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Dopamine and Benzodiazepine-Dependent Mechanisms Regulate the EtOH-Enhanced Locomotor Stimulation in the $GABA_A$ α1 Subunit Null Mutant Mice

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The present study investigated the role of the α1-containing $GABA_A$ receptors in the neurobehavioral actions of alcohol. In Experiment 1, mice lacking the α1 subunit (α1 (−/−)) were tested for their capacity to initiate operant-lever press responding for alcohol or sucrose. Alcohol intake in the home cage was also measured. In Experiment 2, the α1 (−/−) mice were injected with a range of alcohol doses (0.875–4.0 g/kg; i.p.) to evaluate the significance of the α1 subunit in alcohol's stimulant actions. In Experiment 3, we determined if the alcohol-induced stimulant effects were regulated via dopaminergic (DA) or benzodiazepine (BDZ)-dependent mechanisms. To accomplish this, we investigated the capacity of DA (eticlopride, SCH 23390) and BDZ (flumazenil, βCCt) receptor antagonists to attenuate the alcohol-induced stimulant actions. Compared with wild-type mice (α1 (+/+)), the null mutants showed marked reductions in both EtOH and sucrose-maintained responding, and home-cage alcohol drinking. The null mutants also showed significant increases in locomotor behaviors after injections of low–moderate alcohol doses (1.75–3.0 g/kg). βCCt, flumazenil, eticlopride, and SCH 23390 were able to attenuate the alcohol-induced stimulation in mutant mice, in the absence of intrinsic effects. These data suggest the α1 receptor plays an important role in alcohol-motivated behaviors; however, it also appears crucial in regulating the reinforcing properties associated with normal ingestive behaviors. Deleting the α1 subunit of the $GABA_A$ receptor appears to unmask alcohol's stimulatory effects; these effects appear to be regulated via an interaction of both DA- and $GABA_A$ BDZ-dependent mechanisms.
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INTRODUCTION

A number of *in vitro* (Criswell *et al*, 1993, 1995; Duncan *et al*, 1995) and *in vivo* (Harvey *et al*, 2002; June *et al*, 2003) studies employing α1 'efficacy' (eg, zolpidem, CL 218, 872; see Griebel *et al*, 1999), and 'binding' (eg, zolpidem, βCCt, 3-PBC; see June *et al*, 2003) selective ligands suggest the α1-containing $GABA_A$ receptors of the ventral pallidum (VP) play an important role in regulating alcohol's neurobehavioral effects, particularly, alcohol's reinforcing properties. Similar to the α1-selective ligands, the α1-null mutant mice provide researchers the opportunity to investigate the significance of the α1 receptor subunit in regulating alcohol's neurobehavioral effects. Developed in two separate laboratories using different gene-targeting methods (Sur *et al*, 2001; Vicini *et al*, 2001), the α1 (−/−) mice have been reported to have a 50–60% loss in total GABA/BDZ receptor number (Sur *et al*, 2001; Vicini *et al*, 2001), and a compensatory increase in $GABA_A$ receptor α2 and α3 subunit peptide expression (37–39%) (Vicini *et al*, 2001) and immunoprecipitation (45–57%) (Sur *et al*, 2001). In addition to the above, it should be noted that other differences and similarities exists among the two different mice populations. Specifically, both knockout mouse lines were created by gene-targeting and embryonic stem cell technologies. Exon 4 of the Sur *et al* (2001) mice was

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replaced with a neo cassette that remains in the α1 locus, while the mice of Vicini *et al* (2001) lack exon 8 and harbor no marker cassette in the targeted locus. The mice of Sur *et al* (2001) were maintained as separate wild-type and knockout mouse lines by breeding mice of the same genotype. In contrast, the mice of Vicini *et al* (2001) were always maintained by interbreeding of heterozygotes. Lastly, the mice of Sur *et al* (2001) were of a mixed C57BL/6J × Strain 129/SvEv genetic background and those of Vicini *et al* (2001) were of a C57BL/6J × Strain 129Sv/SvJ × FVB/N background. However, despite some of the above differences, both lines display similar changes in GABA$_A$ receptor pharmacology, and an absence of any 'overt' behavioral differences (Sur *et al*, 2001; Vicini *et al*, 2001; Kralic *et al*, 2002a, b).

Recent work by Blednov *et al* (2003a, b) have investigated a number of alcohol's responses in the α1-null mutant mice. Specifically, Blednov *et al* (2003b) demonstrated that the α1 mutants consumed decreased amounts of alcohol and saccharin in the home cage. In contrast, Blednov *et al* (2003b) reported no differences between the mutants and α1 (+/+) mice in a conditioned place preference (CPP) paradigm. These discrepant results are likely due to the different paradigms assessing different reinforcing properties associated with alcohol. In addition, germane to alcohol's reinforcing properties, the α1 (−/−) mice have also been used to investigate alcohol's locomotor stimulant actions. Both developing laboratories report that alcohol (0.5–2.5 g/kg) enhances locomotor behavior in the α1 (−/−) mice (Kralic *et al*, 2003; Blednov *et al*, 2003b), while the α1 (+/+) mice were generally unaffected, or very weakly stimulated (Blednov *et al*, 2003b). The sedative actions of alcohol, however, were not investigated in either study. Nevertheless, both studies suggested that the GABA α1 receptor was important in alcohol's stimulant actions.

Additional studies are warranted, however, to more precisely define the exact role the α1 subunit plays in regulating alcohol's neurobehavioral properties. For example, Roberts *et al* (2000) contend employing the home cage as the only reinforcing model that 'is potentially confounded by palatability' (also see June, 2002). Further, as in studies of other abused drugs, the optimal instrument to assess reward efficacy is the operant chamber where the contingency between responding and drinking can be specified and the volume of liquid ingested per completed schedule can be controlled (see June *et al*, 2002). Second, when the 24 h access model is employed as in the Blednov *et al*, study (2003b), it is difficult to determine pharmacologically relevant blood alcohol concentrations (BAC), since the scheduled drinking bout(s) is often difficult to ascertain. BACs are important when trying to determine EtOH's neuromechanism of action (Crabbe *et al*, 1982; Frye and Breese, 1981; Lister, 1987). Third, while low–moderate doses of EtOH (0.50–2.5 g/kg) were used in the previous work (Blednov *et al*, 2003b; Kralic *et al*, 2003), it is possible that stimulation could be detected at higher doses (eg, 3.0–4.0 g/kg) (see Cohen *et al*, 1997), since the null mutants seem highly sensitive to alcohol motor-stimulating effects, and resistant to alcohol's sedative actions (Blednov *et al*, 2003b). In addition, higher doses may permit investigation of the α1 receptor in alcohol's sedative actions. Finally, it is possible that low–moderate EtOH doses may activate different GABA$_A$ receptor subunits and different brain loci compared with higher doses (see Homanics *et al*, 1997; Tauber *et al*, 2003).

While the direct reinforcing actions of alcohol have been investigated in the operant chamber (June, 2002), alcohol's acute reinforcing actions, as with other abused drugs (Di Chiara and Imperato, 1985), have also been indirectly inferred/investigated via the use of locomotor activational effects (Wise and Bozarth, 1987; Koob and Bloom, 1988; Phillips *et al*, 1998). In general, these studies have hypothesized that the locomotor activational effects in mice may be a putative model of alcohol-induced euphoria in humans (Lukas and Mendelson, 1988; Phillips and Shen, 1996; Phillips *et al*, 1998). These studies have suggested the mesoaccumbens-pallidal circuitry mediate alcohol's stimulant actions (see Shen *et al*, 1998). This circuitry comprises dopaminergic neurons that project from the VTA to nucleus accumbens (NAC) where they form connections with neurons possessing GABA$_A$ receptors in the VP. It is well established that GABAergic neurons form a dense reciprocal connection between the NAC and VP (for a review, see Churchill and Kalivas, 1994). Thus, given the above neuroanatomical connectivity between the DA and GABA$_A$ systems, and a plethora of both immunohistochemical and *in situ* hybridization studies confirming the preponderance of mRNA encoding the α1 receptor within the VP (see June *et al*, 2003), it is possible that both dopaminergic and GABAergic mechanisms may play a significant role in the alcohol stimulant actions in the α1-null mutants.

Thus, in the present study, we first tested the hypothesis that the α1 receptor subunit selectively regulated alcohol-motivated behaviors. Secondly, we determined if the ability of alcohol to produce activational effects in the α1 (−/−) mice were regulated via DA, or benzodiazepine (BDZ)-dependent mechanisms. To accomplish this, we evaluated the capacity of DA (eg, eticlopride, SCH 23390) and BDZ (eg, flumazenil, βCCt) antagonists to attenuate the alcohol-induced stimulant actions. The BDZ component of the GABA$_A$ receptor complex was the focus of this study, since Shen *et al* (1998) suggested 'little evidence' supported a role for the GABA$_A$ receptor in mediating the alcohol activational effects in mice highly sensitive to alcohol stimulation using GABA antagonists (eg, bicuculline or picrotoxin).

MATERIALS AND METHODS

Experiment 1

EtOH and sucrose self-administration paradigms.

Subjects: After weaning, male and female GABA$_A$ α1 (−/−) and α1(+/+) mice were obtained from the University of Pittsburgh Medical School. For the alcohol self-administration studies, mice from the F6 and F7 generations were used at an age of 10–13 weeks. A total of 17 α1 (−/−) and 15 α1 (+/+) mice were used (total $N = 32$). Of these, six were female α1 (−/−) and eight were female α1(+/+); while 11 were male α1 (−/−) and seven were male α1 (+/+). Mice were group housed in the vivarium (3–4 per cage) on a 12-h light/dark cycle (lights on at 0700 hours at 21°C). At the beginning of the study, the mice weighed between 20 and 40 g. The animals were group housed in plastic cages in a vivarium at 21°C

on a 12 h light/dark cycle. Food and water were provided *ad libitum* for the animals, except for the conditions noted under the training phase in the two alcohol self-administration studies. The treatment of all subjects was approved by the institutional review board within the School of Science at IUPUI. *All* procedures in Experiment 1, as well as Experiments 2 and 3 below were conducted in strict adherence with the NIH Guide for the Care and Use of Laboratory Animals.

