasons. First, chance fixation of trait-irrelevant alleles may have occurred in one replicate, resulting in a correlated response which does not represent a true genetic correlation. Alternatively, the correlated response may not have occurred in one replicate because of genetic drift, or sampling error when the foundation population was chosen, so that crucial alleles were not available to be fixed. Since it is difficult to determine which of these possibilities is correct, the detection of a correlated response in one replicate is interpreted as

moderate evidence for a genetic correlation.

In addition to investigation of correlated responses, selected lines provide models for investigation of physiological mechanisms which mediate the selection phenotype. In this case, the experimental approach is slightly different. Rather than primarily searching for response differences between the lines, experimental manipulations are focused on alteration of the selection phenotypes. For example, inhibition of EtOH-stimulated activity in FAST mice by a specific receptor ligand would suggest the involvement of that receptor system in mediating the behavior. Although the guidelines for interpretation of selected line data suggested by Crabbe et al. (1990) specifically addressed correlated responses to selection, these guidelines also apply when searching for underlying physiological mechanisms. Involvement of a particular neural substrate in a selection response is most strongly supported when evidence is found in both replicate lines.

With regard to analysis of genetic correlations in inbred strains, a statistically powerful approach involves the use of a large panel of inbred strains. However, for identification of physiological factors that mediate a phenotype, two inbred strains which represent the high and low extremes of the phenotype can be studied. As discussed above, this can be accomplished by manipulations which alter the phenotype of interest. Care must be taken not to overinterpret results from these studies as genetic correlations since they involve only two inbred strains. One way to bolster and/or confirm results observed in inbred strains is to compare them with other genetic models, such as selected lines, which have been independently derived using different procedures.

For example, B6 and D2 strains consistently differ in sensitivity to stimulation by low EtOH doses, as previously demonstrated in Figure 1. In addition, this study compared the locomotor responses of the inbred strains with those of the FAST and SLOW selected lines. B6 mice, like SLOW mice, are less sensitive to the activating effects of EtOH, whereas D2 mice are more similar to FAST mice, and are highly sensitive to the activating effects of EtOH. Unlike differences between the selected lines, the differences between B6 and D2 mice were not systematically produced, but are a result of arbitrary fixation of their individual genotypes during inbreeding. The involvement of a specific physiological mechanism in mediating EtOH-stimulation, e.g. a particular neurotransmitter system, is more strongly supported when evidence is found in all 3 of the highly sensitive genotypes (FAST-1, FAST-2, and D2), than when found in only one of the genotypes. Genetic correlations found in one model are similarly bolstered when also detected in the other model.

Neural Basis of Locomotor Activation by EtOH

Neural Circuitry

The neural circuit most strongly implicated in the control of locomotor activity, especially with regard to activity in response to psychostimulant drugs or novelty, is one that involves neuronal connections between the ventral tegmental area, nucleus accumbens, and the ventral pallidum/substantia innominata (see reviews by Amalric and Koob, 1993; Kalivas, 1993; Koob et al., 1991). A schematic representation of these projections is given in Figure 3. The

Figure 3. Schematic diagram of neural circuitry believed to be important in mediating locomotor responses to novelty, psychomotor stimulants, and converting motivation to action. Mesolimbic dopamine (DA) neurons project from ventral tegmental area (VTA) to the nucleus accumbens (N Acc), and form synaptic connections with GABAergic neurons projecting to ventral pallidum/substantia innominata (VP/SI). The N Acc also sends GABAergic projections to VTA to form a feedback loop, possibly mediated via $GABA_B$ receptors on DA cell bodies. Glutamatergic projections from limbic areas, such as prefrontal cortex (Pfx) and hippocampus terminate in N Acc to modulate the activity of DA and GABA neurons. (Adapted from Kalivas, 1993; Koob, 1992).

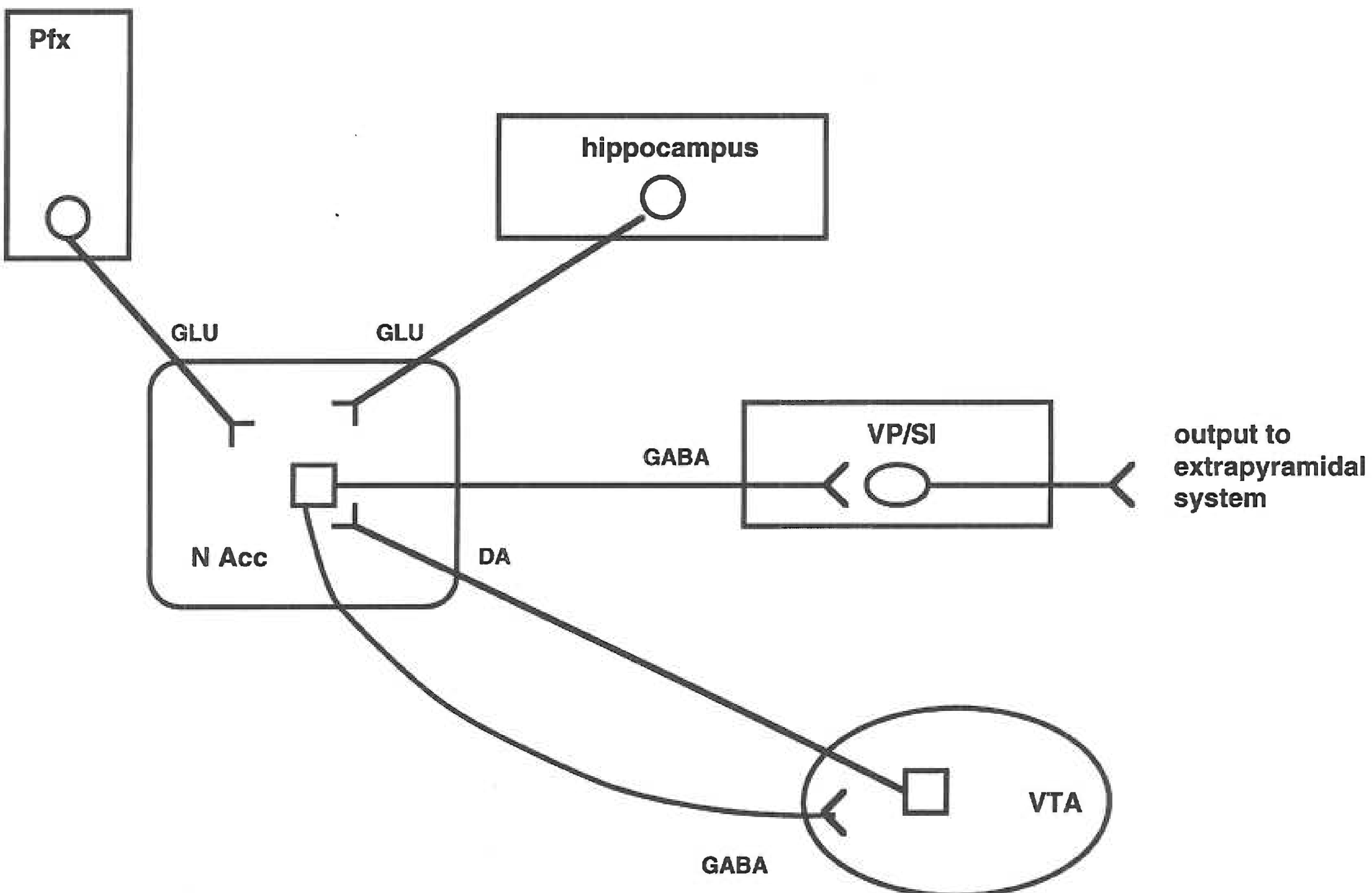


FIGURE 3

dopaminergic mesolimbic pathway, which projects from ventral tegmental area (VTA; also called the A10 region) to nucleus accumbens (N Acc), was previously introduced as the neural substrate of interest in Wise and Bozarth's (1987) psychostimulant theory of addiction. Mesolimbic (mesoaccumbens) neurons synapse on γ-aminobutyric acid-containing (GABA) neurons in the N Acc, which in turn project to ventral pallidum/substantia innominata (VP/SI). The VP/SI, in turn, sends projections to other motor areas to produce changes in locomotor activity. Evidence for the involvement of the mesoaccumbens and accumbens-pallidal projections comes from both lesion and microinjection studies, which have demonstrated that interference with the function of any one of these structures reduced or blocked spontaneous locomotor activity, or locomotor stimulation induced by novelty or administration of psychostimulant drugs (for reviews, see Amalric and Koob, 1993; Koob, 1992; LeMoal and Simon, 1991).

One characteristic of the mesoaccumbens-pallidal circuit is its modulation via positive and negative feedback relationships, both from structures within the circuit and from projections originating outside the circuit. For example, the VP/SI sends GABAergic projections back to N Acc and VTA to regulate activity within these structures. These projections appear to modulate VTA neuron activity via $GABA_B$ receptors located on dopamine cell bodies in the VTA (Kalivas, 1993). Intra-VTA injections of the $GABA_B$ agonist, baclofen, were shown to reduce novelty-induced locomotor activation (Hooks and Kalivas, 1995). In addition, the mesoaccumbens-pallidal circuit is subject to modulation by glutamatergic afferents to N Acc from limbic structures such as

amygdala, prefrontal cortex, and hippocampus. These glutamatergic afferents likely regulate neurotransmission within the accumbens. Thus, it has been proposed that the VTA-accumbens-pallidal circuit is a primary interface between motor and limbic systems, transferring information in order to translate motivation into action (Koob et al., 1991).

Neurochemical Substrates

Because EtOH has a plethora of effects, one hypothesis of EtOH's actions was that it nonspecifically affected cellular membrane fluidity. Several lines of evidence, including the chemical characteristics of alcohols and the proposed mechanisms of general anesthetic actions, contributed to this hypothesis (Hunt, 1985). EtOH is a member of a class of short-chain aliphatic alcohols characterized by one or more hydroxyl (OH^-) groups attached to a hydrocarbon chain. Because of their small size and relatively simple structure, alcohols were not believed to act on receptors in the normal pharmacological sense. However, hydrophillic OH^- groups and hydrophobic hydrocarbons confer polarity to alcohols so that they are soluble in water or lipids, depending on their chain length. Greater chain lengths are associated with increased hydrophobicity due to increased numbers of hydrocarbons, which in turn leads to greater lipid solubility. The classic Meyer-Overton theory suggested that potencies of general anesthetics were positively correlated with their lipid solubilities, and the observations that EtOH shares common effects and properties with general anesthetics led to the suggestion that EtOH's mechanism of actions were similar to those of

anesthetics (see Hunt, 1995, p. 7-8). Thus, it was postulated that anesthetics and alcohols produced their effects by dissolving in the lipid compartments of cell membranes, thereby disordering membrane structure and disrupting normal membrane function. In support of this, Lyon et al. (1981) found that the relative hypnotic potencies of several branched and straight-chain alcohols were positively correlated with their membrane disordering potency, which was also positively correlated with lipid solubility (as determined by membrane/buffer partition coefficients). Currently, it is becoming more widely acknowledged that general anesthetics may selectively interact with membrane-bound proteins, as supported by stereoselectivity of anesthetics (see Franks and Lieb, 1994). Furthermore, based on recent literature, EtOH itself has been proposed to have effects on membrane-bound proteins (e.g. ligand-gated ion channels) that are too specific to be explained by general membrane disordering properties (see Grant, 1994; Nevo and Hamon, 1995).

There are several neurochemical substrates and neurotransmitter systems which have been implicated in the behavioral effects of EtOH. Some of these (e.g. the $GABA_A$ receptor complex and the NMDA-type glutamate receptor) may have specific sites with which EtOH interacts. With regard to the stimulant effects of drugs of abuse, the neural circuitry discussed above suggests three candidate neurotransmitter systems: dopamine, GABA, and glutamate. All three systems have been implicated in EtOH's stimulant effects (Phillips and Shen, in press), and will be the focus of the experiments described here. However, a focus on dopamine, GABA, and glutamate does not imply that other

neurotransmitter systems are unimportant for locomotor stimulation by EtOH.

Dopamine

Dopamine is a biogenic amine which acts as an inhibitory neurotransmitter in the central nervous system (CNS), decreasing the activity of neurons upon which dopaminergic axons terminate (White and Wang, 1986). The actions of dopamine are mediated through second-messenger signaling systems coupled to dopamine receptors. To date, five dopamine receptor subtypes (D_1 - D_5) have been cloned, and have been divided into two classes based on their pharmacological and protein sequence similarities to the D_1 or D_2 receptor subtype. D_3 and D_4 receptors are classified as D_2-like, whereas D_5 receptors are considered to be D_1-like. Although both D_1 and D_2 receptors are coupled to G-protein signaling systems, D_1 receptors interact with stimulatory (G_s), and D_2 receptors interact with inhibitory (G_i) G-proteins, which in turn increase or decrease cyclic AMP (cAMP) concentrations via regulation of adenylate cyclase activity. Like D_1 receptors, D_5 receptors are coupled to G_s; however, the second messenger systems for D_3 and D_4 receptors remain unidentified. Molecular biology and function of dopamine receptors has been extensively reviewed by Civelli, Bunzow and Grandy (1993) and Gingrich and Caron (1993).

Ethanol effects on dopaminergic function. The connection between dopamine and behavioral responses to EtOH has been partly established by evidence that EtOH can alter dopamine neuron function. Although acute EtOH treatment did not cause changes in dopamine levels measured in

mouse brain homogenates (Alari, Lewander and Sjoquist, 1987; Carlsson et al., 1973), Khatib, Murphy and McBride (1988) reported an EtOH-induced increase in dopamine specifically in the posterior striatum (a terminal field of the nigrostriatal pathway). It may be that analysis of whole brain homogenates has not provided evidence for dopamine release in response to EtOH due to regional specificity of EtOH responses. Analysis of whole brain may have "washed out" and masked physiologically relevant changes that occurred in specific areas.

The currently popular method of microdialysis has provided *in vivo* evidence of EtOH-induced dopamine release in specific brain regions of intact animals. Imperato and DiChiara (1986) measured the effect of EtOH administration on metabolite concentrations and dopamine release in freely moving rats by transcerebral dialysis. Ethanol doses which elicited behavioral stimulation in those animals (increases in incidence of rearing, grooming, and ambulation), also stimulated dopamine release and increased the concentrations of the dopamine metabolites, 3,4-dihydroxyphenyl-acetic acid (DOPAC) and homovanillic acid (HVA), in the N Acc. At higher doses (1.0 - 2.5 g/kg), dopamine, DOPAC, and HVA concentrations were increased in the caudate nucleus, as well as the N Acc. These doses produced sedation as well as activation, in accordance with the aforementioned biphasic time course. Interestingly, 5.0 g/kg, the highest dose of EtOH tested, produced a biphasic effect on dopamine release. Dopamine levels decreased during EtOH-induced hypnosis and increased during recovery to a sedated state. These results led the authors to suggest that the inhibitory effects of high doses of EtOH on dopamine release contribute to its sedative-hypnotic effects. In

another study, Yoshimoto et al. (1992) found that either i.p. injection or direct application of EtOH increased extracellular dopamine and DOPAC concentrations within the N Acc. Using *in vivo* voltammetric methods, Diaz and Murgas (1992) showed increases in dopamine release in the corpus striatum of rats for 60 minutes after intragastric administration, which peaked at 30 minutes after administration. Although there is evidence for EtOH interactions with the nigrostriatal system, some of these studies suggest that there are preferential actions on the mesolimbic pathway.

Much of the data providing evidence for EtOH effects on dopamine function come from studies of dopamine synthesis, or dopamine metabolism/turnover. For example, Carlsson and Lindqvist (1973) measured the effect of EtOH on hydroxylation of tyrosine and tryptophan (catecholamine precursors) in rats *in vivo*, and found increased synthesis. However, using the same method as Carlsson and Lindqvist (1973), neither Waldeck (1974) or Bustos and Roth (1976) found EtOH-induced increases in dopamine synthesis in mice or rats, respectively. More consistent findings with regard to EtOH effects on dopamine neuron activity come from studies on dopamine metabolism and turnover. Alari et al. (1987) found that EtOH (2.0 and 4.0 g/kg) caused a dose-dependent increase in DOPAC and HVA, in mouse and rat brain homogenates of telencephalon plus diencephalon. Increases in striatal DOPAC concentrations were found in rats previously given 3.0 g/kg EtOH (Lucchi et al., 1983), as well as in rats given 1, 2 or 4 g/kg EtOH (Bustos and Roth, 1976). Engel et al. (1988) determined that 2.5 g/kg EtOH given to mice caused increases in DOPAC and HVA levels in homogenates of

dopamine-rich limbic regions and striatum, and concomitant increases in locomotor activity. Consistent with these data, Khatib et al. (1988) found increased DOPAC in N Acc of rats as early as 15 minutes after injection of 2.5 g/kg EtOH in rats. Milio and Hadfield (1992) also found evidence for region-specific alteration of dopamine metabolism and turnover. It should be noted that at least one study has found no change in dopamine turnover in rat striatum (Bacopoulos, Bhatnagar and Van Orden III, 1978). Nonetheless the majority of evidence indicates that dopamine synthesis, metabolism, and/or turnover are altered following acute administration of EtOH. The mechanisms by which EtOH produces these changes, and the particular characteristics which confer regional specificity remain to be elucidated.

Electrophysiological data are consistent with the notion that EtOH may increase dopamine neuron activity. For example, low doses of EtOH, administered intravenously to rats, increased the firing rate of both A9 (substantia nigra) and A10 dopamine neurons; however A10 neurons showed a 5-fold greater sensitivity to EtOH compared to A9 neurons (Gessa et al., 1985), again suggesting preferential stimulation of the mesolimbic system. Additional electrophysiological evidence was provided by Brodie, Shefner and Dunwiddie (1990), who demonstrated increased firing of VTA dopamine neurons in vitro. Verbanck et al. (1990) examined the effects of i.v. EtOH (low to high doses) on firing of single cells in VTA and found increased firing *in vivo* and *in vitro*. The increase occurred rapidly upon the infusion of EtOH, and firing rates began to decrease shortly after termination of the infusion, so that they dropped below baseline levels by approximately 10 minutes post-termination.

These EtOH-induced changes in synthesis, metabolism/turnover, or activity were found within the presynaptic neuron that produces and releases dopamine.

In addition to its presynaptic effects, EtOH may have effects on the second messenger systems to which postsynaptic dopamine receptors are coupled. As mentioned previously, adenylate cyclase is coupled to dopamine receptors via G-proteins. Rabin and Molinoff (1981) demonstrated that EtOH caused dose-dependent linear increases in basal and dopamine-stimulated activity of adenylate cyclase in mouse striatal tissue. These effects were significant beginning at 68 mM through 340 mM. The lower concentrations used in this study were physiologically relevant since many signs of intoxication in humans and animals occur at 15 to 150 mM. However, it should be noted that the mouse inbred strain from which tissue was taken (B6) is one that is generally regarded to be relatively insensitive to the locomotor activating effects of EtOH. Therefore, the significance of these changes with regard to EtOH-stimulated activity are unclear. The relevance of neurochemical changes to the behavior of interest should be interpreted with care. Although some studies used physiologically relevant concentrations, or administered doses which produced locomotor stimulation, it was often the case that measurement of dopamine and its metabolites (especially in brain tissue preparations) occurred at some time after peak locomotor stimulation would have been or was observed. Thus, changes seen in these brains after EtOH administration did not necessarily reflect changes that occurred during the time that locomotor stimulation was observed. Methods that circumvent this problem to some extent are those

which measure neurochemical events during the time that the behaviors are taking place, such as *in vivo* microdialysis or voltammetry (see Diaz and Murgas, 1992; Imperato and DiChiara, 1986; Yoshimoto et al., 1992).

Dopaminergic basis of EtOH-stimulated activity. It is clear that dopamine systems underlie a great number of spontaneous locomotor responses, but because the evidence comprises an extremely large literature base, it will not be reviewed here (but see Phillips and Shen, in press). With regard to expression of EtOH-stimulated locomotor activity, some of the earliest direct evidence that catecholaminergic function was necessary came from studies which showed that administration of α-methyl-*p*-tyrosine (AMPT), an inhibitor of tyrosine hydroxylase, blocked EtOH-induced stimulation in mice (Carlsson, Engel and Svensson, 1972; Friedman et al., 1980), and that this effect could be partially reversed by subsequent administration of L-DOPA (Engel et al., 1974). Interestingly, human subjects given AMPT prior to EtOH self-administration reported a shorter duration of EtOH-induced euphoria, and in two of ten cases, a reduced initial euphoria compared to placebo (Ahlenius et al., 1973). In addition, the subjects were rated by themselves and other observers to be less stimulated and more fatigued compared to placebo. Although these experiments using humans and animals did not address whether the effects of AMPT were due to depletion of dopamine, depletion of norepinephrine, or both, they provided evidence for catecholamine modulation of EtOH-induced stimulation in animals, and EtOH-induced stimulation and euphoria in humans. Further, these studies provided some of the impetus for further exploration of the role of catecholamines in general, and the role of

dopamine in particular, in modulating the locomotor stimulant effects of EtOH.

Evidence for dopaminergic mediation of locomotor stimulation induced by low doses of EtOH consists largely of pharmacological studies in which dopamine receptor ligands with varying receptor subtype specificities were administered systemically, and assessed for their effects on EtOH stimulation. For example, Liljequist, Berggren and Engel (1981) found that administration of haloperidol, a dopamine antagonist which binds both D_1 and D_2 receptors, reduced EtOH-stimulated activity of NMRI outbred mice at doses that had no effect on baseline locomotor activity. Higher doses of haloperidol produced further decreases in EtOH-stimulation which were equal in magnitude to their effects on baseline locomotor activity. These data suggested that there can be EtOH-specific actions of a dopamine antagonist at doses that do not affect general motoric behavior. The selective effect of haloperidol on EtOH-stimulated activity has been confirmed in the FAST selected lines of mice. Haloperidol was shown to decrease the locomotor activation produced by EtOH in FAST mice, at doses that had no effect on baseline locomotor activity (Shen, Crabbe and Phillips, 1995). In contrast, the locomotor activity of SLOW mice was not stimulated by EtOH and was unaffected by administration of haloperidol, in the presence or absence of EtOH. In a conditioned place preference paradigm, EtOH's locomotor stimulant effects in the sensitive D2 strain were also decreased by administration of haloperidol (Risinger et al., 1992).

Results from these haloperidol studies strongly suggest that dopamine plays a role in EtOH-stimulated locomotor activity; however,

information about possible differential roles of dopamine receptor subtypes could not be assessed with this "mixed" antagonist. Determination of relative roles of receptor subtypes has been limited to evaluation of D_1 and D_2 receptor subtypes, due to limited availability of ligands specific for D_3, D_4, or D_5 receptors. Several antagonist studies provide strong evidence for the involvement of D_2 dopamine receptors. The D_2 antagonist, pimozide, decreased EtOH-stimulated activity of NMRI (Liljequist et al., 1981) and Swiss-Webster mice (Koechling, Smith and Amit, 1990), at doses that did not affect baseline locomotor activity. In addition, administration of the highly potent and selective D_2 antagonist, raclopride, completely blocked EtOH-stimulated activity of both replicates of FAST mice (Shen, Crabbe and Phillips, 1995), again providing strong evidence for D_2 modulation of this behavior. In contrast, evidence for the involvement of D_1 receptors is less clear. The D_1 antagonist, SCH-23390, dose-dependently decreased or blocked EtOH-stimulation in only one replicate line of FAST mice, providing moderate evidence for the involvement of D_1 receptors in mediating locomotor stimulation of EtOH in these lines (Shen, Crabbe and Phillips, 1995). The studies reported by Shen, Crabbe and Phillips (1995) included an habituation session in the testing apparatus 24 hours prior to drug administration. Koechling et al. (1990) examined the effects of SCH-23390 on EtOH-stimulated activity of nonhabituated Swiss-Webster mice, and found no effect of this antagonist except at doses which depressed baseline locomotor activity. However, in a follow-up study, SCH-23390 was found to reduce EtOH-stimulation without effects on general motoric behavior when the mice had been exposed to the testing

apparatus for four consecutive days prior to administration of the antagonist (Koechling and Amit, 1993). The authors suggested that the involvement of D1 receptors in EtOH-stimulated activity is modulated by stressors, such as a novel testing environment. A recent literature search suggested that no other D_1 specific ligands have been tested for their ability to affect EtOH-stimulated locomotor activity. Testing of more potent and specific D_1 antagonists currently available may shed more light on the role of D_1 receptors in EtOH's stimulant effects. With regard to general motoric behavior, Longoni, Spina and DiChiara (1987) and White et al. (1988) suggested that D_1 receptors are necessary for function, but play a permissive rather than an active role in full expression of locomotor behavior. Others have also found that stimulation of both D_1 and D_2 receptors is necessary for locomotor behavior (Braun and Chase, 1986; Jackson and Hashizume, 1987; Pichler and Pifl, 1989; Rubinstein, Gershanik and Stefano, 1988; Starr and Starr, 1987; Walters et al., 1987). Evidence that D_1 and D_2 receptors may interact in the mediation of EtOH-stimulated activity is provided by the result that coadministration of SCH-23390 and raclopride produced a greater reduction of EtOH-stimulated activity of FAST mice than either drug alone (Shen, Crabbe and Phillips, 1995).

The effects of dopamine agonists on locomotor activity of EtOH-treated animals have been assessed, as well. Apomorphine suppressed EtOH-stimulated activity of NMRI mice (Carlsson et al., 1974; Strömbom, Svensson and Carlsson, 1977) and of Long Sleep (LS) and Short Sleep (SS) selected mouse lines (Dudek et al., 1984). LS and SS mice were bred for differential sensitivities to the sedative effects of EtOH, as measured

by duration of loss of righting reflex (McClearn and Kakihana, 1981), but differential locomotor stimulation by EtOH was also demonstrated in these lines (Dudek et al., 1984). In studies that assessed the effects of various doses of apomorphine on EtOH-stimulated activity (Dudek et al., 1984; Strömbom et al., 1977), dose-dependent biphasic effects of apomorphine were observed. Suppression of EtOH-stimulated activity by apomorphine was greater at lower doses (0.6 - 1.2 mg/kg) compared to the suppression by higher doses (2.0 - 4.8 mg/kg).

Systematic studies of the effects of D_1 and D_2 specific agonists have not been reported. However, the effects of SKF-38393 (a D_1 receptor agonist) and quinpirole (a D_2 receptor agonist) have been assessed in saline and EtOH-treated FAST mice (Phillips and Shen, in press). Locomotor stimulation by EtOH in FAST-1 mice was not as robust as is typically observed in this line, and coadministration of SKF-38393 and quinpirole significantly enhanced locomotor stimulation to a greater degree than either drug alone. These data suggest that the proposed interdependent roles of D_1 and D_2 receptors in mediating baseline locomotor activity, extend to modulation of EtOH-stimulated activity. However, agonist coadministration in FAST-2 mice, who were robustly stimulated by EtOH, did not alter baseline or EtOH-stimulated activity. Thus, only moderate evidence for D_1/D_2 receptor interactions in mediating EtOH stimulation was found. The results in FAST-2 mice may have been due to a "ceiling" effect, which prevented further enhancement of locomotor activity by administration of dopamine agonists.

γ-aminobutyric Acid (GABA)

Another important component of the neural circuitry mediating locomotor and psychomotor activity is the inhibitory neurotransmitter, GABA, which has been a major focus of EtOH research. In particular, the $GABA_A$ receptor complex, consisting of multiple binding sites that modulate an integral Cl^- channel, has been implicated in the sedative, hypnotic, or anxiolytic effects of EtOH. A second GABA receptor, $GABA_B$, has recently become of interest with regard to EtOH-stimulated activity. $GABA_B$ receptors are similar to $GABA_A$ receptors in that they have inhibitory actions in the CNS, but are distinct from $GABA_A$ receptors in that they are insensitive to bicuculline, barbiturates, and benzodiazepines (Bowery, Pratt and Knott, 1990). Rather than being coupled to an integral Cl^- channel, the $GABA_B$ receptor is G_i/G_o-protein coupled, resulting in inhibition of adenylate cyclase. Ca^{2+} and K^+ conductances are also altered as a result of $GABA_B$ receptor activation, possibly through adenylate cyclase-independent mechanisms. These biochemical changes ultimately translate into inhibition of neuronal activity, either through $GABA_B$-receptor-mediated slow inhibitory postsynaptic potentials, or decreases in evoked neurotransmitter release. The release of catecholamines, including dopamine, has been shown to be inhibited by activation of $GABA_B$ receptors (Engberg, Kling-Petersen and Nissbrandt, 1993; Goudreau et al., 1994; Mott and Lewis, 1994; Santiago, Machado and Cano, 1993). Both pre- and postsynaptic localization have been demonstrated for $GABA_B$ receptors in several brain regions, including striatum, caudate-putamen, and hippocampus. Based on neurochemical and electrophysiological data, the

existence of $GABA_B$ receptor subtypes seems likely. While no clear data exist, the possibility that different subtypes are located pre- vs postsynaptically has been discussed (Bowery et al., 1990).

$GABA_B$ receptors and locomotor activity. $GABA_B$ receptors have been postulated to have a role in mediating many behaviors and physiological responses, including analgesia, learning and memory, muscle relaxation, pituitary hormone responses, thermoregulation, and possibly reproductive behavior (Bowery et al., 1990; Paredes and Ågmo, 1992). A possible role of $GABA_B$ receptors in mediating locomotor activity has also been investigated in rats. 3-aminopropanesulfonic acid (APSA) and 4,5,6,7-tetrahydroisoxazolo-[5,4c]-pyridin-3-ol (THIP) are strong agonists for the $GABA_A$ receptor, but weak or completely inactive at the $GABA_B$ site. On the other hand, baclofen is a strong agonist at the $GABA_B$ site, but does not activate the $GABA_A$ receptor (Bowery, 1993). Of these three GABA agonists, baclofen was found to be the most potent and effective in decreasing the locomotor activity of rats (Ågmo and Giordano, 1985). This result was the same whether the drugs were injected systemically or into the cerebral ventricles, suggesting that the effects of baclofen were centrally mediated. Administration of γ-acetylenic GABA (GAG; a specific GABA-transaminase inhibitor), which should increase the amount of endogenous GABA available to receptors, caused a decrease in locomotor activity, as did high doses of the $GABA_A$-selective agonists. These effects were not reversed by administration of the $GABA_A$-selective antagonist, bicuculline, suggesting that the effects of THIP, APSA, and GAG were not mediated by $GABA_A$ receptors. Taken together, this set of experiments suggested that $GABA_B$, rather than $GABA_A$, receptors were

responsible for locomotor reductions produced by GABA or GABA agonists. Demonstration that a $GABA_B$ antagonist could reduce the effects of THIP, APSA, and GAG would have provided strong support for this conclusion; however, a potent and selective $GABA_B$ antagonist was not available at that time. The effects of baclofen on locomotor activity in rats were replicated in another study, which further demonstrated that the stereospecificity of baclofen's effects (Paredes and Ågmo, 1989). These data were consistent with receptor binding studies which showed that (-)-baclofen is the active enantiomer. Locomotor activity of mice has also been demonstrated to be sensitive to the effects of baclofen. Doses of baclofen ranging from 2.5 to 20 mg/kg significantly reduced locomotor activity immediately after injection (Mead and Little, 1995). Effects of higher doses (10 and 20 mg/kg) were observed as long as 3 hours after injection of the drug. A somewhat contradictory result is that the selective $GABA_B$ antagonist, CGP-35348, also produced a decrease in locomotor activity, albeit at a high dose. It is unclear why both the antagonist and the agonist had similar results. In other studies high doses of CGP-35348 have been shown to be ineffective in altering spontaneous behavior (Bowery et al., 1990).

At least one specific brain region, the N Acc, has been implicated as a site of baclofen's effects on locomotor activity. Plaznik, Stefanski and Kostowski (1990) demonstrated that microinjection of baclofen decreased locomotor activity in rats in a concentration-dependent manner, whereas picrotoxin (a blocker of the $GABA_A$ chloride channel) increased locomotor activity. The locomotor decrease produced by baclofen was not observed after chronic desipramine treatment, which

had been shown in a separate study to enhance mesolimbic dopamine function. Therefore, the blockade of baclofen's effects was postulated to be due to changes in dopamine function, which implies an interaction of dopaminergic and GABAergic systems within the N Acc in mediating locomotor activity. Evidence suggests that dopamine/GABA interactions may also occur within the VTA. Baclofen microinjected into VTA has been demonstrated to decrease locomotor stimulation induced by cocaine and amphetamine administration (Kalivas, Duffy and Eberhardt, 1990), and by novelty (Hooks and Kalivas, 1995). These effects may be due to inhibition of mesoaccumbens dopamine neurons by baclofen (Klitenick, DeWitte and Kalivas, 1992).

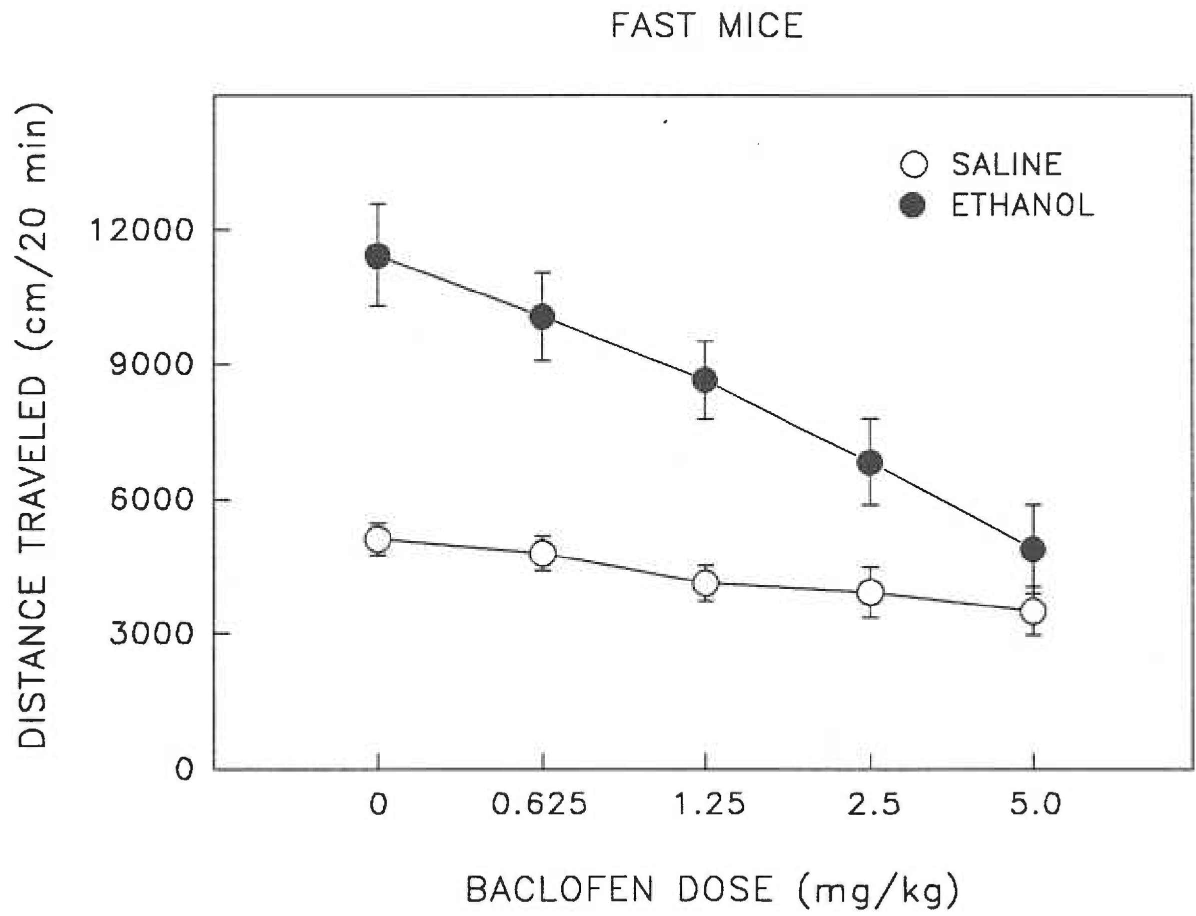
$GABA_B$ receptors and EtOH. In contrast to the amount of data on interactions between dopamine neurons or dopamine receptors and EtOH, there are few studies which have investigated the pharmacological interactions between EtOH and $GABA_B$ receptors, partly due to the relative lack of $GABA_B$-selective ligands. Using microsacs derived from mouse cerebral cortex, Allan, Burnett and Harris (1991) showed that EtOH-enhancement of $GABA_A$-receptor mediated ^{36}Cl-flux was further potentiated in the presence of the $GABA_B$ agonist, baclofen, and blocked by addition of the $GABA_B$ antagonists, phaclofen or 2-OH-saclofen. These effects were suggested to be due to $GABA_B$-mediated second messenger signals affecting $GABA_A$ function. In contrast, Mehta and Ticku (1990) reported no effects of baclofen or phaclofen on EtOH potentiation of $GABA_A$-receptor mediated ^{36}Cl-flux in cultured spinal cord neurons of mice. The differing results of these two studies may be due to differences in the assays used, or may be related to the use of

peripheral vs central tissue. $GABA_B$ activation in the periphery generally leads to reduced Ca^{2+} influx, whereas the predominant response in the CNS is increased outward K^+ flux (Bormann, 1988).

