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GABA_B receptor activation attenuates the stimulant but not mesolimbic dopamine response to ethanol in FAST mice

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Abstract

Neural processes influenced by γ -aminobutyric acid B (GABA_B) receptors appear to contribute to acute ethanol sensitivity, including the difference between lines of mice bred for extreme sensitivity (FAST) or insensitivity (SLOW) to the locomotor stimulant effect of ethanol. One goal of the current study was to determine whether selection of the FAST and SLOW lines resulted in changes in GABA_B receptor function, since the lines differ in sensitivity to the GABA_B receptor agonist baclofen and baclofen attenuates the stimulant response to ethanol in FAST mice. A second goal was to determine whether the baclofen-induced reduction in ethanol stimulation in FAST mice is associated with an attenuation of the mesolimbic dopamine response to ethanol. In Experiment 1, the FAST and SLOW lines were found to not differ in GABA_B receptor function (measured by baclofen-stimulated [³⁵S]GTP[S binding) in whole brain or in several regional preparations, except in the striatum in one of the two replicate sets of selected lines. In Experiment 2, baclofen-induced attenuation of the locomotor stimulant response to ethanol in FAST mice was not accompanied by a reduction in dopamine levels in the nucleus accumbens, as measured by microdialysis. These data suggest that, overall, GABA_B receptor function does not play an integral role in the genetic difference in ethanol sensitivity between the FAST and SLOW lines. Further, although GABA_B receptors do modulate the locomotor stimulant response to ethanol in FAST mice, this effect does not appear to be due to a reduction in tonic dopamine signaling in the nucleus accumbens.

Keywords

alcohol; stimulation; catecholamine; animal model; selected line; drug abuse

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Disclosure Statement

The authors declare they have no actual or potential conflicts of interest associated with this work.

1. Introduction

Recently, support has grown for the use of the GABA_B receptor agonist baclofen for the treatment of alcohol (ethanol) dependence, with several clinical studies reporting baclofen-induced reductions in alcohol consumption and increased abstinence in alcohol-dependent individuals [1–7]. Preclinical studies have predominantly echoed these results, with baclofen reducing ethanol consumption and seeking in mouse and rat models of ethanol consumption and dependence [8–15], although these results have not always been seen [16, 17].

Although rodent models of ethanol consumption have clear translational value, we and others have focused some of our studies on the stimulant properties of ethanol due, in part, to the similarity in neurochemical pathways governing ethanol-induced stimulation and reward [18–21], and because increased stimulant sensitivity is associated with a history of increased ethanol consumption [22] and a family history of alcohol dependence [23, 24]. This measure also avoids some of the complications associated with consumption studies, such as variation in ethanol intake, taste factors that can influence intake, and possible non-specific drug effects, which can be assessed using the locomotor measure. Further, our existing genetic mouse model of increased and decreased sensitivity to the stimulant effects of ethanol (the FAST and SLOW lines [25–27]) permits the evaluation of processes that may be important in determining individual, genetically determined differences in sensitivity to alcohol that may influence genetic risk for alcohol dependence.

Several laboratories, including our own using FAST and SLOW mice, have demonstrated that baclofen potently attenuates ethanol-induced locomotor stimulation in both mice and rats [14, 28–32]. This attenuation of stimulation does not appear to be due to the enhancement of ethanol-induced motor incoordination, as is seen with other GABA mimetic drugs [32], but may rather be due to a direct blockade of a stimulatory effect of ethanol in the brain. This makes baclofen attractive for the pharmacological treatment of alcohol dependence, as this drug decreases the stimulant response to ethanol and reduces ethanol consumption, without shifting the behavioral response to ethanol towards greater intoxication.

GABA_B receptors may contribute to the difference in ethanol-induced stimulation between the FAST and SLOW lines through a difference in the functional state of the receptor. For instance, in limbic brain regions, GABA_B receptor function was significantly lower in rats selectively bred for high ethanol preference compared to rats bred for low ethanol preference [33]. In addition, baclofen may attenuate the locomotor stimulant response to ethanol in FAST mice by diminishing a neurobiological substrate of ethanol. One established neurobiological mechanism associated with some of ethanol's effects is activation of the mesolimbic dopamine pathway [34–40], with dopamine cell bodies located in the ventral tegmental area (VTA) sending projections to the nucleus accumbens (NAc) and other limbic brain regions [41]. Intra-VTA ethanol administration induces a stimulant response in rats [20, 42], and ethanol-induced increases in dopamine function have been correlated with acute ethanol sensitivity in some studies [38, 43–46], including ours, in which FAST mice showed a heightened dopamine response to ethanol compared to SLOW mice [47, 48]. Within the VTA, GABA_B receptors are predominantly localized to dopamine neurons [49], and activation of these receptors regulates dopamine cell firing [50–53] and dialysate dopamine levels in the NAc, both basally [54, 55] and in response to several drugs of abuse, including amphetamine, cocaine, morphine, and nicotine [56, 57]. However, heretofore, the effect of baclofen on ethanol-induced increases in extracellular dopamine levels in the NAc has not been reported.