Details on how the mice were derived have been previously (for a review, see Vicini *et al*, 2001; Kralic *et al*, 2002a) and more recently reported (Kralic *et al*, 2003). However, several phenotypical characterizations should be noted here in relation to the α1 (−/−) mice (Vicini *et al*, 2001; Kralic *et al*, 2002a). *These phenotypical characterizations apply to Experiments 1, as well as 2 and 3 below.* First, the α1 (−/−) mice have a 25-Hz handling-induced tremor. Second, there is a 37 ± 6% reduction in seizure threshold in the α1 (−/−) mice. Third, there is a slight, but nonsignificant weight reduction in the α1 (−/−) KO mice. Finally, there is a 65% decrease in β_2/β_3 subunit peptide expression, a 47% decrease in γ_2-subunit peptide expression, and 37 and 39% increase in α_2- and α_3-subunits, respectively. This molecular compensatory response may have important consequences, since distinct behavioral responses have been associated with specific receptor subtypes (for a recent review, see Vicini and Ortinski, 2004; Rudolph and Mohler, 2004; Boehm *et al*, 2004; Kralic *et al*, 2002b).

Apparatus: Animals were tested in seven standard mice operant chambers (Coulbourn Instruments, Inc., Lehigh Valley, PA) equipped with two levers and two dipper assemblies. While only one lever was active during the sessions, the force required by the mice to depress the active lever was reduced during and after training to compensate for the slight tremor present in some, but not all of the α1 (−/−) mice. Red, yellow, and green cue lights were used to indicate the presence of a reinforcer. The lights were illuminated for 2.5 s. Each reinforced response delivered a 0.02 ml of the reinforcer. The reinforcer was presented for a duration of 3.5 s. However, some data were collected using a 10 s duration for comparison with the 3.5 s data to compensate for the tremor in the α1 (−/−) mice. Operant sessions were 30 min in length; however, a 60 min session was also employed to allow additional time for the α1 (−/−) mice to perform because of the tremor. In addition, while a minimum threshold of 4 g of force/pressure typically activates the response lever for mice, the operant device was modified such that a minimum of 2 g of force/pressure activated the response lever.

Solutions: The EtOH (USP) (2–10% v/v) and sucrose (Fisher Scientific) solutions (2–10% w/v) were prepared in deionized water for the operant chamber as previously described for oral self-administration (June, 2002).

Sucrose as the reinforcer: During the initiation period (Phase I), all mice (total $N = 32$) were water-deprived for 2 weeks using a 23.5 h fluid deprivation schedule to facilitate lever pressing. For 30 min daily at 0010 hours, animals received a 10% (w/v) sucrose solution. Mice lever-pressed for the sucrose under a fixed-ratio 1 (FR1) schedule for the 2-week period. The mice were water-deprived for more than the typical 5 days of training (June *et al*, 2003; June, 2002) because the animals did not initiate lever-press responding after a 5-day period. During the 2-week period, the mice were weighed twice weekly and observed for signs of distress. If there was a greater than 10% reduction of body weight, the water deprivation was discontinued. Approximately 65% of the α1 (+/+) mice began lever pressing at the end of week 1 and the water deprivation was no longer required; however, the remainder required the deprivation throughout the second week. Unlike the α1 (+/+) mice, all α1 (−/−) mice required 2 weeks of water deprivation to initiate even a minimal level of lever-press responding for sucrose (see below). Nevertheless, the deprivation procedure was discontinued after the initial 2 weeks, and animals subsequently lever-pressed for the sucrose solution until their responses stabilized, which was defined as having daily responses within ±20% of the average responses for five consecutive days. In Phase II of the operant training, the sucrose initiation procedures continued, however, the reward cost was increased to an FR4 schedule from the FR1 schedule. As with the FR1 schedule, the mice continued to lever-press for the sucrose solution under the FR4 schedule until their responses stabilized. Hence, the total time under the FR4 schedule was 2 weeks.

EtOH as the reinforcer: During Phase III, mice (total $N = 32$) were trained to lever press for EtOH (10% v/v) using a modified version of the sucrose fading-technique previously used for self-administration of EtOH in rats (Harvey *et al*, 2002; June, 2002). The only exception being that the mice were not deprived since they had already been trained to initiate the sucrose reinforcer above. For 30 min daily, animals received either an EtOH + sucrose cocktail mixture or EtOH solution. Specifically, mice were trained to lever-press for an EtOH + sucrose cocktail mixture under an FR1 schedule. The concentration of sucrose was decreased in a step-wise fashion (10, 8, 6, 4, 2, and 0%) and the EtOH was increased in a similar fashion (2, 4, 6, 8, and 10%) over 7–10 days. Animals were subsequently stabilized on the 10% EtOH solution for 7 days under the FR1 schedule. Following stabilization on the FR1 schedule, the Phase IV stage began. Under Phase IV, the reward cost was increased for the 10% EtOH solution from an FR1 schedule to an FR4 schedule. Mice were then stabilized on the FR4 schedule for the 10% EtOH solution for 2 weeks. Stabilization on the 30 min daily FR4 schedule was subsequently followed by stabilization on a 60 min daily FR4 schedule for 2 weeks (Phase V). Phase V was conducted in an attempt to increase the level of responding in both genotypes, and to further confirm that EtOH was indeed serving as a reinforcer in the mice lines. The 10% EtOH concentration was employed since it is one of the standard concentrations that is used in the literature investigating EtOH neuromechanism of action in rodents, and has been shown to produce significant BAC levels in many murine models (for a review, see Grahame and Grose, 2003). The 10% concentration was also employed because it lends itself to cross comparison within the alcohol literature on operant and home-cage EtOH intake in both mice and rats (Elmer *et al*, 1987; Samson *et al*, 1989; also see Grahame and Grose, 2003).

BAC measurement: To ensure animals were consuming pharmacologically relevant amounts of EtOH during operant sessions, BACs were collected in a subset of animals. A random sample of five female and six male α1 (+/+) mice, and five female and five male α1 (−/−) mice were selected. Specifically, after the 30-min operant session, the mice were placed on a heating pad and anesthetized with ketamine–xylazine cocktail (0.1 ml), in order to restrict their movement during the collection of the blood sample. When the animals no longer responded to a pinch to the tail, a heparin-coated microhematocrit tube was used to pierce the retro-orbital sinus membrane. Approximately, 40–60 μl of whole blood was collected into a heparin-coated microsample tube. Hemostatis was achieved by applying light pressure to the closed eye with cotton until the bleeding stopped. The mice remained on the heating pad until they were able to stand and ambulate normally. Then the mice were returned to their original cages. After collection, the whole blood was immediately centrifuged for 5 min at 1100 r.p.m. The specifics of the BAC analyses have appeared in several previous reports from our laboratory (June *et al*, 2003; Foster *et al*, 2004). The retro-orbital sinus procedure is frequently used with mice and has not been reported to impair subsequent responding/activity in behavioral paradigms (Kralic *et al*, 2003).

2-h limited access home-cage paradigm. The same α1 (−/−) and α1 (+/+) mice ($N=32$) that were used in the operant self-administration experiments above were also used in the limited access experiments. A 1-week period occurred between the end of the operant self-administration study and the beginning of the limited access study. Because the mice were initially group housed during the operant studies, an acclimation phase was necessary to singly house them in the home-cage study. Thus, for 2 h daily, the animals were placed individually in a home cage for 7 days prior to the beginning of the limited access drinking study. Then, for a 2-week period, all mice were given EtOH in one bottle, and water in the other. Mice were initially deprived for 22 h to initiate EtOH drinking, however, after 10 days the deprivation schedule was completely discontinued. Mice were never deprived of food. During the initial 3 days, the mice were given 3% (w/v) EtOH. During the next 4 days, they were given 6% (w/v) EtOH, while during the last 3 days they were given 10% (w/v). A similar ascending procedure has been used previously in outbred rats (for details, see June, 2002). Approximately 10.0 ml of EtOH (10% v/v) was weighed out and placed on the cage in calibrated drinking tubes that had minimal spillage (ie, 0.05 ml) over the 2 h period. A similar volume of water was also presented to the mice. The justification for employing only the 10% EtOH concentration was noted above in the EtOH reinforcement section. Nevertheless, the animals had free access to the drinking tube for the 2-h period. At the end of the drinking session, the animals were placed back into their home cages and the amount of EtOH and water was recorded. The drinking session took place during the light cycle, between 1200 and 1500 hours. After the failure of both the α1 (−/−) and α1 (+/+) mice to consume EtOH levels above 0.047 ± 0.01 ml, the 2 h, two-bottle limited access paradigm was modified to a 2 h, one-bottle limited access EtOH paradigm in both the α1 (−/−) and α1 (+/+) mice. Following stabilization of the 10% EtOH after a period of 7 days, the deprivation schedule was discontinued. Mice were then maintained under the 2 h, one-bottle limited access EtOH paradigm for 7 additional days. The data depicted in Figure 2 represents the average of the final 2 days.

Experiment 2

EtOH-enhanced locomotor stimulation: evaluation of BDZ receptor antagonists.

Subjects: To conduct the initial locomotor activity studies, a second cohort of male and female GABA$_A$ α1 (−/−) and α1 (+/+) mice (total $N=31$) were obtained from the University of Pittsburgh Medical School following weaning. The mice (F6 and F7 generations) were of similar age and maintained under identical conditions as Experiment 1, albeit the mice were never deprived of food or water. Of the 31 mice, five were female α1 (−/−) and six were female α1(+/+), while nine were male α1 (−/−) and 11 were male α1(+/+). At the beginning of the study, the mice weighed between 19 and 36 g.

