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Effects of Vigabatrin, an Irreversible GABA Transaminase Inhibitor, on Ethanol Reinforcement and Ethanol Discriminative Stimuli in Mice

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Abstract

We tested the hypothesis that the irreversible gamma-amino butyric acid (GABA) transaminase inhibitor, γ-vinyl GABA (Vigabatrin; VGB) would reduce ethanol reinforcement and enhance the discriminative stimulus effect of ethanol, effectively reducing ethanol intake. The present studies used adult C57BL/6J (B6) mice in well-established operant, two-bottle choice consumption, locomotor activity and ethanol discrimination procedures, to examine comprehensively the effects of VGB on ethanol-supported behaviors. VGB dose-dependently reduced operant responding for ethanol as well as ethanol consumption for long periods of time. Importantly, a low dose (200 mg/kg) of VGB was selective for reducing ethanol responding without altering intake of food or water reinforcement. Higher VGB doses (>200 mg/kg) still reduced ethanol intake, but also significantly increased water consumption and, more modestly, increased food consumption. While not affecting locomotor activity on its own, VGB interacted with ethanol to reduce the stimulatory effects of ethanol on locomotion. Finally, VGB (200 mg/kg) significantly enhanced the discriminative stimulus effects of ethanol as evidenced by significant left-ward and up-ward shifts in ethanol generalization curves. Interestingly, VGB treatment was associated with slight increases in blood ethanol concentrations. The reduction in ethanol intake by VGB appears to be related to the ability of VGB to potentiate the pharmacological effects of ethanol.

Keywords

alcohol; mouse; reward; ethanol drinking; interoceptive cue

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Conflict of Interest

The authors have no conflicts of interest to declare.

Introduction

Alcohol is one of the most commonly abused drugs in society. A recent publication based on the National Epidemiological Survey on Alcohol and Related Conditions database indicated that almost 18 million adults in the United States meet diagnostic criteria for alcohol abuse or alcohol dependence (Grant *et al.*, 2004). In fact, excessive alcohol (ethanol) consumption is estimated to be the third leading cause of preventable death in the US (Mokdad *et al.*, 2004). For these and a variety of other reasons, considerable effort has been expended to understand the complex nature of this rather simple molecule, which in moderation can have beneficial effects [e.g. (Agarwal, 2002; White, 1996)] but with prolonged and excessive exposure can be quite deleterious [e.g. (Beier *et al.*, 2011; Ringborg, 1998; Seitz *et al.*, 2007; Zakhari *et al.*, 2007)]. A wealth of knowledge regarding the pharmacology and neurobiological effects of ethanol derives from several decades of research using various animal models, in conjunction with pharmacological agents. These studies have established that several central neurotransmitter systems are important for regulating ethanol intake and discrimination, with gamma-amino butyric acid (GABA), glutamate, serotonin and dopamine systems being particularly important. Although all of these neurotransmitter systems are important in the effects of ethanol, the GABAergic system is of particular interest because ethanol directly potentiates chloride conductance through inotropic $GABA_A$ receptors (Burch *et al.*, 1980; Morrow *et al.*, 1988a,b). Additionally, data from the Collaborative Study on the Genetics of Alcoholism (COGA) found that certain single nucleotide polymorphisms in the $GABA_A$ receptor in humans are positively correlated with the development of alcoholism (Edenberg *et al.*, 2004). Importantly, this finding has been substantiated by several additional reports examining the relationship between ethanol-related behaviors and the $GABA_A$ receptor (Bauer *et al.*, 2007; Dick *et al.*, 2006; Xuei *et al.*, 2010).

Using mouse models of ethanol drinking, we have found that ethanol concentrations in nucleus accumbens increase over the course of the drinking session and that licking behavior declines with the increased ethanol concentration (Griffin *et al.*, 2007, 2009). These results suggest that the presence of ethanol within the nucleus accumbens can reduce ethanol intake, perhaps by direct activation of $GABA_A$ receptors and subsequent neuronal inhibition within the nucleus accumbens. Indeed, microrinjection of muscimol, a $GABA_A$ agonist, into the nucleus accumbens can reduce responding for ethanol (Hodge *et al.*, 1995) as well as substitute for the discriminative stimulus effects of ethanol in a classic drug discrimination procedure (Hodge *et al.*, 1998). Together, these findings are consistent with a general hypothesis that ethanol within the nucleus accumbens participates in the ethanol discriminative cue and may serve as a stop signal for ethanol consumption when ethanol reaches sufficient concentrations during a drinking session, perhaps acting through $GABA_A$ receptors. Recent evidence also indicates an important role for $GABA_B$ receptors in regulating ethanol consumption (Colombo *et al.*, 2004; Tanchuck *et al.*, 2011), acting perhaps within the nucleus accumbens (Steffensen *et al.*, 2000) but also the ventral tegmental area (Moore *et al.*, 2009) to affect drinking. Thus, in general, the GABAergic system function appears to be critical for the processes mediating ethanol's reinforcing and discriminative properties which are important for the maintenance of ethanol drinking.

In the present study, we investigated the effects of an indirect GABA agonist, γ-vinyl GABA (vigabatrin; VGB) on several different ethanol-related behaviors of C57BL/6J mice to further elucidate the influence of the GABAergic system on ethanol reinforcement and discrimination. VGB elevates brain GABA concentrations by its irreversible inhibition of GABA transaminase (Valdizan *et al.*, 1992). Upon contact with the transaminase, VGB is transformed into a highly reactive intermediate compound that binds covalently to an active site on the protein resulting in irreversible inhibition. The *in vivo* consequences of this action

are long-lasting increases in GABA concentrations (Gram *et al.*, 1989; Jung *et al.*, 1977; Rey *et al.*, 1992). Previous reports indicate that VGB prevents the rise in extracellular dopamine in nucleus accumbens produced by injections of cocaine (Dewey *et al.*, 1998), nicotine (Dewey *et al.*, 1999) methamphetamine, heroin, or ethanol (Gerasimov *et al.*, 1999) and also reduces ethanol consumption by AA rats (Wegelius *et al.*, 1993). These reports suggest that increasing GABAergic neurotransmission by irreversibly blocking GABA transaminase alters the reinforcing effects of abused drugs. Additionally, activating $GABA_A$ receptors, which occurs with irreversible GABA transaminase blockade, plays a critical role in the discriminative stimulus effects of ethanol (Grant, 1999; Hodge *et al.*, 1998). Therefore, in the present study, we hypothesized that VGB would dose-dependently reduce ethanol reinforcement and enhance the discriminative stimulus effect of ethanol, effectively reducing ethanol intake.

Methods

Subjects

All subjects were male C57BL/6J (B6) mice obtained at approximately 7 weeks of age from the Jackson Laboratory (Bar Harbor, ME) and individually maintained in standard cages with wood-chip bedding in AAALAC accredited colony rooms at the Medical University of South Carolina (MUSC). Mice acclimated to the new environment for at least 1 week prior to beginning any experiment. All procedures were approved for use by the Institutional Animal Care and Use Committee of MUSC.

Apparatus

Self-administration—Mice were tested in chambers with stainless-steel grid floors enclosed in light- and sound-controlled boxes as previously described in detail (Middaugh *et al.*, 1999a,b; Nguyen *et al.*, 2005; Price *et al.*, 2004). Briefly, the computer-controlled chambers recorded the number of presses at a single lever and licking behavior (*e.g.* contacts) at a brass fountain that delivered 60 μL ethanol when response contingencies were met. Unconsumed ethanol was measured and subtracted from the amount delivered to provide the amount of ethanol consumed. Lastly, the sipper tube from a 50 ml water bottle protruded into the chamber to provide free access to water. Licking behaviors were measured at the sipper tube and water volumes consumed from the bottle were determined after adjusting for spillage collected under the spout.