$GABA_B$ receptors and EtOH-stimulated locomotor activity. One of the earliest studies demonstrating that $GABA_B$ receptors may mediate EtOH-induced behaviors used baclofen to inhibit EtOH-stimulated activity (Cott et al., 1976). Interestingly, this study was published prior to the discovery that baclofen acts as a selective $GABA_B$ agonist. It had previously been shown that dopamine function was necessary for expression of EtOH-stimulated activity. Based on evidence that GABA transmission inhibited dopamine function, the authors intended to investigate the ability of GABA systems to alter EtOH-stimulated activity as well. Baclofen decreased the stimulant effects of EtOH in NMRI mice, at doses which had no effect on saline activity. The authors postulated that the effect was due to GABA system modulation of dopaminergic activity. Another important study using inbred ICR mice demonstrated that administration of the $GABA_B$ antagonist, phaclofen, decreased EtOH-stimulated activity and EtOH-induced ataxia, blocked the hypothermic effects of EtOH, and increased the ED_{50} for EtOH-induced loss of righting reflex (Allan and Harris, 1989). Phaclofen had no effects on these behaviors when given alone, and the authors suggested that phaclofen might be regarded as an EtOH antagonist. The results of Cott et al. (1976) and Allan and Harris (1989) appear to be contradictory since both an agonist and an antagonist decreased EtOH-stimulated activity. These results were replicated by Humeniuk, White and Ong (1993), who demonstrated that several $GABA_B$ antagonists

(phaclofen, 2-OH-saclofen, and β-phenyl-β-alanine [BPBA]) decreased the stimulant effects of EtOH in BALB/c mice, as did the agonist, baclofen. However, CGP-35348, which is the most effective $GABA_B$ antagonist developed thus far (Holopainen, Rau and Wojcik, 1992; Ölpe et al., 1990), did not affect EtOH-stimulated activity but reversed the inhibitory effects of baclofen on EtOH stimulation, suggesting that the effects of baclofen were $GABA_B$-specific. The authors suggested that, since baclofen is the most well-characterized $GABA_B$ ligand and has been demonstrated to be an agonist both *in vitro* and *in vivo*, baclofen should be the standard by which to compare other ligands. Thus, the results that phaclofen, 2-OH-saclofen, and BPBA all had effects similar to baclofen suggest that they have partial or full agonist properties *in vivo*, despite their antagonist properties *in vitro*. Another explanation offered by Humeniuk et al. (1993) is the possible existence of multiple $GABA_B$ receptor subtypes with varying affinities for $GABA_B$ ligands, which could account for the differences between *in vitro* and *in vivo* characteristics. Recent experiments in FAST mice have provided further support for $GABA_B$-modulation of EtOH-stimulated activity (Shen, Harland and Phillips, 1995). Baclofen administered 15 minutes prior to EtOH (2.0 g/kg) dose-dependently decreased the stimulant effects of EtOH in both replicates of FAST mice, at doses that had no effect on saline activity (1.25 - 5.0 mg/kg). Because these results formed the basis of much of this dissertation, data are shown in Figure 4. The replicates did not differ in response to baclofen and data are presented with replicates combined.

Figure 4. Effects of the $GABA_B$ agonist, baclofen, on locomotor activity of saline- and EtOH-treated FAST mice. Data are presented collapsed on replicate. Mice were injected with saline or baclofen 15 minutes prior to injection of saline or EtOH (2.0 g/kg) and immediate placement into activity monitors for a 20-minute test. Baclofen doses of 2.5 and 5.0 mg/kg significantly decreased EtOH-stimulated activity of FAST mice ($p < 0.01$); there were no effects of baclofen on activity of saline-injected animals. There was no difference between saline- and EtOH-treated mice given 5.0 mg/kg baclofen, suggesting that baclofen completely blocked EtOH-stimulation. Vertical bars are S.E.M.; n = 10 per line, replicate, and treatment group. (These data were presented in poster form at the annual meeting of the Research Society on Alcoholism in Steamboat Springs, Colorado, June 1995. See abstract: Shen, Harland and Phillips, 1995).


FAST MICE
SALINE
ETHANOL
DISTANCE TRAVELED (cm/20 min)
12000
9000
6000
3000
0
0
0.625
1.25
2.5
5.0
BACLOFEN DOSE (mg/kg)

Glutamate

Glutamate is one of the most abundant amino acids in the nervous system, and is the major excitatory neurotransmitter in the CNS. Glutamate transmission is thought to be involved in a number of phenomena, including long-term potentiation (a molecular model of learning and memory), long-term depression, developmental plasticity, epileptogenesis, and acute and chronic neuropathology. The actions of glutamate are mediated through several receptors, generally classified as ionotropic or metabotropic. Ionotropic receptors are coupled to an integral cation-specific channel, whereas metabotropic receptors are coupled to G-protein mediated second messenger systems. Although there has been some confusion in the literature, at least two ionotropic receptor subtypes exist (Nakanishi, 1992). These are named for their unique agonist ligands: N-methyl-D-aspartate (NMDA), and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). A third ionotropic receptor, the kainic acid receptor (KA) is acknowledged by some investigators (Hollman and Heinemann, 1994).

The ionotropic receptors are currently modeled as a pentameric structure forming a central channel, with each of the 5 subunits containing at least 4 transmembrane domains. In addition to differences in specificity for agonists, NMDA and non-NMDA (KA and AMPA) receptors mediate different components of glutamate stimulated currents across the neuronal membrane. Non-NMDA receptors mediate fast excitatory responses whereas NMDA receptors mediate a slow excitatory component. This is likely due to the unique physiology of NMDA receptors. At resting potentials, NMDA receptor channels are blocked by physiological

concentrations of Mg^{2+}. This blockade is voltage-dependent, and disappears upon cell depolarization. Activation of the NMDA receptor is also dependent on the presence of glutamate and its coagonist, glycine. When activated, NMDA receptor channels allow influx of Ca^{2+}, Na^{+}, and K^{+}.

Several subunits have been cloned for each ionotropic receptor (Hollman and Heinemann, 1994), and likely allow for the existence of receptor subtypes. Molecular cloning experiments have led to the discovery of two NMDA receptor subunits in humans and rats (NMDAR1 and NMDAR2), and in mice (ζ and ϵ, respectively). A total of 8 splice variants have been found for NMDAR1, and a subfamily of NMDAR2 genes includes 4 known subtypes, A - D. Expression of NMDA receptor subunits in *Xenopus* oocytes or transfected cell lines have revealed that homomeric receptors containing only NMDAR1 subunits are fully functional and possess most characteristics of endogenous receptors. On the other hand, NMDAR2 subunits do not form functional ion channels, unless they are coexpressed with NMDAR1 subunits. NMDAR1/NMDAR2 heteromers possess various characteristics depending on the particular NMDAR2 subunit expressed, but are all highly permeable to Ca^{2+}, and are blocked by Zn^{2+} and Mg^{2+} in a voltage-dependent manner. For further detail, see Hollmann and Heinemann (1994).

NMDA receptors and locomotor activity. Data have suggested that glutamatergic systems may be important in mediating locomotor activity in rodents, particularly because glutamate seems to be a major transmitter for basal ganglia related brain regions (Schmidt, Bubser and Hauber, 1992). Although some behavioral effects of non-NMDA glutamate receptor antagonists have been reported (Lalonde and Cote, 1993), the

majority of data has been collected using NMDA receptor ligands, mostly because selective non-NMDA drugs have not been readily available until fairly recently. Danysz et al. (1994) demonstrated that the noncompetitive NMDA antagonists, MK-801 (dizolcipine), phencyclidine (PCP), memantine, and ketamine produced locomotor stimulant effects in rats. However, competitive NMDA antagonists had little effect on baseline locomotor activity. Similar effects of noncompetitive NMDA antagonists have been demonstrated in mice (Diana and Sagratella, 1994; Liljequist et al., 1991). PCP, MK-801, and dextromethorphan all produced increases in locomotor activity. At higher doses, PCP and MK-801 decreased locomotor activity, which may have been due to increased ataxia and stereotypy. Stereotypic behaviors were observed at doses ≥ 0.5 mg/kg, and included rotational behavior, head weaving, body rolling, and head dipping (Liljequist et al., 1991). In addition, genetic factors may determine, at least in part, sensitivity to the locomotor effects of competitive and noncompetitive NMDA receptor antagonists. Two inbred strains (CBA and B6) and an outbred strain (NMRI) were found to differ in locomotor responses to several NMDA antagonists, including MK-801, PCP, ketamine, CGS-19755, CGP-39551, and NPC 12626 (Liljequist, 1991b).

Microinjection studies have shown that injection of glutamate receptor agonists directly into N Acc stimulated locomotor activity in rats. However, NMDA was the least potent agonist (Donzanti and Uretsky, 1983), or was not effective (Kalivas, Duffy and Barrrow, 1989), compared to kainate and quisqualate. Since the N Acc is heavily innervated by dopamine neurons, it is possible that dopaminergic and glutamatergic

systems interact within this brain region. Kalivas et al. (1989) also showed that A10 dopamine neurons may be regulated by glutamate, since glutamate injections into the VTA, which increased locomotor activity, enhanced dopamine release in the N Acc, as measured by *in vivo* microdialysis. However, it has also been shown that i.v. injection of noncompetitive NMDA antagonists increased the firing rate of A10 neurons and increased locomotor activity (French et al., 1991). The similar effects of NMDA receptor agonists and antagonists on dopamine neurons and locomotor activity are perplexing, and further investigations are necessary to resolve these contradictory results.

EtOH and NMDA receptor function. Several lines of research have provided evidence that EtOH is a potent inhibitor of glutamate-mediated events at pharmacologically relevant concentrations. Original observations of EtOH-inhibition of NMDA receptors were made in cultured embryonic mouse hippocampal neurons and in dorsal root ganglia of adult rats using electrophysiological methods (Lovinger, White and Weight, 1989). EtOH was effective in the 5 to 100 mM concentration range, with 30 mM producing 50% inhibition of NMDA-stimulated currents. The inhibitory effects of EtOH on NMDA-activated currents were not inhibited by administration of bicuculline, suggesting that the NMDA effect was not secondary to an effect of EtOH on $GABA_A$ receptors. EtOH also inhibited kainic acid and quisqualate (AMPA receptor agonist)-stimulated currents, but the magnitude and potency of EtOH's inhibitory effects on KA and AMPA-receptor mediated currents, were considerably lower compared to that of NMDA receptors. Hence, the majority of work on EtOH and glutamate receptor interactions has been focused on NMDA receptors. In

additional electrophysiological studies, it was further demonstrated that EtOH inhibited NMDA-receptor mediated currents stimulated by endogenous glutamate release in adult rat hippocampal slices (Lovinger, White and Weight, 1990). Examination of NMDA-activated single channel currents also revealed concentration-dependent effects of EtOH; however, a biphasic response was observed in which low EtOH concentrations (1.74 - 8.65 mM) potentiated, and higher EtOH concentrations (86.5 - 174 mM) blocked NMDA-activated currents (Lima-Landman and Albuquerque, 1989). These effects were due to alterations in mean channel open time (increased at low and decreased at high EtOH concentrations), and in the probability of channel opening (decreased at high concentrations).

Recent work suggests that the effects of EtOH on glutamatergic neurotransmission may involve both NMDA and non-NMDA receptors, and may vary between brain regions. Nie et al. (1993) found that EtOH reduced excitatory glutamatergic transmission evoked by stimulation of the peri-tubercle region in slices of rat N Acc, but found evidence that the effect may be mediated through non-NMDA receptor mediated mechanisms since an AMPA/kainate receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), but not a competitive NMDA antagonist, 2-amino-5-phosphonopentanoic acid (AP-5), abolished the glutamatergic excitatory potentials. Consistent with the *in vitro* work of Nie et al. (1993), Criado et al. (1995) found that systemically administered EtOH reduced the firing rate of spontaneous and glutamate-activated core N Acc neurons in both anesthetized and awake freely-moving rats. However, receptor specificity of EtOH's effects was not investigated. Other *in vivo* electrophysiological work in anesthetized rats demonstrated that

EtOH inhibited NMDA-evoked activity of medial septal neurons following systemic administration of EtOH (Simson et al., 1991). Locally applied EtOH in anesthetized rats also produced this effect in hippocampus and inferior colliculus, but not in the lateral septum (Simson, Criswell and Breese, 1993). Thus, it appears that EtOH's effects on glutamate transmission may be region-specific.

In addition to electrophysiological data, biochemical assays have supported NMDA antagonist-like effects of EtOH. Both NMDA-stimulated $^{45}Ca^{2+}$ uptake and accumulation of cGMP by rat fetal cerebellar granule cells were inhibited by EtOH (10 - 100 mM for Ca^{2+} flux, 25 - 100 mM for cGMP accumulation) in a dose-dependent manner (Hoffman et al., 1989). This inhibitory effect of EtOH on cGMP accumulation was shown to be highly selective for glutamate-stimulated responses of NMDA receptors, rather than stimulation of cGMP by atrial natriuretic factor (Hoffman, Moses and Tabakoff, 1989). In membrane vesicles derived from mouse hippocampi, glutamate-stimulated Ca^{2+}-influx, demonstrated to be mediated by NMDA receptors (Daniell, 1991), was significantly enhanced by low concentrations of EtOH (cited in Daniell and Phillips, 1994a) and inhibited by higher concentrations ranging from 25 - 200 mM EtOH (Daniell, 1995; Daniell and Phillips, 1994a). This biphasic response is consistent with that observed by Lima-Landman and Albuquerque (1989), and has led to the suggestion that the stimulant effects of EtOH may be related to its ability to enhance NMDA function, while the sedative effects of EtOH may be related to inhibition of NMDA receptor function.

In addition to postsynaptic effects of EtOH on the NMDA receptor, there is evidence for presynaptic effects, as suggested by Nie, Madamba

and Siggins (1994). Both basal and NMDA-stimulated glutamate release in striatum were blocked by systemically administered EtOH (2 g/kg, i.p.) or MK-801, as measured by microdialysis in rats (Carboni et al., 1993). It was suggested that this effect of EtOH was due to blockade of NMDA receptors on glutamatergic nerve terminals which modulate glutamate release via a feed-forward mechanism.

Sensitivity of NMDA receptors to EtOH's inhibitory effects may be mediated, at least in part, by genetic factors. Long-Sleep (LS) and Short-Sleep (SS) mice were selectively bred for differences in sensitivity to EtOH-induced narcosis (McClearn and Kakihana, 1981). Microsacs derived from hippocampi of LS and SS mice differed in sensitivity to the inhibition of glutamate-stimulated Ca^{2+}-influx by EtOH (Daniell and Phillips, 1994b). Interestingly, differences were also observed in microsacs derived from hippocampus and cerebrocortex of FAST and SLOW selected lines, in which microsacs derived from SLOW mice were more sensitive to the inhibitory effects of EtOH on glutamate-stimulated Ca^{2+}-influx (Daniell and Phillips, 1994a). The molecular basis of these line differences are unknown, but it has been suggested, based on studies of $GABA_A$ receptor/EtOH interactions, that differences in regional or neuronal sensitivity might be conferred by differences in NMDA receptor subunit composition (Criswell et al., 1993). FAST and SLOW mice may express different NMDA receptor subunits, or may express them in different region-specific patterns, leading to differences in subunit composition and, ultimately, NMDA receptor sensitivity. Consistent with this suggestion, it was found that expression of various NMDA receptor subunits in *Xenopus* oocytes resulted in differential

sensitivity to EtOH's inhibition of NMDA-stimulated currents. Four splice variants of the NMDAR1 subunit, based on the presence (L; long) or absence (S; short) of two independently spliced DNA regions were examined by Koltchine et al. (1993). Homomeric assemblies of the NMDAR1-LL splice variant, containing both DNA regions, were more sensitive to the inhibition of NMDA-activated current by EtOH than homomeric assemblies of the other three splice variants (LS, SL, or SS). Interestingly, the receptor least sensitive to EtOH was the homomeric assembly of the NMDAR1-SS splice variant, which contains neither cassette. Another study compared the relative EtOH sensitivity of homomeric NMDAR1 receptors to heteromeric receptors containing $\zeta 1$ (R1) and $\epsilon 1$ (R2A), $\epsilon 2$ (R2B), or $\epsilon 3$ (R2C) subunits (Masood et al., 1994). In this study, NMDAR1 homomeric receptors were not sensitive to EtOH; however, it was unclear which splice variant was tested. On the other hand, heteromeric receptors displayed varying sensitivities to EtOH-induced inhibition of NMDA-stimulated currents depending upon which NMDAR2 subunit was expressed. NMDA-stimulated currents mediated by NMDAR2A-containing receptors were more sensitive to EtOH than NMDAR2B-containing receptors. NMDAR2C-containing receptors were the least sensitive of the three heteromers. Chu, Anantharam and Treistman (1995) extended these studies to include NMDAR1B/NMDAR2D heteromers, and found them to be the least sensitive of all.

Molecular cloning studies have shown that some modulatory sites are specific to individual receptor subunits, which offers a possible explanation for different EtOH sensitivities of the various NMDA receptor heteromers expressed in oocytes, as described above. However,

the specific mechanisms and sites of EtOH action remain unclear. Chu et al. (1995) failed to find evidence that any known modulators of the NMDA receptor interacted with or altered EtOH effects. Addition of increasing concentrations of glycine, Mg^{2+}, 7-chlorkynurenic acid (7-CK, a glycine site antagonist), or Zn^{2+}, had no effect on EtOH inhibition of heteromeric NMDAR1B/NMDAR2 receptors expressed in *Xenopus* ooctyes. EtOH's inhibitory effects were similarly unaffected by alteration of the channel's redox modulatory site, which enhances or attenuates NMDA responses depending on its reduction/oxidation state. These data suggest that EtOH acts by interacting at a novel hydrophobic site on the NMDA receptor, independent of other modulatory sites. This interaction could lead to a conformational change in the receptor protein, resulting in an alteration in function. The existence of a site of EtOH's actions was further supported by the work of Weight, Lovinger and White (1991) and Weight et al. (1992). In their studies, EtOH did not alter ion selectivity of the channel, failed to induce voltage-dependent blockade of the channel, did not alter agonist affinity or compete with any agonists, and did not interact with any of the modulatory sites on the NMDA receptor.

On the other hand, there is some evidence that EtOH may interact with the glycine coagonist site. Glycine reversed the inhibitory effects of EtOH on NMDA-stimulated Ca^{2+}-influx in brain cells obtained from neonatal rats (Dildy-Mayfield and Leslie, 1991), and similarly reversed the inhibitory effects of EtOH on NMDA-stimulated catecholamine release from cortical and striatal rat brain slices (Woodward, Brown and Gonzales, 1991). In addition, EtOH interfered with the ability of

glycine to enhance NMDA stimulation of cGMP production (Hoffman et al., 1989). Reversal of EtOH-induced inhibition of NMDA-stimulated neurotransmitter release by glycine *in vitro* occurred in rat striatal slices, but not cortical or hippocampal slices (Gonzales and Brown, 1995). Thus, conflicting data with regard to EtOH action on the glycine site may be due to brain regional differences in sensitivity of this site, possibly due to molecular heterogeneity of NMDA receptor subunits.

Some studies have concentrated on binding sites within the channel as possible sites of EtOH interaction. Evidence for the phencyclidine (PCP) site within the channel as a site of EtOH action was obtained by Dildy-Mayfield and Leslie (1991), who showed that PCP, like glycine, inhibited the effect of EtOH on NMDA-stimulated Ca^{2+}-influx; however, Hoffman et al. (1989) did not observe alteration of PCP's inhibition of NMDA-stimulated cGMP production by EtOH, and concluded that the PCP site was not involved in mediating EtOH's actions on the NMDA receptor. The Mg^{2+} binding site within the NMDA receptor channel has generally been excluded as a site of EtOH action, since studies have not demonstrated effects of Mg^{2+} on inhibition NMDA receptor function by EtOH (Chu et al., 1995; Weight et al., 1991; Weight et al, 1992). Other studies demonstrated that EtOH was more effective in inhibiting NMDA receptor function in the presence of physiological concentrations of Mg^{2+}, but also concluded that EtOH was not acting via the site of voltage dependent Mg^{2+} channel blockade because the two antagonists appeared to be acting independently of each other (Martin et al., 1991; Morrisett et al., 1991). Michaelis and Michaelis (1994) recently hypothesized an interaction between EtOH and a second high-affinity Mg^{2+} regulatory site

on the NMDA receptor. This site was postulated because, contrary to what would be expected if Mg^{2+} were acting only as a voltage-dependent channel blocker, low concentrations of Mg^{2+} enhanced the binding of [^{3}H]-TCP, a PCP analog which binds only within an open channel. This high-affinity site was postulated to influence binding of other modulators (e.g. glycine) through conformational changes of the NMDA receptor. Interference of glycine binding and glycine-stimulated [^{3}H]-TCP binding by EtOH may be due to direct interference of EtOH with the glycine site, or may reflect an indirect action via conformational changes induced by interaction with the high-affinity Mg^{2+} site. Michaelis and Michaelis (1994) presented no evidence for this hypothesis, but suggested it as an interesting issue to pursue in future research. Although there is currently disagreement with regard to the mechanism and site of EtOH's inhibitory actions on NMDA receptors, there is little disagreement that EtOH does interfere with NMDA receptor function.

NMDA receptor mediation of EtOH-stimulated activity. Glutamate receptors, particularly NMDA receptors, have generally been associated with the effects of chronic EtOH exposure or consumption (Hoffman et al., 1990; Trujillo and Akil, 1995). Recent data suggest that NMDA receptors may also be involved in the acute locomotor activating effects of EtOH. Carlsson and Engberg (1992) reported that, in a paradigm previously used to assess effects of MK-801 and clonidine (α_2-adrenergic agonist) on locomotor behavior in monoamine-depleted mice, EtOH's effects were similar to those of the noncompetitive NMDA antagonist, MK-801. These data also suggested interactions between catecholaminergic

systems and MK-801 or EtOH. Liljequist (1991a) found that both MK-801 and CGP-39551 (a competitive NMDA antagonist) reduced or blocked EtOH-stimulated activity in male NMRI mice. This effect of CGP-39551 was dose-dependent, with the highest dose (10 mg/kg) completely blocking locomotor activation induced by EtOH. Only one dose of MK-801 was tested (0.05 mg/kg), which completely blocked the stimulant effect of EtOH. In rats, EtOH (0.75 g/kg) slightly decreased, and MK-801 (0.1 mg/kg) robustly increased locomotor activity (Robledo, Kaneko and Ehlers, 1991). When MK-801 and EtOH were administered together, activity levels resembled those of baseline control animals; thus, each drug appeared to cancel the effect of the other. Contrary to the data described above, it was recently reported that, while MK-801 produced a robust stimulation of locomotor activity in rats, subsequent administration of a low EtOH dose did not further alter motor behavior (Criswell et al., 1994).

In contrast to its inhibition of EtOH-stimulated activity, MK-801 has also been shown to enhance EtOH's stimulant effects. In one interesting study, ddY mice were administered several doses of MK-801 and EtOH in combination (Kuribara, 1994). Low EtOH doses which had no significant effect on locomotor activity were used. When combined with low to moderate doses of MK-801 (0.03 - 0.3 mg/kg), activity was stimulated to a greater degree than either drug alone. This same effect was observed with combined administration of ketamine (a noncompetitive antagonist, but less potent than MK-801) and EtOH; however, the effect was not as robust. Interestingly, this potentiation of activity was blocked by interference with dopamine function, either using dopamine

receptor antagonists, or depletion of dopamine using reserpine or AMPT, suggesting that the locomotor stimulation produced by coadministration of noncompetitive NMDA antagonists and EtOH may ultimately be mediated by dopamine systems. Enhancement of MK-801-stimulated activity by EtOH was recently demonstrated in rats (Draski, Liu and Deitrich, 1995). High alcohol sensitivity (HAS) and low alcohol sensitivity (LAS) rats were selectively bred for differences in sensitivity to the sedative effects of EtOH, as measured by loss of righting reflex (Draski et al., 1992; Spuhler, Deitrich and Baker, 1990). Draski et al. (1995) found that, while MK-801 (0.3 mg/kg) increased locomotor activity of both HAS and LAS rats and 1 g/kg EtOH decreased activity, administration of both drugs stimulated locomotor activity beyond the level of MK-801 alone in LAS, but not HAS rats. In summary, NMDA receptor antagonists have been demonstrated to both enhance and inhibit EtOH's stimulant actions in separate studies. These contradictory results may be related to differences in dose, and on the levels of locomotor activity. For example, Kuribara (1994) showed locomotor activity of mice was enhanced when subeffective or slightly effective doses of EtOH and NMDA were coadministered, and that locomotor stimulation produced by higher EtOH or MK-801 doses was reduced when these doses were coadministered. Similarly, locomotor activity of monoamine-depleted mice was enhanced by MK-801 administration when dopamine agonist-induced locomotor activity was relatively low, and was decreased by MK-801 administration when locomotor activity was relatively high (Svensson, Carlsson and Carlsson, 1992).

HYPOTHESES AND SPECIFIC AIMS

Overview

Genetic animal models have gained prominence in the field of drug addiction research (Crabbe, Belknap and Buck, 1994). With regard to EtOH-stimulated locomotor activity, the selectively bred FAST and SLOW lines, and B6 and D2 inbred strains, provide powerful tools in the analysis of two major questions: 1). What are the neural substrates important in mediating sensitivity to the stimulant effects of EtOH? and 2). What are the genetic differences between mice that differ in locomotor response to EtOH? Although these two issues do overlap, it appears that they do not overlap completely. In previous studies, we found that dopamine antagonists blocked or decreased the EtOH-stimulated activity of FAST mice, and had no effect on the activity of EtOH-treated SLOW mice. This suggested that dopamine systems are involved in the stimulant response of FAST mice; however, FAST and SLOW mice did not differ in sensitivity to the locomotor depressant effects of dopamine antagonists (Shen, Crabbe and Phillips, 1995), or the stimulant effects of amphetamine (Phillips et al., 1992). Taken together, these data suggest that, while dopamine systems are important for expression of the stimulant effects of EtOH, selective breeding has not altered dopamine systems, at least with regard to dopamine receptor function in the absence of EtOH. Thus, obtaining an answer to the first question (the identity of important neural substrates), does not necessarily provide an answer to the second (the genetic differences).

Since dopamine is only one of several neurotransmitter systems implicated in mediating locomotor and psychomotor activation, the result

that dopamine antagonist sensitivity did not differ between FAST and SLOW mice led to the suggestion that some other system(s) were involved in determining the difference in locomotor response to EtOH in FAST and SLOW mice, and possibly had been differentially altered during selection of the lines. A role for GABAergic mechanisms in EtOH stimulant response was supported by the observation that baclofen, a $GABA_B$ agonist, dose-dependently reversed and blocked EtOH-stimulation in FAST mice (as seen in Figure 4). In addition, differences between FAST and SLOW mice in sensitivity to baclofen's locomotor effects in the absence of EtOH were found in replicate 1, and thus provided moderate evidence that some aspect of $GABA_B$ function has been altered by selection (Shen, Harland and Phillips, 1995). These data suggest that $GABA_B$ receptors are important in modulation of EtOH-stimulated activity, and it was predicted that activation of $GABA_B$ receptors by baclofen would also interfere with expression of EtOH-stimulated activity in D2 mice. It was further hypothesized that insensitivity of SLOW and B6 mice to the locomotor stimulant actions of EtOH is conferred by enhanced $GABA_B$ receptor activity.

Interactions between dopamine and GABA systems are important aspects of the neural circuitry of drug-induced locomotor activation. Two possible sites of interaction include N Acc (Plaznik et al., 1990) and VTA (Kalivas, 1993). It was hypothesized that GABA and dopamine systems interact to modulate expression of EtOH-stimulated activity in FAST, SLOW mice, and B6 and D2 mice. Experiments that tested this hypothesis are described in greater detail below.

Glutamate is the third neurotransmitter thought to be of primary

importance in mediating locomotor activation. The NMDA-type glutamate receptor appears to be a site of EtOH action in the CNS, and NMDA receptor ligands have been shown to alter locomotor activity, in the presence and absence of EtOH. In addition, differences in sensitivity to EtOH-inhibition of glutamate-stimulated Ca^{2+} influx in cortical and hippocampal microsacs derived from FAST and SLOW mice suggest that selection may have differentially altered NMDA receptor function (Daniell and Phillips, 1994a). Thus, it was hypothesized that FAST and SLOW mice would differ in sensitivity to the locomotor effects of the NMDA receptor antagonist, MK-801. Furthermore, it was hypothesized that NMDA receptor function mediates expression of EtOH-stimulated activity. B6 and D2 strains were tested in addition to FAST and SLOW mice.

Specific Aims

The first specific aim was to test the hypothesis that $GABA_B$ function is important in mediating locomotor responses to EtOH in FAST, SLOW, B6 and D2 mice, and that dopamine/GABA interactions are particularly important. There were 5 experiments included in this specific aim. Although partly based on evidence from neuroanatomical studies, these experiments were not specifically designed as neuroanatomical studies, *per se*, but were intended to be "first-pass" investigations of the relationship between dopamine and $GABA_B$ systems.

1). The effects of the $GABA_B$ agonist, baclofen, on locomotor activity of EtOH-treated B6 and D2 mice were examined. Baclofen had already been demonstrated to inhibit EtOH-stimulated activity of FAST mice, and was predicted to

have the same effect on EtOH-stimulated activity of D2 mice. Baclofen was not expected to affect the activity of B6 mice, since EtOH was not predicted to have stimulant effects in this strain.

2). To confirm the specificity of baclofen's effects on EtOH-stimulated activity, a potent and selective $GABA_B$ antagonist, CGP-35348, was administered to baclofen and EtOH-treated FAST and D2 mice. It was predicted that CGP-35348 would dose-dependently reverse the inhibition of EtOH-stimulated activity by baclofen in both genotypes.

3). It was hypothesized that baclofen's ability to inhibit EtOH's activating effects was due to interactions between dopamine and GABA systems. Specifically, it was hypothesized that the effects of baclofen were ultimately due to inhibition of dopamine neurotransmission. For example, binding of baclofen to $GABA_B$ receptors on VTA dopamine cell bodies would inhibit dopaminergic activity, ultimately resulting in decreased locomotor stimulation in response to EtOH. In this experiment, dopamine agonists administered to FAST and D2 mice treated with baclofen and EtOH were predicted to reverse the inhibitory effects of baclofen on EtOH-stimulated activity, by compensating for the lack of dopamine release.

4). The insensitivity of SLOW and B6 mice to the locomotor stimulant effects of EtOH were hypothesized to be due to a greater sensitivity of $GABA_B$ receptors, or greater

effects of $GABA_B$-mediated events. It was reasoned that blockade of $GABA_B$ receptors with an antagonist, CGP-35348, would render $GABA_B$ receptors inactive, resulting in locomotor stimulation in EtOH-treated SLOW and B6 mice.

5). This experiment was intended to assess the possible interaction between GABA and dopamine in SLOW and B6 mice, and was dependent upon the results of Experiment 4. If $GABA_B$ receptor blockade by CGP-35348 allowed EtOH-induced stimulation in SLOW and B6 mice, this effect was postulated to be due to release of tonic inhibition of dopamine neurons. Thus, it was predicted that administration of a dopamine antagonist would block locomotor stimulation in SLOW and B6 mice produced by CGP-35348 and EtOH administration.

Specific Aim 2 was to determine the possible involvement of glutamatergic systems in mediating locomotor responses to EtOH in FAST, SLOW, B6 and D2 mice. Attention was focused on the NMDA receptor because evidence suggests that EtOH most potently affects this glutamate receptor, and because of the differences in NMDA receptor sensitivity seen in tissue derived from FAST and SLOW mice. The effects of the NMDA noncompetitive antagonist, MK-801, on baseline activity and activity after EtOH administration were tested in FAST, SLOW, B6 and D2 mice.

1). Since evidence suggests that NMDA receptor function was differentially altered between FAST and SLOW mice, it was hypothesized that the lines would also differ

in locomotor responses to the noncompetitive antagonist, MK-801. In addition, it was predicted that B6 and D2 mice would differ in locomotor response to MK-801.

2). The ability of MK-801 to alter locomotor responses to EtOH was assessed in FAST, SLOW, B6 and D2 mice. MK-801 has been shown to either enhance or decrease EtOH-stimulated activity in different studies. However, Kuribara (1994) demonstrated that administration of MK-801 to animals given EtOH at sub-stimulating doses produced a large enhancement of locomotor activity beyond that of MK-801 alone. Thus, it was predicted that MK-801 would enhance locomotor stimulation at low doses of EtOH in genotypes sensitive to EtOH's stimulant effects (FAST and D2 mice). Responses of genotypes relatively insensitive to EtOH's stimulant effects were more difficult to predict. On one hand, administration of MK-801 could confer sensitivity to EtOH's stimulant effects in B6 and SLOW mice. On the other hand, since MK-801 also reduced locomotor activation in some studies, administration could increase sensitivity to the locomotor depressant effects of EtOH.