The purpose of the current study was to examine whether selection for extreme sensitivity and insensitivity to the stimulant effects of ethanol is associated with a change in GABA_B receptor function, and whether the baclofen-induced attenuation of ethanol stimulation is associated with an attenuation of an ethanol-induced increase in dopamine levels in the NAc. In Experiment 1, regional differences in GABA_B receptor function were measured using baclofen-stimulated guanosine 5'-O-(3-[³⁵S]thiotriphosphate) ([³⁵S]GTPγS) binding. Due to the enhanced sensitivity of SLOW mice to the locomotor depressant and sedative effects of baclofen and ethanol [27, 28, 31, 58], we hypothesized that GABA_B receptor function would be significantly higher in SLOW compared to FAST mice, particularly in regions critical for the locomotor stimulant response to ethanol. This hypothesis is also consistent with the decrease in ethanol-induced stimulation seen in FAST mice when GABA_B receptor function is enhanced by baclofen [28, 31, 32], suggesting that reduced GABA_B receptor function may contribute to heightened ethanol stimulant sensitivity. In Experiment 2, the effect of baclofen on ethanol-induced mesolimbic dopamine signaling was examined by measuring extracellular dopamine levels in the NAc using *in vivo* microdialysis in FAST mice. Due to the attenuation of the stimulant response to ethanol in FAST mice by intra-anterior VTA baclofen administration [28], and the reduction of other drug-induced increases in dopamine levels in the NAc that have been found after baclofen treatment using microdialysis [56, 57], we hypothesized that a baclofen-induced attenuation of acute ethanol stimulation would be associated with an inhibition of ethanol-induced increases in mesolimbic dopamine.

2. Materials and Methods

2.1 Subjects

Details regarding the creation of the replicated FAST and SLOW selected lines have been published [25–27, 59]. The selection phenotype was an activation score (ACT), determined by subtracting baseline locomotor activity measured after saline from locomotor activity measured after 1.5 g/kg (first 6 generations) or 2.0 g/kg (all subsequent generations) ethanol. Mice with high ACT scores served as parents of FAST mice and mice with low ACT scores served as parents of SLOW mice. Mice were selectively bred for 37 generations and then maintained under relaxed conditions (random breeding with measures taken to reduce inbreeding). The two pairs of replicate lines (FAST-1 and SLOW-1; FAST-2 and SLOW-2) were initiated from independent populations of heterogeneous stock (HS/Ibg) mice, which were created by interbreeding 8 inbred strains [60].

Male FAST and SLOW mice were used for consistency with previous experiments [28, 32, 48]. However, both male and female mice of these lines have been shown to markedly differ in sensitivity to the stimulant effect of ethanol [26, 27]. Subjects were housed 2–4 per cage with littermates, or with non-littermates of the same approximate age (± 5 d) and the same replicate line; this practice avoided single housing. The vivarium was maintained on a 12:12-h light-dark cycle (lights on at 0600) at 21 $\pm$ 2°C with food (Purina Laboratory Rodent Chow #5001; Purina Mills, St. Louis, MO) and water available ad libitum, except during testing. Precautions were taken to minimize animal discomfort and pain. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the Portland Veterans Affairs Medical Center. All injections were given intraperitoneally and group sizes for each study are given in the relevant tables and figure legends.

2.2 Drugs and Reagents

[³⁵S]GTPγS (1250 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). Ethanol was obtained from Pharmco Products (Brookfield, CT) and diluted in 0.9% physiological saline (Baxter Healthcare Corporation, Deerfield, IL) to a 20% v/v solution for *in vivo*

ethanol testing. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO). ($\pm$) Baclofen, guanosine 5'-diphosphate (GDP), and ethanol for in vitro testing were prepared in 50 mM Tris-HCl buffer (pH 7.4 at room temperature). ($\pm$) Baclofen for in vivo testing was prepared in saline. Tris-EDTA-Dithiothreitol (TED) buffer was comprised of 5 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% w/v sucrose, pH 7.4 (at 4°C). Artificial cerebral spinal fluid (aCSF) was comprised of 145 mM NaCl, 2.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, and 5.4 mM D-glucose. Mobile phase was comprised of 10% acetonitrile, 90 mM NaH₂PO₄, 50 mM citric acid, 1.7 – 2.1 mM octanesulfonic acid, and 50 μ M EDTA.