Ethanol Discrimination—Mice were tested in six gray Plexiglas two-lever chambers with food pellet dispenser as previously described (Groseclose *et al.*, 1997; Middaugh *et al.*, 1988, 1999, 2000a). Food pellet (20 mg A/I Rodent Pellets, Noyes Co., Lancaster, NH) reinforcers were delivered to a tray located at floor level. Levers were located on each side of the tray.

Self-administration

Experiment 1 (Exp-1) was conducted in three phases conducted over a 9-month period as we have previously described (Price *et al.*, 2004). These procedures will reliably yield BECs in excess of 100mg% and the mice are visibly intoxicated, once responding has stabilized, as previously reported (Middaugh *et al.*, 1999a, 2000a,b). The mice were maintained on a 12-h light cycle (on 07.00 h). At all times, mice were maintained at 85% [Range: 82 to 87%] free-feeding body weights by restricting feeding to a single daily ration. Mice were tested in 15 minute sessions five days per week Mon-Fri) between 08.00 and 12.00 h. During the first phase, the mice acquired the lever response and the fixed ratio requirements were increased from FR1 to FR4 over 12 weeks with mice being reinforced with a final ethanol concentration of 12% under the pre-feeding condition (*i.e.* testing before the daily food

ration). The final stage of Phase 1 included one week of testing under the FR4 schedule to examine the influence of systematic changes in ethanol concentration (0%, 3%, 6%, and 12% in ascending order) compared with water on lever pressing.

Phase 2 of Exp-1 determined the effects of VGB on responding for ethanol during pre-feeding tests with 12% ethanol delivered on an FR4 schedule. Mice were habituated to SC injections of vehicle for one week. Over the next five weeks, the effects of VGB were examined. During the first and fifth weeks, mice were injected with vehicle prior to each daily test. During weeks 2-4, mice were injected with VGB (200, 400, & 600 mg/kg) each Wednesday and vehicle the other 4 days of the week. The VGB dosing order was counterbalanced across three subgroups of mice (n=6/Group).

Phase 3 of the study evaluated the selectivity of the effects of VGB on ethanol responding by examining lever responding for water and then food. In the first part of this phase, mice were maintained on the same ethanol reinforcement schedule (12% EtOH on FR4) but switched to post-feeding test sessions (*i.e.* tested after being fed their daily food ration without water availability to increase thirst). After 3 weeks habituation to this new schedule, the effects of VGB were evaluated when reinforcement was either 12% ethanol or water. During these tests, occurring on Wed of two successive weeks, mice were divided into two groups with equivalent mean response output on the preceding day (Tues). One of the groups was injected with VGB (200 mg/kg) and the other with vehicle. Water served as the reinforcer on Wednesday first week and 12% EtOH on Wednesday during the second week (all other days of the week, 12% EtOH was the reinforcer). During the tests in which water served as the reinforcer, we originally intended to include 12% EtOH as the alternative liquid, available through the sipper tube. However, this was discontinued after two mice overdosed on the freely available ethanol during the first test and no alternative fluid was available for the remainder of Phase 3. To assess the effects of VGB on food reinforcement, mice were acclimatized over a 3 week period to another similar set of self-administration chambers but lever responded for 45 mg Noyes food single food pellets from a pellet dispenser. After one day with food pellets delivered for each response, the schedule was increased to FR4 and for 3 weeks of adaptation to food reinforcement sessions. The mice were then divided into two groups with equal mean response output and injected with either saline or VGB (200 mg/kg) 2.5 hr prior to testing on Weds. Data generated on these tests were compared with data generated by the same mice during their VGB 200 mg/kg tests with 12% ethanol as the reinforcement during Phase 2.

Ethanol Consumption (Exp-2)

For this experiment, mice were individually housed and maintained on a 12-hr reverse light/dark cycle, lights off from 11.00 to 23.00h and given 21 h of access daily to ethanol (12% v/v) with water as the alternative choice, in a procedure previously described in detail (Nguyen *et al.*, 2005). The amounts of ethanol, water and food consumed were determined by weighing the food and the tubes containing either water or ethanol before and after the daily 21 h observation periods (08.00 h). Fluid volumes were corrected for spillage and/or evaporation, based on weight loss of control tubes on maintained on empty cages. Unconsumed food was not determined but was not obvious on the floor of the cages.

After 2 weeks of access in the 2-bottle choice procedure, the mice were distributed into three groups (n=10 per group) with equivalent daily ethanol intake levels. Over the following nine days, mice were injected (s.c.; 2.5 h prior to access), according to group assignment, with vehicle, VGB (200 mg/kg) or VGB (600 mg/kg). Because the results of Exp-1 suggested that VGB was effective for 48-72 h, mice were injected with vehicle or VGB on alternate days.

Locomotor Activity (Exp-3)

The effects of VGB on locomotor activity were examined on the same mice used for Exp-2. The most selective dose of VGB on ethanol reinforcement (200mg/kg) was contrasted with the highest VGB dose (600 mg/kg). Locomotor activity was recorded in 15- min intervals using Omni-Tech activity monitors as previously described (Griffin *et al.*, 2010; Halberda *et al.*, 1997). The initial assessment began the week following the consumption experiment (Exp-2) while the mice were still maintained on the ethanol consumption schedule. The mice were randomly assigned to 3 groups (Vehicle, VGB 200 and VGB 600 mg/kg) and locomotor activity tests began 2.5 h after the injections. Two additional assessments were conducted at three and five weeks, respectively, after termination of ethanol consumption using the same mice to evaluate the interactive effects of VGB and ethanol on locomotion. Mice were injected (s.c.) with vehicle or VGB 2.5 h prior to injection (i.p.) with ethanol or saline. Five minutes after the ethanol injection, mice were evaluated for locomotor activity.

Ethanol Discrimination (Exp-4)

We have previously described our ethanol discrimination procedures (Groseclose *et al.*, 1997; Middaugh *et al.*, 1999). In brief, mice were individually housed and maintained on a 12-h light cycle (lights on 06.00 h) and testing occurred 13.00 -16.00h, Mon-Fri. Bodyweights were maintained at 85% [Range: 82 to 87%] of free- feeding weight and the single daily food ration was given after testing.

After reducing body weights, lever responding was established and the reinforcement schedule gradually increased to FR20. After stable responding, ethanol discrimination training was initiated with mice injected i.p, five minutes prior to the session. Ethanol (1 g/kg) and vehicle injections were alternated on a semi-random schedule with no more than 2 successive days of either ethanol or vehicle, and that the number of injections of each be equal over a 10 day period. Responses on a designated lever were reinforced following ethanol injections and responses on the opposite lever were reinforced after vehicle injections, which was counterbalanced across mice. Responses were recorded and a discrimination index (DI) calculated (DI = the number of responses on the correct lever/total responses prior to delivery of the first reinforcer).