Itemization of the experiments for each specific aim are listed in Table 1. For greater methodological detail, see "Methods" and "Results" sections.

Table 1. Summary of experiments, including experiment number, purpose of experiment, and the genotypes tested in each experiment.

TABLE 1

Experiment #	Purpose	Genotype
Specific Aim 1: Assessment of GABA and dopamine interactions in mediating EtOH-stimulated activity.		
1.1	Assess effects of $GABA_B$ receptor activation on EtOH-stimulated activity of B6 and D2 mice.	B6, D2
1.2	Determine $GABA_B$ specificity of baclofen-induced inhibition of EtOH's stimulant effects.	FAST, D2
1.3	Assess dopamine involvement in baclofen effects on EtOH-stimulated activity.	FAST, D2
1.4	Assess importance of $GABA_B$ receptor in conferring insensitivity to EtOH stimulant effects.	SLOW, B6
1.5**	Assess dopamine involvement in EtOH-stimulated activity produced by $GABA_B$ receptor blockade.	SLOW, B6
Specific Aim 2: Assess importance of NMDA receptors in expression of EtOH- stimulated locomotor activity.		
2.1	Assess genotype differences in locomotor responses to a range of MK-801 doses.	FAST, SLOW, B6, D2
2.2	Assess effects of EtOH and MK-801 coadministration.	FAST, SLOW, B6, D2

**Performance of this experiment was contingent upon positive results in Experiment 1.4, which were not observed. Thus, Experiment 1.5 was not attempted.

MATERIALS AND METHODS

Animals

C57BL/6J and DBA/2J inbred mice, and mice from both replicates of the FAST and SLOW selected lines were tested in these experiments. The origin of C57BL/6J (B6) and DBA/2J (D2) mice, and the derivation of FAST and SLOW mice have been discussed in detail above (see "Genetic Animal Models"). FAST and SLOW mice were bred at the Portland VA Medical Center (Portland, Oregon), and B6 and D2 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). In some cases, all 6 genotypes were tested in an experiment; however, for some experiments, a subset of the genotypes was studied as appropriate for the hypothesis being tested. For example, in experiments to determine the effects of a pharmacological agent on EtOH-stimulated activity, only the responses of stimulated genotypes (FAST-1, FAST-2 and D2 mice) were assessed (see Table 1). Male mice were used in all experiments. Selection generation of FAST and SLOW mice used in these experiments varied depending upon availability and is presented with the individual description of each experiment.

B6 and D2 mice were 6 - 8 weeks of age when shipped from Jackson Laboratory to Portland. Upon arrival, mice were housed 4 - 5 per cage, and habituated to the Portland VA animal care facility for 1 - 2 weeks prior to testing. FAST and SLOW mice were housed with littermates, sire, and dam until 21 ± 1 days of age, and subsequently housed 3 - 4 per cage with animals of the same sex, line, and replicate until testing. All animals were kept on a 12 hour light/dark cycle, with lights on between 6:00 a.m. and 6 p.m. Cages were made of clear

polycarbonate (28 x 18 x 13 cm) and contained corn cob bedding that was changed twice weekly. Cages were never changed on a test day. Food and water were available *ad libitum*, except during activity testing. Ambient temperature was controlled at 21 ± 2°C.

Measurement of Locomotor Activity

Locomotor activity was assessed between 9:00 a.m. and 5:00 p.m. in Omnitech Digiscan Animal Activity Monitors (Model CCDIGI; Columbus, OH). Each apparatus comprised a clear acrylic plastic box (40 cm x 40 cm), in which mice were placed for the duration of the activity test. This plastic box was set inside a 40 cm x 40 cm activity monitor, with 8 photocell beams and receptors equally spaced along its 4 sides, approximately 2 cm from the floor. An additional set of 8 photocell beams and detectors were placed 8 cm above the floor along two parallel sides of the box for detection of vertical movements. The monitor and testing chamber were housed inside a sound-attenuating chamber made of opaque black acrylic plastic, which had a ventilation fan mounted on the rear right wall. Fluorescent lights, which were mounted high on the back wall of the sound-attenuating chamber, were turned off so that locomotor activity was assessed in darkness. Eight locomotor activity chambers were used during an experiment, and genotypes and treatment groups were counterbalanced so that each treatment group of each genotype was tested at least once in each monitor. Duration of the activity tests depended on the drug or drugs being tested and the experimental manipulation, as described in greater detail below.

Data were automatically recorded by an IBM-compatible computer in

5-minute samples during activity testing. Several activity variables were recorded, including measures of horizontal activity, vertical activity, stereotypy, direction of travel (clockwise vs anticlockwise; not rotational behavior), and time spent or distance traveled in specific locations in the monitors. However, distance traveled was used as the measure of locomotor activity for these experiments, in part because of its easily interpretable metric character. While horizontal activity counts also provide a measure of locomotor activity, high activity counts do not necessarily reflect increased forward locomotion, since activity counts also accrue when an animal engages in stereotypic behavior (repeated sniffing or grooming) but does not move forward. In contrast, distance accrues only when 2 or more consecutive photocell beams are broken by an animal's movement. Because it is uncontaminated by stereotypy, distance traveled is a more accurate measure of forward locomotion.

Drug Sources and Preparation

(±)-Baclofen (lot# GL-991B), (±)-SKF-38393 N-allyl hydrochloride (lot# WY-IV-18), quinpirole hydrochloride (lot# LE-289A), and (+)-MK-801 hydrogen maleate (lot#s QGK-1094A and LG-391A) were purchased from Research Biochemicals International (Natick, MA). Apomorphine hydrochloride (lot# 73H0225), (+)-methamphetamine (lot# 93H1056), and sodium metabisulfite (lot 64H1038) were purchased from Sigma Chemical Company (St. Louis, MO). CGP-35348 was a generous gift from Ciba-Geigy Limited (Basel, Switzerland). Ethanol (200 proof) was purchased from Pharmco Products Incorporated (Brookfield, CT). All drugs, with the

exception of apomorphine, were dissolved in 0.9% saline. Apomorphine was prepared in a solution of 0.1% sodium metabisulfite in saline to enhance drug stability. Both apomorphine and SKF-38393 were kept on ice and protected from light during experiments to inhibit degradation. Ethanol was diluted to 20% v/v with saline.

Determination of Blood Ethanol Concentrations

Retroorbital sinus (ROS) blood samples (20 μl) were obtained from all EtOH-treated animals immediately upon removal from the activity testing chamber. Samples were immediately placed on ice in microcentrifuge tubes containing 50 μl cold $ZnSO_4$. After all samples were collected on each test day, 50 μl $Ba(OH)_2$ and 300 μl deionized water were added to each microcentrifuge tube. Samples were mixed by brief agitation and centrifuged for 5 minutes at high speed (Beckman Microfuge 12). Supernatant was transferred to glass vials, capped, and assessed for blood ethanol concentrations (BECs) by gas chromatography (Hewlett-Packard 5890) with flame ionization detection. On some test days, the gas chromatograph was not immediately available for use. In that case, the glass vials containing supernatant were stored at 0°C until samples could be analyzed. Samples were never stored for more than two weeks, and were slowly thawed at room temperature prior to assessment for BEC. These methods were previously described by Phillips, Limm and Crabbe (1989).

Statistical Analyses

Total distance traveled was calculated for each animal as the sum

of distance traveled during the entire activity test. When time course data were needed, distance traveled was not summed but was analyzed as a repeated measure, sample by sample. Analysis of variance (ANOVA) was the statistical test used in most cases (CRUNCH4 statistical package). Grouping factors varied for experiments depending on the experimental design, and are explained in more detail for each experiment. Significant main effects were further analyzed by post-hoc Tukey mean comparisons, when appropriate. Significant 2-way interactions were characterized by simple effects analysis followed by Tukey mean comparisons. Some preliminary pilot studies were not designed as full factorial experiments, and were therefore not appropriate for ANOVA. However, these pilot studies were designed with specific comparisons in mind, and were therefore analyzed by planned t-test. In addition, Experiment 2 was designed as a full factorial experiment, but contained one control group that was not part of the factorial design. In that case, differences from control were analyzed by planned t-tests. Significance levels were $p < 0.05$ for all statistical tests.

EXPERIMENTS

Specific Aim 1

Experiment 1.1: $GABA_B$ Mediation of EtOH-Stimulated Activity

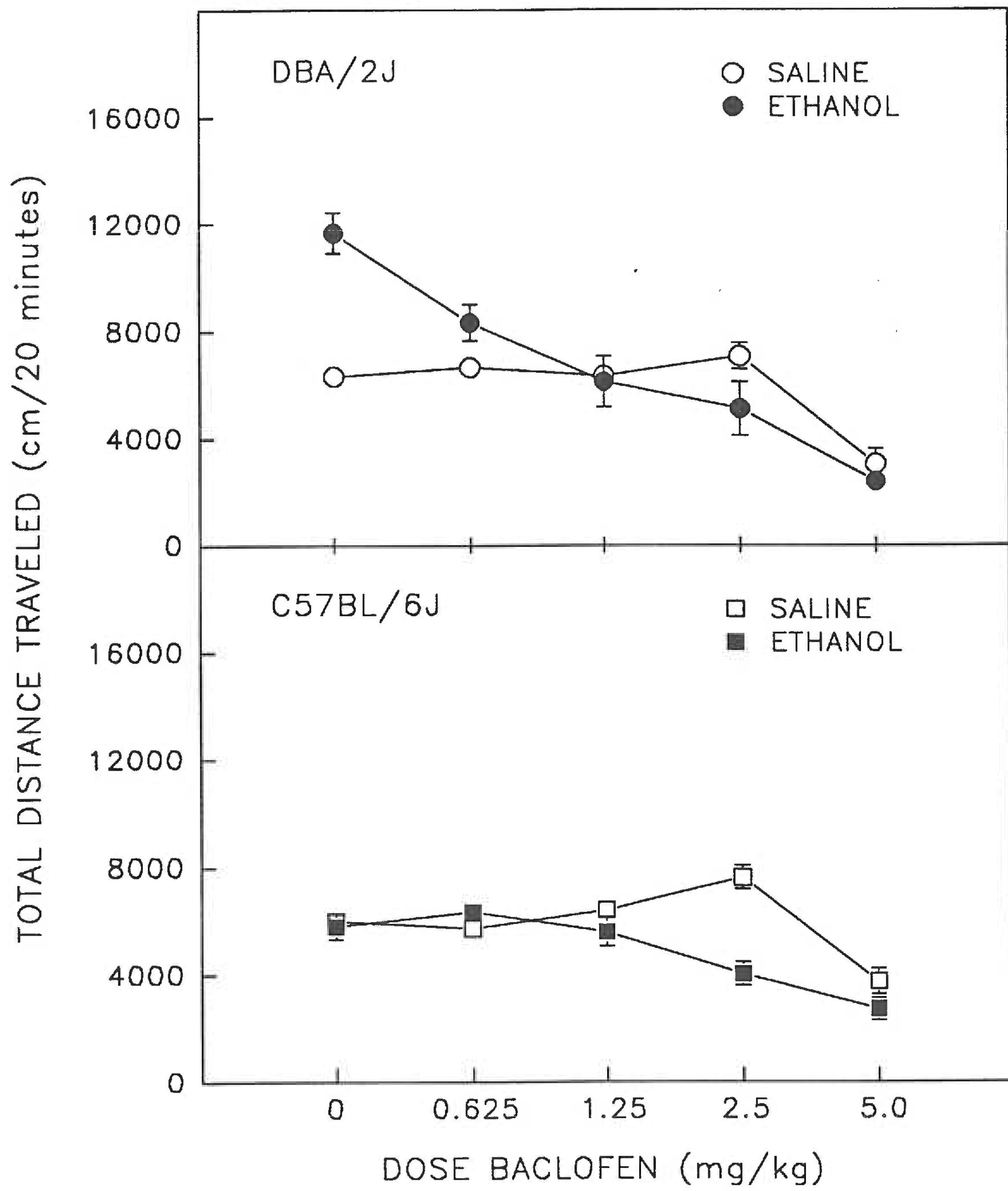
It was previously found that the $GABA_B$ agonist, baclofen, decreased EtOH-stimulated locomotor activity in FAST mice, suggesting that $GABA_B$ receptors are important mediators of EtOH-induced locomotor activation (see "$GABA_B$ receptors and EtOH-stimulated activity"). To determine whether this effect was specific to the FAST selected lines,

or could be found in another genotype sensitive to EtOH's stimulant effects, the effects of baclofen on the locomotor activity of saline- or EtOH-treated D2 inbred mice were assessed in this experiment. As described above, the locomotor activity of D2 mice is stimulated by EtOH, and it was predicted that baclofen would reduce the locomotor stimulant effects of EtOH in D2 mice. In contrast, B6 mice are relatively insensitive to the stimulant effects of EtOH. In order to assess whether baclofen specifically altered genotypes sensitive to EtOH-stimulated activity, or would alter less robust locomotor responses to EtOH as well, B6 mice were included in this experiment.

Methods. The procedure followed that of a comparable experiment using FAST mice, described previously (see Figure 4 and legend). Mice were injected with saline or baclofen (0.625 - 5.0 mg/kg), followed 15 minutes later by a second injection of saline or EtOH (1.5 g/kg, 20% v/v). This dose of EtOH was chosen because it had previously been shown to produce peak stimulation in D2 mice in our testing conditions. The activity test began immediately after the second injection and lasted 20 minutes. After the activity test, mice were removed from the chambers, and ROS blood samples obtained from EtOH-treated animals. EtOH-treated mice were euthanized after blood samples were taken, and saline-treated mice were euthanized after the activity test. Ten mice per strain per treatment group were tested; however, data for one B6 mouse were lost due to experimenter error.

Results. Locomotor activity data are presented in Figure 5, as total distance traveled (cm) during the 20-minute activity test. Visual examination of the data suggested that the strains differed in their

Figure 5. Effects of baclofen on locomotor activity of saline- and EtOH-treated D2 (top panel) and B6 mice (bottom panel). Animals were injected with saline or baclofen 15 minutes prior to i.p. injection of saline or EtOH (1.5 g/kg; 20% v/v). Measurement of locomotor activity lasted for 20 minutes, beginning immediately after the second injection. S.E.M. larger than symbol size are shown; n = 9 - 10 mice per treatment group.


DBA/2J
SALINE
ETHANOL
C57BL/6J
SALINE
ETHANOL
TOTAL DISTANCE TRAVELED (cm/20 minutes)
16000
12000
8000
4000
0
0
0.625
1.25
2.5
5.0
DOSE BACLOFEN (mg/kg)

responses to baclofen and EtOH coadministration. The presence of a significant 3-way interaction between strain, baclofen, and EtOH ($F[4,179] = 4.0$, $p < 0.01$) was consistent with this observation. Data were further analyzed for each strain separately, using 2-way ANOVA grouped on baclofen dose and EtOH treatment. For D2 mice, there were significant effects of baclofen and EtOH, as well as a significant interaction between these variables ($F[4,90] = 10.4$, $p < 0.001$). Characterization of the interaction using simple effects analysis showed that, as expected, D2 mice were stimulated by EtOH when no baclofen was present. Baclofen produced a dose-dependent reduction of EtOH-stimulated activity, in which 0.625 mg/kg baclofen significantly decreased activity of EtOH-treated mice, and 1.25 mg/kg completely blocked EtOH stimulation. Higher doses of baclofen further reduced activity of EtOH-treated D2 mice to below saline baseline levels. The locomotor activity of saline-treated mice was significantly decreased by 5 mg/kg baclofen, but was not altered by any other dose. Saline and EtOH-treated mice differed in locomotor responses at 0, 0.625 and 2.5 mg/kg baclofen, but not at any other dose.

In contrast to D2 mice, B6 mice were not stimulated by EtOH. The locomotor activity of saline- and EtOH-treated mice was significantly decreased by 5 mg/kg baclofen, and there was no difference between these groups. Interestingly, 2.5 mg/kg decreased the activity of B6 mice given EtOH, and slightly but significantly increased saline activity, so that there was a significant difference between saline- and EtOH-treated mice.

Analysis of BECs revealed a significant difference between strains

($F[1,88] = 34.5$, $p < 0.001$) in which D2 mice had higher BEC than B6 mice (mean ± S.E.: 1.64 ± 0.02 and 1.52 ± 0.01 mg/ml for D2 and B6 mice, respectively). There was no effect of baclofen, and no interaction of strain and baclofen on BEC, suggesting that alterations in locomotor activity by baclofen were not due to alterations in absorption or metabolism of EtOH (data not shown).

Summary. Baclofen reduced or blocked EtOH's stimulant effects in D2 mice at doses that did not significantly reduce saline activity. These data, together with similar data from FAST mice, provide strong evidence that $GABA_B$ receptors are important mediators of the stimulant effects of EtOH. The doses of baclofen required to reduce or block EtOH stimulation in D2 mice were lower than those necessary in FAST mice, suggesting that D2 mice were more sensitive to $GABA_B$ ligands. However, FAST and D2 mice were tested at different times with different EtOH doses, and direct comparison was not possible. The higher doses of baclofen tested in this experiment produced locomotor depressant effects in both B6 and D2 EtOH-treated mice. Paradoxically, 2.5 mg/kg baclofen produced slight increases in locomotor activity when given on its own, but significantly decreased the activity of EtOH-treated animals in both strains. These results suggest that baclofen increased sensitivity to EtOH's locomotor depressant effects, particularly in B6 mice; however, the result that 5 mg/kg baclofen did not further decrease the activity of EtOH-treated mice is not consistent with this notion. On the other hand, it is possible that a floor effect occurred at the highest dose of baclofen so that further reductions could not be detected. Strain differences in BEC (D2 > B6) are consistent with previously reported

BECs (Crabbe, 1983; Crabbe et al., 1982; Crabbe et al., 1994), except in one case in which B6 mice had higher BEC than D2 after 2.0 g/kg EtOH (Crabbe et al., 1994). The effects of baclofen on the locomotor activity of ETOH-treated mice could not be explained by changes in EtOH pharmacokinetics, since BECs did not differ as a function of baclofen dose.

Experiment 1.2: Specificity of Baclofen Effects

To confirm that the effects of baclofen on EtOH-stimulated activity in FAST and D2 mice were mediated by $GABA_B$ receptors, the potent $GABA_B$ antagonist, CGP-35348, was coadministered with baclofen and EtOH to FAST-1, FAST-2, and D2 mice. Preliminary pilot studies using other $GABA_B$ antagonists (5-aminovaleric acid or phaclofen) in conjunction with baclofen and EtOH did not yield promising results (see Appendix A). These preliminary data were consistent with the current understanding that these ligands are neither as potent or selective as originally thought.

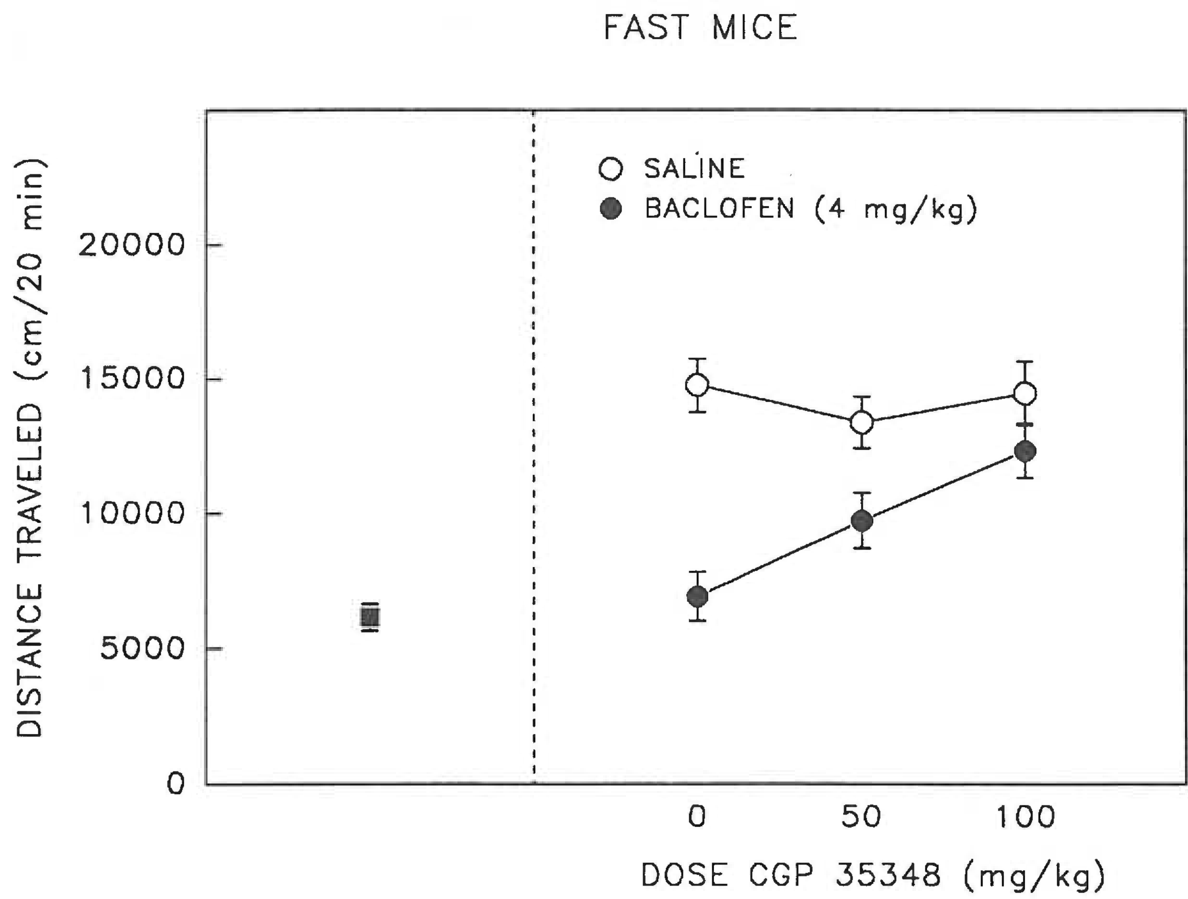
Methods. The genotypes were tested in separate experiments, so that FAST mice (both replicates) were tested in one experiment, and D2 mice were tested in another experiment. The experimental design resulted in 6 treatment groups which yielded data on the effects of CGP-35348 on EtOH-treated animals given saline or baclofen. FAST-1 and FAST-2 male mice from $S_{36}G_{37}$ were injected with saline or CGP-35348 (50 or 100 mg/kg) and saline or baclofen (4 mg/kg). Drugs were combined into one solution so that coadministration of CGP-35348 and baclofen occurred in a single injection. The dose of baclofen was chosen based

on the result that doses up to and including 5 mg/kg decreased EtOH-stimulated activity of FAST mice without effects on saline activity (see Figure 4). Fifteen minutes after the first injection, all mice were injected with 2.0 g/kg EtOH, and tested for 20 minutes in activity monitors. D2 mice were tested in the same manner, except that the dose of baclofen was 1.5 mg/kg and the EtOH dose was 1.5 g/kg. In addition to the 6 treatment groups described above, a baseline activity control group was added to assess the magnitude of EtOH-stimulation and the extent to which baclofen blocked EtOH-stimulation. This control group was given 2 saline injections, 15 minutes apart, and tested for locomotor activity as described above. ROS blood samples were obtained from all EtOH-treated animals after activity testing. The effects of CGP-35348 on baseline locomotor activity (no EtOH present) were not assessed in this experiment; however, it was determined in a separate pilot study that doses of CGP-35348 as high as 200 mg/kg had no effects on baseline activity (see Appendix B).

Results in FAST mice. FAST mice were significantly stimulated by EtOH, as assessed by t-test between the baseline activity group and animals that received EtOH only ($t_{(38)} = 10.1$, $p < 0.01$). The locomotor activity of baclofen + EtOH-treated mice did not differ from baseline, indicating that the stimulant effects of EtOH were completely blocked by baclofen. Three-way ANOVA grouped on replicate, CGP-35348, and baclofen revealed a significant main effect of replicate ($F[1,108] = 7.4$, $p < 0.01$), in which FAST-2 mice traveled greater distances than FAST-1 mice. There were no interactions involving replicate; thus, data are presented collapsed on this variable in Figure 6. Further characterization of a

Figure 6. Reversal of the inhibitory effects of baclofen (4 mg/kg) on EtOH-stimulated activity of FAST mice by the $GABA_B$ antagonist, CGP-35348. Data are presented with replicates combined. The locomotor activity of a group of mice that received only saline injections prior to activity testing (baseline activity) is represented by the data point to the left of the dashed vertical line. Mice to the right of the vertical dashed line were injected with EtOH (2.0 g/kg) 15 minutes after injection with saline or CGP-35348 in combination with saline or baclofen. CGP-35348 and baclofen combinations were mixed in a single solution. After EtOH injection, mice were immediately placed in activity monitors for 20 minutes. Comparison of the baseline activity group with animals that received 0 mg/kg CGP 35348 and saline reveals the magnitude of EtOH-stimulated activity. Vertical bars are S.E.M.; n = 10 mice per replicate and treatment group.

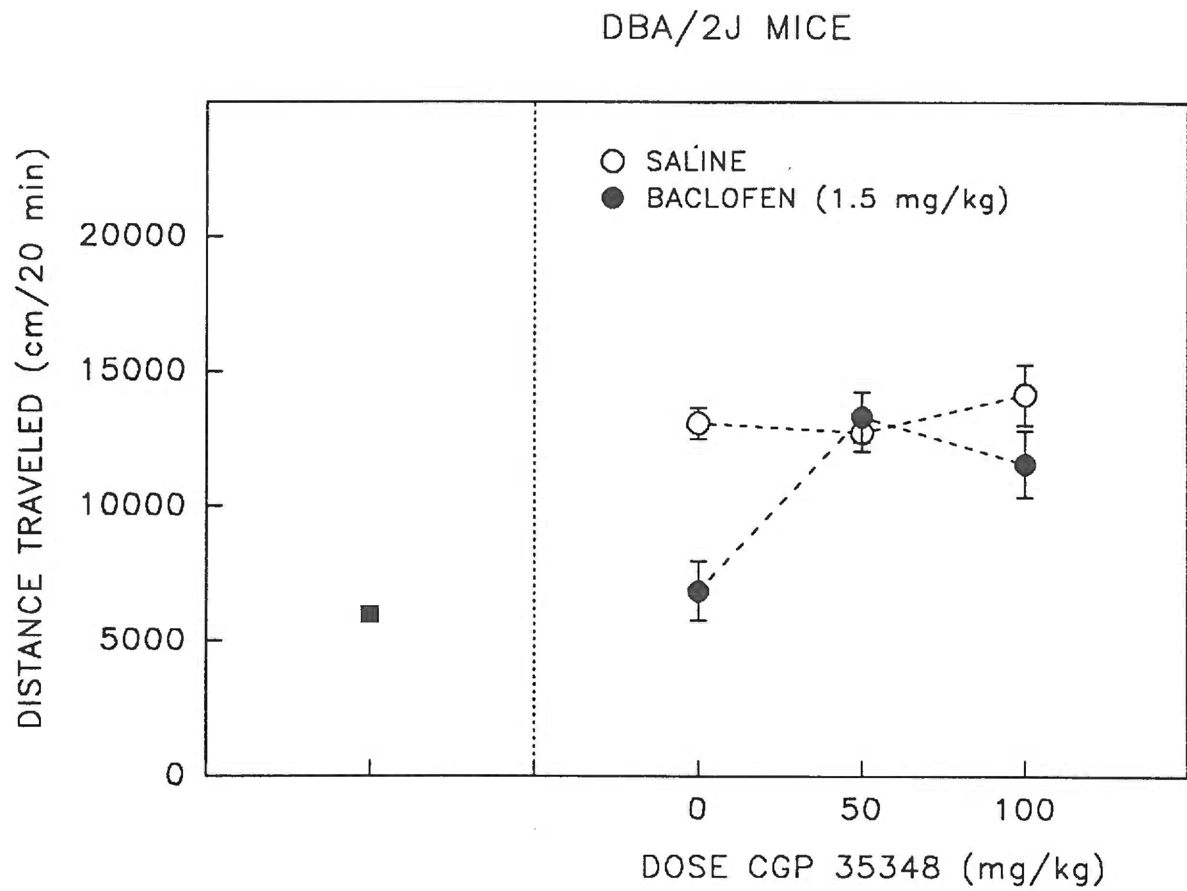
FIGURE 6



significant interaction between CGP-35348 and baclofen ($F[2,108] = 4.5$, $p < 0.05$) showed that CGP-35348 dose-dependently reversed the inhibition of EtOH-stimulated activity by baclofen. This effect of CGP-35348 was significant at 100 mg/kg, but not 50 mg/kg CGP-35348. The activities of saline- and baclofen-treated FAST mice were significantly different when either saline or 50 mg/kg CGP-35348 was administered, but were not different after 100 mg/kg CGP-35348. Since CGP-35348 alone did not significantly alter EtOH-stimulated activity, the similar activity levels between saline- and baclofen-treated mice suggest that CGP-35348 completely blocked baclofen's effects. There were no effects of baclofen, CGP-35348, or the drug combinations on BECs of FAST mice (mean ± S.E. = 2.18 ± 0.01 mg/ml; data not shown).

Results in D2 mice. The effects of baclofen and CGP-35348 coadministration on EtOH-treated D2 mice were similar to those found in FAST mice, and are presented in Figure 7. D2 mice were significantly stimulated by EtOH when no $GABA_B$ ligands were present, compared to baseline control ($t_{(18)} = 2.84$, $p < 0.01$). Administration of 1.5 mg/kg baclofen blocked the stimulant effects of EtOH, as supported by the lack of significant difference between the baseline control group and baclofen + EtOH-treated mice. Two-way ANOVA on EtOH-treated animals, grouped on CGP-35348 and baclofen, revealed a significant CGP-35348 and baclofen interaction ($F[2,54] = 6.2$, $p < 0.01$). CGP-35348 significantly reversed the inhibition of EtOH-stimulated activity by baclofen, but did not alter the activity of saline + EtOH-treated animals. BECs of D2 mice were not altered by administration of CGP-35348, baclofen, or the combination of drugs (mean ± S.E. = 1.77 ± 0.02 mg/ml; data not shown).

Figure 7. Reversal of the inhibitory effects of baclofen (1.5 mg/kg) on EtOH-stimulated activity in D2 mice by CGP-35348. Baseline locomotor activity is depicted by data symbol to the left of the vertical dashed line (see text and Figure 6 legend for more details). All mice to the right of the dashed line received saline or CGP 35348, and saline or baclofen, followed 15 minutes later by an EtOH injection (1.5 g/kg) and a 20-minute activity test. CGP 35348 and baclofen combinations were mixed in a single solution. Vertical bars are S.E.M.; n = 10 per treatment group.


DBA/2J MICE
SALINE
BACLOFEN (1.5 mg/kg)
DISTANCE TRAVELED (cm/20 min)
20000
15000
10000
5000
0
0
50
100
DOSE CGP 35348 (mg/kg)

Summary. These data replicated the findings of the previous studies in which the $GABA_B$ agonist, baclofen, reduced the locomotor activating effects of EtOH in FAST and D2 mice. The selective $GABA_B$ antagonist, CGP-35348, blocked the effects of baclofen in all 3 genotypes, which argues that the effects of baclofen were specifically mediated by $GABA_B$ receptors. The results that 50 mg/kg CGP-35348 partially reversed the effects of baclofen in FAST mice, but completely blocked baclofen's effects in D2 mice, are consistent with the notion that D2 mice are more sensitive to $GABA_B$ ligands; however, as previously noted, FAST and D2 mice were tested at different times with different EtOH doses, and direct comparisons were not possible.

Experiment 1.3: Effects of Baclofen and Dopamine Agonist Coadministration

This experiment was undertaken to characterize the possible interaction between $GABA_B$ and dopamine receptor systems in mediating locomotor stimulation produced by EtOH in FAST and D2 mice, genotypes that consistently show an EtOH stimulant response. The rationale, as outlined previously, was that $GABA_B$ receptors, either directly or indirectly, decrease dopaminergic activity, which has been shown to be important for expression of EtOH-stimulated activity. Thus, restoration of dopamine function by administration of dopamine agonists was expected to reverse the inhibition of stimulation produced by baclofen. Results of several preliminary studies using different dopamine agonists did not support the hypothesis, but are presented below. Because this line of investigation was not promising, a full parametric study was not attempted. The particular replicate of FAST mice used in the individual

studies was solely dependent upon availability.

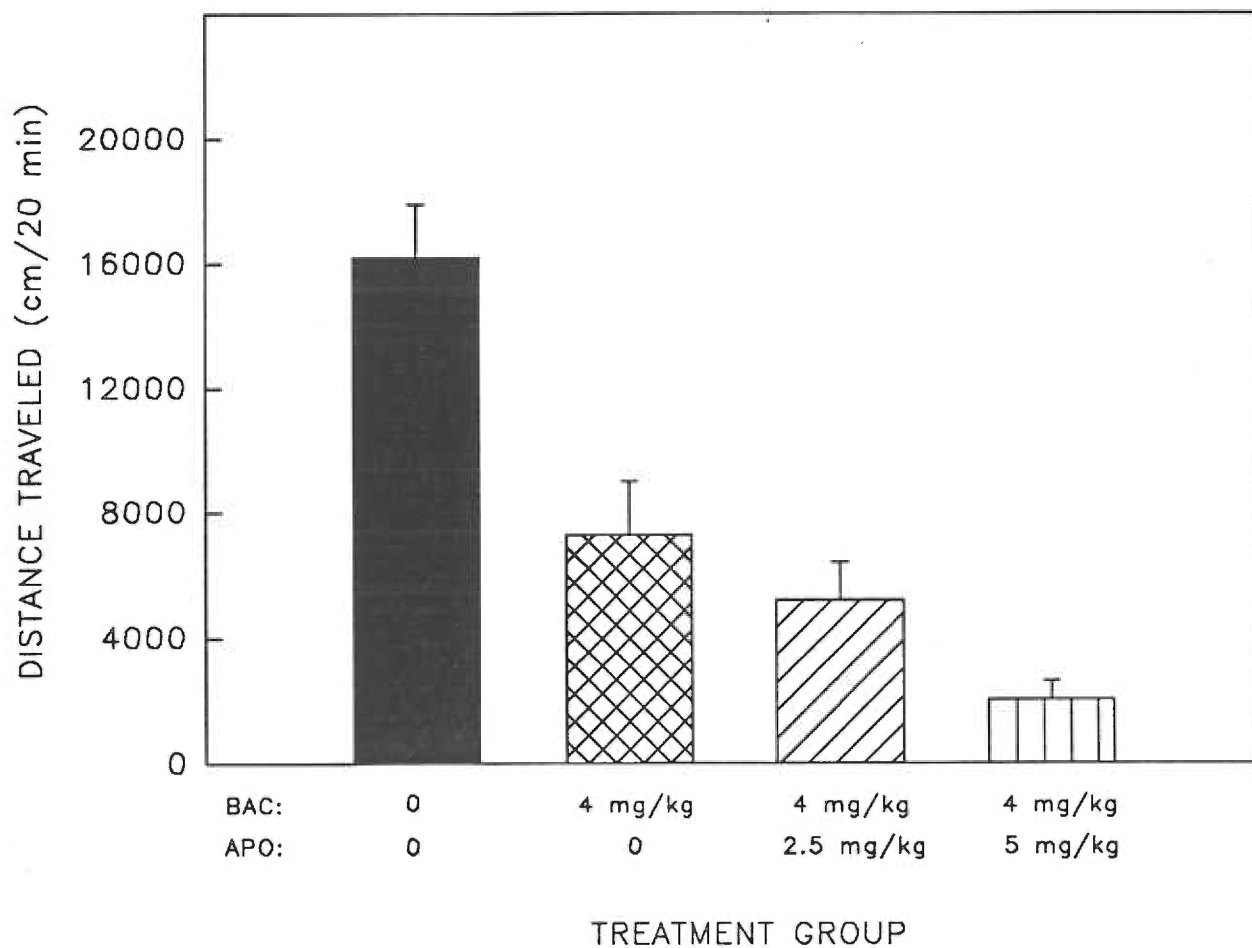
Preliminary Study A: Apomorphine

Methods. The effects of the mixed D_1/D_2 receptor agonist, apomorphine, on locomotor activity of baclofen- and EtOH-treated S_{36} FAST-2 male mice (84 - 102 days old) were assessed in this study. Six mice were tested in each of four treatment groups. All groups received 3 injections; the first injection was administered 15 minutes prior to the second and third injections. One group, which provided information on the activity of EtOH-stimulated animals, was injected with saline, followed by injections of apomorphine vehicle (0.1% sodium metabisulfite) and 2.0 g/kg EtOH. A second group provided information on baclofen's effects on locomotor activity of EtOH-treated animals. This group was first injected with baclofen (4 mg/kg), followed by apomorphine vehicle and EtOH. The effects of apomorphine on baclofen's inhibition of EtOH-stimulated activity were assessed with two additional groups, which were injected with baclofen, followed by apomorphine (2.5 or 5 mg/kg) and EtOH. These doses of apomorphine were chosen based on previously collected data, in which 2.5 mg/kg apomorphine had no significant effect on EtOH-stimulated activity (see Appendix C). Immediately following the third injection, mice were placed in activity monitors, and tested for 20 minutes.