2.3 Experiment 1: Analysis of GABA_B receptor function in the FAST and SLOW selected lines

2.3.1 Membrane Preparation—Methods for membrane preparation and the [³⁵S]GTP γ S binding assay were modified from [61]. Whole brain tissue was obtained from FAST and SLOW mice that were 52–67 days of age and from generations S₃₇G₈₅₋₈₇ (where S_{xx} refers to the number of selection generations, and G_{yy} refers to the total number of breeding generations that have elapsed since the beginning of selection). Subjects were euthanized by cervical dislocation and brain tissue was rapidly removed and placed in tubes on ice. Tissue was homogenized in 5 ml ice-cold TED buffer using a Polytron Homogenizer (20 strokes; Kinematica, Newark, NJ). All subsequent steps were conducted at 4°C. Tissue homogenates were centrifuged at 1000 $\times$ g for 10 min, the supernatant was decanted into a separate tube, and the pellet was resuspended in 10 ml TED buffer and centrifuged again at 1000 $\times$ g for 10 min. The supernatant was again isolated, was combined with the previous supernatant, and this sample was then centrifuged for 20 min at 9000 $\times$ g. The supernatant was then discarded and the resulting tissue pellet was resuspended in 10 ml TED buffer and centrifuged at 18000 $\times$ g for 20 min. This step was repeated again, and the tissue pellet was resuspended in 10 ml TED buffer and kept on ice for 30 min, and then centrifuged at 35000 $\times$ g for 10 min. The final tissue pellet was resuspended in 2 ml of 50 mM Tris-HCl buffer (pH 7.4) and aliquoted prior to storage at –80°C. Protein concentration (μ g/ μ l) was determined using the bicinchoninic acid [62] method (Pierce, Rockford, IL). This repeated tissue washing was performed to remove endogenous GABA from the membrane preparation.

Microdissected brain regions were obtained from FAST and SLOW mice from generations S₃₇G₈₆₋₈₉ that were 51–73 days of age. Cerebellum, hippocampus, prefrontal cortex, striatum (caudate putamen and NAc combined), and ventral midbrain (including the VTA) were rapidly dissected on ice. These regions were chosen based on their well-published roles in the neurobiological and behavioral effects of ethanol [63–65], including the locomotor stimulant, motor-incoordinating and sedative effects of ethanol [18, 19, 66–68], traits for which the FAST and SLOW lines dramatically differ [27, 58, 69]. Prefrontal cortex tissue from 2 mice of the same line and replicate was pooled, whereas ventral midbrain tissue from 3 mice of the same line and replicate was pooled for the membrane preparation to provide enough protein for analysis. Tissue was homogenized in 1 ml (cerebellum) or 500 μ l (remaining brain regions) of ice-cold TED buffer. The methods for membrane preparation matched those used for whole brain, except that a volume of 500 μ l was used for all brain regions except the cerebellum (for which 1 ml TED buffer was used) for the initial centrifugation steps (at 1000 $\times$ g). All remaining steps were performed with 1 ml TED buffer. The final tissue pellet was resuspended in 50 mM Tris-HCl buffer, at a volume of approximately 9 μ l/mg wet tissue weight. An aliquot was taken for protein quantification, and the remaining samples were stored at –80°C until use.

In an additional study, the effect of *in vivo* ethanol pre-exposure was examined on GABA_B receptor function. FAST and SLOW mice were administered an injection of 2 g/kg ethanol

(20% v/v in saline) or an equivalent volume of saline. Mice were 53–69 days of age at the time of treatment and were from generations S₃₇G₈₈₋₈₉. Fifteen minutes after treatment, cerebellar tissue was rapidly dissected on ice and prepared as described above.

2.3.2 [³⁵S]GTPγS Binding—Thawed membranes (10 ! g for regional preparations, 20 ! g for whole brain preparations) were incubated for 60 min at 30!C in 500 !l of 50 mM Tris-HCl (pH 7.4) containing 5 mM MgCl₂, 100 mM NaCl, 0.1 mM EDTA, 0.2 mM EGTA, 0.2 mM dithiothreitol, 20 ! M GDP, 0.2 nM [³⁵S]GTP!S and 0.32 μM – 1 mM baclofen. For the analysis of the effect of *in vitro* ethanol exposure on GABA_B receptor function, cerebellar homogenates (10 μg) were incubated under similar conditions, except that an approximate EC₅₀ dose of baclofen (70 μM) was used alone and in combination with a range of ethanol concentrations (1 – 100 mM). Ethanol (in 50 mM Tris) was first added to the tissue and allowed to incubate for 10 min prior to the addition of assay buffer in order to facilitate an ethanol effect on GABA_B receptor function prior to [³⁵S]GTPγS binding. All reactions were run in triplicate, with each plate comprised of a dose-response for a FAST mouse and a SLOW mouse of the same replicate (or with a baclofen dose-response from a saline- and ethanol-treated subject of the same line and replicate for the *in vivo* ethanol study). The reaction was terminated by rapid filtration through a glass fiber filter (GF/B; PerkinElmer, Waltham, MA), using a 96-well Tomtec cell harvester (Hamden, CT), and washed with ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 4°C). Bound radioactivity was measured using a PerkinElmer BetaPlate 1205 scintillation counter (Waltham, MA). Non-specific binding was measured in the presence of 100 ! M unlabeled GTP!S; these values were then subtracted from total binding to define specific [³⁵S]GTP!S binding. Average basal binding of [³⁵S]GTP!S (in the absence of baclofen) was 12800 ± 980 CPM for whole brain tissue (20 μg tissue), and 4120 ± 130 CPM for regional tissue microdissections (10 μg). Average maximal binding above baseline was 18100 ± 1390 for whole brain tissue and 8980 ± 340 for regional microdissections.