After meeting the discrimination criterion (DI = 85% on 3 consecutive days), ethanol dose-response functions were established using a cumulative dosing procedure involving a series of injections and tests within the same session (Becker *et al.*, 2004; Crissman *et al.*, 2004), although correct responses were not reinforced, consistent with our previous reports (Groseclose *et al.*, 1997; Middaugh *et al.*, 1999). To accomplish this, mice were initially injected with vehicle and five minutes later placed in the operant chamber for a 2-min test with no reinforcement for responses on either lever. The first test was followed rapidly by 5 additional 2-min tests each occuring 5 min after an ethanol injection of 0.5 g/kg, producing a total cumulative dose of 2.5 g/kg (Series 1). After returning to training and meeting criterion performance on subsequent sessions, the mice experienced a second series of tests as before using 0.25 g/kg ethanol (Series 2; for a total cumulative dose of 1.25 g/kg). Thus, during either Series 1 or 2, including the initial injection of vehicle, each mouse had a series of 6 tests within 45 minutes designed to produce increasing blood ethanol concentrations, as previously described (Becker *et al.*, 2004; Crissman *et al.*, 2004). The cumulative dosing procedure was conducted 2.5 h after s.c. injections of either vehicle or VGB (200 mg/kg).

Blood Ethanol Concentration (BEC) Measurements

Blood was collected from the retro-orbital sinus of mice injected with either saline or VGB five minutes after the final 0.25 g/kg ethanol injection in the ethanol discrimination experiment. Blood was not collected after the 0.5 g/kg test (Series 1). The blood sampling

and gas chromatography assay procedures to determine ethanol concentration were according to previously published techniques (Griffin *et al.*, 2009; Middaugh *et al.*, 1992c).

Drugs

Ethanol (95%) was obtained from AAPER (Shelbyville, KY). When used for oral consumption, ethanol was mixed in a volume/volume ratio to yield the desired concentration. When ethanol was given i.p., it was mixed in water at a concentration of 12.3% and administered in a volume of 0.02 ml/g bodyweight. Vigabatrin (VGB), was manufactured by Aventis Pharma, Inc (Laval, Quebec) and was obtained from Murray Shore Pharmacy (Toronto, Ontario) in sachets containing 500mg of powder. VGB was mixed with 0.9% NaCl (normal saline) and administered s.c. in a volume of 0.01 ml/g bodyweight. In all experiments, VGB was given 2.5 h prior to testing based on previous reports (Jung *et al.*, 1977; Kushner *et al.*, 1999).

Statistical Analysis

The experiments described in the present report contain a variety of dependent variables, which were analyzed using analysis of variance (ANOVA), incorporating repeated-measures (RM) on some of the factors as necessary. Significant factor interactions were followed up using post-hoc analysis as described in the results section. Non-linear curve fitting to calculate ED50 values was done using GraphPad Prism® Version 5. For all analyses, significance was taken as $p<0.05$.

Results

Experiment 1 (Exp-1): Effects of VGB on Ethanol Self-administration

This experiment used 24 male B6 mice. During the acquisition phase, 4 mice of 24 that started did not acquire the lever pressing behavior and were excluded from further testing.

Phase 1—In the final stage of training, lever responses and licks were evaluated during separate test days for water, 3 ethanol concentrations and again for water (*i.e.* 0%,3%,6%, 12% and 0%). The lever presses (mean ±SEM) were 62 ± 4 for the combined water days, and 64 ± 3, 73 ± 9, and 86 ± 8, respectively, for the 3%, 6%, and 12% ethanol concentrations. Comparable data for the lick measure were 83 ± 11 for the water days, and 123 ± 20, 172 ± 30, and 298 ± 32 for the increasing ethanol concentrations. One-way RM ANOVA across the four concentrations (0,3,6,12%) indicated significant increases in response frequency for lever presses [$F_{(3,60)}= 4.84$, $p< 0.005$] and licks [$F_{(3,60)}= 36.06$, $p< 0.001$]. Post hoc Duncan's tests indicated that lever responses and licks at the fountain were greater for 12% ethanol than for the other solutions [both *p*'s < 0.01]. Thus, lever presses and licks increased with increasing ethanol concentration and then decreased when water was provided again. These data are consistent with our previous report (Middaugh *et al.*, 1999a) indicating that ethanol, rather than fluid *per se*, influenced appetitive behavior.

Phase 2. Effects of VGB on Responding for Ethanol—Once ethanol reinforced responding was established, we evaluated the effects of VGB on ethanol-reinforced responding over a 5-week period. Our first analysis was to determine if the different dose orders (see methods; I-200, 400, 600; II-400, 600, 200; and III-600, 200, 400) impacted any of the measures. The data collected on the drug test days (Weds) were evaluated with 3 (Vigabatrin Dose Order) x 4(Dose) mixed-factor ANOVAs, with RM on the Dose factor. ANOVA results, summarized in Table 1**,** indicated no effect of the dose order on any of the dependent variables but ethanol intake. Additional post-hoc analyses on the intake data did not reveal specific reasons for this, although the effect of dose order on the 200 mg/kg dose did approach significance in one category [Order II (2.88 ± 0.45) > Order III (1.20 ± 0.33),

Newman-Keuls, p= 0.056]. Thus, it was concluded that dosing order did not meaningfully influence the dependent variables measured in this experiment.

The data from Experiment 1 are summarized in Figure 1. The left panels (Fig 1A-C) summarize data collected on Drug Test days (Weds, Day 3) of each week. Inspection of the graphs indicates clear dose-responsive reductions in lever responses [Fig. 1A; $F_{(3,51)}$= 25.37, p≤0.001], fountain licks [Fig. 1B; $F_{(3,51)}$= 15.84, p≤ 0.001] [, and ethanol intake[Fig. 1C; $F_{(3,51)}$= 23.88, p≤ 0.001] with increasing doses of VGB. Follow-up comparisons of Vehicle means with Drug Dose means via Dunnett's Test established significant VGB-induced reductions for all three doses [p≤ 0.01] for lever presses, fountain licks and ethanol intake.

Interestingly, and in contrast to ethanol, with increasing dose there was an increase in licking behavior at the sipper tube in the chamber that allowed free access to water (see Methods Exp-1), as follows: 0 mg/kg VGB 16 ± 4; 200 mg/kg VGB 10 ± 2; 400 mg/kg VGB 33 ± 12; and 600 mg/kg VGB 37 ± 10. The one-way ANOVA was significant [$F_{(3,48)}$= 4.25, p≤ 0.05] and post hoc Dunnett's tests indicated a significant elevation for the highest dose compared to the vehicle mean (p≤ 0.01). Mean water intake (ml) from the sipper tube also tended to increase (ranging from 0.5 ml to 1.8 ml) but this did not reach statistical significance [$F_{(3,48)}$=1.97, NS]. Together, these data indicate that mice shifted their preference away from ethanol reinforcement to water reinforcement when VGB was administered.

We also specifically examined whether VGB could alter ethanol self-administration during subsequent sessions of ethanol access by analyzing data from test sessions following VGB administration. First, the data collected during daily sessions one week prior to and one week after the three VGB test weeks were analyzed with a 2 (Test Week) x 5 (Days) RM ANOVAs to determine if VGB exposure during the intervening test weeks affected behavioral measures of interest. These analyses (Table 2) indicate that data collected during the weeks of vehicle injections before vs. after the drug tests did not differ for any measures except for average amounts of ethanol consumed, which were slightly greater during the post-test week. Therefore, the average of these two weeks of data was taken as a baseline measure for subsequent analysis. Data generated on daily sessions during each week were examined with 4 (Dose-Week) x 5 (Days) RM ANOVAs. The analyses are summarized in Table 3 and the data are summarized in Fig. 1 D-F. As shown, lever responses (Fig. 1D), fountain licks (Fig. 1E), and ethanol intake (Fig. 1F) varied across days depending on the particular VGB dose administered (Dose Week x Days interaction). These data were further analyzed with one-way RM ANOVAs, followed by Dunnett's Tests comparing responses on the day prior to drug tests with data collected on the Drug test session and post-drug test sessions. Significant VGB effects (p<0.05) were noted for two days after the drug test for the lever response (Fig. 1D), and on the following day for fountain licks (Fig. 1E) and ethanol intake (Fig. 1F).