Results. Data are presented in Figure 8a. Although a group that provided information on baseline activity was not included in this preliminary study, the distance traveled by the EtOH-alone group was comparable to other results from FAST-2 male mice which were stimulated compared to their baseline controls. Differences between groups were

Figure 8a. Effects of apomorphine (APO) on inhibition of EtOH-stimulated locomotor activity by baclofen (BAC) in FAST-2 mice. Apomorphine vehicle was 0.1% sodium metabisulfite. Saline or baclofen (4 mg/kg) was administered 15 minutes prior to injection of apomorphine (0, 2.5 or 5 mg/kg), and 2 g/kg EtOH. Activity test duration was 20 minutes. The solid bar represents the activity of animals given EtOH alone; coadministration of baclofen reduced the activity of these mice. Vertical lines are S.E.M.; n = 5 - 6 per treatment group.

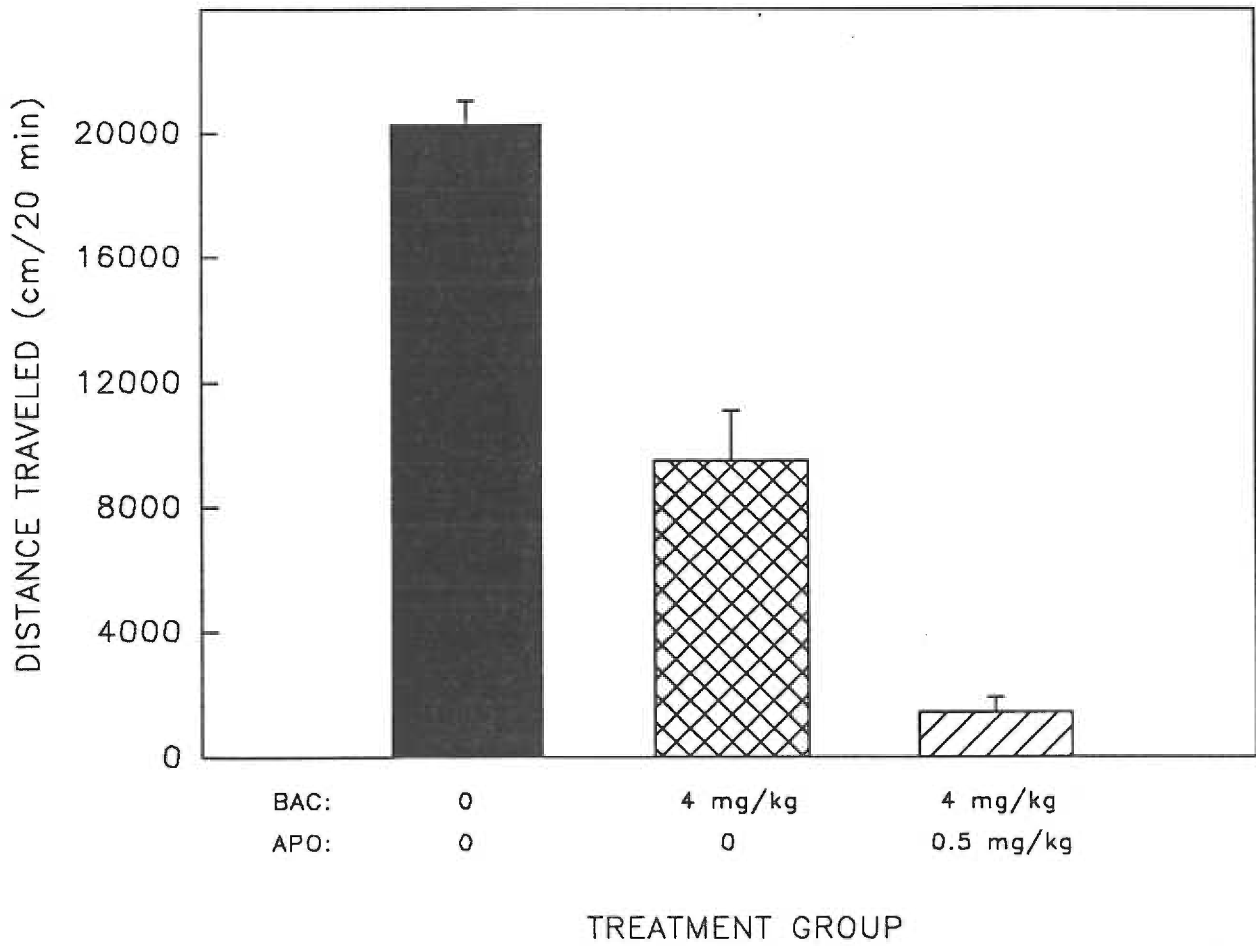
FIGURE 8A



evaluated using planned t-tests. Baclofen significantly decreased activity compared to the activity of the EtOH-alone group ($t_{(9)} = 3.7$, $p < 0.01$). Contrary to the predicted results, apomorphine did not reverse the effects of baclofen. There was no effect of 2.5 mg/kg, but 5.0 mg/kg apomorphine significantly enhanced the effects of baclofen ($t_{(10)} = 2.8$, $p < 0.05$). To determine whether a lower dose could produce a change in the expected direction, a second study was done using the same treatment regimen, except that the effect of 0.5 mg/kg apomorphine was assessed in FAST-1 male mice (S_{36}, 84 - 95 days of age) given baclofen and EtOH (see Figure 8b). Baclofen significantly decreased the activity of EtOH-treated mice, compared to mice treated with EtOH alone ($t_{(7)} = 5.6$, $p < 0.001$). This dose of apomorphine did not reverse the effects of baclofen, but significantly enhanced locomotor decreases produced by baclofen in EtOH-treated animals ($t_{(9)} = 5.3$, $p < 0.05$).

Summary. In these preliminary studies involving apomorphine, the inhibitory effects of baclofen on EtOH-stimulated activity were not altered in the predicted direction. Apomorphine is a mixed D1 /D2 dopamine agonist, which has been demonstrated to have a biphasic U-shaped dose-effect profile for locomotor activity. This profile is thought to be due to preferential binding to dopamine autoreceptors at low doses, followed by binding to post-synaptic receptors at higher doses (DiChiara et al., 1978). Given this profile, it is not completely surprising that the lowest dose of apomorphine further decreased locomotor activity. In addition, as described in Appendix C (Figure C.1), apomorphine decreased the locomotor activity of both FAST and SLOW mice at both 1 and 5 mg/kg. Given this response of FAST and SLOW mice

Figure 8b. Effects of 0.5 mg/kg apomorphine (APO) on inhibition of EtOH-stimulated locomotor activity by baclofen (BAC) in FAST-1 mice. Procedures were as described in Figure 8a legend. Vertical lines are S.E.M.; n = 4 - 6 per treatment group.


DISTANCE TRAVELED (cm/20 min)
20000
16000
12000
8000
4000
0
BAC: 0 4 mg/kg 4 mg/kg
APO: 0 0 0.5 mg/kg
TREATMENT GROUP

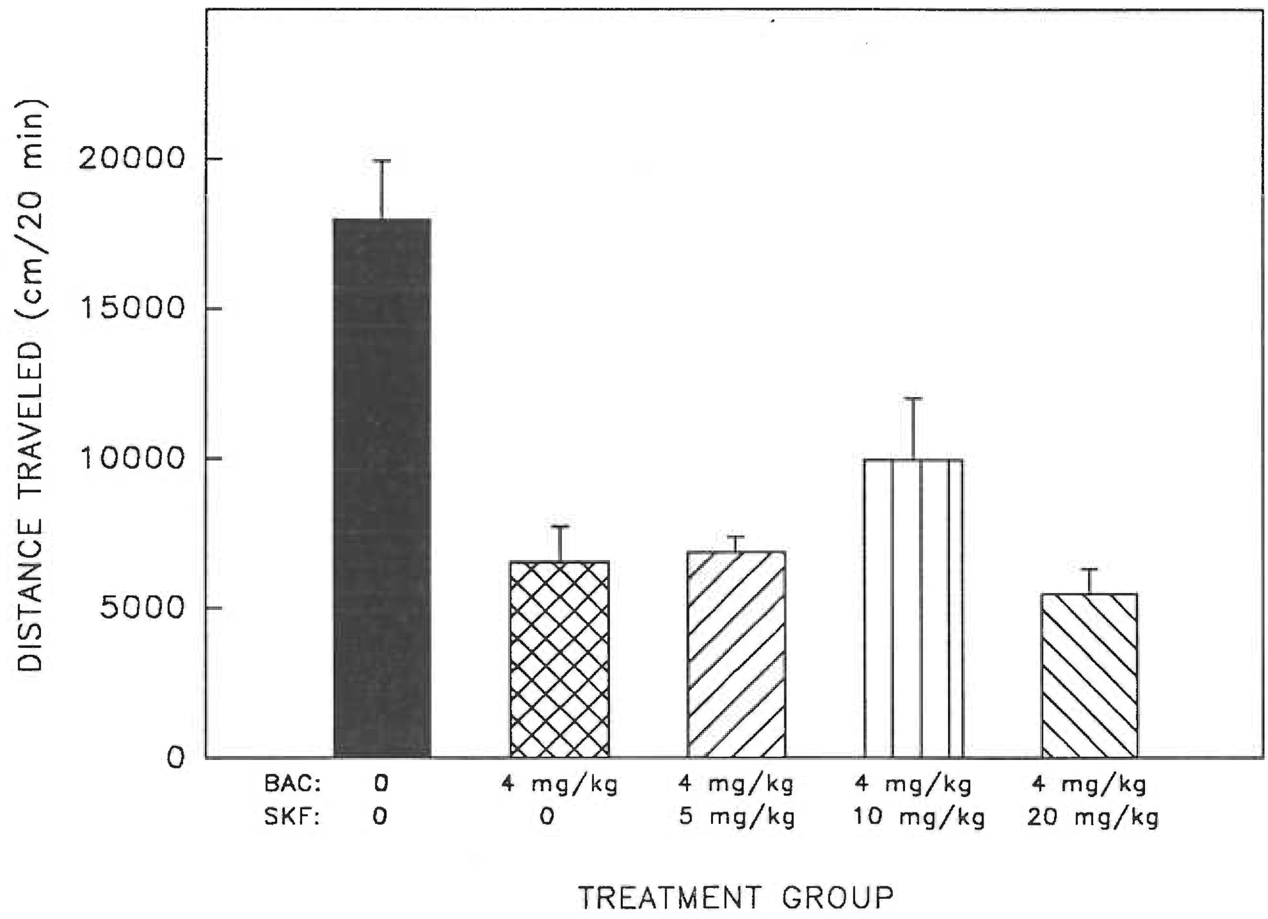
to apomorphine, it is also perhaps not surprising that higher apomorphine doses also decreased locomotor activity of EtOH-treated mice. Since apomorphine did not have the predicted effects, and because interpretation of these data was complicated by the nonspecificity of apomorphine for dopamine receptor subtypes, it was thought that use of ligands more selective for a particular receptor subtype would yield data that were more informative.

Preliminary Study B: SKF-38393

Methods. The effects of SKF-38393, a D1 agonist, on inhibition of EtOH-stimulation by baclofen were assessed in this study, using the same treatment groups as those outlined in Preliminary Study A, with the addition of a third agonist dose. Thus, one group received EtOH alone, one group received baclofen and EtOH, and 3 groups received baclofen, EtOH, and SKF-38393. FAST-2 mice (S_{36}), 97 - 105 days of age, were injected with saline or baclofen 15 minutes prior to SKF-38393 (5, 10, or 20 mg/kg) and EtOH, followed by a 20-minute activity test. There were 6 animals tested in each of the 5 treatment groups.

Results. Data were analyzed by planned t-tests, and are presented in Figure 9. Baclofen significantly reduced the stimulant effects of EtOH ($t_{10} = 5.1$, $p < 0.001$). Although it appeared that 10 mg/kg SKF-38393 increased activity of baclofen + EtOH treated animals, a scatterplot of total horizontal distance showed that one animal in this treatment group displayed much higher activity levels than all others, which may have been due to a misplaced injection. Statistical analyses, both including and excluding this particular animal, were performed, and neither t-test detected significant effects of 10 mg/kg SKF-38393 on

Figure 9. Effects of the D1 agonist, SKF-38393 (SKF), on inhibition of EtOH-stimulated activity by baclofen in FAST-2 mice. Animals were injected with saline or baclofen (4 mg/kg), followed 15 minutes later by saline or SKF-38393 (5, 10, or 20 mg/kg) and EtOH (2 g/kg). Test duration was 20 minutes. Vertical lines are S.E.M.; n = 6 per treatment group.


DISTANCE TRAVELED (cm/20 min)
20000
15000
10000
5000
0
BAC: 0 4 mg/kg 4 mg/kg 4 mg/kg 4 mg/kg
SKF: 0 0 5 mg/kg 10 mg/kg 20 mg/kg
TREATMENT GROUP

the locomotor activity of baclofen + EtOH-treated mice. Administration of other SKF-38393 doses (5 and 20 mg/kg) also had no effect on the locomotor activity of baclofen + EtOH-treated animals, as supported by the lack of significant differences between groups which received SKF-38393 and the group which received baclofen and EtOH. The results of this experiment indicated that SKF-38393 was unlikely to produce informative results.

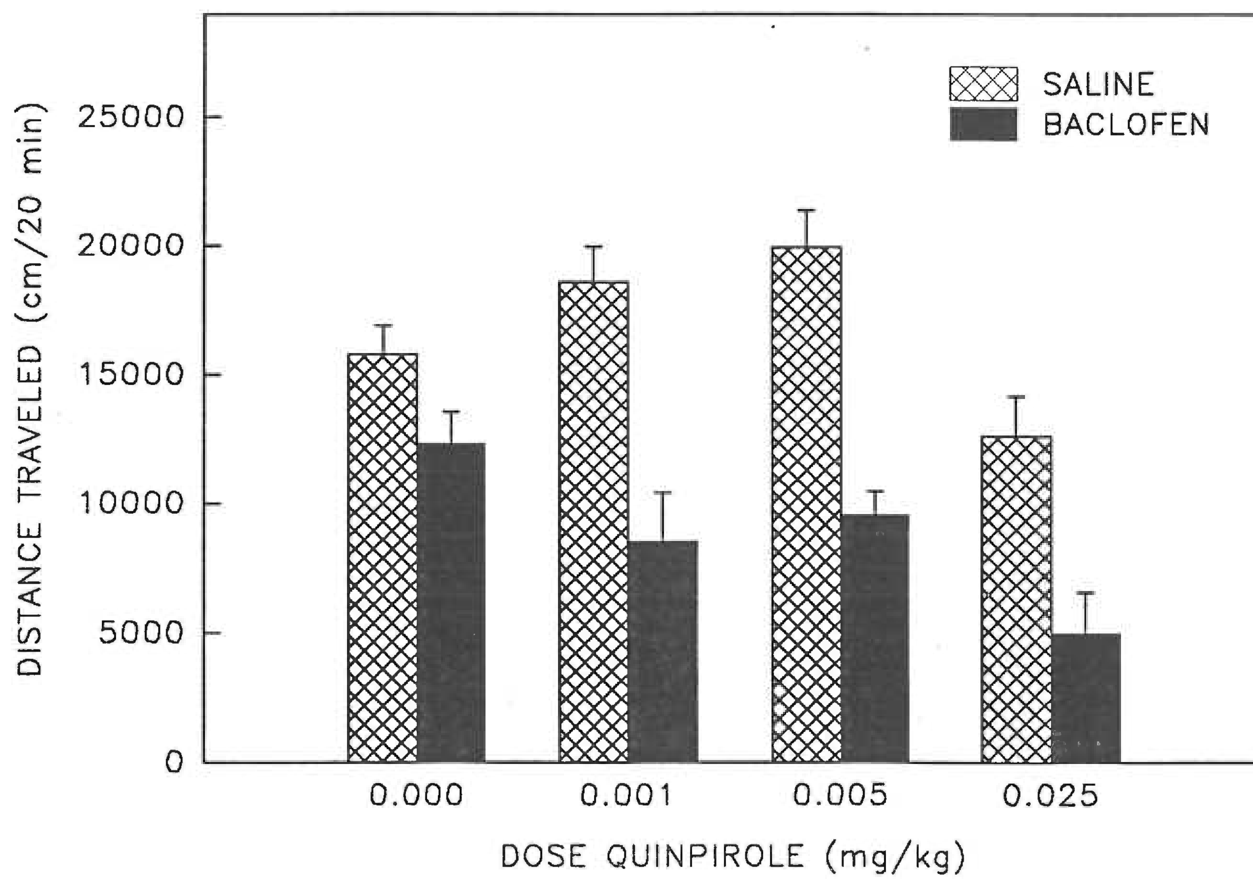
Preliminary Study C: Quinpirole

Neither the mixed agonist, apomorphine, or the D_1 agonist, SKF-38393, had effects on baclofen's inhibition of EtOH-stimulated activity. To investigate whether an agonist acting at D_2-like receptors might have some effect, the effects of the D_2/D_3 agonist, quinpirole, were examined. This drug had previously been tested in drug-naive FAST and SLOW mice, and was found to decrease activity at doses as low as 0.0625 mg/kg. In other studies, quinpirole had no effect on the locomotor activity of saline or EtOH-treated FAST and SLOW mice when administered in doses of 0.001 and 0.005 mg/kg.

Methods. This experiment was designed as a 4 x 2 factorial, in which $S_{36}G_{39}$ - $S_{36}G_{40}$ FAST-2 male mice (51 - 72 days old) received saline or 1 of 3 quinpirole doses (0.001, 0.005, or 0.025 mg/kg), immediately followed by saline or baclofen (4 mg/kg). Fifteen minutes later, all mice were injected with 2.0 g/kg EtOH, and tested for locomotor activity for 20 minutes.

Results. Results of this preliminary study are presented in Figure 10. Two-way ANOVA grouped on quinpirole and baclofen revealed a significant main effect of quinpirole ($F[3,72] = 6.4$, $p < 0.001$) which

Figure 10. Effects of the D2 agonist, quinpirole, on baclofen's reduction of EtOH-stimulation in FAST-2 mice. Saline or quinpirole (0.001, 0.005, or 0.025 mg/kg), and saline or baclofen (4 mg/kg) were injected 15 minutes prior to EtOH (2.0 g/kg) and a 20-minute activity test. Vertical lines are S.E.M.; n = 10 per treatment group.



SALINE
BACLOFEN
DISTANCE TRAVELED (cm/20 min)
25000
20000
15000
10000
5000
0
0.000
0.001
0.005
0.025
DOSE QUINPIROLE (mg/kg)

was due to significantly decreased locomotor activity of animals injected with 0.025 mg/kg quinpirole compared to vehicle-treated animals. No other quinpirole dose significantly affected activity. The locomotor activity of baclofen-treated animals was significantly decreased compared to saline-injected mice ($F[1,72] = 55.2$, $p < 0.001$). Quinpirole appeared to produce differential effects in saline- vs baclofen-treated mice, in which quinpirole and baclofen coadministration produced slightly decreased activity relative to baclofen alone; however, the interaction of quinpirole and baclofen was not significant, and further statistical analyses were not appropriate. Therefore, coadministration of the D_2/D_3 agonist, quinpirole, did not reverse the effect of baclofen on EtOH-stimulated activity; rather, there appeared to be an enhancement of baclofen's effect, especially at the highest dose of quinpirole tested.

The inhibition of EtOH-stimulated activity by baclofen in the absence of other dopamine ligands observed in this study was not as robust as that seen in other studies presented here (see Figures 4 and 6). Examination of data from individual animals by scatterplot showed that the locomotor activity of 3 animals was approximately twice that of all others tested (total $n = 10$). Exclusion of those three animals reduced the mean locomotor activity of the group to a level (9007.1 ± 1238) equivalent to that of animals coadministered baclofen and quinpirole (0.001 and 0.005 mg/kg). ANOVA results, and the conclusion that quinpirole did not alter effects of baclofen, were not altered by exclusion of these animals from data analyses.

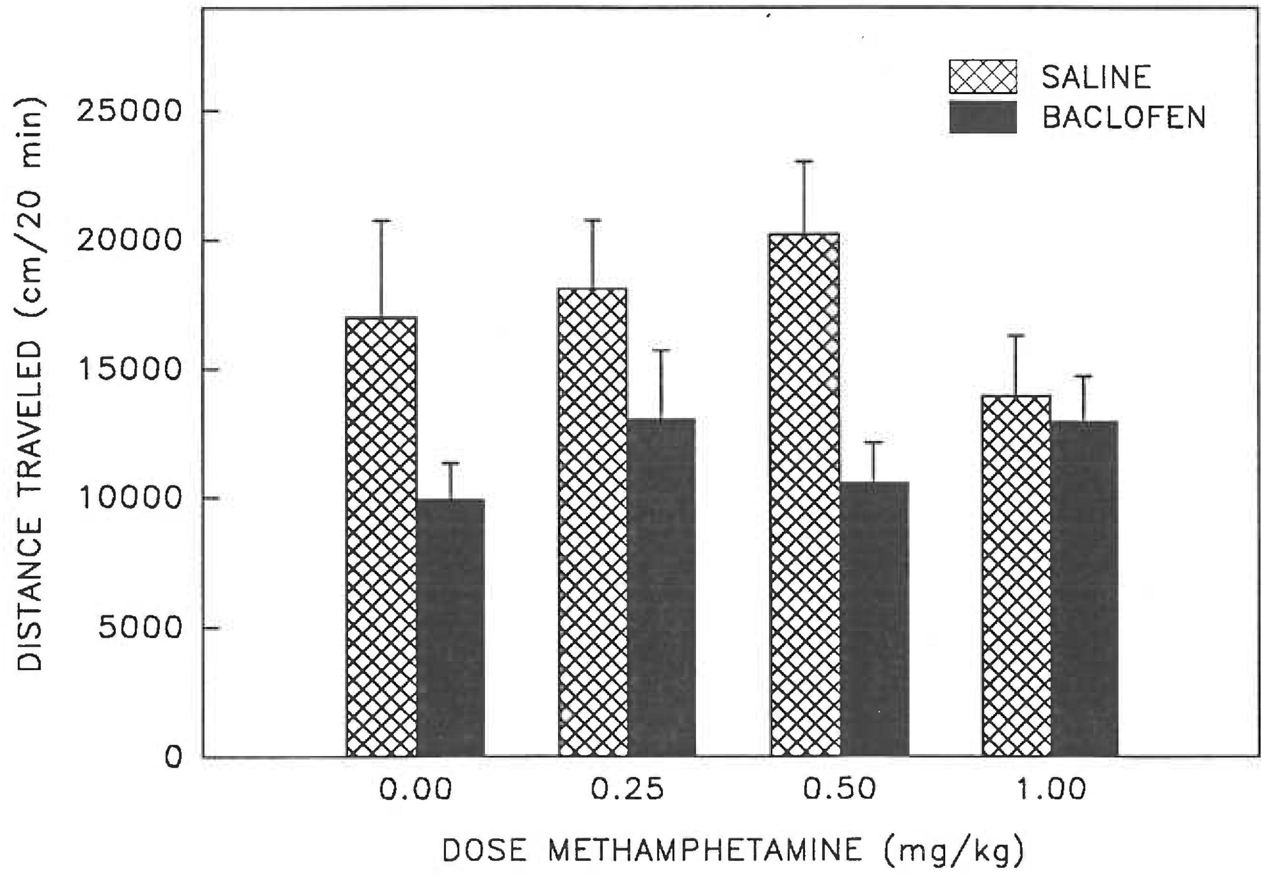
Preliminary Study D: Methamphetamine

Amphetamine is a psychostimulant drug which increases synaptic dopamine concentrations. Dopamine agonists did not produce the hypothesized results, but it was speculated that increasing the concentration of endogenous ligand in the synapse might alter baclofen's effects on EtOH-stimulated activity.

Methods. The design of this experiment is the same as that used in Preliminary Study C. In a 4 x 2 factorial design, male $S_{36}G_{38}$ FAST-2 mice (76 - 103 days) were injected with saline or 1 of 3 doses of methamphetamine (0.25, 0.5 or 1.0 mg/kg), followed immediately by an injection of saline or baclofen. Fifteen minutes later, all animals received an injection of EtOH, and were immediately tested for locomotor activity for 20 minutes. These doses of methamphetamine were chosen based on other pilot studies in drug-naive animals, in which both 1 and 2 mg/kg methamphetamine stimulated activity compared to saline controls. Lower doses were chosen so that results could be interpreted independently of stimulant effects of methamphetamine itself.

Results. Two-way ANOVA with methamphetamine dose and baclofen dose as grouping factors revealed a significant effect of baclofen ($F[1,31] = 10.0$, $p < 0.01$) in which all baclofen-treated mice had lower activity compared to saline-treated animals (see Figure 11). There were no significant effects of methamphetamine, nor was there a significant interaction between methamphetamine and baclofen treatment. Therefore, methamphetamine did not alter or reverse the inhibitory effects of baclofen on EtOH-stimulated activity. One interesting result, though not statistically supported, is the slight decrease in activity produced

Figure 11. Effects of methamphetamine on locomotor activity of EtOH-treated FAST-2 mice given saline or baclofen. Saline or methamphetamine (0.25, 0.5, or 1 mg/kg) and saline or baclofen (4 mg/kg) were administered 15 minutes prior to EtOH injection (2 g/kg) and a 20-minute activity test. Vertical lines are S.E.M.; n = 5 per treatment group.


SALINE
BACLOFEN
DISTANCE TRAVELED (cm/20 min)
25000
20000
15000
10000
5000
0
0.00
0.25
0.50
1.00
DOSE METHAMPHETAMINE (mg/kg)

by coadministration of methamphetamine (particularly 1 mg/kg) and EtOH. Since stimulant effects of EtOH are mediated to some extent by dopaminergic systems, one might have expected that coadministration would enhance EtOH stimulation. On the other hand, one might also predict that, 1) high concentrations of synaptic dopamine would result in inhibitory feedback to decrease dopaminergic activity, or 2) the administration of methamphetamine resulted in depleted dopamine stores, or somehow compromised the ability of the dopamine neuron to function normally, thus resulting in a reduction of the stimulant response to EtOH.

Summary. None of the dopamine agonists tested reversed the inhibitory effects of baclofen on EtOH-stimulated activity. Apomorphine, SKF-38393 (D_1 agonist), and quinpirole (D_2/D_3 agonist) were administered in low to moderate doses. Apomorphine enhanced the inhibitory effects of baclofen at several doses, as did the highest dose of quinpirole. This quinpirole dose also decreased the locomotor activity of mice given EtOH only. However, SKF-38393 had not effect on the locomotor activity of baclofen + EtOH-treated mice. Administration of methamphetamine, which increases synaptic dopamine concentrations, also did not reverse the effects of baclofen. These pilot studies did not provide evidence to support a role of $GABA_B$/DA interactions in mediating EtOH's locomotor stimulant effects, and complete parametric studies in all genotypes were not performed.

Experiment 1.4: Effects of CGP-35348 in EtOH-treated SLOW and B6 mice

Since administration of a $GABA_B$ agonist decreased the stimulant

effects of EtOH in FAST and D2 mice, it was hypothesized that administration of a $GABA_B$ antagonist might confer stimulant responses to animals otherwise insensitive to EtOH's locomotor increasing effects. Prior to undertaking a full study, pilot data were first collected using SLOW and B6 mice. Due to unavailability of replicate 2 mice, only SLOW-1 mice were tested in these preliminary studies.

Methods. SLOW-1 mice were tested for locomotor activity after injection of saline or CGP-35348 (100 mg/kg) 15 minutes prior to injection of saline or EtOH (2.0 g/kg). This dose of CGP-35348 was effective in reversing the effects of baclofen as described in Experiment 2, and was therefore thought to be sufficient to block $GABA_B$ receptors. B6 mice were tested in the same way in a separate pilot study, but an additional dose of CGP-35348 was also tested (200 mg/kg), and the dose of EtOH was 1.5 g/kg.

Results. Data are presented in Figures 12 and 13 for SLOW-1 and B6 mice, respectively. As apparent in Figure 12, EtOH significantly decreased the locomotor activity of SLOW-1 mice (main effect of EtOH: $F[1,20] = 166.6$, $p < 0.01$). CGP-35348 did not affect the activity of saline-treated SLOW-1 mice, nor did it alter EtOH's depressant effects. Consistent with results seen previously under these testing conditions (see Figure 5, experiment 1), 1.5 g/kg EtOH did not alter the locomotor activity of B6 mice. CGP-35348 did not alter the activity of saline- or EtOH-treated B6 mice, even at a higher dose of 200 mg/kg.

Summary. Administration of the $GABA_B$ antagonist, CGP-35348, clearly did not alter activity of SLOW-1 or B6 mice in these pilot studies; thus, a full study was not pursued. It is possible that the

Figure 12. Effects of CGP-35348 on locomotor activity of SLOW-1 mice given saline or EtOH. CGP-35348 was administered 15 minutes prior to saline or EtOH (2 g/kg). Animals were tested for activity for 20 minutes immediately following saline or EtOH injection. Vertical lines are S.E.M.; n = 6 per treatment group.

FIGURE 12

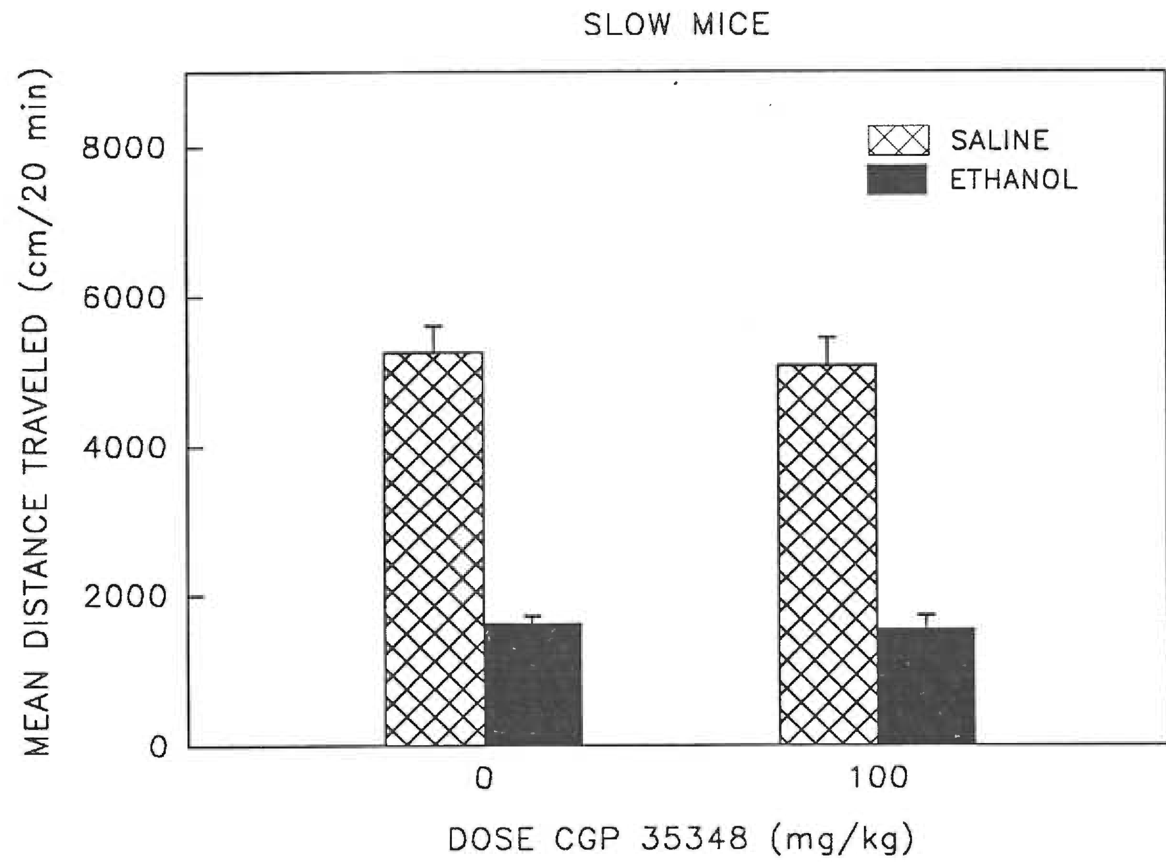
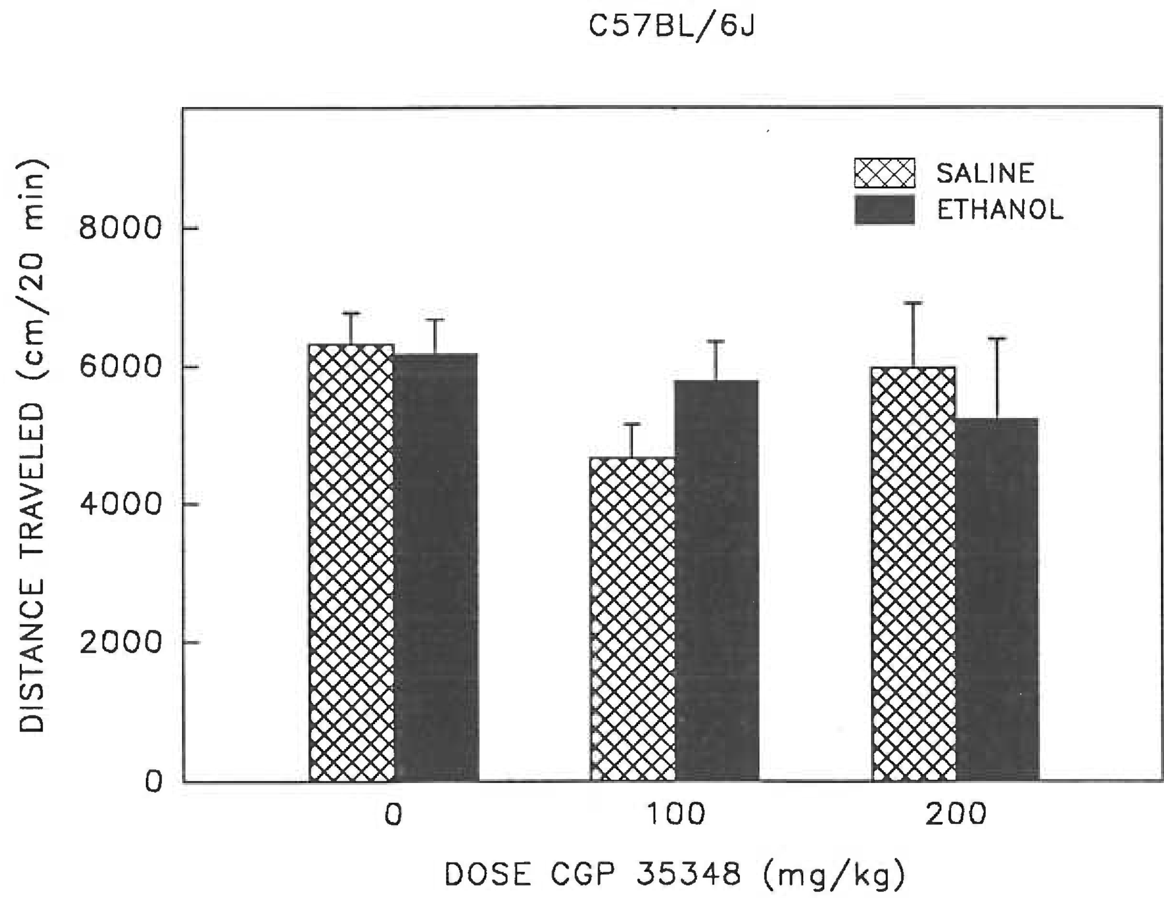


Figure 13. Effects of CGP-35348 on locomotor activity of B6 mice given saline or EtOH. Saline or CGP-35348 (100 or 200 mg/kg) was administered 15 minutes prior to saline or EtOH (1.5 g/kg). Animals were tested for activity for 20 minutes immediately following saline or EtOH injection. Vertical lines are S.E.M.; n = 5 - 6 per treatment group.