Drugs: For the open-field studies, EtOH (15% v/v) was prepared daily by mixing 95% pure ethanol (U.S.P.A.) with a 0.90% sodium chloride solution in an injection volume sufficient to produce doses of 0.875–4.0 g/kg. βCCt (1.0–15.0 mg/kg) and flumazenil (1.0–15.0 mg/kg) were prepared as an emulsion in 1% Tween-20 vehicle (Sigma-Aldrich, St Louis, MO) and mixed with a 0.90% sodium chloride solution to a volume of 10 ml/kg. When necessary, some drug treatments were sonicated. βCCt was synthesized by several of the authors (WY and JMC) using previously published procedures (see Cox *et al*, 1998; June *et al*, 2003), while Ro15-1788 (flumazenil) was a gift from Hoffman La Roche (Nutley, NJ). The α1-selective mixed agonist–antagonist βCCt (Griebel *et al*, 1999; June *et al*, 2003) was selected since it has been shown to block the reinforcing, and the locomotor depressant actions of EtOH (June *et al*, 2003). Flumazenil, the reference ligand (File and Pellow, 1986) was used since it has also been reported to antagonize some of alcohol's neurobehavioral effects (Lister, 1988; Scollo-Lavizzari and Matthis, 1985; Klotz *et al*, 1986; Knapp *et al*, 2004), albeit, this antagonism has been somewhat controversial (Koob *et al*, 1986; Lister, 1988; June and Lewis, 1994). The two BDZ antagonists were also selected since their *in vitro* efficacy profile has recently been characterized at the α1–α5 receptor subunits (Harvey *et al*, 2002; June *et al*, 2003). The efficacy profile of BDZ receptor ligands has been suggested to be important in determining the precise neuromechanism of action in which ligands antagonize alcohol neurobehavioral properties (for a review, see Jackson and Nutt, 1995; June *et al*, 2003; McKay *et al*, 2004).

Apparatus: Horizontal activity (ie, ambulatory behaviors), total distance, and stereotypy (eg, repetitive grooming, etc.) were recorded individually for 10 min in a Plexiglas chamber ($42 \times 42 \times 30$ cm) using a Digiscan Activity Monitoring System (Accuscan Electronics, Columbus, Ohio, USA). Movement was detected by two sets of four infrared perpendicular photo-beams in the walls of the chamber with 16 beams along each axis. Ambulatory counts were defined as the breaking of the beams in the

X (left–right) or *Y*-axis (front–back). Total distance was measured using the total number of centimeters (cm) traveled. Measurement of stereotypic behavior comprised repetitive breaking of the same beam in a given plane of the open field. All experiments were conducted under dim lighting (25 W) conditions. Immediately after each mouse had completed its session, the entire activity chamber was cleaned to eliminate odors and related stimuli to prevent the next subject from following the path of the prior mouse. Other specific details of these procedures and apparatus have previously and recently been reported (June *et al*, 1998a, b; McKay *et al*, 2004).

Study 1: evaluation of EtOH dose response.

Procedures: To habituate the mice to the activity monitor prior to any drug treatment, mice were given 3-daily 10 min sessions (June *et al*, 1998a, b; McKay *et al*, 2004). These sessions thoroughly habituated the animals to the open-field arena. Activity measurements collected between drug injection days were evaluated to determine any baseline shifting during the testing phase. Following the 3-day acclimation phase, mice received in a randomized sequence 'control' saline pretreatment injection volumes appropriate to doses of 0.875–4.0 g/kg of EtOH. Then, following the saline treatments, mice received in another randomized sequence injections of EtOH alone (0.875–4.0 g/kg). To confirm the reliability of the EtOH injections, each of the EtOH doses (0.875, 1.75, 3.0, and 4.0 g/kg) were randomly administered twice. The two EtOH injections were subsequently averaged for comparison with the control condition. EtOH was administered 5 min prior to the mice being placed in the open field. To control for residual carryover effects, each drug pretreatment was separated by at least 3–5 days and subsequent pretreatments were never administered until activity levels returned to baseline levels (see McKay *et al*, 2004; Cook *et al*, 2005). All injections were administered by the i.p. route.

Study 2: evaluation of BDZ antagonists on EtOH's (3.0 g/kg) actions.

Procedures: Following evaluation of the EtOH dose response, mice received a randomized sequence of EtOH (3.0 g/kg) alone, or in combination with βCCt (3.0 mg/kg + 3.0 g/kg; 7.5 mg/kg + 3.0 g/kg, or 15 mg/kg + 3.0 g/kg). Mice also received in a randomized sequence injections of flumazenil in combination with EtOH (3.0 mg/kg + 3.0 g/kg, 7.5 mg/kg + 3.0 g/kg, or 15 mg/kg + 3.0 g/kg). To determine the intrinsic actions of the BDZ antagonists, the highest dose of βCCt (15 mg/kg) and all three flumazenil doses (3.0, 7.5, 15 mg/kg) were given alone in a randomized sequence. Only the highest dose of βCCt was employed due to the limited amount of this compound at the time of experimental testing. The 3.0 g/kg EtOH injection was randomly given a third time for evaluation of its interaction with the two BDZs. The 3.0 g/kg EtOH dose was selected as the combination EtOH dose with βCCt and flumazenil since it like the 1.75 g/kg dose was the 'optimal' stimulating EtOH dose in the α1 (−/−) mice. In addition, the 3.0 g/kg EtOH dose was selected as the combination dose since it, unlike the 1.75 g/kg dose, produced a marked reduction in locomotor behaviors in the α1 (+/+) mice. Hence, this differential genotype effect of the 3.0 g/kg EtOH dose permitted the investigation of the α1 receptor and its interaction with the BDZ receptor complex in modulating EtOH's stimulant and depressant actions using an established alcohol antagonist (June *et al*, 2003). As noted above, EtOH was administered 5 min prior to being placed in the open field. When βCCt or flumazenil was given in combination with EtOH, they were given *10 min* prior to the EtOH; however, when given alone, they were administered *15 min* prior to being placed in the open field. All mice received their drug treatment in a randomized design to control for order and sequence effects. To control for residual carryover effects, each drug pretreatment was separated by at least 3–5 days and subsequent pretreatments were never administered until activity levels returned to baseline levels (for additional details, see June *et al*, 1998a, b; McKay *et al*, 2004; Cook *et al*, 2005). The rationale for using a 5 min prior to behavioral testing for EtOH was based on the literature showing that this period represents the ascending limb of the BAC curve, which corresponds to the activational/euphoric action of alcohol (Frye and Breese, 1981; Lewis and June, 1990). The rationale for using the 15 min interval was based on extensive work in our laboratory (June *et al*, 1998a, b; June *et al*, 2003; Harvey *et al*, 2002) and those of Breese *et al* (2004) demonstrating that BDZ antagonist can attenuate/block a number of EtOH's neurobehavioral effects. All drug injections were given i.p.

Experiment 3

Evaluation of dopamine receptor antagonists on EtOH's (1.5 g/kg) actions.

Subjects: A third cohort of post weaned α1 (−/−) and α1 (+/+) mice were obtained from the University of Pittsburgh Medical School for the EtOH alone, in combination with the DA antagonists locomotor activity studies. The mice were from the F8 and F9 generations and their ages were between 10 and 13 weeks. They were maintained and derived (see Vicini *et al*, 2001; Kralic *et al*, 2002a, 2003) under identical conditions as Experiments 1, and 2, but were never deprived of food or water. In addition, similar phenotypical characterizations were substantiated in the F8 and F9 generations as in prior generations. A total of 23 α1 (−/−) and 27 α1 (+/+) mice were used (total $N = 50$). Of these, 14 were female α1 (−/−) and 18 were female α1 (+/+), while nine were male α1 (−/−) and nine were male α1 (+/+). At the beginning of the study, the mice weighed between 27 and 42 g.

Drugs: Eticlopride and SCH 23390 were obtained from Sigma-Aldrich, (St Louis, MO). The two DA antagonists were mixed with a 0.90% sodium chloride solution to a volume of 10 ml/kg. The D1 receptor antagonist SCH 23390 and the D2 receptor antagonist eticlopride were selected because of the high concentration of D1 and D2 DA receptors that have been reported in the mesoaccumbens-pallidal circuitry (White and Wang, 1984; Napier and Chrobak, 1992; Lu *et al*, 1998). These ligands were also selected due to their selectivity (Seeman and Ulpian, 1988), and their established roles in blocking the reinforcing actions of alcohol (Hodge *et al*, 1997; McBride and Li, 1998;

Liu and Weiss, 2002; Eiler *et al*, 2003; for a review, see Melendez *et al*, 2005). However, as with flumazenil, this antagonism has been somewhat controversial (Linseman, 1990; Brown *et al*, 1982; for a recent review, see June and Eiler, in press).

Procedures: Animals were habituated in an identical manner as the BDZ study as noted above. Following habituation, mice received a randomized sequence of saline, EtOH alone (1.5 g/kg), eticlopride/SCH 23390 in combination with EtOH (0.01 mg/kg + 1.5 g/kg; 0.02 mg/kg + 1.5 g/kg; 0.08 mg/kg + 1.5 g/kg) or eticlopride/SCH 23390 alone (0.01; 0.02; 0.08 mg/kg). EtOH alone was administered 5 min prior to being placed in the open field. When eticlopride or SCH 23390 was given in combination with EtOH, they were given 2 h prior to the EtOH. When the DA antagonists were given alone, they were administered 2 h and 5 min prior to being placed in the open field. All mice received their drug treatment in a randomized design to control for order and sequence effects. To control for residual carryover effects, each drug pretreatment was separated by at least 3–5 days and subsequent pretreatments were never administered until activity levels returned to baseline levels (see June *et al*, 1998a, b; McKay *et al*, 2004). The DA receptor antagonist doses used in the present study were based on prior reports in the literature demonstrating their effectiveness in blocking the locomotor stimulant actions of EtOH in mice (Shen *et al*, 1995; Cohen *et al*, 1997). The rationale for using the 2 h interval was also based on an extensive search of the alcohol self-administration (see Pfeffer and Samson, 1988; Samson and Hodge, 1996) and locomotor activational (Shen *et al*, 1995; Cohen *et al*, 1997) studies demonstrating that a 0.5–2.5 h interval is needed to avoid untoward/nonspecific effects of the DA antagonist on behaviors. In addition, the 2 h time period was used for administration of the DA receptor antagonists based on preliminary work from our laboratory showing little if any intrinsic effects being observed on the three parameters of locomotor behaviors (ie, ambulatory count, total distance, stereotypy counts). However, this was not the case with shorter intervals such as the 0.5–1.0 h in the mutant or wild-type mice. The 2 h interval is also consistent with previous work evaluating the role of D1 and D2 receptors in motivational related task, particularly where locomotor behaviors are an integral component of the dependent variable measure (CTA learning, place conditioning learning) (Hoffman and Beninger, 1988). All drug injections were given i.p.