Phase 3. Effects of VGB on Ethanol vs. Food and Water Reinforcement

Ethanol vs. Food: The comparative effects of VGB on ethanol *vs.* food reinforcement was determined by comparing lever response data for food reinforcement with data generated by the same mice for 12% ethanol during the VGB dose-response phase noted above (Phase 2). Test conditions were identical for the two experiments except for the difference in reinforcers. These data are summarized in the Pre-feeding portion of Fig 2, and were analyzed with a 2 (VGB) x 2 (Reinforcer) RM ANOVA. The ANOVA supported the interaction noted in the figure ($F_{(1,18)}$ = 12.585, p< 0.01). Post hoc analysis using pair-wise comparisons found that VGB significantly reduced responses for EtOH, but not food (p<0.05).

Ethanol vs. Water: The comparative effect of VGB on ethanol *vs.* water reinforcement under post-feeding test conditions is summarized on the right side of Fig 2**.** Mice used in the dose-response experiment were tested in a 2 (VGB) x 2 (Reinforcer) mixed-factor design with RM on the Reinforcer factor. On the test day, 9 mice were injected with VGB (200 mg/kg), and 8 with saline. An ANOVA confirmed the interaction of VGB and Reinforcer type apparent in four bars on the right side of Fig 2 ($F_{(1,15)} = 17.67$, $p < 001$). Pairwise post hoc analysis indicated that the 200 mg/kg dose of VGB significantly reduced responses for ethanol but not water ($p<0.05$).

Experiment 2 (Exp-2): VGB Effects on Ethanol Consumption

Figure 3 summarizes ethanol (g/kg), food (g), and water (μl/g) consumption on days when the drug was injected (noted as DD: Drug Days) or not (noted as ND: Non-drug days). The 5 days of drug injections versus the 4 days of no injection were averaged for each animal to provide the group averaged data presented in Figure 3. Inspection of the graph indicates a dose responsive reduction in ethanol consumption on the days of VGB injection (DD) as well as the intervening days (ND). In contrast, mice injected with either dose of VGB had no reduction in food and water consumption. In fact, mice injected with the highest VGB dose had elevated water consumption, and to a lesser extent food consumption on non-drug days (ND). Mixed-factor 3 (VGB Dose) x 2 (Injection Day) ANOVAs with RM on the Injection Day factor were used to analyze the data. ANOVA confirmed the VGB-induced reduction of ethanol consumption (VGB Dose: $F_{(2,25)} = 30.78$, $p < 0.001$). Importantly, the VGB-Dose x Injection Day interaction did not approach significance ($F < 1.0$) indicating that ethanol consumption was reduced for at least 48 h following injection. Comparison across means of the three dose groups in Figure 3 confirmed the differences between all three groups (Newman-Keuls tests, $p < 0.01$). In contrast, the ANOVA on water consumption indicated a significant Vigabatrin-Dose x Injection Day interaction ($F_{(2,25)} = 21.56$, $p < 0.001$). Post-hoc analysis indicated that mice injected with the 600 mg/kg dose of VGB consumed more water than the other groups on days when the drug was not injected (ND). The ANOVA on food consumption also indicated a significant VGB Dose x Injection Day interaction ($F_{(2,25)} = 4.25$, $p < 0.05$). Post hoc comparisons indicated a significant increase in food intake on the days following drug tests (ND) for mice injected with VGB 600 mg/kg compared to vehicle treatment the day before (Dunnett's test, $p < 0.02$). Thus, treatment with VGB produced a long-lasting reduction in ethanol consumption that were coupled to increases in water and food consumption on non-treatment days.

Experiment 3 (Exp-3): Effects of VGB and ethanol on locomotor activity

The results of this experiment are summarized in Figure 4. Total distance (cm) data were analyzed with a 3 (VGB Dose) x 4 ($Time_{15\text{-Min Interval}}$) mixed-factor ANOVA with RM on the time factor. Fig. 4A indicates that at doses of 200 & 600 mg/kg, given s.c. 2.5 h prior to the assessment, VGB did not alter total distance traveled over the hour according to either Dose ($F_{(2,21)} = 1.20$, NS) or its interaction with Time ($F_{(6,67)} = 1.47$ NS).

Fig. 4B & 4C summarize the results of two experiments conducted three (**4B**) and five (**4C**) weeks, respectively, after termination of ethanol consumption. For the first test, summarized in Fig. 4B, the VGB and ethanol doses were 200 mg/kg and 2 g/kg, respectively. The 3 (Drug Condition) x 4 (Time Interval) RM ANOVA on these data indicated significant effects of Drug Condition [$F_{(2,15)} = 3.48$, $P \leq 0.05$] and its interaction with Time Interval [$F_{(6,45)} = 4.59$, $P \leq 0.001$]. Post hoc one-way ANOVAs and mean comparisons at the four time points (Dunnett's Test) comparing the two drug groups with (SAL+VEH) controls indicated that the 2 g/kg ethanol dose (SAL+EtOH 2) increased total distance traveled during the first 15-min interval ($p \leq 0.01$) indicative of ethanol-induced stimulation as previously reported (Griffin *et al.*, 2010; Middaugh *et al.*, 1992a). This ethanol-induced

locomotor stimulation was blocked by pre-treatment with the 200 mg/kg VGB dose (VGB +EtOH 2) that also significantly reduced motor activity during the second 15-minute interval (VGB+EtOH <SAL+VEH). These results suggest that VGB can enhance the biphasic action of ethanol on locomotor activity toward its suppressive effects observed at higher ethanol doses.

To assess further the possibility that VGB enhanced biphasic ethanol effects toward suppression, a subsequent experiment summarized in Fig. 4C was completed using a borderline suppressive dose of ethanol (2.5 mg/Kg) for B6 mice (Middaugh *et al.*, 1992a) and the highest dose of VGB (600 mg/kg) used in the current experiments. The 3 (Drug Condition) x 4 (Time Interval) AVOVA of these data also indicated significant effects of Drug Condition [$F_{(2,15)}$= 34.94, P≤ 0.001] and its interaction with Time Interval [$F_{(6,45)}$= 4.59, P≤ 0.001]; however, the pattern of change differed from that observed at the lower doses of EtOH and VGB. Subsequent post hoc analyses indicated that, as expected, the 2.5 g/kg ethanol dose given by itself was neither stimulatory or suppressive under the test conditions, compared to the SAL+VEH group. However, the combination of VGB and ethanol (VGB 600 + EtOH 2.5) produced a strong reduction in in motor activity ($p< 0.05$) compared to either EtOH 2.5 by itself or the vehicle controls. These results indicate that: **1)** over the entire range doses tested for its effect on ethanol consumption, VGB alone does not affect locomotion under the conditions of the experiment; **2)** the most selective dose of VGB for ethanol reinforcement can interact with ethanol to shift the biphasic effects of ethanol on motor activity from stimulation toward depression without gross impairment of locomotion and **3)** high VGB doses can interact with slightly higher, but still non-activity suppressing doses of ethanol to profoundly reduce locomotor activity.