C57BL/6J
SALINE
ETHANOL
DISTANCE TRAVELED (cm/20 min)
8000
6000
4000
2000
0
0
100
200
DOSE CGP 35348 (mg/kg)

dose of EtOH used for SLOW-1 mice produced such a robust depressant response that CGP-35348 could not counteract it. However, the activity of B6 mice, whose locomotor activity was not decreased by EtOH, did not change with CGP-35348. Thus, it is unlikely that using a less effective dose of EtOH in SLOW-1 mice would have altered the results. As described in "Hypotheses and Specific Aims", a study was planned to assess the interaction of dopamine with $GABA_B$ effects in SLOW and B6 mice (Experiment 1.5 in Table 1). However, this experiment was contingent upon successful stimulation of these genotypes by CGP-35348, and was not performed.

Specific Aim 2

Experiment 2.1: Effects of MK-801 on Locomotor Activity

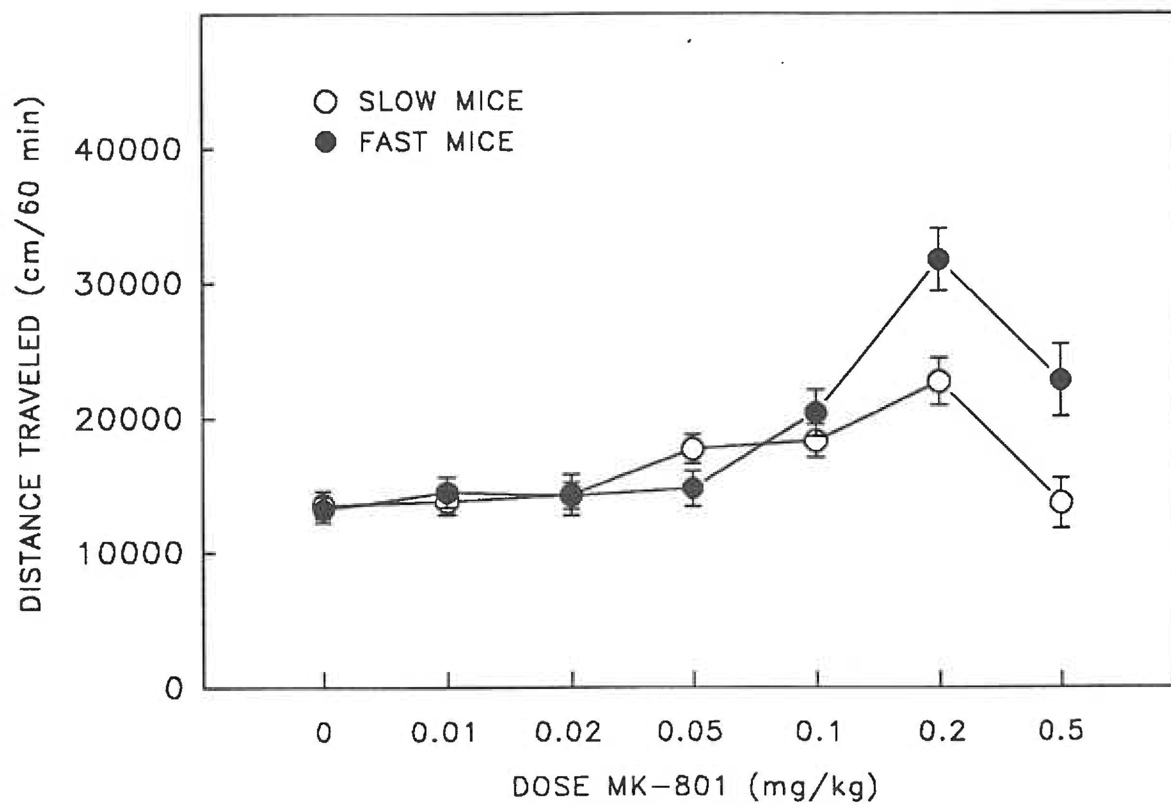
As reviewed above, several lines of evidence suggest an interaction of EtOH with glutamatergic systems to produce its effects. Behavioral effects of EtOH can be modulated by NMDA glutamate receptor ligands, and electrophysiological and biochemical data suggest that EtOH directly interacts with NMDA receptors. As previously noted, the inhibitory effects of EtOH on glutamate-stimulated Ca^{2+} influx differed in microsacs obtained from brains of FAST and SLOW mice (Daniell and Phillips, 1994 (Daniell and Phillips, 1994a). These data suggest that NMDA receptor function was differentially altered by selection of FAST and SLOW mice. Experiment 2.1 tested this hypothesis by examining locomotor responses to the noncompetitive NMDA receptor antagonist, MK-801, which has locomotor stimulant properties in rodents. It was further hypothesized that differences in EtOH stimulant sensitivity

between D2 and B6 mice are due to differences in NMDA receptor function, and that these strains would also differ in locomotor responses to MK-801.

Methods. Locomotor effects of the noncompetitive NMDA receptor antagonist, MK-801, were assessed in FAST and SLOW mice ($S_{36}G_{37}$ - $S_{36}G_{38}$; 55 - 90 days of age) in one experiment, and B6 and D2 mice (65 - 73 days of age) in another experiment. The experimental protocol was identical for both studies. Mice were injected with saline or 1 of 6 doses of (+)-MK-801 hydrogen maleate (0.01, 0.02, 0.05, 0.1, and 0.2 mg/kg), and immediately placed in activity monitors for 60 minutes. Data were collected in 12 5-minute epochs. In addition to assessing possible genotypic differences in locomotor response to EtOH, the time course of MK-801 effects on locomotor activity was determined in order to establish injection time parameters for the subsequent experiment.

Results in FAST and SLOW mice. In order to assess genotypic differences in locomotor response to MK-801, total distance traveled during the 60-minute test session was calculated for each animal, and analyzed by 3-way ANOVA grouped on line, replicate, and MK-801 dose. There was a significant main effect of replicate, but no significant interactions involving this variable, and data are presented collapsed on replicate in Figure 14. Simple effects analysis was performed to characterize the significant interaction between line and MK-801 ($F[6,252] = 6.6$, $p < 0.001$). Doses up to 0.05 mg/kg MK-801 had no effects on locomotor activity in either line. However, 0.1, 0.2 and 0.5 mg/kg MK-801 significantly increased total distance traveled by FAST mice. Although the highest dose of MK-801 produced locomotor activation

Figure 14. Effects of increasing doses of the noncompetitive NMDA receptor antagonist, MK-801, on locomotor activity of SLOW and FAST mice. Data are presented collapsed on replicate. Mice were injected with saline or a single dose of MK-801 and immediately placed in activity monitors. Cumulative distance traveled during the 60-minute test duration are presented. Vertical lines are S.E.M.; n = 10 per line, replicate, and dose.



SLOW MICE
FAST MICE
DISTANCE TRAVELED (cm/60 min)
40000
30000
20000
10000
0
0
0.01
0.02
0.05
0.1
0.2
0.5
DOSE MK-801 (mg/kg)

relative to saline activity, 0.5 mg/kg was on the descending limb of a biphasic dose-response curve, which was characterized by peak activation in FAST mice administered 0.2 mg/kg. SLOW mice were significantly stimulated by 0.2 mg/kg MK-801, but not by any other dose. There were significant differences between the lines at 0.2 and 0.5 mg/kg MK-801. SLOW mice were significantly less stimulated by 0.2 mg/kg than FAST mice. In addition, whereas FAST mice were stimulated by 0.5 mg/kg MK-801, the activity of SLOW mice given this dose was not different from saline activity. Thus, SLOW mice could be characterized as having a "flattened" biphasic dose-response curve relative to FAST mice, with decreased sensitivity to the stimulant effects of MK-801.

The effects of MK-801 were assessed during the course of the 60-minute test, largely to aid in determining injection time parameters for a subsequent experiment (Experiment 2.2). Time course data were analyzed by mixed 4-factor ANOVA with line, replicate, and MK-801 dose as between- groups factors, and time (epoch) as a within-groups factor. Replicate 1 mice were significantly more active compared to replicate 2 mice (main effect $F[1,252] = 113.9$, $p < 0.001$), and there was a significant 3-way interaction of replicate, MK-801, and time ($F[66,2772] = 3.1$, $p < 0.01$). This interaction was largely due to the higher activity of replicate 1 mice, not differences in pattern of MK-801 response; thus, data are shown collapsed on replicate in Figure 15a for SLOW mice and Figure 15b for FAST mice. In addition to replicate effects, there was a significant 3-way interaction between line, MK-801, and time ($F[66,2772] = 3.2$, $p < 0.01$). Further characterization of each line's locomotor response to MK-801 was accomplished by 2-way ANOVA,

Figure 15a. Time course of MK-801 effects on locomotor activity in SLOW mice (collapsed on replicate). Each 5-minute data sample is shown for each MK-801 dose, relative to saline, in panels A - F. For procedural details, see Figure 14 legend.

FIGURE 15A

SLOW MICE

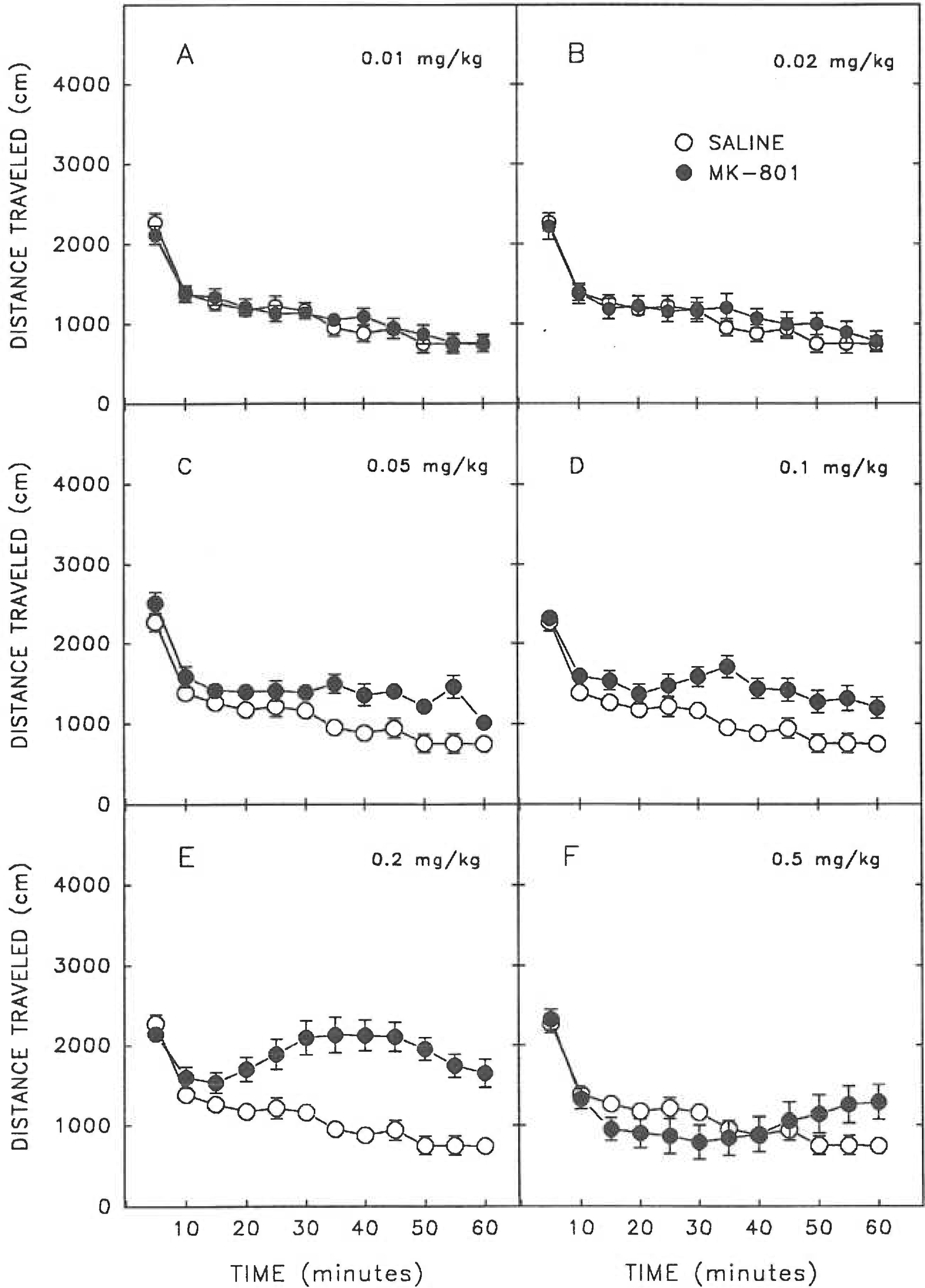
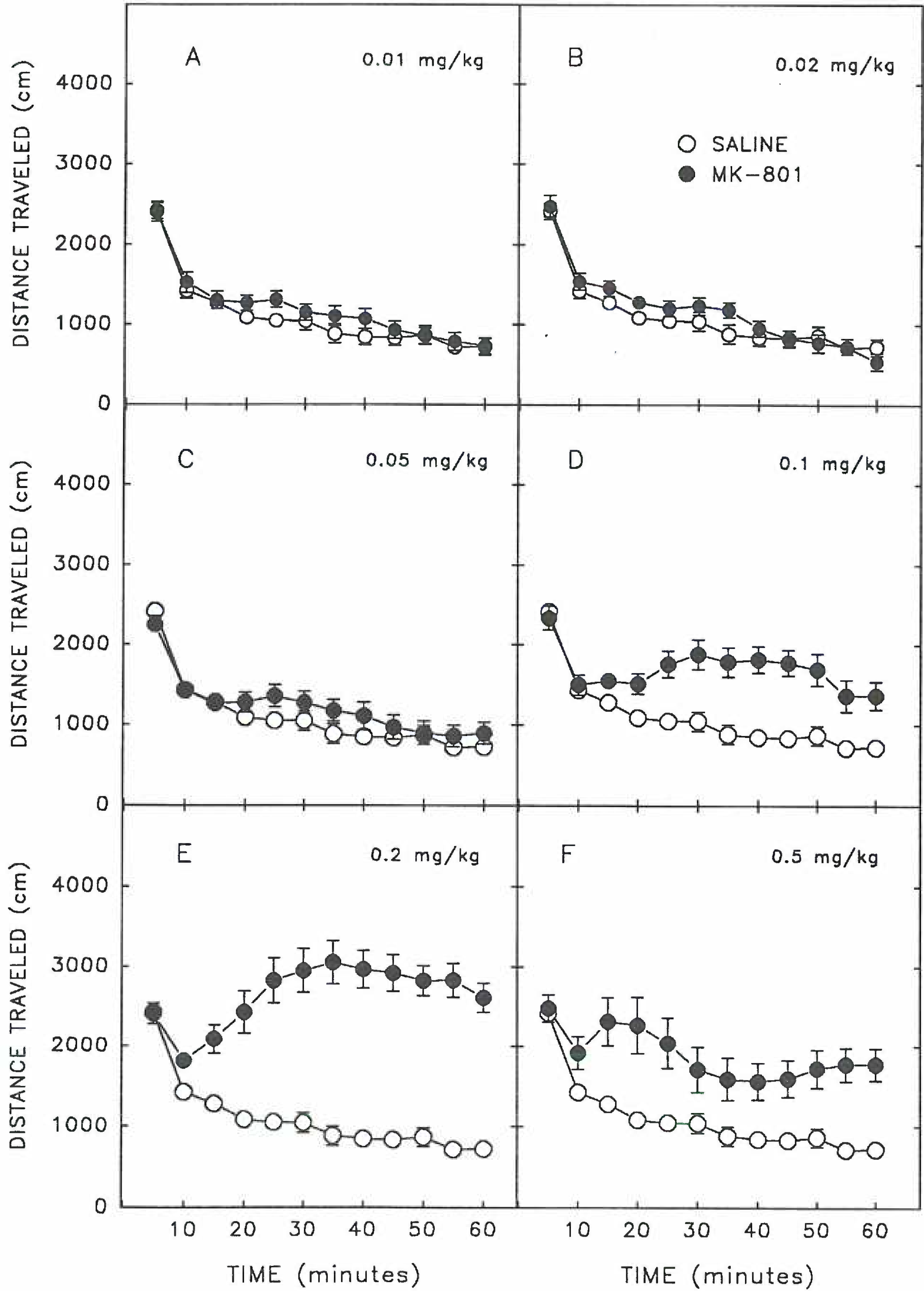


Figure 15b. Time course of MK-801 effects on locomotor activity in FAST mice (collapsed on replicate). See Figure 14 and 15a legends for procedural details. Vertical lines are S.E.M.

FIGURE 15B

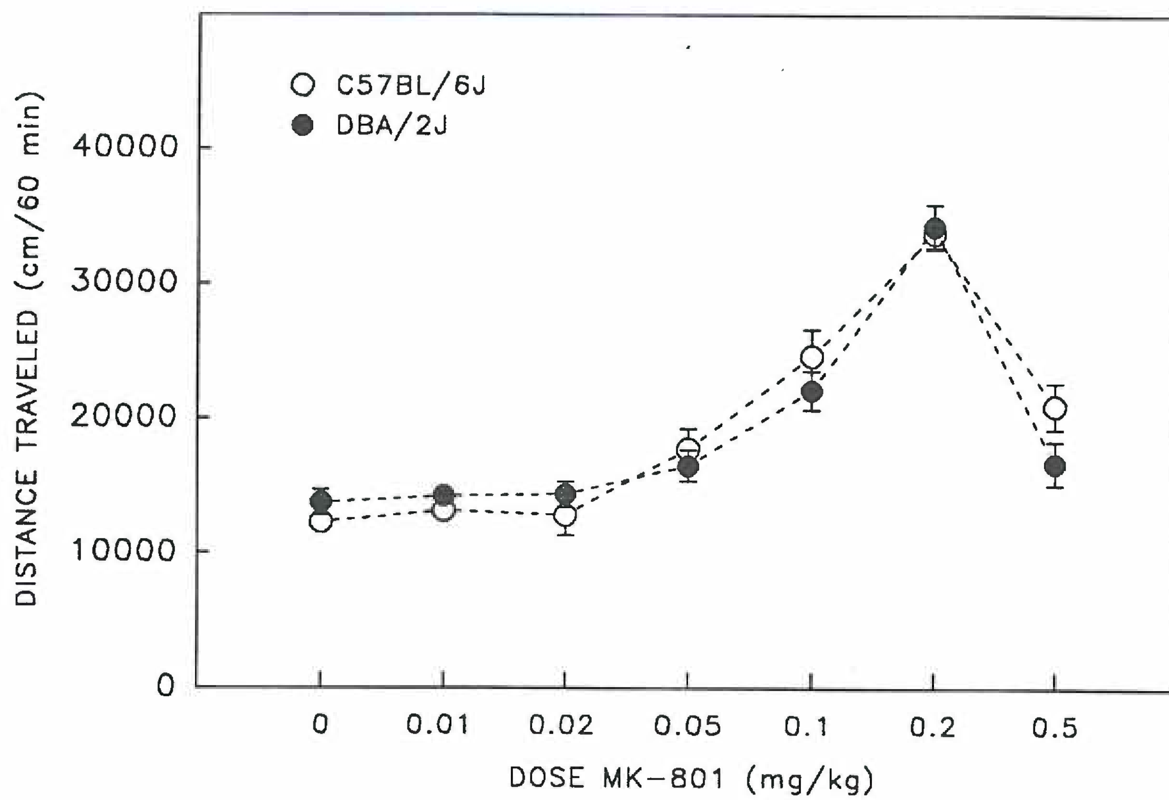


grouped on MK-801 as a between- and time as a within-groups factor (collapsed on replicate). These analyses revealed significant MK-801 x time interactions for both SLOW ($F[66,1463] = 5.0$, $p < 0.001$) and FAST mice ($F[66,1463] = 11.2$, $p < 0.001$). Only 0.2 mg/kg significantly increased activity of SLOW mice (relative to saline-treated mice) beginning at T = 25 minutes post-injection and lasting through T = 60; 0.1 mg/kg significantly increased activity of SLOW mice at T = 35, but this dose had no effects at any other time point. There were no effects of any other dose at any time point for SLOW mice. In FAST mice, 0.2 and 0.5 mg/kg MK-801 significantly increased activity relative to saline controls at every time point from T = 15 to T = 60 (except T = 30 at which 0.5 mg/kg barely missed significance). In addition, locomotor activity was significantly increased by 0.1 mg/kg MK-801 between T = 25 and T = 50. These time course results were consistent with cumulative data with regard to MK=801 doses which produced significant locomotor activation. Overall, evaluation of time course effects showed that, the onset of MK-801's stimulant effects, when they occurred, was generally between 15 and 20 minutes post-injection.

Results in B6 and D2 mice. Similar to its effects in FAST and SLOW mice, MK-801 altered the locomotor activity of B6 and D2 mice in a dose-dependent biphasic manner. Unlike the results observed in FAST and SLOW mice, there were no differences between the inbred strains in magnitude of stimulation, as shown in Figure 16. Two-way ANOVA for total distance traveled, grouped on strain and MK-801 dose, revealed a significant main effect of MK-801 treatment ($F[6,123] = 57.3$, $p < 0.001$), but no effect of strain, and no strain x MK-801 interaction.

Figure 16. Effects of MK-801 on locomotor activity of B6 and D2 mice. Animals were injected with saline or one dose of MK-801 and immediately placed into locomotor activity test chambers for 60 minutes. Data are presented as total distance traveled during the 60-minute test. Vertical lines are S.E.M.; n = 9 - 10 per strain and dose group.

FIGURE 16



Large increases in locomotor activity, which resulted from administration of 0.1, 0.2, and 0.5 mg/kg MK-801, were significantly different from saline activity. Similar to FAST and SLOW mice, peak activation occurred at 0.2 mg/kg MK-801, with 0.5 mg/kg falling on the descending limb of a biphasic dose-response curve.

Time course data were analyzed by mixed 3-factor repeated measures ANOVA grouped on strain and MK-801 as between-groups factors, and time as a within-groups factor. A significant 3-way interaction of strain, MK-801, and time ($F[66,1353] = 3.6$, $p < 0.01$) was further characterized for each strain by 2-way repeated measures ANOVA, grouped on MK-801 as a between- and time as a within-groups factor. Significant MK-801 x time interactions were found for D2 ($F[66,682] = 12.4$, $p < 0.001$) and B6 mice ($F[66,671] = 9.7$, $p < 0.001$). Time course data are presented in Figures 17a (B6) and 17b (D2). Beginning around T = 15 and T = 20, 0.2 mg/kg and 0.1 mg/kg MK-801 (respectively) produced locomotor activation in B6 mice that lasted the duration of the activity test. The locomotor activity of B6 mice given 0.5 mg/kg was significantly increased at T = 15, and T = 45 through T = 60. Although not significantly different from saline-treated mice, the locomotor activity of mice given 0.5 mg/kg MK-801 was still slightly elevated between T = 20 through T = 40. The pattern of results was only slightly different in D2 mice. Beginning at T = 10, 0.2 and 0.5 mg/kg MK-801 significantly increased activity of D2 mice compared to the activity of saline-injected mice at the same time points. Activity levels of mice administered 0.2 mg/kg MK-801 remained elevated relative to saline controls throughout the 60-minute test; however, beginning at T = 25, the locomotor activity of mice injected

Figure 17a. Time-course of MK-801 effects on locomotor activity in B6 mice. Each 5-minute data sample is presented for each dose (panels A - F) relative to saline-injected animals. Vertical lines are S.E.M.

FIGURE 17A

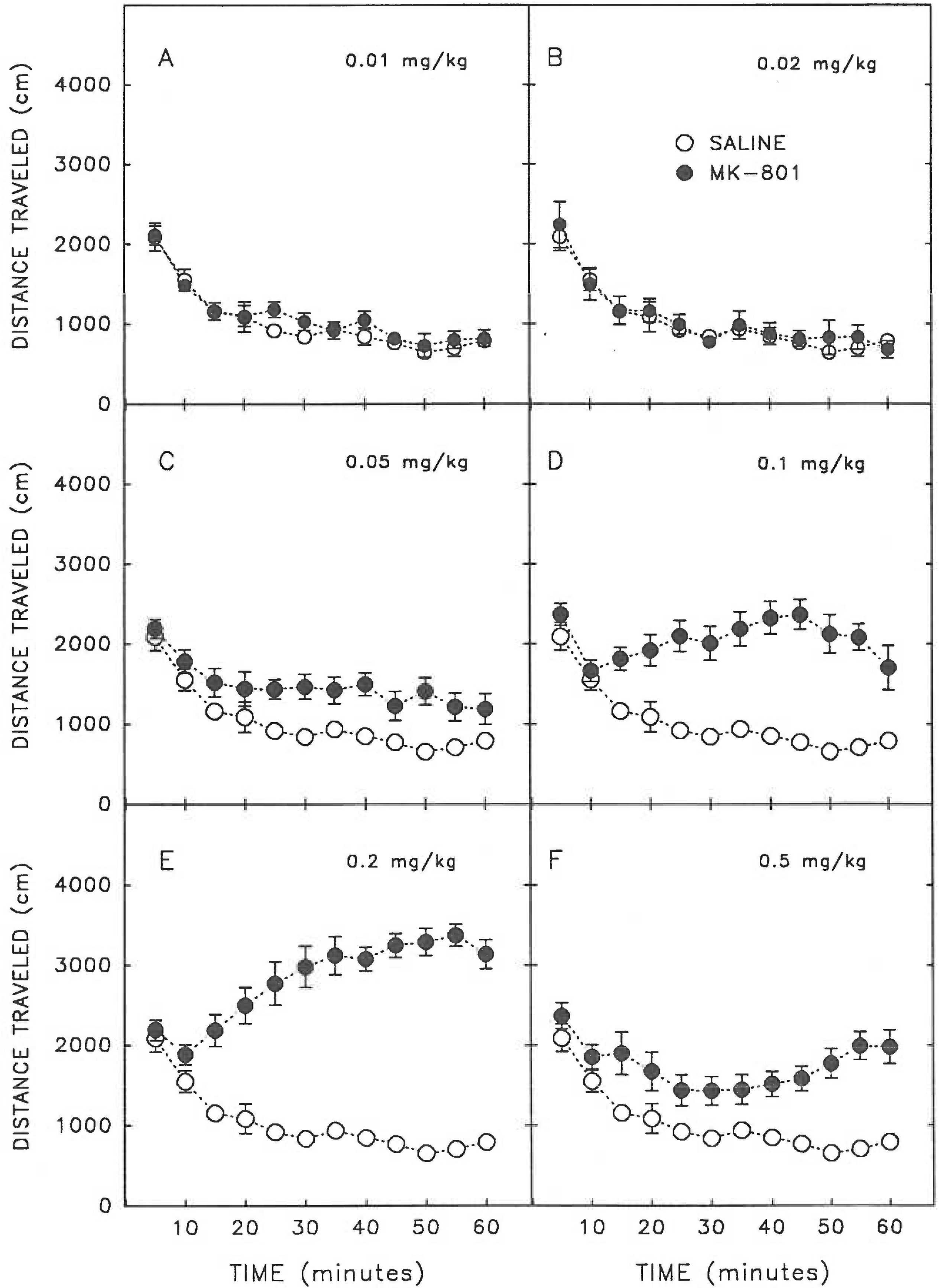
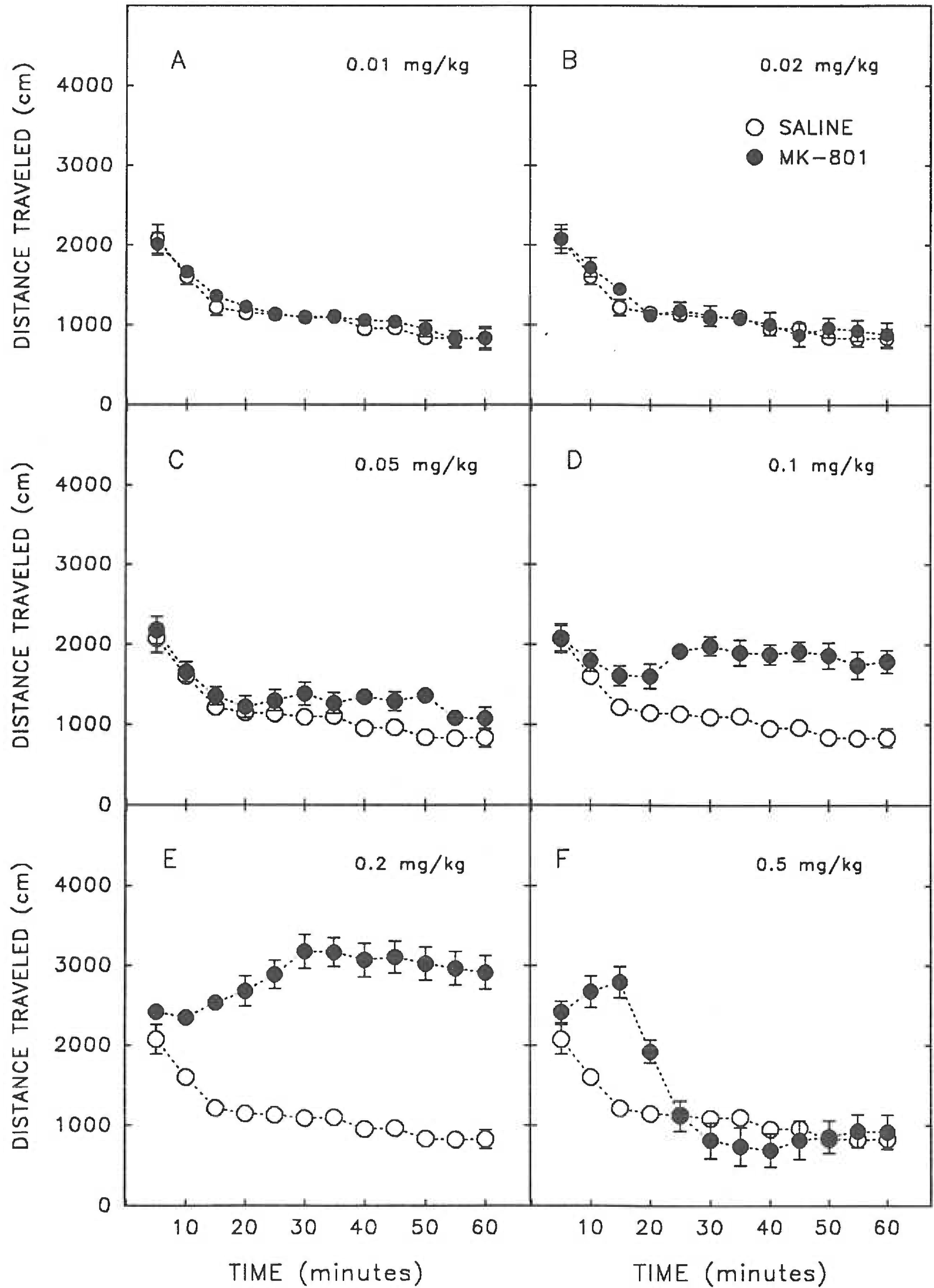


Figure 17b. Time-course of MK-801 effects on locomotor activity of D2 mice. See Figure 16 and Figure 17a legends for more detail.


DBA/2J
A
0.01 mg/kg
B
0.02 mg/kg
SALINE
MK-801
C
0.05 mg/kg
D
0.1 mg/kg
E
0.2 mg/kg
F
0.5 mg/kg
DISTANCE TRAVELED (cm)
0
1000
2000
3000
4000
10
20
30
40
50
60
TIME (minutes)

with 0.5 mg/kg dropped dramatically to levels roughly equal to that of saline-injected mice. At the same time (T = 25), 0.1 mg/kg MK-801 produced significant increases in locomotor activity of D2 mice that lasted through the rest of the test session. Overall, these results suggested slight strain differences with regard to onset of MK-801 effects around 10 - 15 minutes post-injection (D2 before B6), and strain differences in pattern of response to 0.5 mg/kg during the course of the activity test; however, the clear differences in MK-801 sensitivity observed in FAST and SLOW selected lines were not observed in these inbred strains.