Statistical Analyses

Data are reported as the mean ± SEM value. To evaluate differences between groups, analysis of variance (two-way ANOVA) with Newman–Keuls *post hoc* test analyses were carried out in all experiments. In Experiment 3, the averages of the no injection and saline injection conditions were pooled and used as the 'control condition' for comparison with the other drug treatment conditions. Because the study employed both male and female mice of both genotypes, the initial analyses across experiments 1, 2, and 3 were investigated using gender as a factor; however, since no effect of gender was found in any of the experiments, the data were collapsed for analyses in all three experiments.

RESULTS

Experiment 1

Etoh and sucrose self-administration paradigms.

Operant self-administration: EtOH. Figure 1a shows EtOH-maintained responding (10% v/v) for α1 (−/−) and α1 (+/+) mice during a 30-min operant session under an FR4 schedule of reinforcement. The data depicts five consecutive test days following stabilization after the mice had undergone the sucrose-fading procedure. The α1 (−/−) mice lever-pressed profoundly less for EtOH compared with the α1 (+/+) mice (two-way ANOVA; genotype (GT): $F_{(1,144)} = 74.6$, $p < 0.001$; Day: $F_{(4,144)} = 1.49$, $p > 0.05$ with no interaction: $F_{(4,144)} = 1.72$, $p > 0.05$). This profound difference was observed on all days tested (eg, days 1–5), ($p \leqslant 0.05$).

Sucrose. Figure 1b shows sucrose-maintained responding (10% w/v) for α1 (−/−) and α1 (+/+) mice during a 30-min operant session under an FR4 schedule of reinforcement. The data depicts five consecutive test days following stabilization after the mice had undergone the sucrose-training procedures. Compared with the α1 (+/+) mice, the α1 (−/−) lever-pressed for profoundly less sucrose (10% w/v) (two-way ANOVA; GT: $F_{(1,144)} = 192.7$, $p < 0.01$; day: $F_{(1,144)} = 0.89$, $p > 0.05$; with no interaction: $F_{(1,144)} = 0.8724$, $p > 0.05$). Similar to the responding maintained by alcohol, the α1 (−/−) mice consumed markedly less sucrose on all days tested (days 1–5), relative to the α1 (+/+) mice ($p < 0.01$).

EtOH. Figure 1c shows EtOH-maintained responding (10% v/v) for α1 (−/−) and α1 (+/+) mice during a 60-min operant session under an FR4 schedule of reinforcement. The data depicts four consecutive test days following stabilization after the mice had undergone the sucrose-fading procedure. Again, the α1 (−/−) mice lever-pressed profoundly less for EtOH compared with the α1 (+/+) mice (two-way ANOVA; GT: $F_{(1,26)} = 38.25$, $p < 0.0001$; day: $F_{(2,52)} = 1.22$, $p > 0.05$ with no interaction: $F_{(2,52)} = 1.16$, $p > 0.322$). This marked difference in responding was observed on test days 1–5 ($p < 0.01$). Compared with the 30 min session, responding during the 60 min session was markedly greater in the α1 (+/+) mice across each of the 4 test days ($p < 0.01$); however, it was similar in the α1 (−/−) mice ($p > 0.05$).

BAC determination. Body weights of the α1 (+/+) mice ($N = 11$) used for BAC determination ranged from 25 to 40 g. EtOH (10% v/v) responding for the α1 (+/+) mice yielded intakes of 0.28–7.17 g/kg of absolute EtOH. Consumption in milliliters was 0.10–2.95. BACs ranged from 11.7 to 34.4 mg/dl. Body weights of the α1 (−/−) mice ($N = 10$) used for BAC determination ranged from 22 to 28 g. EtOH responding for the α1 (−/−) mice yielded intakes of 0.0–0.3 g/kg of absolute EtOH. Consumption in milliliters was 0.0–0.10. BACs ranged from 1.8 to 4.45 mg/dl. There was a significant difference in the BACs of the α1 (+/+) in comparison with the α1 (−/−) mice (mean = 28 ± 2.3 *vs* 2.6 ± 0.13 mg/dl) ($t = 8.84$; df = 19, $p < 0.01$, two-tail *t*-test).

2-h limited access paradigm: *EtOH*. Figure 2 shows EtOH (10% v/v) intake in g/kg for the α1 (−/−) and α1 (+/+) mice during the 2-h limited access home-cage paradigm.

Following stabilization on the EtOH only availability paradigm for 7 days, average intakes on the 6–7th day were recorded for comparison between the two genotypes.

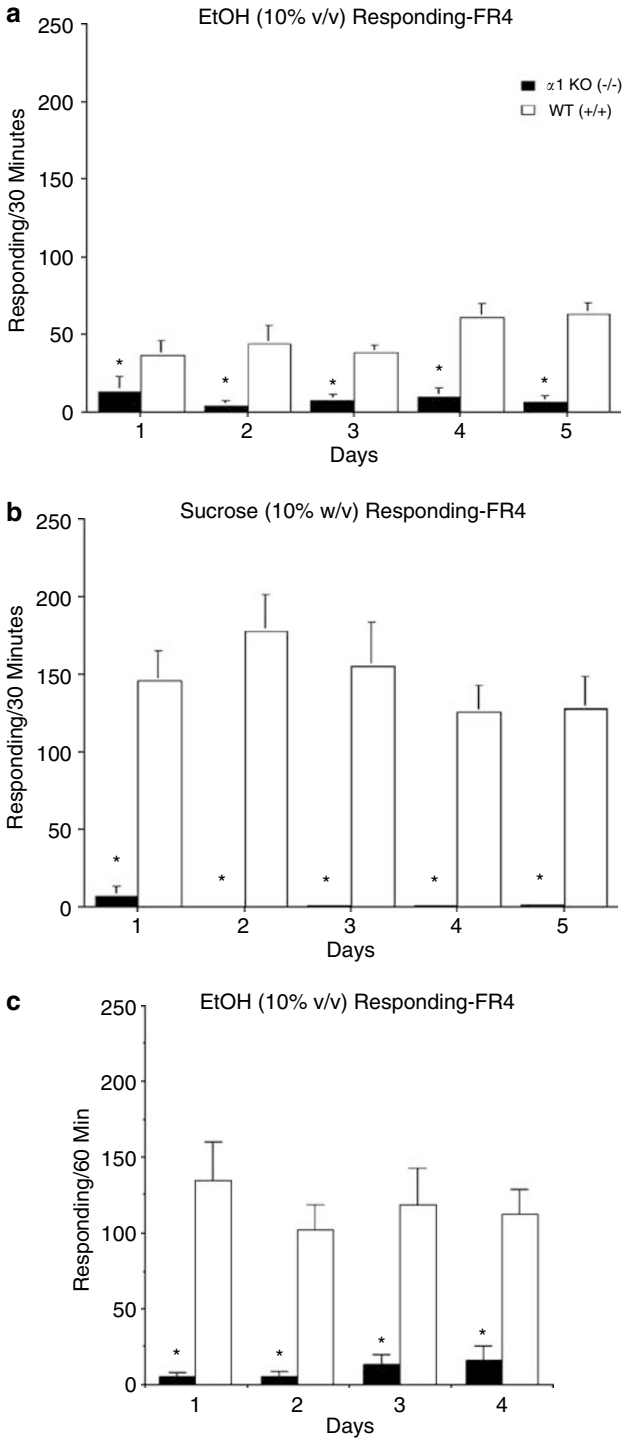

Figure 1 (a) The rate of operant responding for EtOH (10% v/v) for the GABA$_A$ α1 (−/−) KO and WT (+/+) mice on an FR4 schedule during a 30 min session for 5 days. (b) The rate of operant responding for sucrose (10% w/v) for the GABA$_A$ α1 (−/−) KO and WT (+/+) mice on an FR4 schedule during a 30 min session for 5 days. The GABA$_A$ α1 (−/−) KO mice consumed significantly less ethanol and sucrose. (c) The rate of operant responding for EtOH (10% v/v) for the GABA$_A$ α1 (−/−) KO and WT (+/+) mice on an FR4 schedule during a 60 min session for 4 days. *$p<0.01$.

A significant difference in EtOH (10% v/v) intake between the α1 (−/−) and α1 (+/+) mice was observed ($F_{(1,16)}=7.9$, $p\leqslant 0.013$). EtOH intake in g/kg for the α1 (−/−) mice was 0.583± 0.34 *vs* 1.84± 0.29 for the α1 (+/+) ($p<0.01$).

Experiment 2

Saline pretreatments. As noted above in the procedure section, before the drug treatments began, the mice were acclimated to the open-field testing and given randomized saline pretreatments in injection volumes sufficient to produce EtOH doses of 0.875–4.0 g/kg. A within genotype evaluation of these data across each of the three locomotor parameters, at the four different saline injection dose volumes (ie, 0.875–4.0 g/kg), revealed that each of the saline dose volumes were statistically similar ($p>0.05$). The sole exception was with the stereotypy count parameter in the α1 (−/−) ($F_{(3,42)}=3.98$, $p<0.01$) (data not shown). In addition, a between-genotype evaluation across each of the three locomotor parameters, at the four different saline injection dose volumes revealed, except for the stereotypy parameter at the 0.875 g/kg dose level ($p<0.05$), that none of the other saline pretreatments were significantly different ($p>0.05$) between the α1 (−/−) and α1 (+/+) mice (data not shown). Hence, because the different saline injection dose volumes were similar within and between each genotype across the three locomotor parameters (exception noted above), the saline data were pooled across each activity parameter for each genotype and used as a baseline value for comparison with the EtOH alone (Figure 3a–c), and EtOH in combination with the BDZ antagonists data (Figure 4a–b). The pooled data between the α1 (−/−) and α1 (+/+) mice across the three locomotor parameters were not statistically significant ($p>0.05$).