Experiment 4 (Exp-4): The effects of VGB on the discriminative stimulus of ethanol

The impact of VGB on ethanol discrimination was tested using the most selective dose of VGB found to reduce ethanol reinforcement (200 mg/kg). The analysis was conducted using data from 8 mice (of 17) that routinely demonstrated DI = 85% during training sessions, indicating successful discrimination of 1g/kg ethanol from vehicle. The primary data was percent responding on the ethanol-paired lever and is summarized in Figure 5. The 0.5 g/kg dosing series, giving a total cumulative dose of 2.5 g/kg, was tested first and produced a discriminative stimulus that fully generalized to the ethanol-paired lever, evidenced by response ratios well above criterion performance levels (80%). In contrast, although the 0.25 g/kg dosing series produced a total cumulative dose of 1.25 g/kg (greater than the 1 g/kg training dose) did not fully generalize to the ethanol paired lever, indicated by mean responding below the criterion performance levels. Interestingly, pretreatment with VGB (200 mg/kg) increased discrimination ratios in both cumulative dosing series. For the 0.5 g/kg series, VGB enhanced responding on the ethanol paired lever occurred early in testing when ethanol cumulative doses were low whereas for the 0.25 g/kg series, VGB increased discrimination ratios above criterion performance (Fig. 5A & 5B) later in the dosing series. These observations were confirmed by a 2 (VGB Dose) x 2 (EtOH Dose) X 6 (Cumulative Dose) RM ANOVA, revealing a significant 3-way interaction [$F_{(5,35)}$= 3.32, $p < 0.02$]. The 3-way interaction was further analyzed with 2-way ANOVAs and post-hoc analysis with pair-wise comparisons. VGB administered 2.5 h prior to testing produced a left-ward shift in the generalization curve for the 0.5 g/kg dosing series (VGB Dose X Cumulative Dose Interaction: $F_{(1,7)}$= 11.94, ≤ 0.01). Similarly, pretreatment with VGB prior to testing in the 0.25 g/kg dosing series resulted in full generalization to the subthreshold 0.25 g/kg ethanol dosing series (VGB Dose X Cumulative Dose Interaction: $F_{(1,7)}$= 8.22, p≤ 0.01). In both cases, VGB enhanced the ethanol discriminative stimulus.

These data were further analyzed by calculating ED_{50} values for individual mice using non-linear regression analysis. The effects of VGB on ED_{50} values (Fig. 5C) were analyzed

using paired t-tests according to the ethanol dosing series. Although VGB reduced the ED_{50} of the 0.5 g/kg dosing series, the effect did not reach statistical significance [t(7)=2.61, p=0.07]. On the other hand, VGB did significantly reduce the ED_{50} for the 0.25 g/kg dosing series ($p<0.05$), indicative of a left-ward shift in the ethanol generalization function. Taken together, these results indicate that VGB enhances the discriminative stimulus effects of ethanol.

Finally, at the conclusion of testing with the 0.25 g/kg injection series, blood ethanol concentrations (BEC) were measured. Surprisingly, it was found that BEC's were 36% higher in mice injected 2.5 h earlier with VGB (55.2 ± 2.24 mg/dl) compared to saline (40.3 ± 2.4 mg/dl), [$t_{(8)}$= 7.43, p≤ 0.01]. Because these results suggested that VGB increased BEC, an additional experiment was conducted to confirm this result using a different group of B6 mice that were age-matched, but ethanol naïve and not food restricted, as well as a higher VGB dose (400 mg/kg), followed by a single i.p. injection of ethanol (2 g/kg). Blood samples were collected at 5, 30 and 60 min following the ethanol injection. BEC values were as follows for vehicle versus VGB: 5 min (224.6 ± 10.3 vs 248 ± 7.7 mg/dl), 30 min (171.9 ± 3.6 vs 195.3 ± 5.7 mg/dl) and 60 min (140.4 ± 11.4 vs 154 ± 17.4 mg/dl). Again, s.c. VGB given 2.5 h earlier, were associated with elevated BEC (14% to 18%) at all time points, compared to saline controls [$F_{(1,34)}$= 13.400, p≤ 0.01]. These results indicate that VGB can slightly increase plasma concentrations of ethanol.

Discussion

The present experiments indicate that, under several different conditions, vigabatrin (VGB) can dose-dependently and selectively reduce ethanol consumption compared to water or food. The VGB-induced reduction in ethanol consumption was accompanied by a shift toward water preference as evidenced by increased water consumption when the drug was present. This result indicates a bidirectional effect of VGB on ethanol compared to water reinforcement, underscoring the selectivity of VGB for reducing ethanol reinforcement. Further, because the lower, more selective doses of VGB (200 mg/kg) only reduced, rather than abolishing ethanol consumption, it is reasonable to hypothesize that VGB reduced ethanol consumption by potentiating the pharmacological effects of ethanol. To examine this possibility, the effects of co-administration of VGB and ethanol on locomotor activity and the discriminative–stimulus effects of ethanol were also examined. While not affecting locomotor activity if administered by itself, VGB (200 mg/kg) attenuated the locomotor-stimulating effects of a moderate ethanol dose, and a higher VGB dose (600 mg/kg) profoundly reduced locomotion when co-administered with a slightly higher ethanol dose. The combined effects of these two results suggests that VGB enhances the pharmacological action of ethanol, moving it from the excitatory toward the depressive effects of its biphasic action. Consistent with its enhancement of ethanol effects on motor activity, we found that VGB (200 mg/kg) enhanced the discriminative-stimulus effects of ethanol as evidenced by left-ward and upward shifts in ethanol generalization curves. The combined experiments indicate that irreversible inhibition of GABA transaminase can selectively reduce ethanol reinforcement, and that this may occur by an enhancement of the pharmacological effects of ethanol.

Using well-established operant procedures (Middaugh *et al.*, 1999a, 2000b; Nguyen *et al.*, 2005; Price *et al.*, 2004), we found that VGB dose-dependently reduced responding for ethanol reinforcement and ethanol intake. Importantly, a lower dose of VGB (200 mg/kg) for B6 mice selectively reduced ethanol, compared to food or water intake during short access periods (*e.g.* 15 minutes). In the present study, the motivational state of the mice was manipulated by testing under in a pre-feeding condition in which they were likely more hungry than thirsty and also tested in a post-feeding condition in which they were more

thirsty than hungry. B6 mice will lever press for ethanol reinforcement in the absence of hunger or thirst (Middaugh *et al.*, 2000b), but some degree of deprivation is necessary to assess responding for food and water over short periods of time. Additionally, testing the same mice responding for ethanol, food or water under the similar motivational and experimental conditions as done in Exp-1 (Methods) strengthens conclusions about the comparative effects of VGB on responding for ethanol *vs* food or water reinforcement. Because the indirect mechanism of action of VGB is to cause a general increase GABA levels and there is the potential for a broad suppression of behavior, the use of identical procedures in the current study was critical to determine the selectivity of VGB for ethanol reinforcement.