Summary. MK-801 increased locomotor activity in FAST, SLOW, B6 and D2 mice, but only at the higher doses tested here. Differences in sensitivity to these locomotor activating effects were found between FAST and SLOW mice, with FAST mice showing greater stimulation in response to MK-801. B6 and D2 mice did not differ in sensitivity to MK-801's activating effects. In addition to dose-response curves, the effects of MK-801 during the 60-minute test duration were examined. Locomotor responses were most noticeable approximately 20 minutes post-injection in FAST and SLOW mice, and approximately 10 - 15 minutes post-injection in the B6 and D2 inbred strains. When present, MK-801 effects were long-lasting, and were observed through the duration of the 60-minute activity test. Injection time parameters for subsequent experiments, which assessed MK-801 effects on locomotor responses to EtOH, were established based upon these time course data.

Experiment 2.2: Effects of MK-801 and EtOH Coadministration

Administration of MK-801 has been demonstrated to potentiate or alter several behavioral effects of EtOH, including anticonvulsant effects (Deutsch et al., 1991), sedation (Draski et al., 1995; Ferko, 1992), and locomotor activity (Draski et al., 1995; Kuribara, 1994). In the following experiments, the effects of MK-801 on locomotor activity responses to several doses of EtOH were assessed in FAST, SLOW, B6, and D2 mice. As discussed previously (see "Hypotheses and Rationale"), MK-801 was predicted to potentiate or enhance the locomotor stimulant effects of EtOH in FAST and D2 mice. On the other hand, it was speculated that MK-801 could either potentiate or reverse the locomotor effects of EtOH in SLOW and B6 mice.

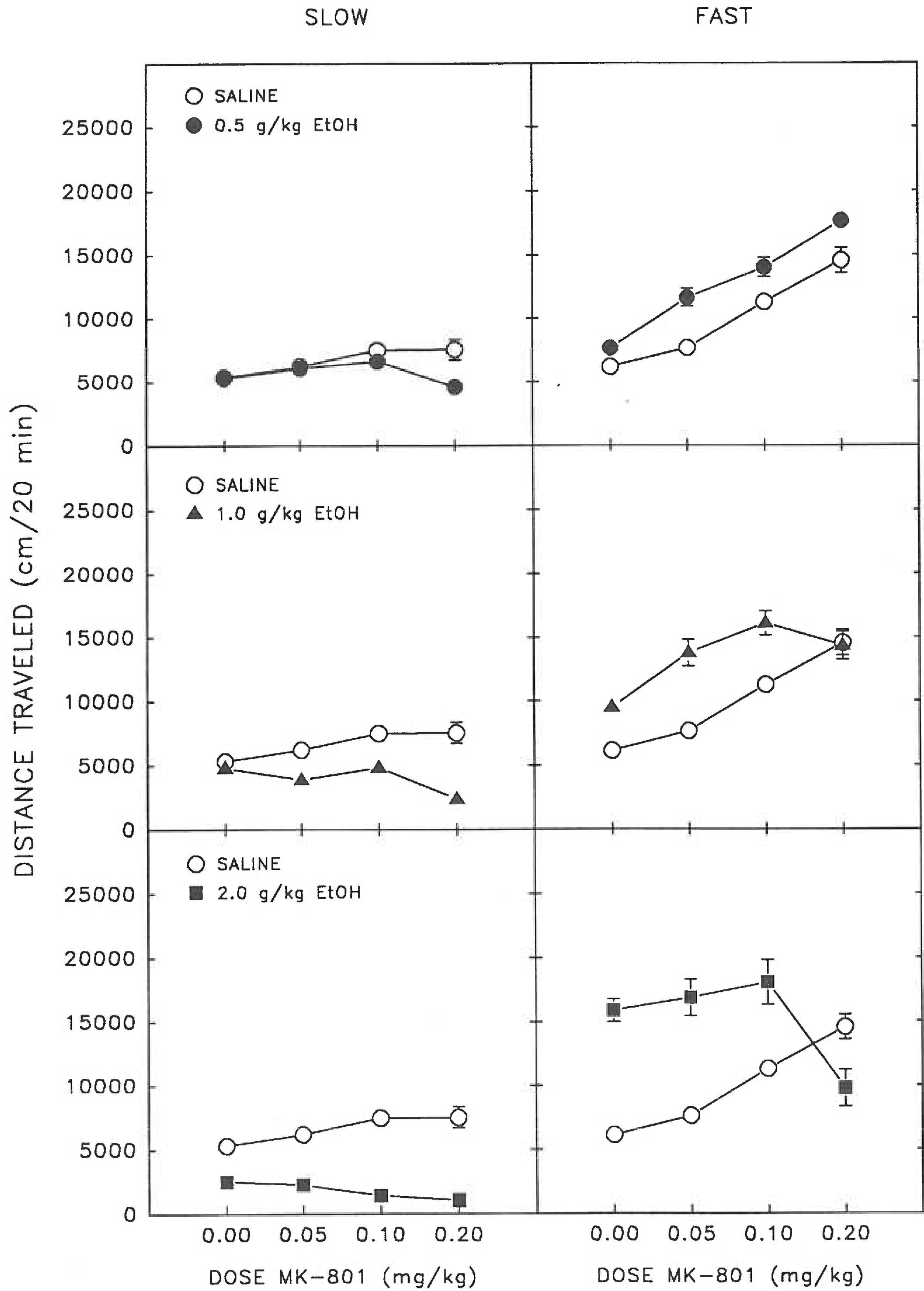
Methods. Male $S_{36}G_{38}$ - $S_{36}G_{40}$ FAST and SLOW mice (48 - 83 days of age), and male B6 and D2 mice (53 - 67) days of age, were tested for locomotor activity in response to coadministration of MK-801 and EtOH. B6 and D2 mice were tested simultaneously in one experiment; FAST and SLOW mice were tested simultaneously in another experiment. However due to availability of animals, it was not always possible to test both replicates of FAST and SLOW mice at the same time. Saline or (+)-MK-801 hydrogen maleate (0.05, 0.1, or 0.2 mg/kg) was injected 20 minutes prior to injection of saline or 1 of 3 doses of EtOH. A 20-minute activity test began immediately after the second injection. Doses of MK-801 used in this experiment were chosen, on the basis of data collected in the previous experiment, to be noneffective, moderately effective, or highly effective in producing locomotor effects on their own. Similarly, doses of EtOH were chosen so that a noneffective, a moderately effective, and

a highly effective dose was used in each experiment. Thus, FAST and SLOW mice were tested with 0.5, 1.0, or 2.0 g/kg, whereas B6 and D2 mice were injected with 0.5, 1.0, or 1.5 g/kg EtOH. The 20-minute pretreatment time was chosen based data reported in Experiment 2.1, in which MK-801 effects on locomotor activity were more apparent approximately 20 minutes post-injection. Although the onset of MK-801 effects occurred earlier in B6 and D2 mice than in FAST and SLOW mice, the differences were slight. Injection time parameters were kept constant between the experiment using inbred strains and the experiment using selected lines in order to maintain consistency. Ideally, 10 mice per strain were tested in each of the sixteen treatment groups; however, due to experimenter error, 9 FAST-1 mice were tested in one of the treatment groups and 11 FAST-1 mice were tested in another. After the activity test, EtOH-treated mice were removed from the testing apparatus and retroorbital sinus blood samples were obtained. All mice were humanely euthanized after the activity test.

Results in FAST and SLOW mice. Blood ethanol concentrations obtained from EtOH-treated animals were in the expected range for most animals; however, two SLOW-1, two FAST-1, and one FAST-2 mouse had BECs that were at least 2.5 standard deviations away from the mean, indicating either a misplaced injection or an incorrect dose. These animals were subsequently excluded from analysis of locomotor activity and BEC.

Locomotor activity data are presented in Figure 18, and show the effects of increasing doses of MK-801 on locomotor activity responses to EtOH in SLOW and FAST mice. Data were first analyzed by 4-way ANOVA

Figure 18. Effects of MK-801 administration on locomotor activity of EtOH-treated SLOW (left panels) and FAST mice (right panels). S.E.M. larger than symbol size are shown; n = 9 - 11 per line, replicate, and treatment group.


SLOW
FAST
SALINE
0.5 g/kg EtOH
SALINE
1.0 g/kg EtOH
SALINE
2.0 g/kg EtOH
DISTANCE TRAVELED (cm/20 min)
25000
20000
15000
10000
5000
0
0.00
0.05
0.10
0.20
DOSE MK-801 (mg/kg)

grouped on line, replicate, MK-801 dose, and EtOH dose. There was a main effect of replicate ($F[1,571] = 9.7$, $p < 0.01$), and nearly all two- and three-way interactions involving replicate were significant (all p's < 0.01), with the exception of the line x replicate interaction, and the three-way interaction between replicate, MK-801, and EtOH. The 4-way interaction just reached significance ($F[9,571] = 1.87$, $p = 0.054$). These results suggested the presence of replicate-dependent differences in total distance traveled; however, comparison of results of 2-way ANOVA (grouped on MK-801 and EtOH) for each line and replicate showed that the overall responses of SLOW-1 and SLOW-2 mice did not differ, and the responses of FAST-1 and FAST-2 mice did not differ. Thus, data are presented collapsed on replicate. Because characterization of the responses of each set of lines was desired, data were further analyzed by 2-way ANOVA grouped on MK-801 and EtOH treatment for FAST and SLOW mice separately. Simple effects analysis of a significant MK-801 x EtOH interaction ($F[9,302] = 4.5$, $p < 0.001$) in SLOW mice, followed by Tukey mean comparisons, showed that EtOH did not alter locomotor activity of SLOW mice, except at the highest dose (2.0 g/kg), which significantly decreased locomotion ($p < 0.01$). In contrast, MK-801 (0.1 and 0.2 mg/kg) significantly increased the activity of SLOW mice when given alone ($p < 0.01$). Given this stimulant effect of MK-801, one might expect that the activity of EtOH-treated SLOW mice would also be increased by MK-801; however, this was not the case. Rather, MK-801 appeared to potentiate the locomotor depressant effects of EtOH in SLOW mice, particularly at subeffective doses of EtOH. For example, while baseline activities were equivalent for saline- and EtOH-treated (0.5

g/kg) mice, increasing doses of MK-801 caused the activities of saline- and EtOH-treated mice to diverge so that EtOH-treated mice given 0.2 mg/kg MK-801 were significantly less active than mice that received this dose of MK-801 alone. Furthermore, 0.2 mg/kg MK-801 significantly decreased the locomotor activity of SLOW mice given 1.0 g/kg EtOH compared to animals that received this dose of EtOH alone. MK-801 did not alter the locomotor depression produced by 2.0 g/kg EtOH in SLOW mice, possibly due to a floor effect. An alternative interpretation is that EtOH blocked the locomotor stimulant effects of MK-801, since the activity of SLOW mice given 1.0 or 2.0 g/kg EtOH after each dose of MK-801 was significantly lower than mice given the appropriate MK-801 dose alone.

The effects of MK-801 on EtOH-induced locomotor activity in FAST mice were slightly more complex. Both potentiation and antagonism of EtOH's stimulant effects were observed, depending on the combination of MK-801 and EtOH that was administered. Simple effects analysis of an MK-801 x EtOH interaction in FAST mice ($F[9,301] = 12.3$, $p < 0.001$), showed that 0.1 and 0.2 mg/kg MK-801 alone significantly increased the activity of FAST mice. EtOH administration increased the locomotor activity of FAST mice in a dose-dependent manner in the absence of MK-801. Locomotor activity levels of mice given 2.0 g/kg EtOH (no MK-801) were significantly higher than those of saline-treated mice, but the locomotor stimulation produced by 1.0 g/kg EtOH just missed significance. At substimulant doses of EtOH (0.5 and 1.0 g/kg), pretreatment with MK-801 significantly enhanced stimulation, even at an MK-801 dose which did not alter locomotor activity on its own (0.05

mg/kg). More specifically, each group of mice which received MK-801 prior to 0.5 g/kg EtOH was more active compared to mice given only 0.5 g/kg EtOH. All doses of MK-801 significantly enhanced the activity of mice given 1.0 g/kg EtOH, compared to mice given 1.0 g/kg alone. However, there was a hint of a biphasic effect since the activity of 1.0 g/kg EtOH-treated mice given the highest dose of MK-801 (0.2 mg/kg) did not differ from animals which received this dose of MK-801 alone. This biphasic effect was more clearly observed in FAST mice given 2.0 g/kg. The stimulant effects of 2.0 g/kg EtOH were not significantly potentiated by any dose of MK-801, but were reversed by a dose of MK-801 (0.2 mg/kg) which produced locomotor stimulation on its own. This is supported by the result that the locomotor activity of mice given 0.2 mg/kg MK-801 in conjunction with 2.0 g/kg EtOH was significantly lower than that of mice given either 0.2 mg/kg MK-801 or 2.0 g/kg EtOH alone.

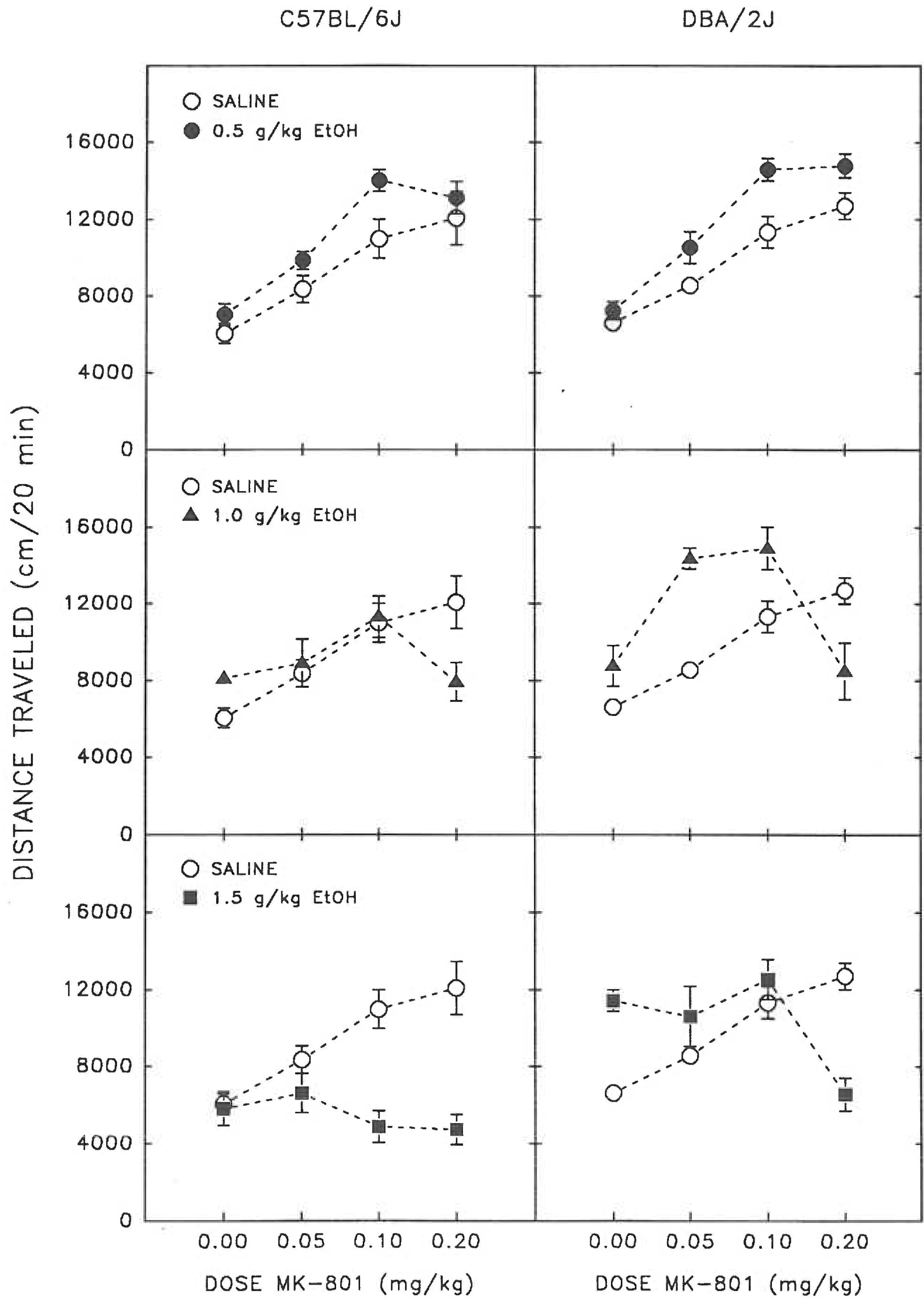
There were EtOH dose-dependent increases in BEC, and SLOW mice consistently had higher BEC than FAST mice. BECs (± S.E.) for SLOW mice were 0.395 ± 0.01, 1.06 ± 0.02, and 2.39 ± 0.02 mg/ml; BECS for FAST mice were 0.38 ± 0.02, 0.97 ± 0.02, and 2.26 ± 0.02 mg/ml, for 0.5, 1.0, and 2.0 g/kg EtOH, respectively, collapsed on MK-801 treatment. There were no effects of MK-801 on BEC; thus, alterations in locomotor responses to EtOH were not due to effects of MK-801 on EtOH pharmacokinetics (data not shown).

Results for B6 and D2 mice. Three-way ANOVA grouped on strain, MK-801, and EtOH treatment revealed a significant 3-way interaction for total distance traveled during the 20-minute test ($F[9,288] = 2.06$, $p < 0.05$), and examination of the data suggested that the strains differed

in their locomotor responses to EtOH and MK-801 administration (see Figure 19). Thus, further statistical analyses were performed separately for each strain, grouped on MK-801 and EtOH. Two-way ANOVAs revealed significant MK-801 x EtOH interactions for both B6 ($F[9,144] = 5.0$, $p < 0.01$) and D2 mice ($F[9,144] = 10.3$; $p < 0.01$). B6 mice, whose locomotor activity was not significantly affected by any dose of EtOH, were significantly activated by 0.1 and 0.2 mg/kg MK-801 alone. Coadministration of 0.1 mg/kg MK-801 with 0.5 g/kg appeared to cause locomotor stimulation that was higher compared to either drug alone; however, there were no significant differences detected, likely due to variability of the measure in B6 mice. In general, administration of MK-801 prior to 1.0 g/kg EtOH resulted in locomotor activity levels of saline- and EtOH-treated mice that were equivalent, and increased with increasing doses of MK-801. One interesting exception was that the highest dose of MK-801 (0.2 mg/kg) injected prior to 1.0 g/kg EtOH decreased locomotor activity to levels similar to 1.0 g/kg EtOH alone, resulting in a biphasic response profile, and indicating that potentiation in the direction of locomotor depression may have occurred in this case. The locomotor activity of B6 mice treated with 1.5 g/kg EtOH was unaffected by administration of MK-801. However, similar to SLOW mice, it appears that EtOH blocked the locomotor stimulant effects of MK-801.

In contrast to B6 mice, MK-801 pretreatment did result in robust alterations of the activity of EtOH-treated D2 mice. MK-801 alone significantly increased locomotor activity (0.1 and 0.2 mg/kg) and, consistent with results of Experiment 5, the magnitudes of stimulation

Figure 19. Effects of MK-801 administration on locomotor activity of EtOH-treated B6 (left panels) and D2 mice (right panels). Saline or MK-801 was administered 20 minutes prior to injection of saline or EtOH, immediately followed by a 20-minute activity test. S.E.M. larger than symbol size are shown; n = 10 per strain and treatment group.


C57BL/6J
DBA/2J
SALINE
0.5 g/kg EtOH
SALINE
1.0 g/kg EtOH
SALINE
1.5 g/kg EtOH
DISTANCE TRAVELED (cm/20 min)
0
4000
8000
12000
16000
0.00
0.05
0.10
0.20
DOSE MK-801 (mg/kg)
DOSE MK-801 (mg/kg)

in D2 mice were similar to those observed in B6 mice. There were dose-dependent increases in locomotor activity as a result of EtOH administration, but this effect was significant only at the highest dose (1.5 g/kg). Overall, the effects observed in D2 mice were similar to those seen in FAST mice: potentiation of EtOH-stimulation occurred in response to combinations of low doses of MK-801 and EtOH, whereas antagonism of EtOH-stimulation occurred in response to combinations of high doses of MK-801 and EtOH. Potentiation of stimulation was significant and dose-dependent for D2 mice treated with 0.5 g/kg EtOH, and reached a plateau at 0.2 mg/kg MK-801. A biphasic effect of MK-801 was observed for D2 mice given 1.0 g/kg EtOH. Potentiation of stimulation was robust and significant at 0.05 mg/kg, reached a plateau at 0.1, and reversed at 0.2 mg/kg MK-801, so that locomotor activity of EtOH-treated D2 mice was equivalent to EtOH-alone, and below that of MK-801 alone. The stimulant effects of 1.5 g/kg EtOH were not enhanced by MK-801, but were reduced by 0.2 mg/kg, despite the robust locomotor stimulant effects of this MK-801 dose when given on its own.

There were EtOH dose-dependent increases in BEC, and D2 mice had consistently higher BEC than B6 mice. There were no effects of MK-801 administration on BEC. For 0.5, 1.0, and 1.5 g/kg EtOH, respectively, mean BEC (± S.E.) were 0.34 ± 0.01, 0.94 ± 0.01, and 1.55 ± 0.02 mg/ml for B6, and 0.36 ± 0.02, 1.03 ± 0.01, and 1.70 ± 0.02 mg/ml for D2 mice. Thus, changes in locomotor activity were not due to effects of MK-801 on EtOH pharmacokinetics.

Summary. In FAST and D2 mice, both potentiation and antagonism of EtOH-stimulation occurred in response to MK-801. These effects were

dependent on both MK-801 and EtOH, so that low dose combinations generally resulted in enhancement of activity, and high dose combinations resulted in antagonism of locomotor stimulation by EtOH. Interestingly, the antagonism of EtOH-stimulation occurred at a dose of MK-801 which robustly increased locomotor activity when given alone. In contrast to D2 and FAST mice, MK-801 did not increase locomotor activity of B6 or SLOW mice beyond its own stimulant effects. Rather, there was potentiation of locomotor depression, which was most evident in SLOW mice.

DISCUSSION

The experiments described here assessed the possible roles of GABA and glutamate systems in EtOH-stimulated activity, and further attempted to ascertain whether dopamine/GABA interactions, suggested by neuroanatomical evidence (e.g. Kalivas, 1993), are important for the expression of EtOH-stimulated activity. Two sets of genetic mouse models were studied: FAST and SLOW selected lines of mice, and D2 and B6 inbred strains. As discussed previously, these genetic mouse models provide unique tools for inquiry into neurochemical mechanisms of EtOH's locomotor effects. Whereas FAST and D2 mice are highly sensitive to EtOH's locomotor stimulant effects, SLOW and B6 mice are insensitive to activation by EtOH. SLOW mice are further characterized as being more sensitive to the locomotor depressant effects of EtOH. Genetic factors account for differences between FAST and SLOW mice, and between D2 and B6 mice. However, differences between FAST and SLOW mice were systematically produced through selective breeding, while differences

between the inbred strains arose by chance. The use of both sets of genetic models in these experiments allowed assessment of the generality of a particular result. When one neurotransmitter system is implicated in mediating responses of both replicates of FAST mice and in D2 mice, whose high sensitivity to EtOH's stimulant effects arose through different processes, the conclusion is that the importance of this particular mechanism is not specific to only one special case, but may be universally important.

Specific Aim 1

The experiments in Specific Aim 1 were designed to test hypotheses regarding the role of $GABA_B$ receptors in mediating sensitivity to the acute effects of EtOH. Results of these experiments provided strong support for the involvement of $GABA_B$ systems in mediating EtOH-stimulated locomotor activity, but did not support the involvement of an interaction between dopamine and $GABA_B$-mediated processes in mediating these effects.

Role of $GABA_B$ Receptors

Previous studies had demonstrated that the $GABA_B$ agonist, baclofen, dose-dependently reduced and blocked the stimulant response of FAST mice to EtOH (Shen, Harland and Phillips, 1995), suggesting that $GABA_B$ receptors are important in mediating EtOH's activating effects. The results of Experiment 1.1, in which baclofen decreased the EtOH-stimulated activity of D2 mice, but had little effect on baseline activity, suggest that $GABA_B$ receptors mediate EtOH-stimulation in this

inbred strain as well. Together with data from FAST mice, these results provide strong evidence for $GABA_B$ involvement in EtOH's locomotor stimulant effects. Specific $GABA_B$ receptor mediation of baclofen's effects was supported by the results that CGP-35348, a $GABA_B$ antagonist, reversed the inhibitory effects of baclofen on EtOH-stimulated activity in both FAST and D2 mice. These data are consistent with other studies which have demonstrated $GABA_B$ involvement in EtOH stimulation in other mouse genotypes (Cott et al., 1976; Humeniuk et al., 1993). A possible confound is that baclofen affects muscle tone (Nevins, Nash and Beardsley, 1993; Wolfarth et al., 1992), which may also affect locomotor activity. However, baclofen doses required to reduce muscle tone in mice (as measured by grip strength) were relatively high (10 and 17.8 mg/kg), and caused significant decreases in baseline locomotion (Nevins et al., 1993). In the studies described here, EtOH-stimulated activity was decreased by much lower doses of baclofen (0.625 - 5.0 mg/kg) which had no effect on baseline activity, suggesting that these doses likely were not high enough to decrease muscle tone. On the other hand, EtOH also has muscle relaxant effects, and a synergistic effect of baclofen and EtOH administration may have occurred. A test of muscle tone in FAST and D2 mice given dose combinations of baclofen and EtOH used in the experiments described herein may help to resolve this possible confound.

Since activation of $GABA_B$ receptors by baclofen blocked EtOH-stimulated activity in FAST and D2 mice, it was postulated that administration of a $GABA_B$ antagonist could produce locomotor stimulation in response to EtOH in mice relatively insensitive to EtOH's activating

effects. However, preliminary studies in SLOW-1 and B6 mice showed that the $GABA_B$ antagonist, CGP-35348, did little to alter the activity of EtOH-treated mice, suggesting that blockade of $GABA_B$ receptors did not confer sensitivity to EtOH's stimulant effects. It has been suggested that, as a result of selection, FAST mice have generally become more sensitive to EtOH's stimulant effects, and SLOW mice have become more sensitive to EtOH's sedative effects (Shen et al., in press). It may be that sensitivity to the sedative effects of EtOH is mediated by neurochemical substrates separate from those which mediate sensitivity to EtOH's stimulant effects, so that blockade of $GABA_B$ receptors has no effect on SLOW or B6 mice even though activation blocked EtOH-stimulation in FAST and D2 mice. Another possible explanation is that the $GABA_B$ system is only one of several modulatory inputs in the complex system underlying expression of locomotor activation, so that alteration of $GABA_B$ function alone is not sufficient to confer sensitivity to EtOH's stimulant effects. On the other hand, the hypothesis that administration of a $GABA_B$ receptor antagonist would have confer a locomotor stimulant response to EtOH rests on the assumption that $GABA_B$ receptors provide tonic inhibition. Blockade of receptors which are not tonically active would not be expected to produce any effect. The lack of $GABA_B$ antagonist effects on locomotor activity in the studies reported herein suggest that $GABA_B$ receptors may not be tonically active, especially given the high doses of CGP-35348 that were used (up to 200 mg/kg). These results are consistent with other data in which high doses of CGP-35348 had no effect on rotarod performance in rats or mice (Bittiger et al., 1990). However, Bittiger et al. (1990) provided

electrophysiological evidence that CGP-35348 increased firing frequencies of hippocampal neurons and increased neuronal excitability. Behaviorally, higher doses of this antagonist induced intensive grooming, hyperactivity, and stereotypy, but the doses (up to 1000 mg/kg) and concentrations used were so high that the specificity of these effects is a concern, especially since 300 mg/kg CGP-35348 has also been demonstrated to decrease locomotor activity in mice (Mead and Little, 1995). Thus, while the data are somewhat contradictory, some evidence suggests that $GABA_B$ receptor-mediated tonic inhibition does not occur, which may provide one explanation with regard to the inability of a $GABA_B$ antagonist to confer EtOH stimulant responses in SLOW and B6 mice.

In conclusion, $GABA_B$ receptors appear to be involved in EtOH's stimulant effects, but not in determining insensitivity to EtOH stimulation.

$GABA_B$/Dopamine Interactions

A second set of experiments in Specific Aim 1 addressed the hypothesis that $GABA_B$ and dopamine-mediated events interact as part of the neural circuitry which mediates EtOH-stimulation. $GABA_B$ receptors have been shown to have inhibitory effects on release of several neurotransmitters, including dopamine (Goudreau et al., 1994; Mott and Lewis, 1994; Santiago et al., 1993). Dopaminergic system involvement in locomotor stimulation by EtOH has been demonstrated in FAST mice (Shen, Crabbe and Phillips, 1995), and in other mouse genotypes (e.g. Dudek et al., 1984; Koechling et al., 1990; Liljequist et al., 1981). It was

hypothesized that inhibition of EtOH-stimulated activity by baclofen was due to inhibition of dopaminergic activity, and that administration of a dopamine agonist would reverse this inhibition. Contrary to this hypothesis, reversal of baclofen's inhibitory effects was not observed in response to any of the dopamine agonists tested. Administration of methamphetamine, which causes release of endogenous dopamine, also did not have the predicted effect. Thus, these data do not support the involvement of dopamine/$GABA_B$ interactions in mediating locomotor responses to EtOH.

Specific Aim 2

Genotypic Differences in Sensitivity to Locomotor Effects of MK-801

Specific Aim 2 addressed the possible involvement of the NMDA-type glutamate receptor in mediating locomotor responses to EtOH. FAST and SLOW mice were stimulated by MK-801, and showed differential sensitivity to this effect at the higher doses tested, suggesting that selection has altered NMDA receptor function between the lines. On one hand, these data are somewhat surprising given that NMDA receptor-mediated glutamate-stimulated Ca^{2+} flux did not differ between FAST and SLOW mice in the absence of EtOH (Daniell and Phillips, 1994a). However, Ca^{2+} flux was measured in FAST and SLOW mice from S_{21} - S_{22} (T.J. Phillips, personal communication), and current data were collected from $S_{36}G_{37}$ - $S_{36}G_{38}$. Basal glutamate-stimulated Ca^{2+} influx may differ in current generations of FAST and SLOW mice, as a result of continued selection. Data from FAST and SLOW mice suggest that NMDA receptor function is an important component of expression of EtOH-stimulated activity (discussed

below). Since all trait-relevant genes should be fixed in alternative allelic form between the lines, it might be expected that NMDA receptor sensitivity would be differentially altered between the lines.

In contrast to FAST and SLOW mice, B6 and D2 mice did not differ in their sensitivities to the locomotor stimulant effects of MK-801. As previously discussed, B6 and D2 mice were included in these studies as confirmation of results seen in FAST and SLOW selected lines. However, a different outcome in inbred strains does not necessarily negate the results seen in selected lines. One possible explanation for a discrepancy between these genetic animal models is that chance fixation of trait-irrelevant gene loci in FAST and SLOW mice has resulted in a false genetic correlation; however, the differences in MK-801 sensitivity were observed in both replicates, providing strong evidence for a correlated selection response. A second alternative is that chance fixation of alleles at trait-irrelevant loci in B6 and D2 mice masked strain differences in MK-801 sensitivity. Since the origins of FAST and SLOW mice involved 8 inbred strains, another alternative is that there are allelic variants (e.g. extreme increaser or decreaser alleles) in FAST and SLOW mice not present in B6 or D2 mice. Finally, it may be that B6 and D2 mice simply are not polymorphic at the critical locus or loci. A complex trait such as sensitivity to EtOH's stimulant effects is likely to be polygenically mediated, with each gene locus accounting for a small to moderate amount of phenotypic variance. Recent estimates indicate that EtOH stimulant sensitivity is mediated by 3 - 5 gene loci (Dudek and Tritto, 1994; Dudek and Underwood, 1993). As a result of systematic matings, all trait-relevant loci are

differentially fixed between bidirectionally selected lines. In contrast, even robust phenotypic differences between two inbred strains are largely due to arbitrary differences in genotype, and inbred strains are unlikely to be different at all trait-relevant loci. For example, D2 and B6 mice may differ at other gene loci mediating sensitivity to EtOH-induced activation, but not at the relevant NMDA receptor locus, especially if it does not account for a large proportion of phenotypic variance. One prediction is that, for a given phenotype, differences between selected lines are likely to be larger in magnitude compared to differences between two inbred strains. This is true for FAST and SLOW mice, compared to B6 and D2 mice, as seen in Figure 1. In addition, FAST mice are much more stimulated than D2 mice, whereas SLOW mice are much less sensitive to EtOH's stimulant effects compared to B6 mice.

In addition to effects on locomotor activity, high doses of MK-801 have been demonstrated to elicit stereotypic behaviors (Liljequist et al., 1991; Willets, Balster and Leander, 1990). The mechanisms underlying stereotypic effects are unknown, and may be related to nonspecific effects of MK-801. In rodents, these MK-801 induced-stereotypies are characterized by head weaving, ataxia, and body rolling, and were observed after administration of doses ≥ 0.5 mg/kg (Liljequist et al., 1991). It is possible that the biphasic effects of MK-801 on locomotor activity observed in these experiments were due to increased stereotypy at the highest dose tested (0.5 mg/kg). Analysis of stereotypy measures recorded by Omnitech activity monitors did not support this suggestion. However, the ability of automated monitors to accurately reflect traditionally experimenter-rated stereotypic

behaviors is unclear. In general, stereotypy and forward locomotion are competing behaviors so that increased stereotypy results in decreased forward locomotion. Thus, one would expect a negative correlation between measures of these behaviors. However, correlational analyses of data from the MK-801 dose-response study revealed either no relationship, and sometimes a positive relationship between stereotypy measured in the Omnitech monitors and distance traveled. Thus, analysis of stereotypy measures collected by Omnitech provided inconclusive results. An obvious method of determining whether biphasic dose-effects were due to stereotypic behaviors at higher doses is direct visual observation. Because the testing apparatus used in these studies did not allow visual access to the animals, behavior could not be observed during the activity test. However, no obvious stereotypic behavior was observed when animals were removed from the apparatus 60-minutes after MK-801 administration.

In conclusion, differences in sensitivity to MK-801 were found between FAST and SLOW mice of both replicates, but were not confirmed in the comparison of D2 and B6 mice. These data suggest that innate differences in NMDA receptor function, at least with respect to the MK-801 binding site, are not necessary for differential sensitivity to the stimulant effects of EtOH.

MK-801 Effects on EtOH-treated Mice

In addition to effects on baseline activity, MK-801 altered locomotor responses to EtOH in all genotypes. In FAST and D2 mice, combinations of MK-801 and EtOH increased locomotor activity when given

in low doses which alone had no effect on activity. A most striking result is that high dose combinations of EtOH and MK-801, which alone significantly increased locomotor activity of FAST and D2 mice, resulted in dramatic reductions in locomotor activity to near baseline levels. In contrast to its effects in FAST and D2 mice, MK-801 did not increase locomotor activity of SLOW and B6 mice, but appeared to potentiate the locomotor depressant effects of EtOH at higher dose combinations. Furthermore, the highest dose of EtOH tested in SLOW and B6 mice appeared to block the locomotor stimulant effects of MK-801. The consistency of the results among the FAST lines and D2 mice, and among B6 and SLOW mice suggests that these effects are not specific to just one inbred strain or one selected line, and suggest that NMDA receptors are important sites of EtOH effects on locomotor activity.