The initial presentation of the locomotor activity data following the EtOH treatment depicts three locomotor activity parameters (Figure 3a–c). The rationale for this stems from the fact that previous studies evaluating EtOH

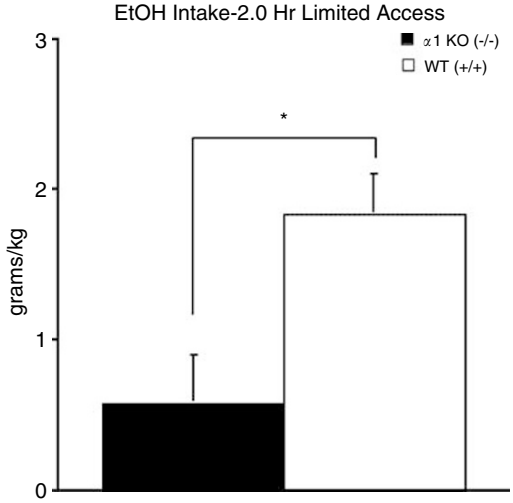

Figure 2 EtOH (10% v/v) consumption for the GABA$_A$ α1 (−/−) KO and WT (+/+) mice during a 2-h limited access session. The GABA$_A$ α1 (−/−) KO mice consumed significantly less ethanol. *$p<0.01$.

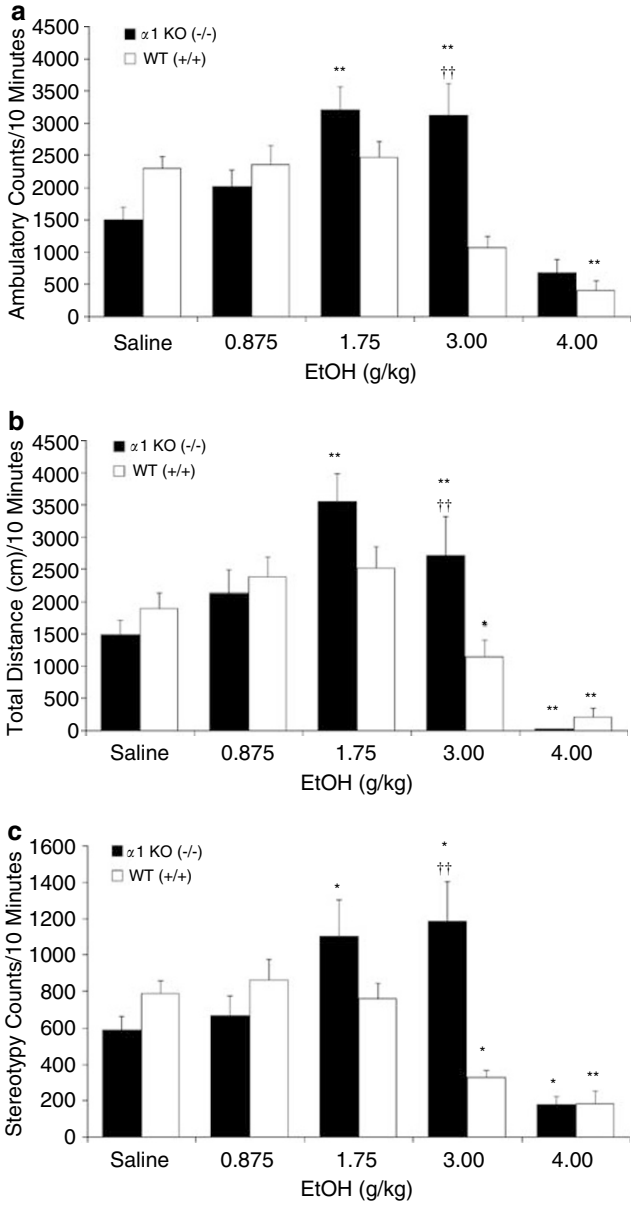

Figure 3 (a) Horizontal activity in the GABA$_A$ α1 (−/−) KO and WT (+/+) mice following i.p. administration of EtOH (10% v/v) (0.875–4.0 g/kg). (b) Effects of EtOH (10% v/v) (0.875–4.0 g/kg) on total distance traveled by the GABA$_A$ α1 (−/−) KO and WT (+/+) mice in a 10-min activity monitor session. (c) Stereotypy in GABA$_A$ α1 (−/−) KO and WT (+/+) mice following i.p. administration of EtOH (10% v/v) (0.875–4.0 g/kg). $^{**}p<0.01$, $^{*}p<0.05$ EtOH *vs* Saline control; $^{\dagger\dagger}p<0.01$ α1 (−/−) KO *vs* WT (+/+) mice. Data are shown as mean (±SEM).

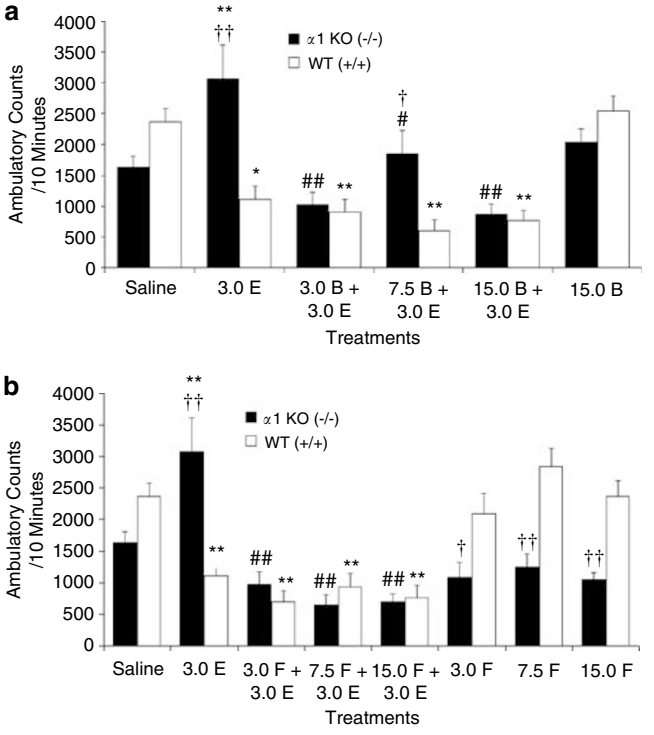

Figure 4 (a) Horizontal activity in the GABA$_A$ α1 (−/−) KO and WT (+/+) mice following i.p. administration of the 3.0 g/kg dose of EtOH (10% v/v) alone and in combination with various doses of βCCt (3.0–15.0 mg/kg). The effect of the 15.0 mg/kg dose of βCCt alone is also depicted. $^{**}p<0.01$ treatment *vs* Saline control; $^{\dagger\dagger}p<0.01$ α1 (−/−) KO *vs* WT (+/+) mice; $^{\#\#}p<0.01$, $^{\#}p<0.05$ combination dose *vs* EtOH alone. Data are shown as mean (±SEM). (b) Horizontal activity in the GABA$_A$ α1 (−/−) KO and WT (+/+) mice following i.p. administration of the 3.0 g/kg dose of EtOH (10% v/v) alone and in combination with various doses of flumazenil (3.0–15.0 mg/kg). The effects of all doses (3.0–15.0 mg/kg) of flumazenil alone are also depicted. $^{**}p<0.01$ treatment *vs* Saline control; $^{\dagger\dagger}p<0.01$, $^{\dagger}p<0.05$ α1 (−/−) KO *vs* WT (+/+) mice; $^{\#\#}p<0.01$ combination dose *vs* EtOH alone. Data are shown as mean (±SEM).

or BDZ actions on locomotor behaviors in the α1 mutant and wild-type mice have employed ambulatory counts (Blednov *et al*, 2003b), total distance (Kralic *et al*, 2003), and stereotypy (Reynolds *et al*, 2003) measurements. Thus, by illustrating all three parameters in the present study, a comparison across the three studies can be made before (ie, basal activity) and following drug treatment. However, because of the relatively similar profile of effects observed across the three locomotor activity parameters following the BDZ and DA interactional studies with EtOH, only the ambulatory count parameter will be illustrated. The authors selected the ambulatory count parameter (also referred to as horizontal activity) to illustrate the BDZ and DA interactional studies with EtOH since this parameter is one of the most frequently presented locomotor activity measures in the pharmacology literature (see Lister, 1988; Phillips and Shen, 1996; June *et al*, 1998a, b), and hence, lends itself to comparisons across many studies in the literature.

Study 1: evaluation of EtOH dose response.

Ambulatory counts: Figure 3a shows ambulatory counts for the α1 (−/−) and α1 (+/+) mice following EtOH pretreatments (0.875–4.0 g/kg). A two-way ANOVA revealed a significant dose ($F_{(4,112)}=35.56$, $p<0.01$) and dose × genotype (GT) interaction ($F_{(4,112)}=47.73$, $p<0.01$); however, the main effect of GT was not significant ($F_{(1,28)}=0.39$, $p>0.05$). While basal activity rates in the α1 (−/−) mice were reduced relative to the α1 (+/+) mice, these effects did not reach statistical significance ($p>0.05$). *Post hoc* tests revealed the 1.75 and 3.0 g/kg EtOH doses significantly increased ambulatory counts in the α1 (−/−) mice ($p<0.01$); however, the activation seen with the 0.875 g/kg dose was not significant ($p>0.05$). The reduction seen with the 4.0 g/kg dose in the α1 (−/−) mice also

reached significance ($p<0.05$). In the α1 (+/+) mice, both the 3.0 and 4.0 g/kg doses produced marked reductions in ambulation ($p\leqslant 0.05$). In addition, a significant genotype effect was apparent at the 3.0 g/kg dose level with the α1 (−/−) mice exhibiting a markedly greater activation compared with the α1 (+/+) mice ($p<0.01$).

Total distance (cm): Figure 3b shows total distance in centimeters for the α1 (−/−) and α1 (+/+) mice following EtOH pretreatments (0.875–4.0 g/kg). A two-way ANOVA revealed a significant dose ($F_{(4,112)}=49.63$, $p<0.01$), and dose × GT interaction ($F_{(4,112)}=68.78$, $p<0.01$); however, the main effect of GT was not significant ($F_{(1,28)}=0.19$, $p>0.05$). *Post hoc* analyses confirmed that the 1.75 and 3.0 g/kg doses significantly increased total distance in the α1 (−/−) mice, while the 4.0 g/kg doses significantly reduced it ($p<0.01$). Similar to the ambulation parameter, *post hoc* analyses confirmed that both the 3.0 and 4.0 g/kg doses produced marked reductions on distance traveled in the α1 (+/+) mice ($p<0.01$). Finally, at the 3.0 g/kg dose level, a significant genotype effect was observed with the α1 (−/−) mice exhibiting a significantly greater activation than the α1 (+/+) mice ($p<0.01$).