2.3.3 Data Analysis—Percent stimulation of basal [³⁵S]GTP!S binding by baclofen was calculated by nonlinear regression analysis (variable slope) using GraphPad PRISM software (GraphPad 5, San Diego, CA). The concentration of baclofen eliciting half-maximal stimulation of [³⁵S]GTP!S binding (EC₅₀) and maximal stimulation of [³⁵S]GTP!S by baclofen (E_{max}) were then calculated for each subject. Descriptive statistics were obtained using GraphPad Prism software, and statistical outliers were identified within each replicate line, defined as having a value (EC₅₀ and/or E_{max}) of 3 standard deviations or greater from the mean. EC₅₀ and E_{max} data were subsequently analyzed by analysis of variance (ANOVA) using GraphPad Prism software. Replicate was included in initial analyses. In the absence of significant interactions with replicate, reanalysis considered data for the two replicates combined. For the *in vivo* ethanol experiment, data were similarly analyzed by ANOVA for the effects of line, replicate, and ethanol dose. [³⁵S]GTP!S binding in response to *in vitro* ethanol exposure was expressed as percent change from baclofen (70 μM) and analyzed by repeated measures ANOVA, with ethanol dose as the repeated measure. Significance levels were set *a priori* at α = 0.05.

2.4 Experiment 2: Effect of GABA_B Receptor Activation on Mesolimbic Dopamine Activity

2.4.1 Stereotaxic Surgery—Surgical methods were adapted from previous studies in our laboratory [28, 48]. FAST-1 and -2 mice were 49–71 days of age at the time of surgery and from selection generations S₃₇G₈₉₋₉₄. Mice were administered an anesthetic cocktail comprised of 140 mg/kg ketamine, 14 mg/kg xylazine, and 2.8 mg/kg acepromazine, diluted 1:6 in saline (VA Medical Center pharmacy, Portland OR), and then the dorsal scalp was shaved and the cranial surface was exposed and cleaned. Once secured in the stereotaxic apparatus (Cartesian Research, Inc, Sandy, OR; David Kopf Instruments, Tujunga, CA), a

CMA/7 guide cannula with a stainless steel obturator (CMA Microdialysis, Stockholm, Sweden; shaft length 7 mm, outer diameter 0.38 mm) was implanted 1 mm dorsal to the left NAc, using bregma and lambda as landmarks (coordinates relative to bregma: +1.4 mm anterior, 0.8 mm lateral, 3.8 mm ventral [70]). An anchor screw (1/8 inch; Small Parts, Miami Lakes, FL) was implanted through the right side of the skull to better secure the head mount to the skull. The guide cannula and anchor screw were secured in place with duralon carboxylate cement (3M, St. Paul, MN). Subjects were returned to the colony room in rat-sized cages with food and water available *ad libitum*. Mice were group housed with their original cage mates until the time of testing, which we have found aids recovery and leads to fewer problems with the surgical preparation. A minimum of 4 days elapsed after surgery before the microdialysis probe was implanted.

2.4.2 Locomotor Activity Apparatus—For measurement of locomotor activity, mice were tested in clear acrylic plastic chambers measuring 40 × 40 × 30 cm (l × w × h; AccuScan Instruments, Inc., Columbus, OH) that were transected by 8 pairs of photocell beams and detectors positioned 2 cm above the chamber floor. Each apparatus was housed in a light-proof, sound-attenuating cabinet (Flair Plastics, Portland, OR), with a fluorescent white light and ventilation fan. Photocell beam interruptions were recorded and translated to horizontal distance traveled in centimeters by AccuScan software (Versamax Version 1.80).