In addition to attaining access to ethanol by lever-pressing, mice had free access to water during operant test periods in Exp-1 (see Methods), providing an additional opportunity to evaluate the selectivity of VGB for reducing ethanol reinforcement. Previous studies indicated that mice tested one hour after being given their daily food ration, during which time water was removed to increase 'thirst', will immediately drink from the water spout during the first 3 minutes of an operant session but then shift to nearly exclusive lever-responding for ethanol during the remainder of the session (Price *et al.*, 2004). In contrast, when mice are tested before being fed their daily ration, the amount of "free" water consumed is negligible (Price *et al.*, 2004). Consistent with these prior observations, we found that mice consumed very little free water following vehicle injection and this also was not affected by VGB 200 mg/kg which significantly reduced ethanol intake. However, the higher VGB doses (400 & 600 mg/kg), while producing larger reductions in ethanol intake, increased the amount of water consumed from the sipper tube. Thus, the mice shifted their preference from ethanol to water.

The increases in appetitive behavior for water reinforcement following VGB treatment in our experiments with B6 mice are consistent with other reports. For example, the increased water consumption that accompanied the reduction in ethanol intake was also reported for AA rats (Wegelius *et al.*, 1993). Further, clinical reports indicate that some patients taking VGB for treatment of seizures experience gain weight (Jallon *et al.*, 2001; Tartara *et al.*, 1992). In contrast to the clinical reports, VGB (300 mg/kg) administered daily reduced food consumption and bodyweight of adult and adolescent obese Zucker Fatty rats and adolescent Sprague Dawley rats (DeMarco *et al.*, 2008). The varied outcome of experiments across laboratories, species, and drug doses indicates the importance of these factors when comparing the effects compounds on drug vs natural reinforcers. The current study and several previous reports indicate that VGB effects on responding for cocaine (Kushner *et al.*, 1999), nicotine (Paterson *et al.*, 2002), and ethanol (Current study) all occurred at lower doses than required to reduce (or increase) responding for reinforcers such as food and water. Moreover, it was recently reported that microinjection of the $GABA_A$ agonist muscimol into the shell of the nucleus accumbens reduced ethanol consumption, but the same muscimol dose, given in the same location, actually increased food and sucrose consumption (Stratford *et al.*, 2011). From these reports as well as the current study, it is clear that the relative impact of GABAergic modulation using VGB (or other manipulations) on ethanol versus natural reinforcers is complex and further experimentation is necessary to understand the differences in these processes.

An important observation from the operant experiment indicated that VGB effects on ethanol reinforcement carried over to the next session, and even to the next 2 sessions for the highest VGB dose. This prolonged effect is likely due to the irreversible inhibition of GABA transaminase by VGB (Gram *et al.*, 1989; Jung *et al.*, 1977; Rey *et al.*, 1992), resulting in long-term effects on ethanol reinforcement. These observations suggested that VGB would have long-term effects on ethanol consumption when evaluated using the free access (21-h),

two-bottle choice procedure. Indeed, when administered every other day, VGB dose-dependently reduced ethanol intake on days when drug was administered, as well as the intervening days when VGB was not administered, indicating a long duration of action for VGB. Similar to Exp-1, VGB dose-dependently increased water consumption; however, the increase was only noted on non-treatment days (Figure 3). The results of the free access, 2-bottle choice experiment clearly indicate that there was a shift in preference from ethanol to water with VGB treatment. In this experiment, there was also a small, but statistically significant, increase in food consumption with increasing VGB dose on non-treatment days. The bidirectional nature of VGB effects on ethanol versus food and water reinforcement suggests the intriguing possibility of distinct, but perhaps over-lapping, GABAergic-mediated regulatory processes involved with pharmacological (drug) versus reinforcers such as food and water.

Given that VGB effects on GABA transaminase lead to increases in GABA, it is reasonable to postulate that some of the observed effects on the self-administration and consumption of ethanol might reflect an enhancement of the pharmacological effects of ethanol. The present set of experiments did provide evidence that the pharmacological effects of ethanol were enhanced by VGB. The biphasic action of ethanol, ranging from stimulation to depression of activity with increasing dose (Middaugh *et al.*, 1989, 1992a,b), appeared to be shifted toward depression by administration of VGB. The stimulation produced by a 2 g/kg dose of ethanol, commonly observed in B6 mice, was attenuated by pretreatment with VGB 200 mg/kg (Figure 4), the dose most selective for reducing ethanol reinforcement. It is important to note that the combination of 2 g/kg ethanol and 200 mg/kg VGB did not grossly impair locomotion and mice given this combination were still quite ambulatory. In contrast, using a 2.5 mg/kg ethanol dose, which is on the borderline of stimulatory/depressive effects of ethanol for B6 mice (Middaugh *et al.*, 1992a), pretreatment with 600 mg/kg VGB greatly enhanced the depressive effects of ethanol. Although a complete dose-response function experiment for different combinations of VGB and ethanol will be necessary for firm conclusion, our data suggest that VGB and ethanol interact to shift the locomotor effects of ethanol from stimulatory towards depressive.

Based on the argument that the discriminative stimulus effects of ethanol can provide an discriminative stimulus regarding the organism's level of intoxication (Hodge *et al.*, 2006) another possible explanation for the observed VGB-induced reduction in ethanol self-administration is that VGB enhances the discriminative stimulus effects of ethanol. This possibility was tested in our evaluation of VGB effects on ethanol discrimination using a cumulative dosing series (0.5 g/kg) that produced response ratios above criterion performance standards (*i.e.* >80% on the ethanol-paired lever). VGB enhanced the discriminative stimulus effects of ethanol at the beginning of the series when cumulative doses were low. At this EtOH dose, VGB appeared to shift the generalization curve to the left; although this was not statistically supported by analysis of ED_{50} values. To evaluate further whether VGB enhanced the discriminative-stimulus effects of ethanol, we used a lower cumulative dosing series of ethanol (0.25 g/kg) that produced sub-criterion levels of ethanol discrimination (*i.e.* <80% on the ethanol-paired lever). Under the lower dosing conditions, VGB produced a significant leftward and upward shift in the generalization curve and significantly reduced the ED_{50} values for ethanol discrimination, which strengthens the evidence that VGB can enhance the discriminative stimulus effects of ethanol.

Because ethanol functions as an agonist at $GABA_A$ receptors (Burch *et al.*, 1980; Morrow *et al.*, 1988a,b) and VGB increases GABA concentrations (Gram *et al.*, 1989; Jung *et al.*, 1977; Rey *et al.*, 1992) that act on these receptors, the interaction of VGB-induced increases in GABA concentrations with ethanol at $GABA_A$ receptors appears to be an especially

important mechanism for the pharmacological effects we observed. This interaction explains the reduced stimulatory effects and the enhanced discriminability of ethanol by B6 mice, and both of pharmacological effects are consistent with reduced ethanol consumption following VGB pretreatment. Additionally, we found that VGB produced slightly elevated blood ethanol concentrations in the B6 mice used in these studies. For example, when co-administered with VGB, ethanol injections increased BEC by approximately 36% in the food-restricted mice used in the discrimination experiment, and 18% in non-deprived mice. The mechanism for the altered ethanol pharmacokinetics following bolus injections is unclear and we are not aware of previous reports describing this effect. A more thorough examination of ethanol levels in brain, liver and perhaps other tissues will be required to establish possible mechanisms for these modest increases, especially in the context of self-administered ethanol. Clearly, the small increase in blood ethanol concentrations provides an additional mechanism to explain some of the pharmacological effects we observed. Nevertheless, the altered pharmacokinetics of ethanol in combination with VGB do not completely explain the reduction noted in ethanol consumption. At the onset of a self-administration session, ethanol is not yet in the brain and brain ethanol concentrations rise gradually, peaking after consummatory behavior has ceased (Griffin *et al.*, 2007, 2009). This gradual rise is in contrast to the bolus injections given in the experiments in which we observed increased BEC with VGB. Therefore, it is plausible that the action of VGB to increase GABAergic neurotransmission in critical regulatory brain regions such as the nucleus accumbens may set the stage for a shift in preference away from ethanol to water (or food) before ethanol is present in the brain in pharmacologically relevant amounts. Thus, the VGB-induced increase in GABA concentrations that impact $GABA_A$ and perhaps also $GABA_B$ receptors remains an important mechanism in the effects reported in the present experiments.