Stereotypy counts: Figure 3c shows stereotypy counts in the α1 (−/−) and α1 (+/+) mice following EtOH pretreatments (0.875–4.0 g/kg). A two-way ANOVA revealed a significant dose ($F_{(4,112)}=16.32$, $p<0.01$) and GT × dose interaction ($F_{(4,112)}=30.56$, $p<0.01$). However, the main effect of GT was not significant ($F_{(1,28)}=0.28$, $p>0.05$). *Post hoc* test revealed that the 1.75 and 3.0 g /kg EtOH doses significantly increased stereotypy in the α1 (−/−) mice ($p<0.05$), while the 4.0 dose reduced it ($p<0.05$). In addition, both the 3.0 and 4.0 g/kg doses reduced stereotypy in the α1 (+/+) mice ($p<0.01$). As with the ambulatory and distance travel parameters, a simple effect analysis at the EtOH dose level revealed that at the 3.0 g/kg dose, a significant effect of genotype was apparent, with the α1 (−/−) mice exhibiting a profound activational effect compared with the α1 (+/+) mice ($p<0.01$).

Study 2: evaluation of BDZ antagonists on EtOH's (3.0 g/kg) actions.

βCCt and EtOH: Figure 4a shows the effects of EtOH alone, and in combination with the various doses of βCCt (3.0–15.0 mg/kg) on ambulation in the α1 (−/−) and α1 (+/+) mice. The highest βCCt dose given alone is also depicted. Compared with the EtOH alone condition, all βCCt treatments attenuated the EtOH-induced stimulation in the α1 (−/−) mice; however, none of the βCCt treatments were effective in altering the EtOH-induced sedation in the α1 (+/+) mice. A two-way ANOVA revealed a significant dose ($F_{(4,80)}=957.96$, $p<0.01$) and GT × dose interaction ($F_{(4,80)}=120.86$ ($p<0.01$)); however, the main effect of GT was not significant ($F_{(1,20)}=0.009$, $p>0.05$). Newman–Keuls *post hoc* analyses confirmed that the 3.0 and 15.0 mg/kg doses of βCCt given immediately prior to the EtOH attenuated the EtOH-induced stimulation in the α1 (−/−) KO mice ($p<0.01$), while the 7.5 mg/kg dose resulted in a complete reversal of the EtOH-induced stimulantion ($p<0.05$). In further support of the βCCt attenuation, the combination doses were either indistinguishable from the saline control condition as with the 7.5 mg/kg βCCt combination ($p>0.05$), or slightly lower as with the 3.5 and 15 mg/kg combination conditions (albeit statistically similar) ($p>0.05$). In contrast, *post hoc* test showed that none of the three βCCt doses (3.0–15.0 mg/kg) attenuated the EtOH-induced sedation in the α1 (+/+) mice ($p>0.05$); ambulatory counts for the combination conditions were not significantly different from the 3.0 g/kg EtOH alone condition in the α1 (+/+) mice ($p>0.05$). Newman–Keuls *post hoc* test further revealed that given alone, the 15.0 mg/kg βCCt dose was without effect on ambulatory counts in the α1 (−/−) and α1 (+/+) mice ($p>0.05$). Hence, the highest dose of βCCt was devoid of intrinsic effects in both genotypes on ambulatory behaviors.

Flumazenil and EtOH: Figure 4b shows the effects of EtOH alone (3.0 g/kg), and in combination with the various doses of flumazenil (3.0, 7.5, 15.0 mg/kg) on ambulation in the α1 (−/−) and α1 (+/+) mice. The three flumazenil doses given alone are also depicted. A two-way ANOVA revealed a significant dose ($F_{(7,140)}=114.73$, $p<0.01$) and GT × dose interaction ($F_{(7,140)}=47.99$, $p<0.01$); however, the main effect of GT was not significant ($F_{(1,20)}=0.08$, $p>0.05$). *Post hoc* analyses confirmed that the combination doses of flumazenil (3.0–15.0 mg/kg) given immediately prior to the EtOH attenuated the EtOH-induced stimulation in the α1 (−/−) mice ($p<0.01$); however, flumazenil did not alter the sedation in the α1 (+/+) mice ($p>0.05$). While the flumazenil combinations in the α1 (−/−) mice were reduced relative to the control condition, none were statistically lower than the control condition ($p>0.05$). In contrast, in the α1 (+/+) mice, each of the three flumazenil combinations were significantly lower compared with the control condition ($p<0.01$). Flumazenil did not significantly alter ambulatory behaviors in the α1 (−/−) or α1 (+/+) mice relative to their respective control conditions ($p>0.05$). However, a genotype comparison at each of the three flumazenil dose levels shows that activity in the α1 (+/+) mice was markedly enhanced relative to the α1 (−/−) ($p\leqslant 0.05$). This starkly contrasted the 15 mg/kg βCCt dose condition given alone in the α1 (−/−) and α1 (+/+) mice ($p>0.05$) (see Figure 4a). Hence, while flumazenil alone did not significantly alter ambulation relative to control levels in either genotype ($p>0.05$), it reduced activity levels in the α1 (−/−) mice/or elevated activity in the α1 (+/+) mice to such an extent that it produced a 'profound' separation between the genotypes on ambulatory behaviors at the 3.0 ($p<0.05$), 7.5 ($p<0.01$), and 15 mg/kg ($p<0.01$) dose levels.

Experiment 3

Evaluation of dopamine receptor antagonists on EtOH's (1.5 g/kg) actions.

Eticlopride and EtOH: Figure 5a illustrates the effects of EtOH alone (1.5 g/kg), and in combination with the various doses of eticlopride (0.01–0.08 mg/kg) on ambulation in the α1 (−/−) and α1 (+/+) mice. The three eticlopride doses given alone are also depicted. A two-way ANOVA revealed a significant dose [$F_{(7,\ 30)}=174.99$, $p<0.01$] and dose × GT interaction ($F_{(7,30)}=191.03$ ($p<0.01$)) effects; however, the main effect of GT only approached significane

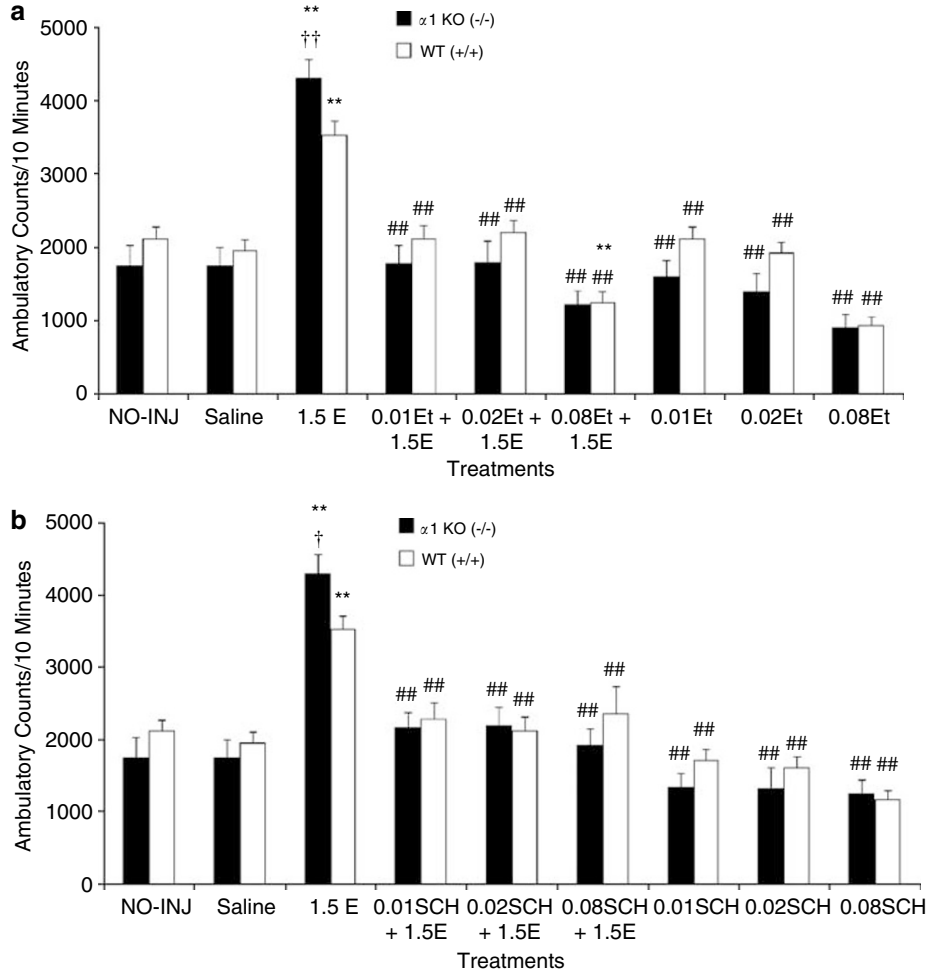

Figure 5 (a) Horizontal activity in the GABA$_A$ α1 (−/−) KO and WT (+/+) mice following i.p. administration of the 1.50 g/kg dose of EtOH (10% v/v) alone and in combination with various doses of eticlopride (0.01–0.08 mg/kg). The effects of all doses (0.01–0.08 mg/kg) of eticlopride alone are also depicted. $^{**}p<0.01$ treatment *vs* Saline control; $^{\dagger\dagger}p<0.01$ α1 (−/−) KO *vs* WT (+/+) mice; $^{\#\#}p<0.01$ combination dose *vs* EtOH alone. Data are shown as mean (±SEM). (b) Horizontal activity in the GABA$_A$ α1 (−/−) KO and WT (+/+) mice following i.p. administration of the 1.50 g/kg dose of EtOH (10% v/v) alone and in combination with various doses of SCH 23390 (0.01–0.08 mg/kg). The effects of all doses (0.01–0.08 mg/kg) of SCH 23390 alone are also depicted. $^{**}p<0.01$ treatment *vs* Saline control; $^{\dagger}p<0.05$ α1 (−/−) KO *vs* WT (+/+) mice; $^{\#\#}p<0.01$ combination dose *vs* EtOH alone. Data are shown as mean (±SEM).