The effects of MK-801 on locomotor activity in HAS and LAS rats, bred for differential sensitivity to the sedative effects of EtOH as measured by loss of righting reflex (Draski et al., 1992; Spuhler et al., 1990), were investigated in a study similar to the experiments described here (Draski et al., 1995). MK-801 increased locomotor activity, and EtOH produced locomotor depression in both rat lines. Coadministration of MK-801 and EtOH caused an increase in the activity of LAS rats, above levels of MK-801 alone; however, the activity of HAS rats given MK-801 was not significantly altered by additional administration of EtOH. Locomotor activity of LAS rats has been reported to be activated (Krimmer, 1991) or unchanged (Schechter and Krimmer, 1992) by administration of low EtOH doses. In contrast, HAS rats have consistently been more sensitive to the locomotor depressant

effects of EtOH compared to LAS rats (Krimmer, 1991; Schecter and Krimmer, 1992). Comparison of results between HAS and LAS rats and FAST and SLOW mice is somewhat difficult, given the differences in species and selection phenotypes. On one hand, FAST mice could be comparable to LAS rats because they have been found to be less sensitive to the sedative effects of EtOH (Shen et al., in press), and LAS rats are relatively more active after EtOH administration. In this view, the enhancement of locomotor activity by the combination of MK-801 and EtOH in FAST mice is consistent with results in LAS rats, and the lack of further effect in SLOW mice by coadministration of the lower doses of EtOH and MK-801 are consistent with results in HAS rats. On the other hand, more recent data in LAS rats showing no change in locomotion produced by EtOH suggest a greater similarity to SLOW than to FAST mice. If comparison of LAS rats and SLOW mice is more appropriate, MK-801 and EtOH coadministration in HAS/LAS and FAST/SLOW selected lines did not produce consistent results. B6 and D2 mice, whose locomotor activities after administration of MK-801 and EtOH were consistent with those of FAST and SLOW mice, have been found to be both similar and different in sensitivity to loss of righting reflex, with B6 mice showing either greater or lesser sensitivity in different studies (see Phillips and Crabbe, 1991).

Administration of either MK-801 or EtOH alone produces a biphasic pattern of locomotor response, with an ascending limb of activation at lower doses and a descending limb of activation at higher doses (shown here for MK-801; for EtOH, see Pohorecky, 1977; Dudek et al., 1991; Shen et al., 1995). The enhancement of EtOH effects by MK-801 can be

conceptualized as a shift to the left of a biphasic EtOH dose-response curve, so that EtOH doses which generally have no effect now produce stimulation, and EtOH doses which were stimulating in the absence of MK-801 are now on the descending limb of the biphasic curve. As discussed previously, evidence suggests that EtOH has NMDA antagonist effects, much like MK-801, resulting in inhibition of glutamate-stimulated Ca^{2+} flux. In addition, EtOH's site of interaction appears to be separate from that of the MK-801 binding site, and may be a novel hydrophobic site on the NMDA receptor. The increased potency of EtOH in the presence of MK-801 could be due to increased numbers of bound NMDA receptors, or a greater functional inhibition, by administration of both ligands.

The absence of a stimulant response to EtOH in SLOW and B6 mice in the presence of MK-801 is consistent with the notion that MK-801 can shift the dose-response curve to the left, if one assumes that NMDA receptor sensitivity to EtOH is higher than that of FAST mice, resulting in behavioral responses that are already on the descending limb of the EtOH locomotor dose-response curve. Thus, further administration of MK-801 would potentiate the locomotor depressant or sedative effects of EtOH. Other data are consistent with this idea. It has been demonstrated that the sedative-hypnotic effects of high EtOH doses can be potentiated by high doses of MK-801 (Ferko, 1992; Wilson, Bosy and Ruth, 1990). The assumption that SLOW mice are more sensitive to EtOH-inhibition of NMDA receptors is supported by the demonstration that hippocampal and cerebral cortical microsacs derived from SLOW mice were more sensitive to EtOH's inhibitory effects on L-glutamate-stimulated

Ca^{2+} influx compared to microsacs derived from FAST mice (Daniell and Phillips, 1994a).

If it is true that insensitivity to EtOH's locomotor stimulant effects is due to high EtOH sensitivity of NMDA receptors in SLOW and B6 mice, no combination of MK-801 and EtOH doses would be likely to produce stimulation in these genotypes. The effects of MK-801 and EtOH coadministration in FAST mice seem to support this, since stimulation was not observed for any of the dose combinations tested, including those involving substimulant doses of MK-801 and EtOH. However, it is possible that even lower doses of MK-801 and EtOH would produce stimulation. In B6 mice, combinations of MK-801 with a low EtOH dose produced stimulation, suggesting that EtOH sensitivity of NMDA receptors in B6 mice is lower than that of SLOW mice.

<u>In conclusion, these data suggest an interaction between EtOH and NMDA systems to produce alterations in locomotor activity</u>. <u>Furthermore, the biphasic behavioral profiles suggest a complexity in this interaction that requires further investigation</u>.

Neural Circuitry of EtOH-Stimulated Locomotor Activity

The neural connections between VTA, N Acc, and VP/SI constitute what is now often referred to as the mesoaccumbens-pallidal circuit (Hooks and Kalivas, 1995). This circuit has previously been demonstrated to be important in regulating spontaneous, novelty- and psychostimulant-induced locomotor activity. As previously reviewed, there is evidence that EtOH administration activates the mesoaccumbens (mesolimbic) dopamine projection, as measured by increased firing rates

(Gessa et al., 1985), and increased dopamine transmission in N Acc (Diaz and Murgas, 1992; Imperato and DiChiara, 1986; Yoshimoto et al., 1992). Previous studies demonstrated the importance of dopamine (Shen, Crabbe and Phillips, 1995) and $GABA_B$ function (Shen, Harland and Phillips, 1995) in FAST mice. Furthermore, glutamatergic transmission has been implicated as an important factor in mediating locomotor responses to EtOH (Draksi et al., 1995; Kuribara, 1994; Liljequist 1991a; Robledo et al., 1991). These lines of evidence are consistent with mediation of EtOH-stimulated activity by the mesoaccumbens-pallidal circuit.

The $GABA_B$ receptor-mediated system appears to be an important modulatory influence of the mesoaccumbens dopamine projection, and this interaction has been demonstrated to mediate the activating effects of psychomotor stimulants. Intra-VTA injections of the $GABA_B$ agonist, baclofen, resulted in blockade of cocaine-, amphetamine- and novelty-induced locomotor stimulation (Hooks and Kalivas, 1995; Kalivas, Duffy and Eberhardt, 1990). This effect was postulated to be mediated via $GABA_B$ receptors located on dopamine neurons, as supported by the result that intra-VTA application of baclofen reduced dopamine concentrations in N Acc (Kalivas, Duffy and Eberhardt, 1990). Data presented in this thesis provided evidence for the involvement of $GABA_B$ systems in EtOH's stimulant effects in FAST and D2 mice, and previous data have suggested the importance of dopamine systems (Shen, Crabbe, and Phillips, 1995). However, evidence for $GABA_B$/dopamine interactions in modulating expression of EtOH-stimulated activity was not found. If a neural circuit for EtOH-stimulated activity exists, it may be that dopamine and $GABA_B$ systems are critical components of the circuit, but are not

directly linked. Alternatively, it is possible that, rather than being mediated by one neural circuit comprising several neurotransmitter systems, EtOH-stimulated activity is mediated by two or more parallel and independent pathways with different transmitter systems. Finally, baclofen has been demonstrated to reduce the transmission of several neurotransmitters, including dopamine (Bowery et al., 1990). Thus, it may be that baclofen administration reduced the activity of additional neurotransmitter systems important in expression of EtOH-stimulated activity (e.g. acetylcholine (Blomqvist, Soderpalm and Engel, 1992); serotonin (Blomqvist, Söderpalm and Engel, 1994), and α-adrenergic receptors (Seppala et al., 1994)), so that compensation of only one of these systems (dopamine) is not sufficient to restore the behavior.

Another possibility is that the methodology employed did not provide a good means of addressing the hypothesis. These studies involved systemic injection of drug solutions, which did not allow for control of the site of CNS action of the drug. With regard to specific interactions between neurotransmitter systems, microinjection of drugs into putatively important specific neuroanatomical locations may be a better approach. While microinjections had been considered prior to undertaking these studies, several issues led to the decision to use systemic injections. First, these experiments were mostly intended as "first-pass" experiments to determine whether or not these specific neurotransmitter systems were involved. Second, microinjection techniques have not been fully developed in mice, and time constraints would not allow development of these methods for these studies. Furthermore, the relative inefficiency of microinjection was

incompatible with the large numbers of animals required for testing in these experiments.

Glutamatergic inputs from several limbic areas (medial prefrontal cortex, hippocampus, and amygdala) are also thought to be important modulators of the mesoaccumbens-pallidal circuit and locomotor behavior via actions within the N Acc. The result that administration of the NMDA receptor antagonist, MK-801, altered the activity of EtOH-treated mice in a dose-dependent manner in these studies is consistent with this notion, and consistent with the hypothesis that NMDA receptors are important in expression of EtOH-stimulated activity. In FAST and D2 mice, enhancement of stimulation was observed at low dose combinations and reduced activity was observed at high dose combinations. Only decreases in psychomotor stimulant induced-locomotion in response to intra-N Acc injection of glutamate receptor antagonists have been reported (e.g. Puliverenti, Swerdlow and Koob, 1989), but substimulant doses of cocaine and amphetamine were not tested. Since the ability of MK-801 to enhance or inhibit locomotor activity seems to be activity-level-dependent, it is possible that enhancement of locomotion would be seen after administration of substimulant doses of cocaine or amphetamine and MK-801.

A simple model to account for MK-801 effects on locomotion and EtOH's stimulant effects is presented in Figure 20. This model simply provides more detail with regard to glutamate receptor mediation of locomotor activity, and does not differ from the neural circuitry reviewed previously. Dopamine (from VTA) and glutamate neurons (from hippocampus and prefrontal cortex) are conceptualized as mediators of

Figure 20. Schematic model of neuroanatomical circuitry responsible for MK-801 effects on locomotion and EtOH/MK-801 interactions. Glutamate neurons project from hippocampus and prefrontal cortex and provide excitatory input to N Acc neurons. Inhibitory dopaminergic input comes from mesoaccumbens neurons originating in VTA. GABAergic N Acc neurons project to VP/SI and inhibit pallidal neurons via the $GABA_A$ receptor complex (GRC). Locomotor behavior is altered by manipulations of N Acc and VP/SI via additional circuitry that is currently not well-characterized (black box).

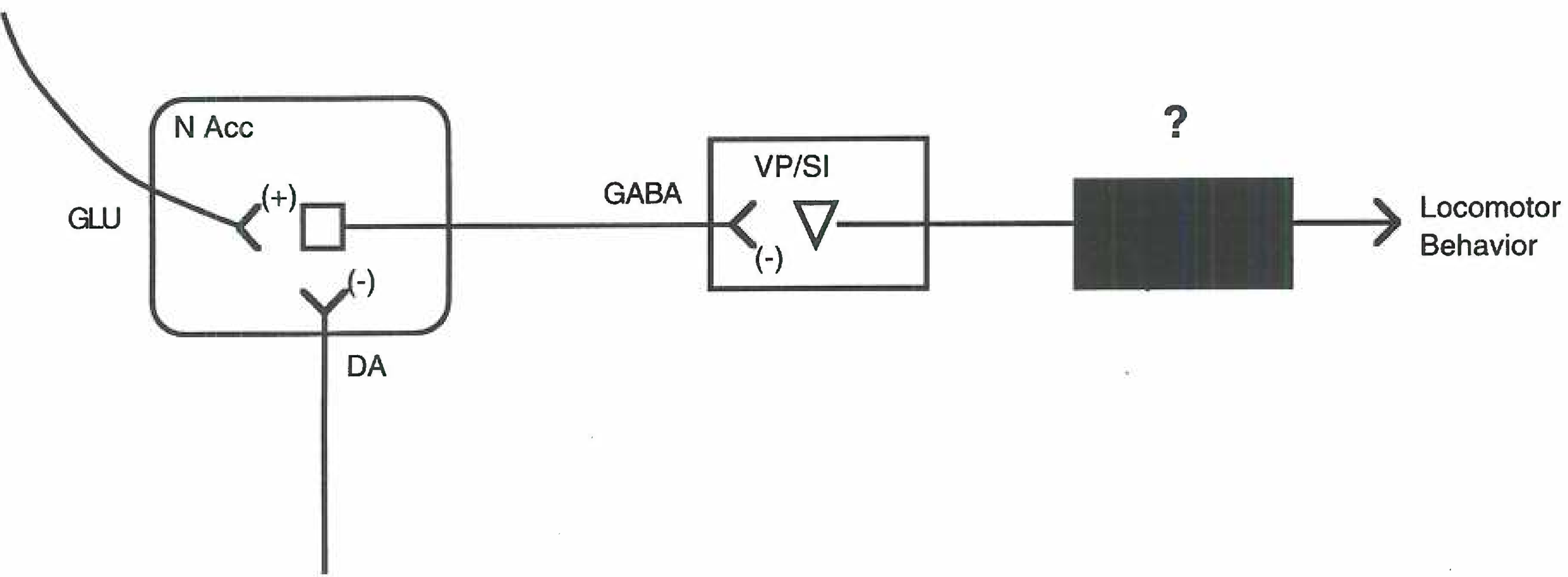


FIGURE 20

GABAergic N Acc output neurons. Dopamine provides inhibitory input (White and Wang, 1986) to decrease the activity of GABAergic neurons projecting to VP/SI, while glutamate provides excitatory input to the same GABAergic neurons. Increases in N Acc dopamine release have consistently been shown to result in increased locomotion (LeMoal and Simon, 1991), presumably via decreased GABA activity in VP/SI (mediated by $GABA_A$ receptors), and additional pathways still to be determined. Administration of glutamate antagonists, such as MK-801, would reduce the normal excitatory input, and decrease the activity of GABA neurons. Thus, the behavioral result of MK-801 administration is locomotor stimulation. This model, though currently speculative, could be tested by determining the effects of a $GABA_A$ agonist on MK-801-stimulated activity. According to the model, $GABA_A$ receptor (also known as the GABA receptor complex, or GRC) activation in the VP/SI would counteract the decrease in GABAergic activity produced by MK-801 administration, leading to a reduction in the stimulant effects of MK-801.

A possible mechanism of EtOH's actions on NMDA receptors has already been discussed above. Briefly, enhancement of the stimulant effects of EtOH and MK-801 by administration of both drugs may be due to the ability of each drug to inhibit NMDA receptor function, possibly at different sites on the receptor, resulting in greater effectiveness in producing locomotor stimulation. The ability of EtOH to block MK-801-stimulated activity in SLOW and B6 mice is an interesting result. Though there are no data to support this possibility, this effect of EtOH could be mediated by a negative feedback mechanism, perhaps via pallidothalamic and thalamocortical connections (not shown in Figure

20). In addition, use-dependent desensitization of NMDA receptors has been demonstrated (Zorumski and Thio, 1992), and negative feedback mechanisms could occur at the level of the receptor. Alternatively, EtOH has been demonstrated to enhance GRC-mediated Cl^{-}-flux (Allan and Harris, 1987), a mechanism by which EtOH's ataxic and sedative effects might occur (Frye and Breese, 1982; Lijequist and Engel, 1982; Martz, Deitrich and Harris, 1983). Therefore, EtOH's inhibitory effects on MK-801 stimulation may be due to activation of the GRC. The data here do not suggest a specific anatomical location for this effect, but activation of GRC in VP/SI could theoretically produce the observed results.

Theories of Addiction

The psychomotor stimulant theory of addiction proposed by Wise and Bozarth (1987) was provided as one justification for investigation of the neurochemical substrates underlying EtOH-stimulated activity. According to this theory, locomotor stimulation and reinforcement are mediated by a common neural substrate, the mesolimbic dopamine pathway. Furthermore, the theory postulates that the reinforcing, presumably pleasurable, effects of drugs promote continued drug-seeking behavior and eventually lead to addictive behavior. Thus, an understanding of the mechanisms by which drugs produce their locomotor stimulant actions would lead to an understanding of the mechanisms by which drug addiction develops. In general, data have provided mixed support for the ability of the psychomotor stimulant theory of addiction to account for alcohol addiction. Cunningham et al. (1992) observed that the EtOH-stimulated

D2 strain demonstrated greater place preference for the EtOH-paired environment than the non-stimulated B6 strain, providing support for the positive relationship between stimulation and reward predicted by the psychomotor stimulant theory. On the other hand, B6 and D2 mice also provide a well-characterized example of the dissociation of sensitivity to EtOH's locomotor stimulant effects from its motivational properties, as measured in a two-bottle choice (water vs EtOH) consumption paradigm. Whereas B6 mice readily consume EtOH solutions and show preference for EtOH over water, D2 mice consistently consume very little EtOH and prefer to drink water (see Phillips and Crabbe, 1991). Interestingly, results from conditioned place preference and EtOH consumption studies in FAST and SLOW mice are opposite to those found in B6/D2 comparisons. FAST and SLOW selected lines did not differ in magnitude of conditioned place preference for EtOH; however, FAST mice drank more EtOH than SLOW mice in a forced consumption paradigm (both replicates), and had higher preference ratios than SLOW mice (replicate 1 only) in a two-bottle choice paradigm (Risinger et al., 1994). Thus, evidence from genetic models of EtOH stimulant sensitivity has been both consistent with and contrary to the predictions of the psychomotor stimulant theory.

Rat lines selectively bred for high (Alcohol-Preferring; P) vs low (Alcohol-Nonpreferring; NP) alcohol consumption may provide a genetic animal model of differences in sensitivity to EtOH's reinforcing effects. P rats were demonstrated to be more sensitive to the locomotor stimulant effects of several low doses of EtOH, compared to NP rats (Waller et al., 1986), providing evidence for a positive genetic correlation between sensitivity to EtOH's reinforcing and stimulant

effects. However, negative genetic correlations have also been detected. Locomotor responses to acute and chronic EtOH exposure, as well as EtOH consumption and preference have been measured in BXD recombinant inbred strains, for determination of quantitative trait loci (QTL) which mediate these phenotypes (Phillips et al., 1994; Phillips et al., 1995). Comparison of the locomotor activity and consumption data sets revealed negative correlations between sensitivity to the locomotor activating effects of EtOH and EtOH consumption/preference (Phillips et al., 1995). Thus, greater locomotor stimulation was correlated with lower levels of EtOH consumption. Furthermore, only one common QTL marker, which was positively correlated with activity and negatively correlated with consumption, was found between the phenotypes, suggesting little overlap in the genetic factors which mediate EtOH consumption and EtOH stimulation.

A great deal of evidence supports the involvement of dopamine in expression of EtOH-stimulated activity (see Introduction; and Phillips and Shen, in press). Dopaminergic involvement in the rewarding effects of EtOH is also supported by studies in which EtOH self-administration in an operant paradigm was altered by systemic injection (Rassnick, Pulvirenti and Koob, 1993) or N Acc microinjection (Samson et al., 1992) of dopaminergic drugs. In microdialysis studies, N Acc dopamine levels increased during EtOH self-administration (Weiss et al., 1992). In contrast, administration of the mixed dopamine antagonist, haloperidol, prior to each EtOH conditioning trial, decreased locomotor activity but had no effect on conditioned place preference in D2 mice (Risinger, Dickinson and Cunningham, 1992). These data suggest a dissociation of

locomotor stimulant effects and reward with regard to dopaminergic modulation of these phenomena.

The experiments presented in this dissertation did not directly test the psychomotor stimulant theory of addiction; however, previous data demonstrating the importance of dopamine function for expression of EtOH-stimulated activity in FAST mice are consistent with the theory (Shen, Crabbe and Phillips, 1995). The implications of the current demonstration that GABA and glutamate systems are important are currently unknown. These systems may be secondarily important for expression of EtOH-stimulated activity, functioning either as afferents to the mesolimbic projection or as efferents modulated by dopamine transmission in the N Acc. On the other hand, the lack of support for GABA/DA interactions in the current studies suggests the possibility that neurotransmitter systems act independently to affect locomotor stimulation by EtOH, and interactions with a primary dopamine projection are unnecessary. Further research is required to determine which of these possibilities most accurately reflects the mechanisms of EtOH action.

A theory of addiction which has received much recent attention is the incentive-sensitization theory proposed by Robinson and Berridge (1993). This theory postulates that repeated drug use results in progressive sensitization of neural systems which mediate "wanting" and "craving", ultimately leading to addiction. In animals, neural sensitization induced by repeated drug exposure is thought to be reflected, in part, by enhanced responsiveness to the locomotor stimulant properties of the drug. Locomotor sensitization has been most

extensively studied for the psychomotor stimulants; however, sensitization to the stimulant effects of EtOH has also been demonstrated in various mouse genotypes (Masur et al., 1986; Phillips, Burkhart-Kasch and Crabbe, 1991; Phillips, Dickinson and Burkhart-Kasch, 1994; Phillips et al., 1995). Not surprisingly, some of the neural substrates thought to be involved in mediating sensitization of locomotor stimulation share common components with those involved in acute stimulant effects (Kalivas and Stewart, 1991; Kalivas, Sorg and Hooks, 1993). However, the mechanisms underlying these phenomena may be distinct since recent QTL and correlational analyses did not support a genetic relationship between initial sensitivity and sensitization to EtOH (Cunningham, 1995; Phillips et al., 1995), or cocaine (Tolliver et al., 1994). Furthermore, contrary to predictions of the incentive-sensitization theory, Cunningham (1995) found no relationship between expression of EtOH conditioned place preference and sensitization to the EtOH's stimulant effects. Thus, further testing of the importance of sensitization processes in animal models of drug reward or drug craving (Markou et al., 1993) is required to evaluate the ability of the incentive-sensitization theory to explain addiction.

Future Directions

Characterization of FAST and SLOW mice

FAST and SLOW mice have been characterized for a few of the neurochemical substrates thought to be involved in locomotor responses to EtOH. There are still many systems yet to be investigated in these lines, however, and many avenues of investigation (e.g. biochemistry,

electrophysiology, molecular genetics) remain open. The $GABA_A$-receptor complex (GRC) is one candidate neurotransmitter system for further study. Behaviorally, FAST mice were found to be more sensitive to the locomotor stimulant effects of pentobarbital, phenobarbital, and several alcohols, but less sensitive to the depressant effects of diazepam (Phillips et al., 1992). The differences in sensitivity to the locomotor effects of GRC ligands suggests that selection has altered GRC function. This possibility is currently being pursued by examining relative sensitivities to the convulsant effects of several drugs which interact with the GRC, such as pentylenetetrazole and picrotoxin. Other neurotransmitter receptors implicated in mediating EtOH's stimulant effects include central nicotinic acetylcholine receptors (Blomqvist, Soderpalm and Engel, 1992), serotonin receptors (Blomqvist, Söderpalm and Engel, 1994), and α-adrenergic receptors (Seppala et al., 1994). The possibility that these receptor systems have been differentially altered between FAST and SLOW mice, and/or that these receptor systems mediate expression of EtOH-stimulated activity in FAST and SLOW mice remains to be investigated.

Characterization on biochemical and electrophysiological levels would be helpful in elucidating differences between selected lines, as well as the mechanisms by which these differences occur. For example, evidence for differences in GRC function may be found by measuring Cl^- flux in vesicles obtained from brains of FAST and SLOW mice, which may also provide hypotheses with regard to EtOH's mechanism of action. In addition, the currently observed differences in locomotor activity in response to MK-801 in FAST and SLOW mice, and the differences in

sensitivity to EtOH inhibition of Ca^{2+} influx mediated via the NMDA receptor in these lines, suggest that the lines differ in NMDA receptor function. Further characterization of these line differences in NMDA receptor function should include electrophysiological analyses and additional biochemical analyses, such as sensitivity to inhibition of glutamate-stimulated cGMP by EtOH, in an effort to more accurately determine the sources of the line differences, and determine the cellular mechanisms by which EtOH alters NMDA receptor function.

Recent QTL analyses in recombinant inbred strains have identified chromosomal regions of importance in mediating acute stimulant responses to EtOH (Cunningham, 1995; Phillips et al., 1995), and can generate hypotheses to be tested in FAST and SLOW mice using molecular techniques. Both Cunningham (1995) and Phillips et al. (1995) detected significant correlations of acute EtOH activity with several markers on chromosome 3 and chromosome 18, and FAST and SLOW mice could be screened for marker polymorphisms in those regions. In some cases, chromosomal regions detected by QTL analyses include candidate genes, whose products have a known function, which may provide the bases for hypotheses regarding specific biological substrates mediating the phenotype. For example, markers on chromosome 3 that were significantly correlated with sensitivity to acute EtOH lie near genes for the alcohol dehydrogenase enzymes, *Adh-1* and *Adh-3*, suggesting involvement of EtOH metabolism or metabolites. Consistent with this suggestion, decreases in spontaneous locomotor activity were observed in mice after administration of the EtOH metabolite, acetate, which upon further processing, leads to production of adenosine (Carmichael et al., 1991). The adenosine

receptor blocker, 8-PT (8-phenyltheophylline), blocked the depressant effects of acetate and enhanced the stimulant effects of EtOH, thus providing a possible mechanism by which EtOH metabolism may affect sensitivity to acute EtOH.

Behavioral and biochemical studies may also suggest candidate gene loci that were differentially fixed by selection. Recent studies suggest subunit specificity for the potency of EtOH's effects on NMDA receptor function (Chu et al., 1995; Criswell et al., 1993; Koltchine et al., 1993; Masood et al., 1994), and it is possible that FAST and SLOW mice differ in allelic forms, or in expression of a particular subunit. A molecular basis for variations in GRC sensitivity to EtOH has also been demonstrated (Criswell et al., 1993), and may provide an explanation for differences between FAST and SLOW mice in sensitivity to the locomotor effects of GRC ligands (Phillips et al., 1992). These studies await the inbreeding of FAST and SLOW mice, since molecular genetic techniques are currently amenable only for genotypes which are homozygous at all loci.

Determination of Neural Circuitry

Further studies on the integrative nature of neurotransmitter systems to influence activity should still be pursued to determine whether locomotor activation by EtOH is mediated by a neural pathway. This neural circuitry may or may not be the same as the mesoaccumbens-pallidal circuit implicated in psychostimulant-induced activation. However, some methodological issues need to be addressed. *In vivo* microinjection and microdialysis techniques, which have been developed

primarily in rats, require further refinement and fine-tuning for use in mice because of comparatively small mouse brain sizes. These techniques would be beneficial because they circumvent the uncertainty associated with systemic injections by providing a more precise method of targeting specific neuroanatomical sites. With regard to genetic analyses, there is a greater advantage to using mice because of the availability of a much greater number of different inbred strains and selected lines of mice compared to rats. Active pursuit of refinements of these *in vivo* methods is highly encouraged, since information obtained from genetic mouse models will be invaluable.

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APPENDICES

Appendix A: $GABA_B$ Antagonists

Prior to the use of the $GABA_B$ antagonist, CGP-35348, two other ligands with $GABA_B$ antagonist properties were tested for efficacy in altering locomotor activity in mice. 5-aminovaleric acid (5-AVA) and phaclofen had been demonstrated to antagonize the effects of baclofen (Dutar et al., 1988; Kerr et al., 1987; Schwarz et al., 1988) both centrally and peripherally. In addition, phaclofen antagonized several behavioral effects of EtOH (Allan and Harris, 1989), although it failed to do so in later studies (Humeniuk et al., 1993). Phaclofen and 5-AVA were thus considered to be good candidates for selective antagonism of baclofen's effects on EtOH-stimulation; however, in a series of preliminary experiments, described below, it was found that these compounds had little effect.

Since no data had previously been collected on the locomotor effects of 5-AVA in the experimental conditions used in these experiments, pilot studies which assessed the effects of 5-AVA alone, as well as in combination with EtOH or baclofen and EtOH, were performed.

Preliminary Studies on 5-Aminovaleric Acid

5-AVA effects on baseline activity were investigated in S_{35} FAST male and female mice from both replicates (81 - 98 days of age) using an up-down method. Initially, the locomotor activity of mice given 20 mg/kg 5-AVA was compared to that of mice injected with saline (n = 4/dose). Mice were tested for locomotor activity for 1 hour. Since it appeared that 20 mg/kg had slight depressant effects, one lower (10

mg/kg) and one higher dose (40 mg/kg) were subsequently tested for effects on locomotor activity. Comparison of all 3 doses and saline activity in Figure A.1 did not strongly suggest that 5-AVA had robust effects on activity in a clear dose-dependent manner, and ANOVA did not reveal significant dose effect. In support of this, a much higher dose of 5-AVA (100 mg/kg) had no effect compared to saline activity, when tested in a separate session (see Figure A.2).

5-AVA effects on EtOH activity were tested, in spite of its lack of effect on baseline activity. Male FAST mice from both replicates (S_{35}; 83 - 95 days old) were injected with saline or 100 mg/kg 5-AVA 15 minutes prior to injection of 2.0 g/kg EtOH, and a 15-minute activity test. There were 6 mice tested per treatment group. The 15-minute pretreatment time was used to determine the effect of 5-AVA within time parameters that were used in the baclofen experiments. 5-AVA had no effect on EtOH-stimulated activity under these conditions (see Figure A.3).

Although 5-AVA did not affect EtOH-stimulated activity in FAST mice, the primary question was whether a $GABA_B$ antagonist could reverse the effect of the $GABA_B$ agonist, baclofen, on EtOH-stimulated activity. Thus, 5-AVA effects on the inhibition of EtOH-stimulation by baclofen were investigated in S_{35} FAST-2 male mice (60 - 65 days old). Mice were injected with saline, baclofen (5 mg/kg), or a combination of baclofen (5 mg/kg) and 5-AVA (25, 50, or 100 mg/kg); all mice were injected 15 minutes later with 2.0 g/kg EtOH. The activity test duration was 30 minutes, but data are presented in Figure A.4 as distance traveled in the first 20 minutes of the test, for comparison with data presented

Figure A.1. Effects of 5-aminovaleric acid (5-AVA) on spontaneous locomotor activity of FAST mice (both replicates). Animals were tested using an up/down method, beginning with a session in which saline or 20 mg/kg was injected, followed by a 60-minute activity test. In a subsequent testing session, animals were injected with saline, 10 or 40 mg/kg 5-AVA, and tested for 60 minutes. n = 4 - 6 mice per treatment group; vertical lines are S.E.M.

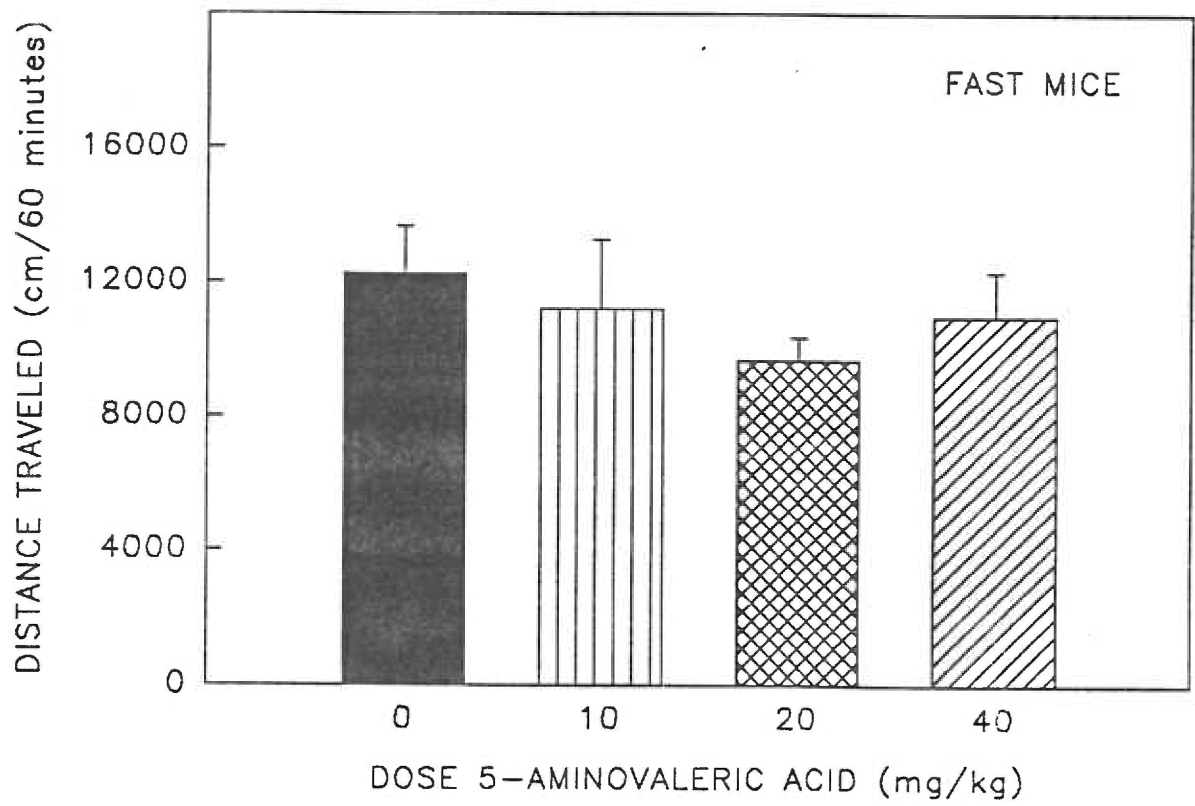

FAST MICE
DISTANCE TRAVELED (cm/60 minutes)
16000
12000
8000
4000
0
0
10
20
40
DOSE 5-AMINOVALERIC ACID (mg/kg)

Figure A.2. Effects of saline or 100 mg/kg 5-AVA on locomotor activity of FAST mice (both replicates). Mice were injected and immediately placed in activity monitors for 60 minutes. n = 4 per treatment group; vertical lines are S.E.M.