2.4.3 Microdialysis Procedure—Details specific to this procedure have been published elsewhere [48]. Briefly, a CMA/7 microdialysis probe (probe specifications: shaft length 7 mm, 1 mm exposed cuprophane membrane, 0.24 mm outer diameter, 6 kDa cut-off; CMA Microdialysis, Stockholm, Sweden) was implanted the evening before testing, at which point a tethering post (Instech Laboratories, Plymouth Meeting, PA) was also cemented to the head mount. The mouse was then secured to a dual-channel microdialysis swivel (Instech) and counterbalanced lever arm (Instech) that permitted free movement. Each mouse was housed overnight in a rat cage with free access to food and water, and aCSF was infused through the microdialysis probe at a rate of 2 µl/min. An automated light timer maintained the appropriate L:D cycle.

On the following morning (~15 h following probe implantation), the rat cage, food and water were removed and subjects were placed directly into the locomotor activity chambers. Locomotor activity and dialysate samples were simultaneously collected in 15-min time bins, with dialysate collected in glass microvials (Agilent Technologies, Palo Alto, CA) that contained 2 µl of a concentrated antioxidant (20 mM oxalic acid, 2 M glacial acetic acid) to prevent the spontaneous oxidation of dopamine. Habituation to the testing procedure occurred for 2 h prior to drug administration, with 4 baseline samples (collected every 15 min) and 4 samples after an IP injection of saline (collected every 15 min). Each mouse was then briefly removed from the activity monitor and injected with saline or one of two doses of baclofen (1.25 or 2.5 mg/kg) and returned to the monitor. After 15 min, each mouse was then injected with saline or 2 g/kg ethanol, and ethanol-induced changes in locomotor activity and dialysate dopamine were collected every 15 min for 1 hour (consistent with [48]). Baclofen doses and pretreatment interval were chosen from a previous study that showed a dose-dependent reduction in ethanol-stimulated activity in FAST mice [31].

Dialysate samples were stored at −80°C until analyzed. Subjects were euthanized by cervical dislocation and brain tissue was rapidly removed, flash frozen in ice-cold isopentane, and stored at −80°C until sectioned for histology. Brains were sectioned into 40 µm coronal slices using a cryostat (Leica CM1850; Nussloch, Germany), and thaw mounted on Superfrost Plus slides (VWR, West Chester, PA). Slices were imaged using a Leica DMLB light microscope (Model LB30T, Wetzlar, Germany) connected to a SPOT Insight digital camera and software (Diagnostic Instruments, Sterling Heights, MI). Probe

placement was identified by methylene blue (1–2 μ l, 10 mg/ml) infusion through the probe or observation of the probe track. Data from only those subjects in which the majority of the microdialysis probe was located within the boundaries of the NAc were included in the analysis.

2.4.4 High-Performance Liquid Chromatography (HPLC)—Dialysate concentrations of dopamine were measured by HPLC with electrochemical detection (5014B, ESA Inc.; reducing electrode –100 mV; oxidizing electrode +200 mV). Mobile phase (pH 5.6) was pumped at a rate of 0.35–0.5 ml/min using an ESA 582 isocratic solvent delivery system (ESA Inc., North Chelmsford, MA). Samples (20 μ l) were delivered by a refrigerated (4–6°C) autosampler (ESA 542) for separation on a C18 column (ESA model MD-150, 3 mm inner diameter, 150 mm long, 3 μ m particle size). The column temperature was maintained at 28°C. Dialysate concentrations were determined from peak area, using ESA Coullarray for Windows software, and calculated from an external standard curve prepared at the time of analysis (0.15 – 8 nM dopamine HCl). The sensitivity for dopamine was in the sub-nM range, and average signal-to-noise ratios were as follows: 51.4 ± 2.2 , 48.2 ± 1.7 , 34.7 ± 1.3 , 18.3 ± 0.8 , 9.5 ± 0.4 , and 2.2 ± 0.2 for the 8, 6, 4, 2, 1, and 0.15 nM standards, respectively, and 9.3 ± 0.7 for baseline samples. Methods for HPLC followed those of Meyer and colleagues [48].

2.4.5 Data Analysis—All statistical analyses were conducted using Statistica Software (version 10, StatSoft, Inc., Tulsa, OK). Locomotor activity data were first analyzed by ANOVA grouped on replicate, baclofen pretreatment dose, and ethanol dose, with time as a repeated measure. The absence of significant interactions with replicate led to consideration of data collapsed on this factor. Significant time effects led to subsequent baclofen by ethanol dose ANOVAs within specific time bins. Dialysate dopamine levels were expressed as percentage of the baclofen response and analyzed similarly. Significant interactions were further considered by examining simple main effects and post-hoc Newman-Keuls mean comparisons, as appropriate. Significance levels were set *a priori* at $\alpha = 0.05$.