($F_{(1,44)}=3.61$, $p>0.05$). Compared with the control conditions (eg, no injection and saline injection), the 1.5 g/kg EtOH injection led to an increase in ambulation in both the α1 (−/−) and α1 (+/+) mice ($p<0.01$). When eticlopride was given 2 h prior to the EtOH injection, all doses of eticlopride attenuated the increase in ambulation seen with EtOH alone in both genotypes ($p<0.01$). Given alone, eticlopride was without effect in both genotypes ($p>0.05$), except for the highest dose (0.08 mg/kg) that produced a reduction in ambulation ($p<0.01$). Finally, *post hoc* analyses revealed that while the 1.5 g/kg EtOH dose increased ambulation in both genotypes, the enhancement was significantly greater in the α1 (−/−) mice ($p<0.01$).

SCH 23390 and EtOH: Figure 5b illustrates the effects of EtOH alone (1.5 g/kg), and in combination with the various doses of SCH 23390 (0.01–0.08 mg/kg) on ambulation in the α1 (−/−) and α1 (+/+) mice. The three SCH 23390 doses given alone are also depicted. The EtOH alone data are redrawn from Figure 5a. A two-way ANOVA revealed a significant main effect of dose ($F_{(7,168)}=88.55$, $p<0.01$) and GT ($F_{(1,24)}=5.05$, $p<0.05$). A highly significant GT × dose interaction also emerged ($F_{(7,168)}=107.11$ ($p<0.01$)). Compared with the EtOH alone condition, similar to the three eticlopride doses, each of the three SCH 23390 doses attenuated the EtOH-induced locomotor stimulation ($p<0.01$). Given alone, SCH 23390 was without effect in both genotypes, except for the highest dose (0.08 mg/kg) that produced a reduction in ambulatory behaviors in the α1 (+/+) mice ($p<0.01$).

DISCUSSION

The α1-Containing GABA$_A$ Receptor Mediates EtOH and Sucrose-Maintained Responding

The results of the present study demonstrate that the α1-containing GABA$_A$ receptor is necessary for both EtOH and sucrose-motivated behaviors. BAC levels indicated α1 (+/+) mice consumed pharmacologically relevant

amounts of EtOH above and beyond that of the α1 (−/−) mice in the operant chamber (28 ± 2.3 *vs* 2.6 ± 0.13 mg/dl, respectively). The data from the two EtOH self-administration paradigms and the sucrose operant paradigm parallel those of Blednov *et al* (2003b) in the 24 h home-cage study with the 12 and 15% EtOH concentrations. Blednov *et al* (2003b), however, did not differentiate between the two genotypes at the 3, 6, and 9% (v/v) concentrations. This is likely due to the insensitivity of the home-cage paradigm (Roberts *et al*, 2000). It is well documented that a high correlation exists between intake of EtOH and sweet solutions in the P and HAD rats (Murphy *et al*, 2002; Woods *et al*, 2003; Eiler *et al*, 2005). However, it is important to note that as with the P rats (Stewart *et al*, 1994), the α1 (−/−) mice did not show an oral preference for bitter tasting solutions (Blednov *et al*, 2003b); hence, the link between EtOH and reinforcement for sweet palatable solutions in both α1-null mutants and P rats was reinforcer specific. It should also be noted that the study unfortunately did not employ a design using multiple EtOH concentrations; thus, the degree to which concentrations in excess of 10% would differentiate the α1 (+/+) and α1 (−/−) mice in the operant chamber is not clear.

Hypothesized Mechanism of Action in Reducing EtOH and Sucrose-Maintained Responding Following Deletion of the GABA$_A$ α1 Subunit Receptor

While deletion of the GABA$_A$ α1- subunit receptor may be associated with reduction of the positive reinforcing properties associated with EtOH and sucrose, other hypotheses must be considered. First, the behavioral phenotype of the α1 (−/−) mice may be due in part to the compensatory increases and/or decreases of non-targeted subunits (ie, 65% decrease in β_2/β_3 subunit peptide expression; 47% decrease in γ_2-subunit peptide expression; and 37 and 39% increase in α2 and α3 subunits, respectively (Sur *et al*, 2001; Vicini *et al*, 2001; Boehm *et al*, 2004; Rudolph and Mohler, 2004)). It is worth noting, however, that the β_2 (−/−) mice did not show a decreased consumption of EtOH (3–15% v/v) (Blednov *et al*, 2003b), suggesting that there is something unique about the α1 subunit that contributes to the decreased lever pressing for EtOH and sucrose. Second, while the α1 (−/−) mice have normal ataxic and locomotor behaviors, they possess a 25-Hz tremor (Vicini *et al*, 2001; Sur *et al*, 2001). The tremor may have affected the ability of the α1(−/−) mice to lever-press for the available reinforcer, despite modifications to accommodate the tremor (see Materials and methods). However, locomotor activity is less of a consideration in the home-cage paradigm and the α1 (−/−) mice still consumed markedly less EtOH relative to the α1 (+/+). Finally, it should be noted that very low EtOH doses have been shown to eliminate/normalize the tremor (unpublished observations), and thus, a confounding effect of tremor is unlikely to be present after the α1 (−/−) mice consume minute quantities of EtOH. Thus, the 25-Hz tremor does not seem to be a likely hypothesis in explaining the differential ingestive behavioral profile between the genotypes.

Finally, it is clear that the genotype differentiation was most profound in the operant chamber of the present study, irrespective of the reward type. It is possible that deletion of the α1 subunit may directly/indirectly influence the production of instrumental responding rather than directly effect the rewarding stimuli *per se*. Presently, DA is the primary neurotransmitter that has been associated with instrumental responding (for a review, see Salamone and Correa, 2002). Salamone and Correa (2002) have hypothesized that DA may 'promote expenditure of effort in instrumental tasks', particularly in ratio schedules. One way in which the α1 subunit might modulate DA is being localized directly on DA cell bodies in the mesoaccumbens circuitry in loci such as the VTA (Fritschy and Mohler, 1995; Charlton *et al*, 1997), or possibly via an action of GABA interneurons (Johnson and North, 1992) on primary DA cell bodies. Thus, by removing the α1 inhibitory GABAergic tone in the VTA, the integrity of the normal downstream GABA/DA circuitry in reinforcing loci such as the nucleus accumbens (NAcc) and the VP is compromised, resulting in an inability of the α1 (−/−) mice to engage in instrumental responding. It is interesting to note that while Phillips *et al* (1998) found that D2 receptor (−/−) mice were capable of home-cage alcohol drinking (albeit at much lower levels than their D2 (+/+) counterparts), Risinger *et al* (2000) reported that the D2 (−/−) mice demonstrated a profound reduction in EtOH-maintained responding (similar to α1 (−/−) mice in the current study). Hence, a reduction of DA neurotransmission in the α1 (−/−) mice may predispose them to a failure/reduced capacity to initiate lever-press responding for instrumental reinforcers (but see June *et al*, 2003). In contrast to the instrumental responding hypothesis, it is possible that the inhibitory GABAergic tone removal may disinhibit DA efflux, resulting in a downstream elevation of DA in the NAcc, VP, or BST (see below) (see Harvey *et al*, 2002). If this was the case, DA substitute for the EtOH/sucrose reward and the α1 (−/−) mice would not be appetitively motivated to seek out rewards.

The Role of the α1 Containing GABA$_A$ Receptor in the Stimulant and Sedative Properties of EtOH: An Evaluation Across Multiple Locomotor Behaviors

The present study extends prior research demonstrating that deletion of the α1-containing GABA$_A$ receptor enhances the capacity to observe EtOH-induced stimulation in the open field (Blednov *et al*, 2003b; Kralic *et al*, 2003). However, in contrast to the Blednov *et al* (2003b) study, none of the doses tested produced stimulation in the α1 (+/+) mice in Experiment 2. Furthermore, the 3.0 g/kg dose of EtOH produced only sedation in the α1 (+/+) mice (see Figure 3). These findings observed with the α1 (+/+) are not consistent with the existing literature demonstrating EtOH-induced stimulation in 'outbred' mice with doses of 1.0–3.0 g/kg (Frye and Breese, 1981; Phillips and Shen, 1996; Cohen *et al*, 1997). In the α1 (−/−) mice, a dose as high as 4.0 g/kg was required to cause significant suppression. These mice seem resistant to the sedative effects of EtOH. The magnitude of suppression with the 4.0 g/kg dose, however, was similar in both genotypes, albeit it is likely that the effects of such an intoxicating EtOH dose is regulated via multiple α receptor subtypes (see Homanics *et al*, 1997; Tauber *et al*, 2003). In the current study,

however, there is a trend for the α1 (+/+) mice to be more active than the α1 (−/−) mice across the three locomotor parameters (see Figure 3a–c). Blednov *et al* (2003b) also reported that basal activity levels were greater in the α1 (+/+) compared with the α1 (−/−) mice. Thus, basal difference between the genotypes is not a likely explanation of the discrepant results between the two studies. Rather, differences in breeding strategies or genetic backgrounds maybe a more tenable explanation.

Hypothesized Mechanism of Action Regulating the EtOH-Induced Activation Following Deletion of the α1-Containing $GABA_A$ Subunit Receptor

While deletion of the α1-containing $GABA_A$ receptor appears to plays a salient role in EtOH-induced stimulant effects (Kralic *et al*, 2003; Blednov *et al*, 2003b), the exact neuromechanism(s) regulating these effects are not clear. Several potential hypotheses, however, could account for these effects. First, in the absence of the α1 inhibitory sedative influences in CNS brain loci (eg, cortex, thalamus, hypothalamus, hippocampus, amygdala, ventral pallidum, midbrain, cerebellum) (Churchill *et al*, 1991; Fritschy and Mohler, 1995; Pirker *et al*, 2000), the EtOH-induced stimulation maybe unmasked and more readily observed. Second, it is possible elevation of α2 and α3 receptors in mesolimbic loci (eg, cortex, hypothalamus, hippocampus, amygdala, nucleus accumbens, VTA, bed nucleus of the stria terminalis (BST), etc.) (Fritschy and Mohler, 1995; Pirker *et al*, 2000; Kaufmann *et al*, 2003) enhances the capacity of i.p. doses of EtOH to activates/modulate $GABA_A$ receptors. These effects could result in an elevation of DA in putative reward areas (eg, amygdala, nucleus accumbens, BST) and a subsequent increase in locomotor behaviors. It is well established that $GABA_A$ agonists have been reported to release DA and increase locomotion (Kalivas *et al*, 1990). In addition, $GABA_A$ agonists have also been shown to increase DA neuronal firing in the mesolimbic dopamine system (Xi and Stein, 1998). Finally, it is possible that compensatory changes in the α1 mutants may extend beyond the GABAergic or dopaminergic systems (Reynolds *et al*, 2003; Boehm *et al*, 2004; Vicini and Ortinski, 2004).