FIGURE A.2

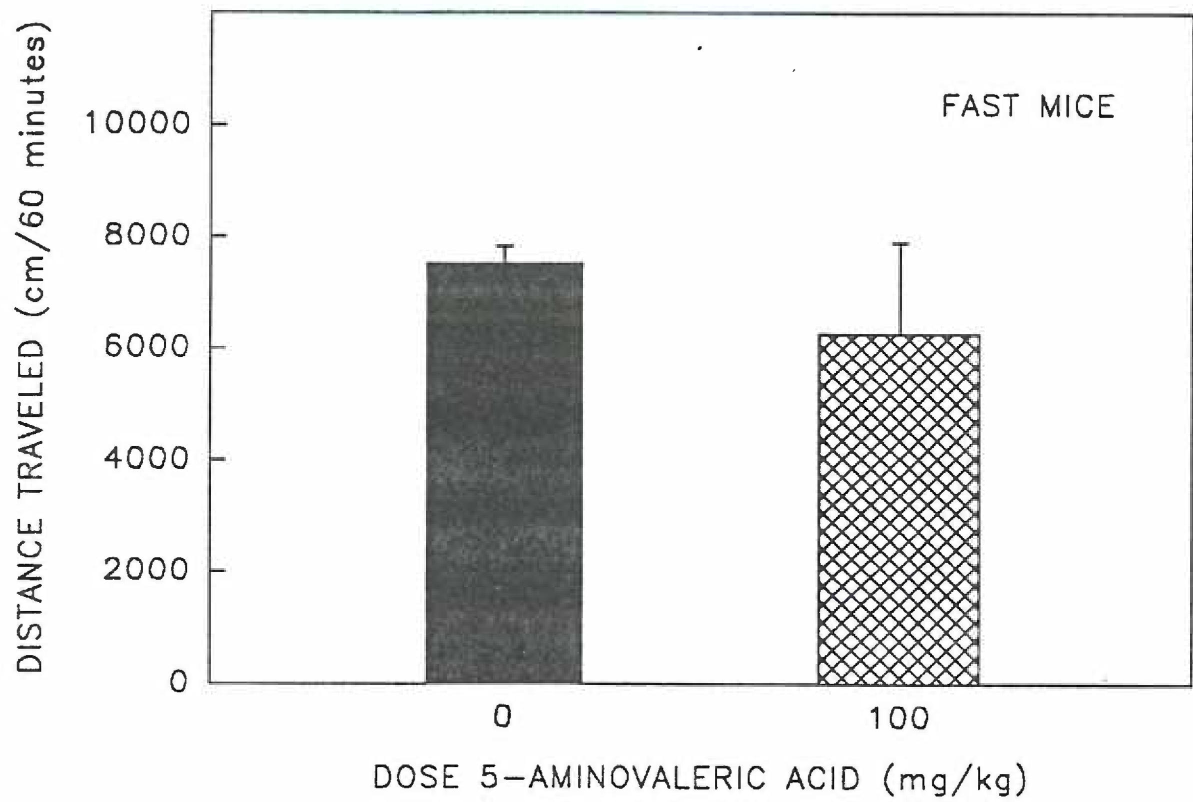


Figure A.3. Effects of 5-AVA on EtOH-stimulated activity of FAST mice (both replicates). Mice were injected with saline or 100 mg/kg 5-AVA, followed 15 minutes later by injection of 2.0 g/kg EtOH. Locomotor activity was assessed for 15 minutes, immediately following the EtOH injection. n = 6 per treatment group; vertical lines are S.E.M.

FIGURE A.3

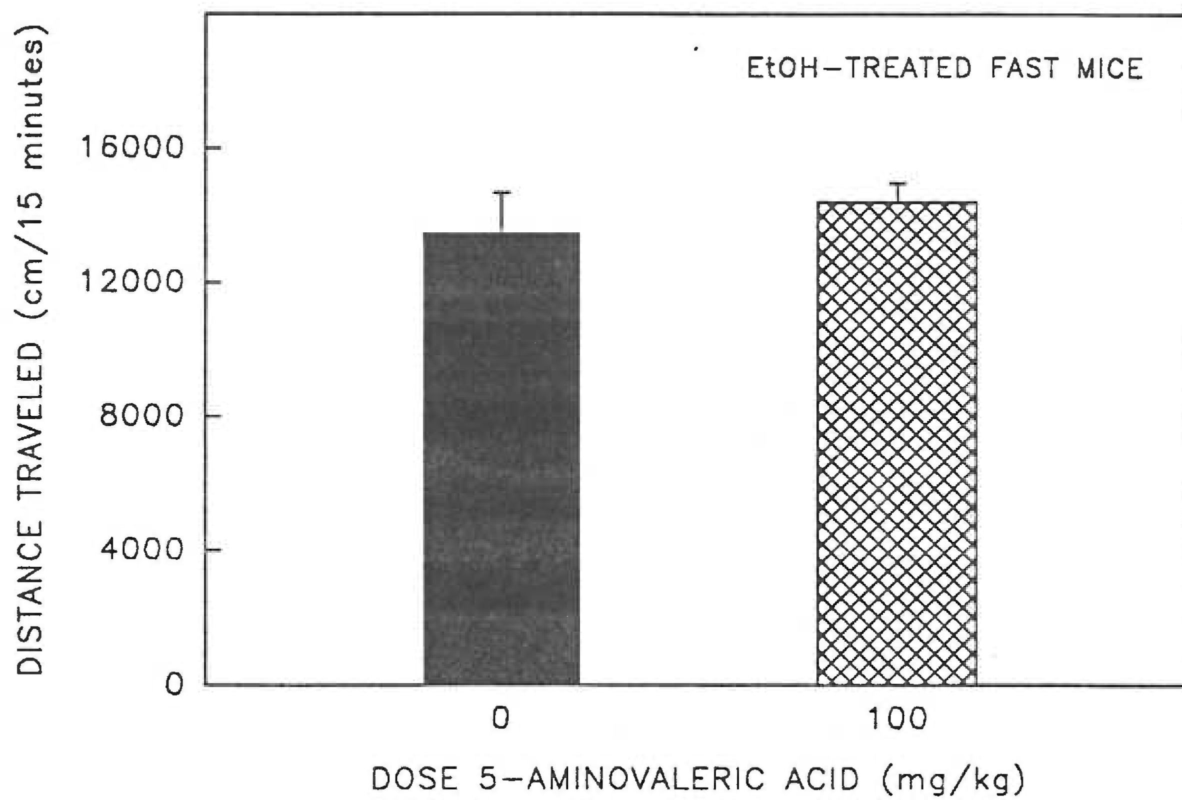
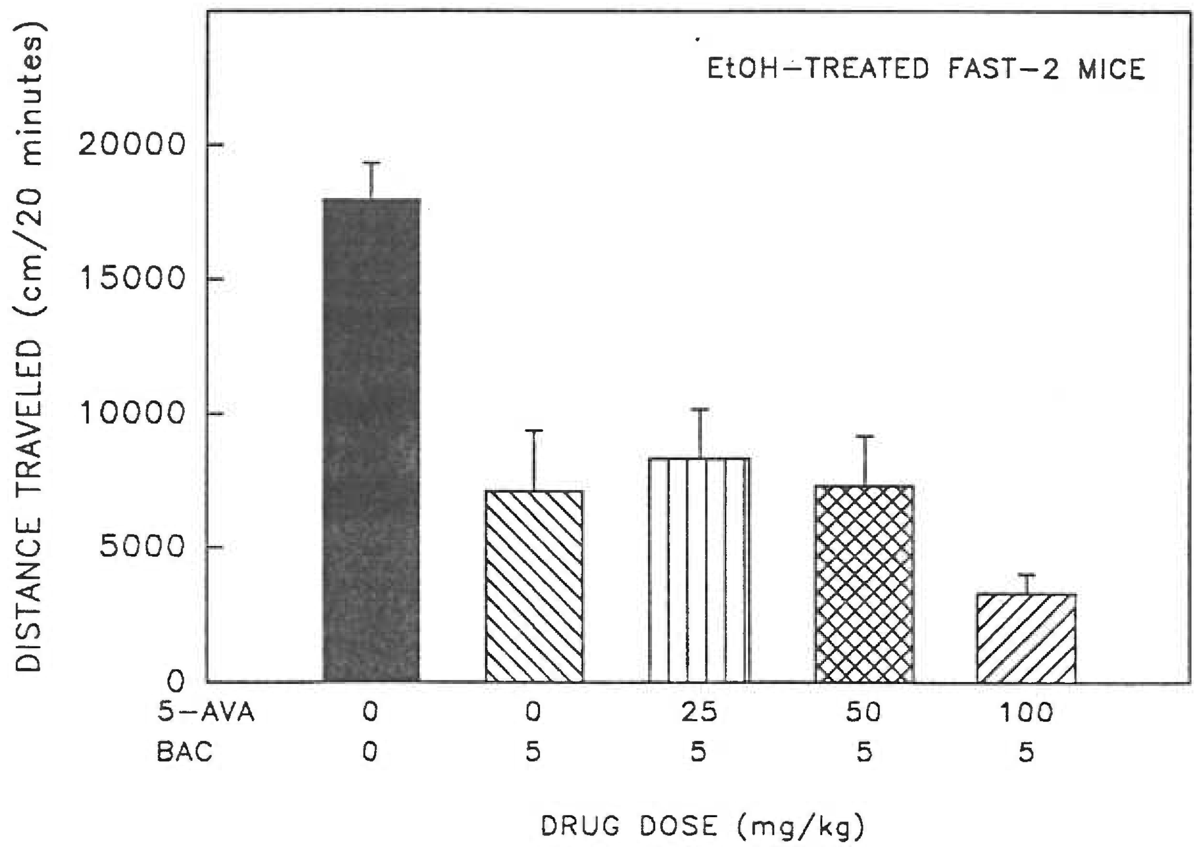


Figure A.4. Effects of 5-AVA on baclofen's inhibition of EtOH-stimulated activity in FAST-2 mice. Animals were injected with saline or baclofen, or a combination of 5-AVA and baclofen, followed by injection of EtOH (2.0 g/kg) 15 minutes later. Activity test duration was 30 minutes, but data for the first 20 minutes of the test are presented. n = 5 per treatment group; vertical lines are S.E.M.

FIGURE A.4



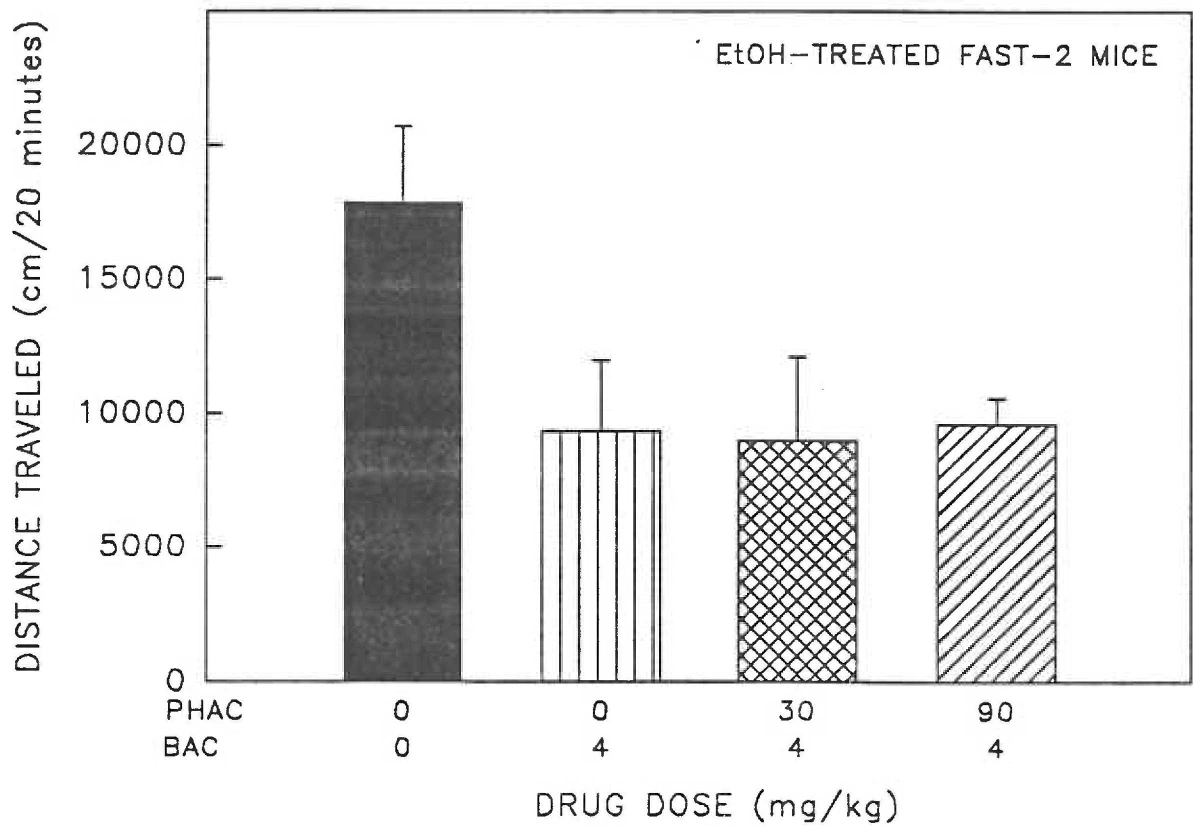
throughout this thesis. Baclofen decreased the activity of EtOH-treated mice, but 5-AVA did not reverse this effect. The highest dose of 5-AVA appeared to slightly exacerbate EtOH's effects, but this effect was not significant.

In summary, 5-AVA did not alter baseline locomotor activity, EtOH-stimulated locomotor activity, or the effects of baclofen on EtOH-stimulated activity. Perusal of the literature suggests that this particular drug is not currently used in research, which is likely due to the demonstration that 5-AVA can also have agonist effects (Holopainen et al., 1992).

Preliminary Studies on Phaclofen

Previous investigations on the effects of phaclofen in FAST mice (S_{33}) showed that phaclofen doses up to 45 mg/kg had no effect on the locomotor activity of either saline or EtOH-treated mice (Tamara J. Phillips, personal communication). The effects of phaclofen on the inhibition of EtOH-stimulated activity by baclofen were further investigated in this preliminary study. S_{35} FAST-2 male mice, 52 - 63 days old, were injected with saline or phaclofen (30 or 90 mg/kg), followed 5 minutes later with saline or baclofen (4 mg/kg). Fifteen minutes after the second injection, all mice were injected with 2.0 g/kg EtOH and tested for locomotor activity for 30 minutes. Data are presented in Figure A.5 as the total distance traveled during the first 20 minutes of the activity test, and show that while baclofen decreased EtOH, neither dose of phaclofen reversed this effect of baclofen. Other preliminary studies which altered injection time parameters were also

Figure A.5. Effects of phaclofen on the inhibition of EtOH-stimulated activity by baclofen in FAST-2 mice. Animals were injected with saline or phaclofen, followed by a second injection of saline or baclofen 5 minutes later. EtOH (2.0 g/kg) was injected 15 minutes following the second injection, and mice were tested for locomotor activity for 30 minutes. Data for the first 20 minutes are presented. n = 4 per treatment group; vertical lines are S.E.M.



EtOH-TREATED FAST-2 MICE
DISTANCE TRAVELED (cm/20 minutes)
20000
15000
10000
5000
0
PHAC
BAC
0
0
0
4
30
4
90
4
DRUG DOSE (mg/kg)

performed, but did little to alter the results, and are not presented here.

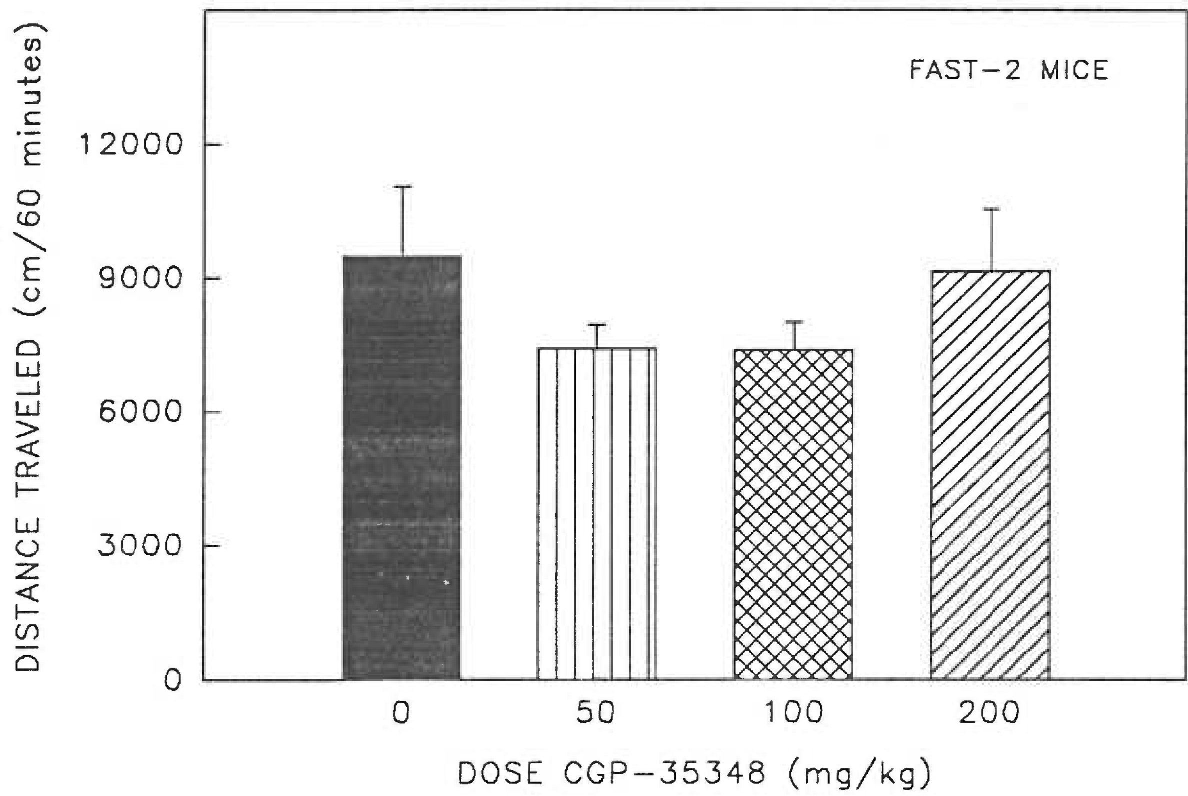
Unlike 5-AVA, phaclofen has consistently been demonstrated to have $GABA_B$ antagonist actions; however, phaclofen has much lower affinity for the receptor compared to baclofen and does not readily cross the blood-brain barrier. Thus, it is not surprising that systemic administration of this drug did little to alter locomotor activity.

Appendix B: CGP-35348

Experiments presented in this thesis provided evidence that the inhibition of EtOH-stimulation by baclofen could be reversed by the $GABA_B$ antagonist, CGP-35348. Although this effect was shown to occur in the absence of CGP-35348 effects on EtOH-stimulation, the design of Experiment 2 did not include treatment groups to assess the effects of CGP-35348 on baseline locomotor activity. Results of Experiment 3 showed that CGP-35348 did not alter baseline activity of SLOW-1 mice; however, it was considered important to demonstrate that this drug also does not alter baseline activity of FAST mice. FAST-2 males from $S_{37}G_{39}$ (52 - 69 days of age) were injected with saline, or CGP-35348 (50, 100, or 200 mg/kg), and immediately placed in activity monitors for a 1-hour activity test. There were n = 6 animals per dose group. Data are presented in Figure B.1. Although it appeared that 50 and 100 mg/kg CGP-35348 decreased locomotor activity of FAST-2 mice, there were no significant effects of dose on locomotor activity. The locomotor activity of mice injected with 200 mg/kg CGP-35348 was nearly equivalent to that of saline-treated mice.

Figure B.1. Effects of CGP-35348 on locomotor activity of FAST-2 mice. Animals were injected with saline or CGP-35348 and immediately tested for locomotor activity for 60 minutes. n = 6 per dose; vertical lines are S.E.M.

FIGURE B.1



Appendix C: Apomorphine

Two doses of apomorphine were tested for their effects on locomotor activity in both replicates of S_{36} FAST male mice, to compare with a larger dose-response study conducted in S_{13} (T. J. Phillips, personal communication). In the current study, FAST mice (81 and 118 days of age) tested for locomotor activity after injection of saline or 1 or 5 mg/kg apomorphine. The replicates did not differ in their apomorphine response, and data are presented collapsed on replicate in Figure C.1 (n = 16 per dose). Both apomorphine doses decreased locomotor activity of FAST mice. These data are consistent with the dose-response study referred to earlier. Although several investigators have reported biphasic effects of apomorphine on locomotor activity, in which activity is decreased by low doses and returns to baseline levels at higher doses, this pattern was not observed in FAST mice in this study, or in FAST and SLOW mice in the prior study.

Since apomorphine effects on baseline activity in this preliminary experiment were consistent with those found earlier, further experiments focused on apomorphine effects on EtOH-stimulated activity in FAST mice. In the first study, apomorphine was injected immediately prior to injection of 2.0 g/kg EtOH in S_{36} FAST male mice from both replicates (81 - 100 days old). Data are presented in Figure C.2, and show that all doses reduced EtOH-stimulated locomotor activity in a 15-minute activity test. There was a hint of return to stimulated levels in animals treated with 7.5 mg/kg apomorphine; however, there was a great deal of variability in this group, and the locomotor activity of animals given 10 mg/kg was reduced to the same levels as animals given 5 mg/kg.

Figure C.1. Effects of apomorphine (1 or 5 mg/kg) on locomotor activity of FAST mice (both replicates). Locomotor activity test duration was 15 minutes, beginning immediately after saline or apomorphine injection. n = 16 per dose; vertical lines are S.E.M.

FIGURE C.1

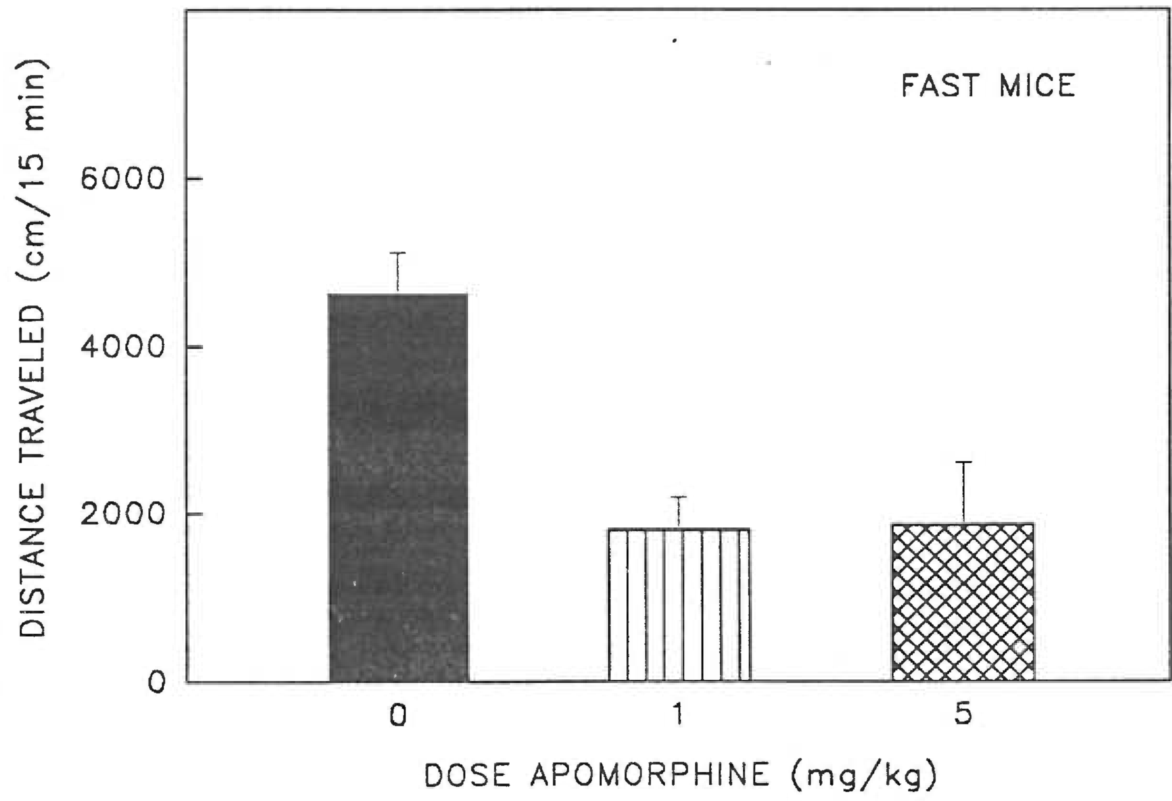
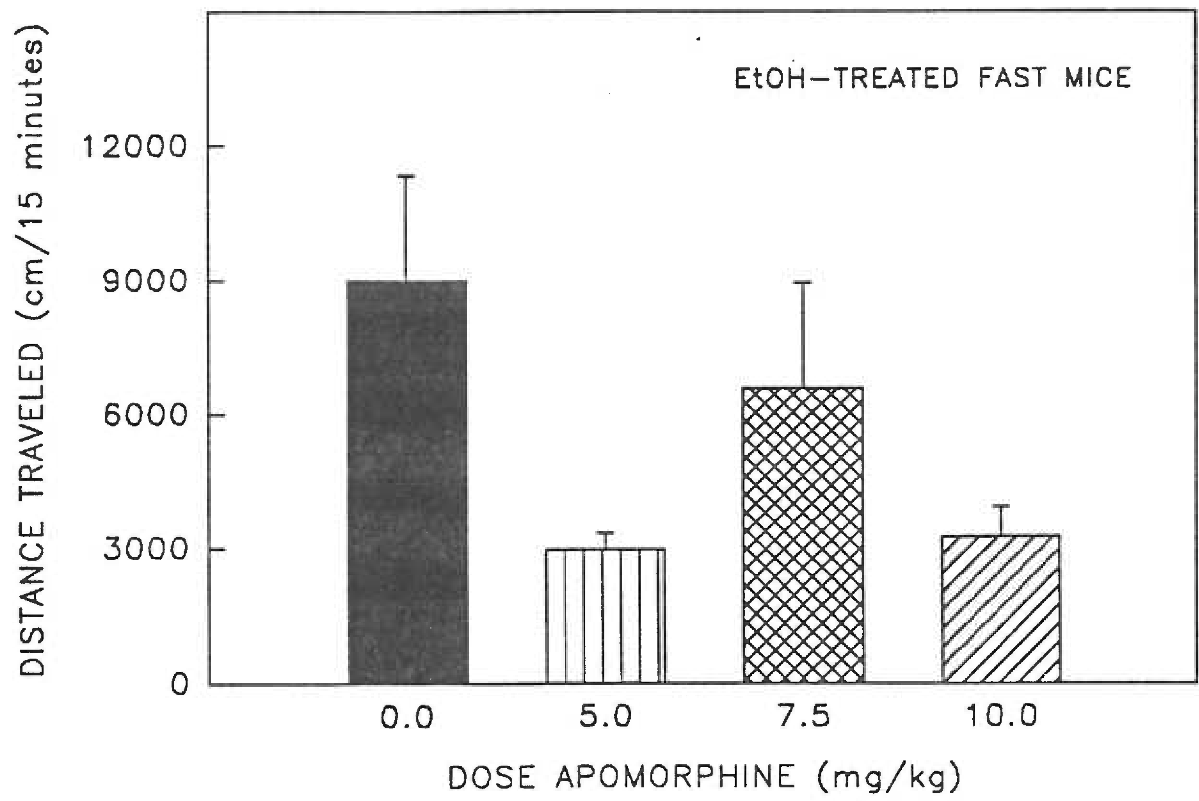


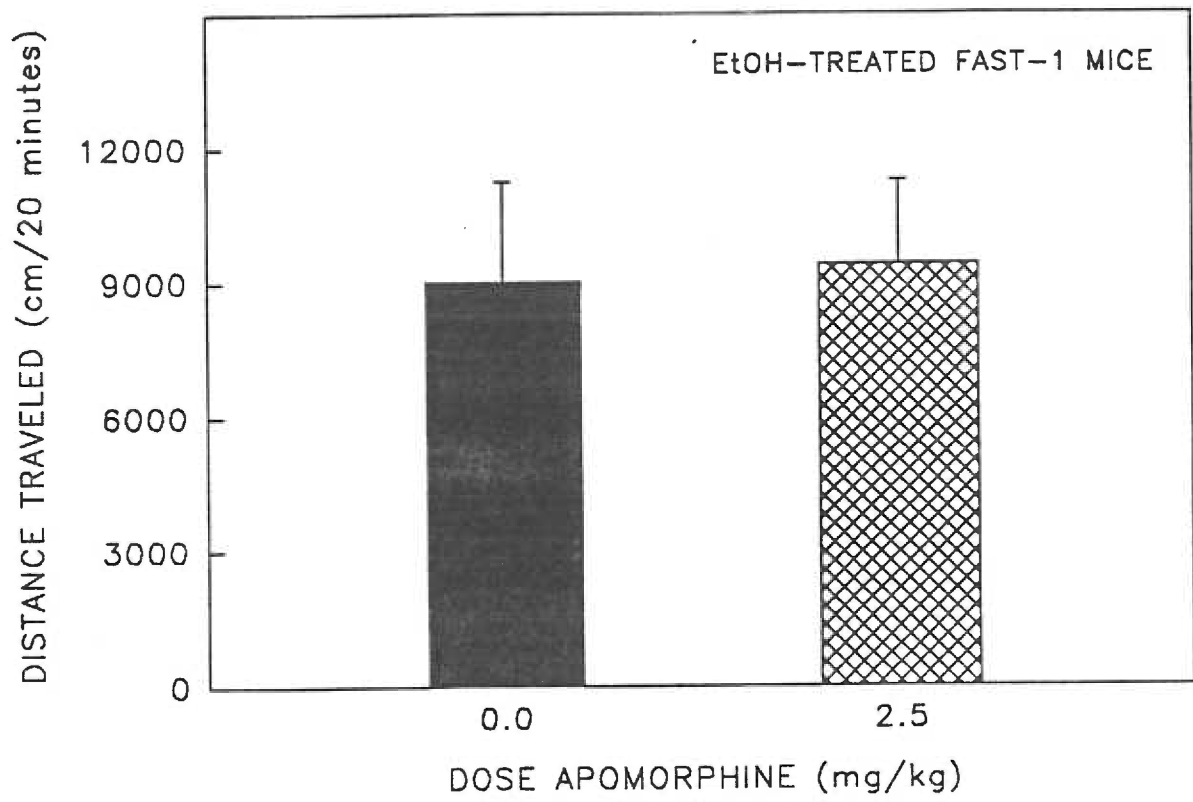
Figure C.2. Effects of apomorphine (5 - 10 mg/kg) on locomotor activity of EtOH-treated FAST mice (both replicates). Saline or apomorphine was injected immediately before injection of EtOH (2.0 g/kg), and a 15-minute activity test. n = 8 per treatment group; vertical lines are S.E.M.


EtOH–TREATED FAST MICE
DISTANCE TRAVELED (cm/15 minutes)
12000
9000
6000
3000
0
0.0
5.0
7.5
10.0
DOSE APOMORPHINE (mg/kg)

One lower dose of apomorphine was tested in combination with EtOH, to attempt to find a dose which did not affect EtOH-stimulated activity. The experimental protocol was the same as the previous study, except that 2.5 mg/kg apomorphine was injected immediately prior to EtOH, followed by a 20-minute activity test. There was no significant effect of 2.5 mg/kg apomorphine on EtOH-stimulated activity in FAST-1 male mice (82 - 91 days of age), as seen in Figure C.3. Since apomorphine at this dose seemed to have little effect on EtOH-treated mice, 2.5 mg/kg was chosen as one dose to be tested in preliminary studies on the effects of apomorphine on baclofen-inhibition of EtOH-stimulated activity, as described in Experiment 3.

Figure C.3. Effects of 2.5 mg/kg apomorphine on EtOH-stimulated activity of FAST-1 mice. Saline or apomorphine was injected immediately prior to EtOH (2.0 g/kg). Activity test duration was 20 minutes. n = 6 per treatment group; vertical lines are S.E.M.

FIGURE C.3

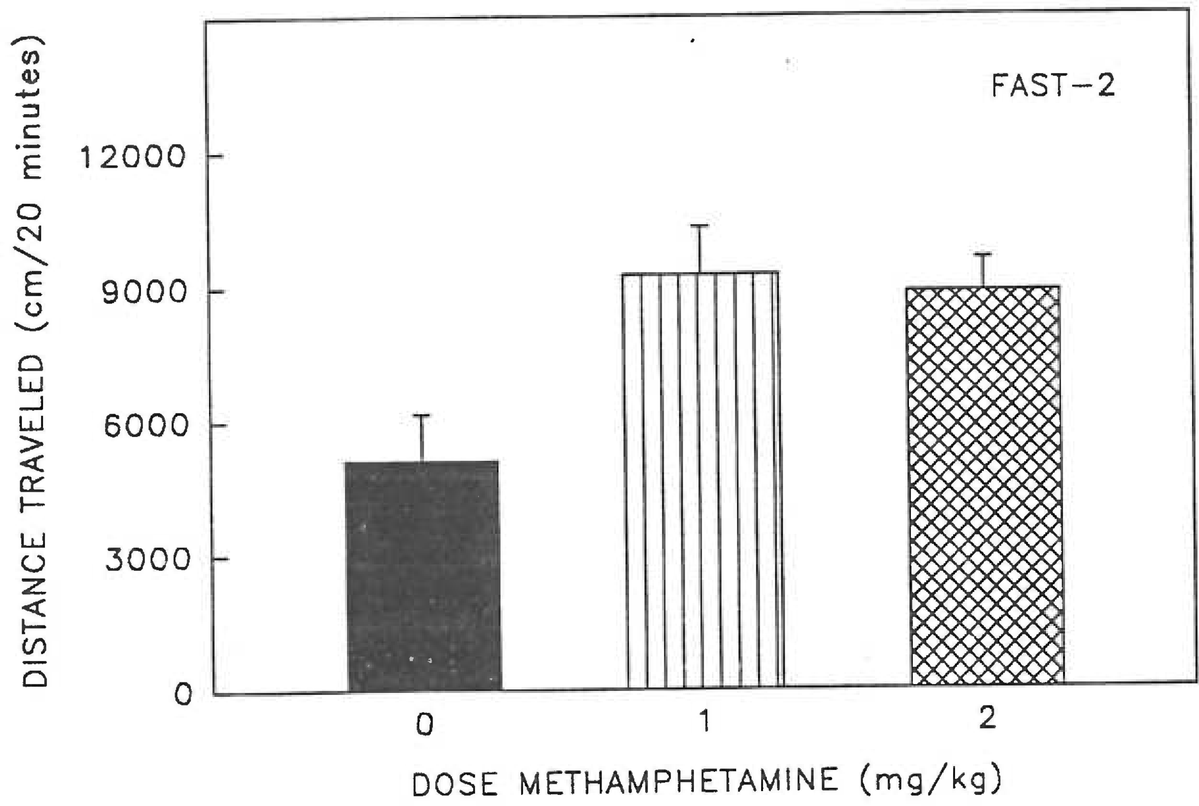


Appendix D: Methamphetamine

FAST-2 male mice ($S_{37}G_{38}$) were 54 - 57 days old when tested for methamphetamine effects on locomotor activity. To mimic the experimental conditions that would occur when baclofen and EtOH were also injected, mice were injected with saline, or 1 or 2 mg/kg methamphetamine, followed by a second injection of saline (equivalent to volume of baclofen injection). After a 15-minute wait, mice were again injected with saline, in a volume equivalent to volume of 20% EtOH needed to give each mouse a 2.0 g/kg dose. Locomotor activity was immediately assessed for 20 minutes. Under these experimental conditions, 1 and 2 mg/kg methamphetamine increased locomotor activity, as shown in Figure D.1.

Figure D.1. Effects of methamphetamine on locomotor activity of FAST-2 mice. Mice were injected with saline or methamphetamine (1 or 2 mg/kg), followed by a second injection of saline (10 ml/kg). After 15 minutes, mice were injected with saline, in a volume equivalent to that required for 2.0 g/kg EtOH (20% v/v). This treatment regimen was used to mimic the experimental conditions when methamphetamine effects of baclofen and EtOH coadministration were tested. Duration of locomotor activity testing was 20 minutes. n = 5 - 6 per treatment group; vertical lines are S.E.M.

FIGURE D.1



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