In conclusion, we comprehensively examined the effects of VGB using several procedures designed to model specific aspects of ethanol-related behaviors using C57BL/6J (B6) mice. The experiments indicated that VGB produced dose-responsive reductions in ethanol self-administration and ethanol consumption. In contrast to the shorter duration of tiagabine in our earlier report (Nguyen *et al.*, 2005), the effect of VGB was observed for at least 48 hours following s.c. injections of the drug. This is consistent with the irreversible inhibition of GABA transaminase by VGB and the enduring effects on GABA metabolism. Interestingly, the effects of VGB on water and food were opposite to the effects on ethanol with increases in water and food consumption found after VGB treatment. VGB treatment also blunted the activity-stimulating effects and discriminative stimulus effects of ethanol, demonstrating that VGB augmented the pharmacological effects of ethanol at the GABAA receptor. Intriguingly, VGB treatment was associated with slight increases in blood ethanol concentrations, providing a possible additional mechanism for its effects. Collectively, the data presented in the present report converges on the idea that VGB reduces ethanol consumption by reducing ethanol stimulation and enhancing the discriminative stimulus properties of ethanol.

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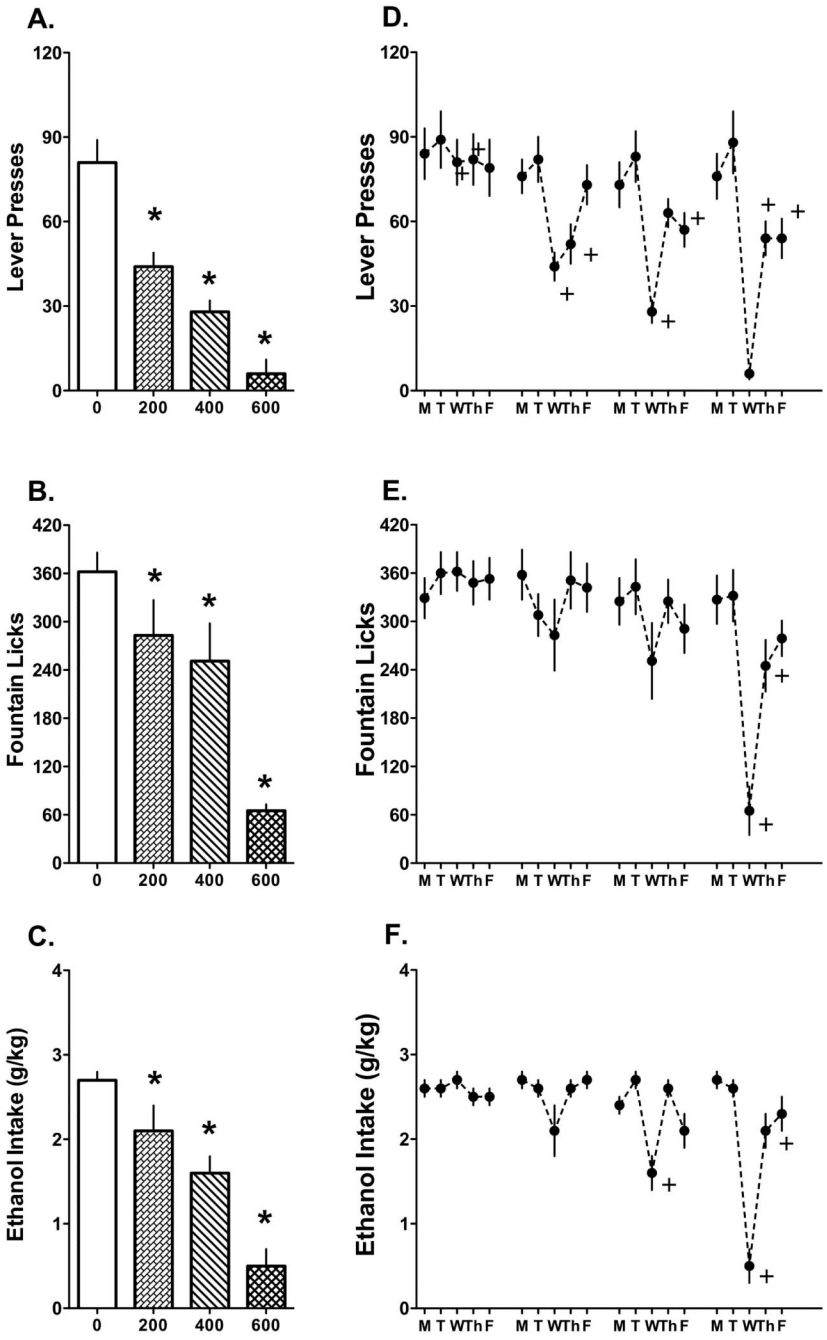

Figure 1.

Effects of vigabatrin (VGB) in B6 mice (n=18) on lever responding for ethanol with concurrent free access to water. VGB was injected subcutaneously 2.5 hr prior to the 15-min tests each Wednesdays over a three week period with vehicle injected prior to the other daily sessions. **Panels A, B, C)** VGB produced dose-responsive reductions in lever responding, licking behavior, and ethanol intake. **Panels D, E, F)** Lever responses for ethanol remained reduced for as long as two days, and licks and ethanol consumption for as many as two days, following VGB injections. Values are means ± S.E.M. (*p<0.05 compared to vehicle, +P<0.05 compared to Mon-Tues average).

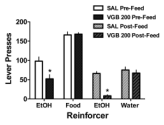

Figure 2.

Effects of VGB on lever responding for food and water in comparison with ethanol (n=18). When responding for 12% ethanol or food pellet reinforcement and tested before the daily food ration (Pre-Feeding Condition), VGB 200 mg/kg selectively reduced responding for ethanol. Further, when responding for 12% ethanol or water reinforcement and tested after being fed the daily food ration (Post-Feeding condition), VGB 200 mg/kg selectively reduced responding for ethanol. All values are means + S.E.M. (*$p<0.05$ compared to vehicle).