3. Results

3.1 Experiment 1: Analysis of GABA_B receptor function in the FAST and SLOW selected lines

3.1.1 Basal and baclofen-stimulated [³⁵S]GTP γ S binding—Data from 6 samples from the following regions were removed for being statistical outliers (defined as being greater than 3 standard deviations from the mean): whole brain (1 FAST-2 and 1 SLOW-1), cerebellum (1 SLOW-1), hippocampus (1 FAST-2), and ventral midbrain (1 FAST-1 and 1 SLOW-2). Initial analyses revealed no significant interaction of line with replicate. For whole brain, and all brain regions except striatum, there were no significant differences in basal binding levels between the FAST and SLOW lines (data not shown). However, within the striatum, basal [³⁵S]GTP γ S binding was significantly higher in SLOW mice (mean $\pm$ SEM: 7650 ± 470 CPM) than in FAST mice (6070 ± 280 CPM) [$F_{1,28}=8.3$; $p<0.01$].

Data reflecting GABA_B receptor function are shown in Table 1. Whole brain tissue was used to confirm that baclofen-induced increases above basal binding were specific to the GABA_B receptor, as inclusion of a GABA_B receptor specific antagonist (CGP-54626) in the incubation buffer reduced baclofen-stimulated binding to basal levels (data not shown). GABA_B receptor function in whole brain or regional tissue preparations was not found to differ for FAST and SLOW mice, except in striatal tissue. For striatal tissue, there was no difference between the lines in EC₅₀ for baclofen; however, there was a significant line by replicate interaction [$F_{1,26}=6.4$; $p<0.05$] for E_{max}. In FAST-1 mice, E_{max} was significantly

higher than that for SLOW-1 mice ($p<0.01$), but there was no similar line difference in replicate 2 (see Fig 1A and Table 1).

3.1.2 Effect of ethanol exposure on GABA_B receptor function within the cerebellum—The effect of *in vivo* ethanol exposure on GABA_B receptor function was assessed in cerebellar tissue due to the high density of this receptor in this brain region, and the critical involvement of cerebellar GABA_B receptors in the motor effects of ethanol [71–74]. Moreover, as shown in Table 1, baclofen had the highest efficacy and potency in the cerebellum in both FAST and SLOW mice, compared to other brain regions tested. Therefore, the ability to detect an ethanol effect on GABA_B receptor function seemed most likely in this brain region. Data from one ethanol-treated FAST-1 mouse were removed as they met the outlier criterion. Although there was no effect of *in vivo* ethanol exposure on the EC₅₀ for baclofen in the cerebellum, ethanol treatment did increase the E_{max} for baclofen [$F_{1,43}=7.3$; $p<0.01$]; there were no line or replicate differences (Fig 1B). Unlike the effects of *in vivo* ethanol treatment, inclusion of ethanol in the assay buffer (1–100 mM) did not significantly affect baclofen-stimulated [³⁵S]GTPγS binding (data not shown).

3.2 Experiment 2: Effect of GABA_B Receptor Activation on Mesolimbic Dopamine Activity

3.2.1 Histology—A summary of microdialysis probe placements classified as “hits” are shown in Fig 2. A hit was defined as over 50% of the 1 mm probe being located within the borders of the NAc. The majority of probe placements were located within the medial NAc, corresponding to the medial NAc shell and core. The final group size was 10–15 FAST mice per baclofen dose and ethanol dose.

3.2.2 Locomotor Activity—As shown in Fig. 3 (A and B), baclofen dose-dependently attenuated the locomotor stimulant response to ethanol in FAST mice, consistent with previous reports [28, 31, 32]. Over the course of the experiment, there was a significant baclofen dose × ethanol dose × time interaction [$F_{24,756}=1.6$; $p<0.05$] that did not interact with replicate, and main effects of ethanol dose and/or interactions of baclofen and ethanol dose were observed for all four time points after ethanol administration (no systematic group differences were observed prior to ethanol administration). As we were interested in examining the effects of baclofen on ethanol-induced locomotor activity and dialysate dopamine levels, we chose to focus our analysis on the time periods immediately after baclofen and ethanol administration.

Baclofen alone, which was administered 15 min prior to ethanol treatment, did not significantly alter locomotor activity; mean ($\pm$ SEM) distance traveled was 302 ± 77 , 595 ± 202 , and 202 ± 28 cm for 0, 1.25 and 2.5 mg/kg baclofen-treated mice, respectively. For the 1 h following ethanol administration, there were significant main effects of baclofen dose [$F_{2,63}=6.0$; $p<0.01$] and ethanol dose [$F_{1,63}=24.0$; $p<0.0001$], and a baclofen dose X ethanol dose interaction [$F_{2,63}=6.1$, $p<0.01$]. There was also an interaction of ethanol dose and time [$F_{3,189}=13.2$; $p<0.0001$]; however, there was no significant 3-way interaction of baclofen dose, ethanol dose, and time. To follow up the significant baclofen dose X ethanol dose interaction, data were collapsed on time (as presented in Fig. 3B). Simple main effects analyses revealed no significant effect of baclofen dose in saline-treated FAST mice, but a significant effect of baclofen on activity in ethanol-treated mice ($p<0.0001$). Both doses of baclofen (1.25 and 2.5 mg/kg) were found to significantly reduce the locomotor stimulant response to ethanol ($p<0.01$).