BDZ Antagonists Attenuate the EtOH-Induced Stimulation in α1 (−/−) Mice, but Fail to Reverse the Sedation in α1 (+/+) Mice

The third major finding of the present study was that both βCCt and flumazenil were capable of significantly attenuating the EtOH-induced stimulant actions in the α1 (−/−) KO mice. Recombinant receptor studies show that βCCt exhibits a >10-fold selectivity for the GABA α1 over the α2 and α3 receptors, and a >110-fold selectivity for the α1- over the α5 subtype (Cox *et al*, 1995). Thus, βCCt exhibits the greatest binding selectivity of the currently available α1 receptor ligands (June *et al*, 2003; McKernan *et al*, 2000; Cox *et al*, 1998). In contrast, flumazenil is a nonselective BDZ-binding antagonist at the diazepam-sensitive sites (Huang *et al*, 2000). In relation to physiological efficacy (ie, potentiation of GABAergic activity), *Xenopus* oocyte studies have reported that both βCCt and flumazenil demonstrate a neutral or low efficacy agonist response profile across the α1, α2, α3, α4, and α5 receptors (June *et al*, 2003). However, despite the qualitatively similar response profile of βCCt and flumazenil, unlike βCCt, flumazenil significantly increased the GABA currents at the α2, α3, and α4 subtypes relative to the control condition. The functional behavioral significance of such low-level GABAergic modulation is not known. The differential interaction of βCCt with the 3.0 g/kg EtOH dose, however, could be due to βCCt's action at different receptor subunits depending on the dose of the drug administered. For example, in the human HEK cell assay, βCCt produced GABA neutral effects at the α1–α5 receptors using 1–10 μM concentrations; however, it produced partial to full agonist effects (23–75%) at 10–100 μM concentrations at these same receptors (June, 2003). It is possible a mixed pharmacological profile (ie, agonist or antagonist) of βCCt may have interacted with the 3.0 g/kg EtOH dose in the present study.

The current findings are in agreement with those of Lister (1988) showing that the classic GABA agonist diazepam, and the BDZ antagonist ZK 93426 attenuate the locomotor stimulant actions produced by EtOH in mice. While ZK 93426 has been reported to be a 'prototype' BDZ antagonist (Jensen *et al*, 1984), we previously reported that at the α1–α4 receptor subtypes ZK 93426 produced a marked potentiation of GABAergic activity in *Xenopus oocytes* (135–145%) (Harvey *et al*, 2002; June *et al*, 2003). The antagonism of the EtOH-induced stimulation by βCCt in the current study, and by diazepam (0.2, 0.5 mg/kg) and ZK 93426 (2, 5 mg/kg) in the Lister (1988) study, occurred in the absence of intrinsic activity. The absence of intrinsic effects on locomotion by βCCt in the present study also parallels our prior work with rats (June *et al*, 2003), and those of Griebel *et al* (2001) with mice using doses of 3–60 mg/kg. However, the data with flumazenil showing that it was an effective antagonist of the EtOH-induced stimulant effects are at variance with the data by Lister (1988). The failure of Lister (1988) to observe antagonism by flumazenil is likely due to the very low doses of the drug employed (2.5, 5.0 mg/kg). While similar doses of ZK 93426 (eg, 2.5, 5.0 mg/kg) were also employed by Lister (1988), flumazenil's efficacy profile in enhancing GABAergic activity is far less than that of ZK 93426 (see Harvey *et al*, 2002; June *et al*, 2003). Taken together, these data strongly suggest that partial–full activation of various $GABA_A$ receptor subtypes may reduce the EtOH-induced activational effects in mice. Given the compensatory increases of the α2 and α3 receptors in the α1 (−/−) mice, these animals would seem highly sensitive to positive BDZ agonists. Nevertheless, *inhibition of DAergic activity via GABA modulation* at various $GABA_A$ receptor subtypes seems plausible to explain the antagonism of the EtOH-induced activational effects in the α1 (−/−) mice.

In contrast to the effects of βCCt and flumazenil on the EtOH-induced activational effects, neither βCCt nor flumazenil antagonized the sedation produced by the 3.0 g/kg EtOH dose in the α1 (+/+) mice. These data contrast recent (June *et al*, 2003) and previous reports (June *et al*, 1998a, b) demonstrating that βCCt and other prototype BDZ antagonists (eg, ZK 93246, CGS 8216) were effective in attenuating the sedative actions of alcohol in rats. However, in these prior studies, a 1.25–1.50 g/kg dose of EtOH was employed (June *et al*, 1998a, b, 2003). Thus, it is possible

that while sedative EtOH doses can effectively be antagonized by BDZ receptor antagonists, higher EtOH doses ($\geqslant$3 g/kg), which effect multiple neurotransmitter systems (Draski and Deitrich, 1996), are not capable of being antagonized via BDZ ligands. Finally, a careful analysis of the flumazenil combination data revealed effects typically lower than the control condition. It is possible that in the presence of alcohol, the α2, α3, and α6 receptors are more sensitive to partial agonist modulation by flumazenil. In the complete absence of alcohol, however, these receptors appear less sensitive to BDZ modulation. Such an interpretation is also compatible with the high compensatory levels of α2,α3, and α6 receptors in the α1 (−/−) mice (Vicini *et al*, 2001; Kralic *et al*, 2002a; Sur *et al*, 2001).

While flumazenil alone did not significantly alter locomotor behaviors in the α1 (−/−) mice, it elevated activity in the α1 (+/+) mice to such an extent that it produced a 'profound' separation between genotypes. This was not the case with βCCt (see Figure 4a *vs* b). Nevertheless, the activational effects seen with flumazenil in the open field in the α1 (+/+) mice are consistent with prior reports with flumazenil ZK 93426, and diazepam (0.2, 0.5 mg/kg) (File *et al*, 1982a, b; File and Pellow, 1986). Further, in the Lister (1988) study when the activational doses of diazepam were combined with a stimulant dose of EtOH (2 g/kg), a profound reduction in exploration, locomotion, and even ataxia were observed. The data of the present study illustrating a 34–75% reduction in locomotor activity in the α1 (−/−) mice following the βCCt, and flumazenil combinations are consistent with the Lister (1988) study. Hence, the combined effects of a stimulant alcohol dose and very low doses of a BDZ agonist exerts ataxia. This effect is even *more* exaggerated in the α1 (−/−) mice where selected receptors (ie, compensatory receptors) may be modulated to a greater degree.

Selective D1 and D2 DA Antagonists Attenuate the EtOH-Induced Stimulation in α1 (−/−) and α1 (+/+) Mice

The fourth major finding of the present study was that SCH 23390 and eticlopride, a selective D1 and D2 DA receptor antagonist, respectively (Seeman and Ulpian, 1988), were both able to attenuate the alcohol-induced stimulation in mutant mice, in the absence of intrinsic effects. The data of the present study are in agreement with a series of studies suggesting a significant role for the involvement of DA in alcohol-induced activational effects in mice (Shen *et al*, 1995; Cohen *et al*, 1997; Le *et al*, 1997). The data of the present study are also consistent with the current dogma in the alcohol field that the D1 and D2 receptors of the mesolimbic, particularly of the extended amygdala circuitry, play a critical role in the reinforcing properties of alcohol (Koob, 1999; Hodge *et al*, 1997; Phillips *et al*, 1992; McBride and Li, 1998; Liu and Weiss, 2002; Eiler *et al*, 2003; Melendez *et al*, 2005). It is interesting to note that unlike Experiment 2 of the current study, and the prior work by Kralic *et al* (2003) using mice of similar genetic background (eg, Kralic *et al*, 2003), a 1.5 g/kg alcohol dose significantly elevated ambulatory behaviors in the α1 (+/+) mice of Experiment 3. The rational for this discrepancy is not totally clear, however, it is possible that genetic/random drift may have enhanced the sensitivity of alcohol in the later generations of α1 (+/+) mice. It has been suggested, however, that random drift may be more salient in earlier, not later generations (Falconer and Mackay, 1996). Despite the enhanced sensitivity of the α1 (+/+) mice in Experiment 3, the magnitude of alcohol-induced activation was still greater in the α1 (−/−) compared with the α1 (+/+) mice (Figure 5). These effects were also observed across the total distance and stereotypy count parameters (data not shown). Together, these data confirm that an enhanced alcohol-induced activational effect is associated with genetic deletion of the α1-containing $GABA_A$ receptor. These activational effects within the DA systems are regulated via both D1 and D2 receptor subtypes.

SUMMARY

The present data provides the first demonstration that the α1-containing $GABA_A$ receptor is necessary for EtOH-motivated behaviors, and motivated responding for a sucrose reinforcer. The degree to which a 'global' deletion of the α1 subunit relates specifically to oral alcohol reinforcement compared with general motivated behaviors is not clear. It is possible that common/overlapping GABAergic mechanisms regulate motivated responding for both EtOH and sweet caloric reinforcers. The α1-null mutants showed increases in motor activity following low–moderate alcohol doses; however, increases were observed at one-fold higher doses (1.5 *vs* 3.0 g/kg) than those previously reported (Kralic *et al*, 2003). Further, the null mutants were resistant to the sedative effects of alcohol. The removal of the inhibitory GABAergic tone on DAergic, and possibly other neurotransmitters systems appear to unmasked alcohol's stimulatory effects. The BDZ antagonists βCCt and flumazenil were able to attenuate the alcohol-induced stimulation in the mutant mice. This attenuation was hypothesized to be due to the partial agonist properties of the BDZ antagonists. Further, compensatory elevations of non-α1 receptors in the null mutants appear to sensitize these animals to the *weak and partial* agonist properties of BDZ antagonists, and in the presence of EtOH induces an exaggerated reduction in locomotor behaviors. Finally, a selective D1 and D2 DA antagonist was also effective in blocking the alcohol-induced stimulation in the absence of intrinsic effects. Thus, alcohol's locomotor stimulant actions appear to be regulated in part, via an interaction of both DA- and $GABA_A$ BDZ-dependent mechanisms.

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