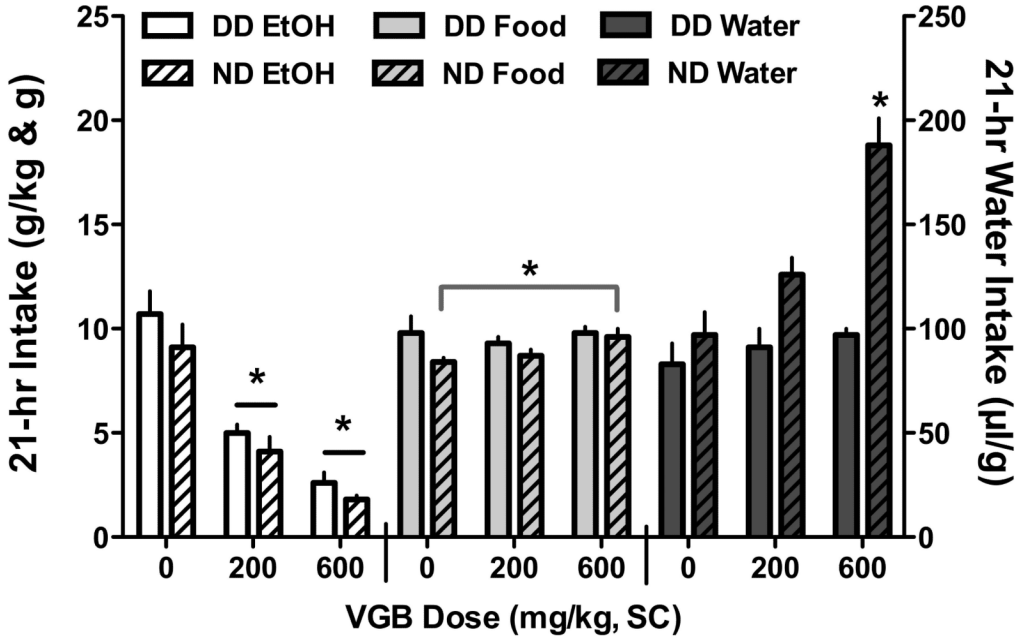

Figure 3.

Twenty-one hour consumption of 12% ethanol (g/kg), water (µl/kg), and food (mg/g). The data are averaged across drug days (DD) when mice were injected with either saline (n=10) or VGB (200, n=8) or 600, n=10) mg/kg) and averaged across non-drug days (ND) when mice received no injections. VGB reduced ethanol consumption on drug and non-drug days; whereas water consumption was markedly increased and food consumption was slightly, but significantly, increased only non-drug days. All values are means + S.E.M. (*$p<0.05$ compared to vehicle).

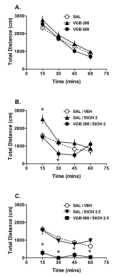

Figure 4.

Total distance traveled in 15-min intervals for B6 mice (n=6/Group) treated with VGB, ethanol or the combination. **A)** summarizes data for mice given vehicle, VGB 200 or VGB 600 mg/kg and shows that VGB did not affect motor activity. **B)** summarizes data following injections with saline or VGB 200 mg/kg given 2.5 hrs prior to vehicle or ethanol (2 g/kg). These data indicate that VGB attenuated the locomotor stimulatory effects of ethanol. **C)** shows the effects of VGB (600 mg/kg) and ethanol (2.5 g/kg): VGB attenuated stimulation produced by the 2 g/kg ethanol dose and at 600 mg/kg, reduced motor activity of mice injected with the 2.5 g/kg ethanol dose. All values are means ± S.E.M. (*$p<0.05$ compared to vehicle).

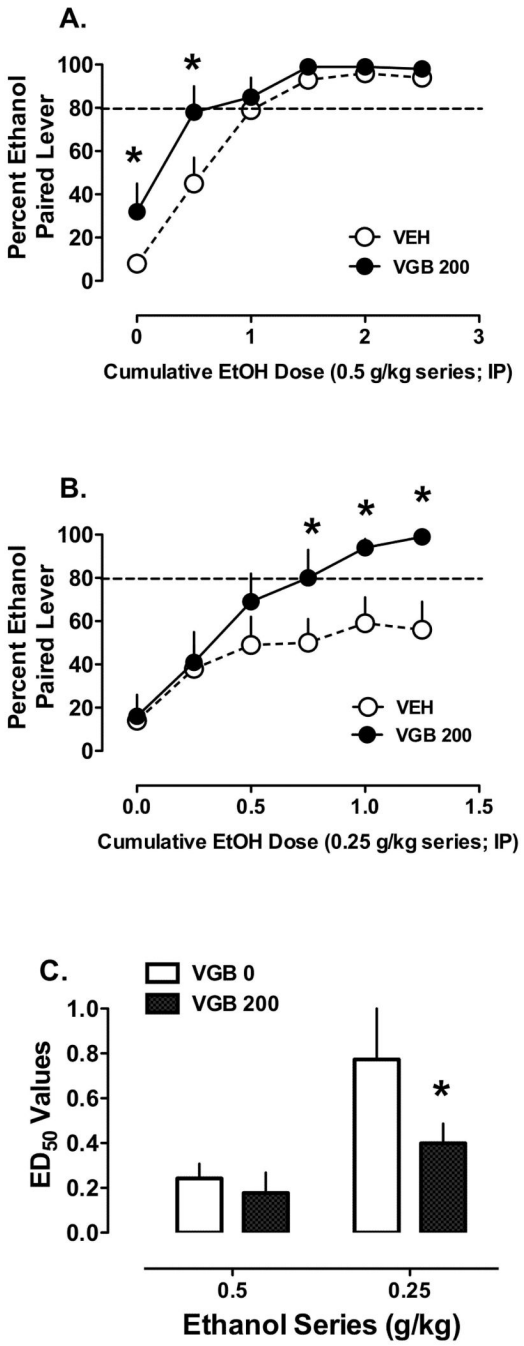

Figure 5.

Ethanol discrimination by B6 mice (n=8) treated with VGB 200 mg/kg. **A)** VGB significantly enhanced ethanol discrimination with the 0.5 g/kg dosing series at the lower doses in the series (*p<0.05). **B)** In contrast, VGB enhanced discrimination near the end of the dosing series (0.25 g/kg). **C)** The ED_{50} values were not significantly lowered by VGB for the 0.5 g/kg dosing series, but VGB significantly reduced the ED50 values for the 0.25 g/kg dosing series (*p<0.05). All values are means + S.E.M. (*p<0.05 compared to vehicle).

Table 1

Exp-1: Order x Dose ANOVAs on Data Collected on Weekly Test Days.

Measure	Dose $F_{(1,68)}$	*p*	Order x Dose $F_{(4,68)}$	*p*
Lever Response	35.22	0.001	0.51	0.736
Fountain Licks	15.33	0.001	1.38	0.252
EtOH Intake (g/kg)	10.99	0.001	3.04	0.024
SipperTube Contacts	6.47	0.008	2.32	0.080
H_2O Intake (μl/g)	3.12	0.062	1.64	0.196

Table 2

Exp-1: Test Week x Days ANOVAs on Data Collected Before and After Vigabatrin Tests.

Measure	Test Time $F_{(1,68)}$	*p*	Test Time x Day $F_{(4,68)}$	*p*
Lever Presses	0.59	0.587	1.99	0.125
Fountain Licks	0.09	0.772	0.66	0.582
EtOH Intake (g/kg)	9.32	0.007	0.64	0.592
Sipper Tube Licks	3.00	0.101	0.65	0.632
H_2O Intake (μl/g)	0.09	0.770	2.27	0.089

Table 3

Exp-1: ANOVA Summary of Dose Week x Day ANOVAs on Data Collected Daily during the each Test Week.

Measure	DoseWeek $F_{(3,51)}$	*p*	DoseWeek x Day $F_{(12,204)}$	*p*
Lever Presses	7.86	0.005	6.70	0.001
Fountain Licks	12.12	0.001	7.17	0.001
EtOH Intake (g/kg)	9.15	0.001	11.35	0.001
Sipper Tube Licks	3.06	0.062	3.67	0.067
H_2O Intake (μl/g)	3.91	0.017	0.89	0.507