3.2.3 Dialysate Dopamine—Despite the attenuation of ethanol-induced locomotor stimulation by baclofen in FAST mice, baclofen did not reduce ethanol-induced increases in NAc dopamine levels in these mice, as shown in Fig. 3C and D. At baseline, average

dialysate dopamine levels were 1.1 ± 0.1 nM, and did not differ significantly between groups. Similarly, no significant group differences were observed during the microdialysis habituation period (baseline and saline injection; data not shown). Therefore, similar to the activity data, we focused our analysis on the time period immediately after baclofen and ethanol administration (see Fig. 3C), with data expressed as percent change from the last sample taken after baclofen (0, 1.25, or 2.5 mg/kg) treatment. This provides a measure of ethanol effects in saline versus baclofen treated mice.

There was no significant effect of baclofen alone on extracellular dopamine levels in the 15 min immediately following baclofen administration (values were 1.1 ± 0.1 nM, 1.2 ± 0.2 nM, and 1.2 ± 0.2 nM for 0, 1.25, and 2.5 mg/kg baclofen, respectively). Analysis of data for the 1-h period following ethanol administration revealed a main effect of ethanol dose [$F_{1,63}=10.7$; $p<0.01$], with 2 g/kg ethanol significantly increasing dialysate dopamine levels, but no significant interactions with baclofen dose or time (Fig 3C). Due to the lack of an effect of time, dialysate dopamine levels were averaged across the 1-h period, for which there was a main effect of ethanol dose [$F_{1,63}=9.1$; $p<0.01$] (Fig 3D). However, similar to the repeated measures analysis, there was no significant effect of baclofen dose on dialysate dopamine, nor was there an interaction of baclofen dose and ethanol dose, supporting the conclusion that baclofen pretreatment did not attenuate the NAc dopamine response to ethanol.

4. Discussion

Although GABA_B receptor activation potently modulates the stimulant response to ethanol in FAST mice, and the FAST and SLOW lines differ in sensitivity to the motor sedative effects of baclofen [28, 31], differences in receptor function between FAST and SLOW mice do not appear to overtly contribute to the differential genetic susceptibility of these mouse lines to ethanol-induced stimulation. In addition, the attenuation of ethanol stimulation by baclofen in FAST mice appears to be independent of changes in tonic mesolimbic dopamine signaling, since there was a reduction in behavioral stimulation in the absence of a similar reduction in ethanol-induced elevated dopamine signaling in the NAc. This suggests that the stimulant response in FAST mice can be attenuated even when ethanol-enhanced dopamine signaling from the VTA to NAc is left intact.

4.1 GABA_B receptor function is not systematically altered in the FAST and SLOW selected lines

The FAST and SLOW selected lines are a unique genetic animal model that has been used to examine the neurochemical systems mediating genetically-determined differences in acute ethanol sensitivity. By examining some genetically correlated line differences (defined as a phenotypic difference between the lines for a trait other than the selection phenotype, aka ethanol-stimulated locomotor activity), the systems that were altered through the process of genetic selection, and that thereby contribute to the selection response, can be inferred [75]. For example, the line difference in sensitivity to the locomotor depressant effect of baclofen [28, 31] suggests that some aspect of GABA_B receptor signaling, possibly receptor density, receptor function, or downstream signaling cascades, was altered during selection. Since the VTA is important for the stimulant effects of ethanol [20, 28, 42], but GABA_B receptor density was not different between the FAST and SLOW lines in this region [28], we hypothesized that GABA_B receptor function was altered by selection. This hypothesis was not strongly supported.

Although no line difference was found for GABA_B receptor function in either whole brain tissue, or in several regional preparations (prefrontal cortex, hippocampus, ventral midbrain, and cerebellum), a line difference in $E_{\max}$ in the presence of baclofen (FAST-1 > SLOW-1)

was found in striatal tissue. This line difference was opposite in direction to our hypothesis. Increased GABA_B receptor function in FAST-1 mice could contribute to the extreme locomotor stimulant response to ethanol, as increased agonist efficacy for striatal GABA_B receptors would be predicted to potentiate ethanol-induced inhibition of the NAc [76], leading to increases in locomotor activity [77–79]. However, there was also an overall line difference in basal [³⁵S]GTPγS binding in striatal tissue (FAST < SLOW), which could have led to an artificial inflation of baclofen-stimulated potentiation of binding in FAST-1 mice. At present, the source of this line difference in basal [³⁵S]GTPγS binding is unknown. Although recent studies have explored the contribution of constitutive, agonist-independent, activity to GPCR function and pharmacology [80, 81], it is unknown how this basal elevation in striatal GPCR function in SLOW mice may affect ethanol sensitivity or GABA_B receptor function.

A line difference in receptor function was found in the replicate 1 but not replicate 2 lines, which provides modest evidence of a genetic correlation [75]. It is possible that this finding is a false positive; this genetic difference may have arisen by chance due to genetic drift (inbreeding resulting in a change in frequency of selection trait irrelevant genes) and therefore may not be relevant to the ethanol sensitivity difference. However, this result could also be meaningful. Replicate selections are not likely to result in identical outcomes. Because more than one gene and more than one mechanism contributes to the magnitude of a quantitative trait, a mechanism that is relevant to the selection trait and contributes to the differentiation of one set of lines may be absent from the differentiation of the other set of lines. This could be due to differences in allelic diversity/composition in the independent founding populations, or could be due to the loss of certain alleles in the finite breeding populations, resulting in the loss of genetic diversity relevant to the selection trait. In fact, the magnitude of difference for the ethanol stimulation trait is not identical between the two sets of replicate FAST and SLOW lines (e.g., see [27]). Thus, the expectation that some genetic differences and relevant mechanisms could be found in one set and not the other is reasonable. Additional research will be needed to determine whether striatal GABA_B receptor function contributes to genetically-determined differential sensitivity to the locomotor stimulant effects of ethanol.

In an *in vivo* analysis of the effect of acute ethanol exposure (15 min) on GABA_B receptor function, agonist efficacy ($E_{\max}$) in the cerebellum was increased by ethanol, supporting the hypothesis that ethanol has ‘GABA-mimetic’ effects [82]. However, this result did not differ between the FAST and SLOW lines in the cerebellum; therefore, it likely does not contribute to the selection phenotype. Contrary to this result, inclusion of ethanol in the incubation buffer did not affect agonist efficacy, suggesting that ethanol may not directly alter GABA_B receptor function, as measured by [³⁵S]GTPγS binding.

Although there was little evidence for systematic, selection-induced alterations in GABA_B receptor function, this does not rule out the contribution of GABA_B receptors to extreme sensitivity to the stimulant effects of ethanol. First, studies examining GABA_B receptor function using positive allosteric modulators [83] might reveal more subtle differences in receptor function between the lines. Alternatively, GABA_B receptors may significantly contribute to the differential sensitivity to ethanol, but at sites downstream of the receptor/g-protein apparatus. For instance, recent studies have found that the K⁺ channel tetramerization domain-containing (KCTD) proteins, which bind to the GABA_{B2} subunit, increase agonist potency and effector signaling [84]. Additionally, the function of the G-protein inwardly rectifying K⁺ channel (GIRK), which is coupled postsynaptically to the GABA_B receptor [85], has been found to be enhanced by ethanol [86–88].

4.2 Activation of GABA_B receptors blocks the stimulant response to ethanol, but not the ethanol-induced increase in dopamine

Although GABA_B receptor activation reduced the stimulant response to ethanol in FAST mice, it did not reverse ethanol-induced increases in extracellular dopamine in the NAc. Initially, we hypothesized that baclofen would reduce the stimulant response to ethanol in FAST mice via a baclofen-induced attenuation of the stimulatory effects of ethanol on mesolimbic dopamine signaling. This hypothesis was based on the finding that intra-VTA administration of baclofen altered the locomotor stimulant response to ethanol in FAST mice [28]. Previous studies, some using similar methodology as the current study, reported that baclofen decreased VTA dopamine cell firing and dopamine release in the VTA and NAc [50–55], and attenuated amphetamine-, cocaine-, morphine-, and nicotine-induced increases in dopamine levels in the NAc in rats, as measured by *in vivo* microdialysis [56, 57]. Previous studies have not considered this baclofen effect for ethanol, and our results did not support a similar baclofen-dependent attenuation of ethanol-induced extracellular dopamine in the NAc in FAST mice.

There are several potential explanations for this outcome. First, in the case of ethanol-induced increases in dopamine levels, microdialysis may not be highly sensitive for measuring spatially or temporally restricted reductions in dopamine levels induced by baclofen. Due to the predominant use of *in vivo* microdialysis to examine baclofen and other drug effects (including ethanol) on dopamine levels, it was logical for us to choose this technique to determine whether similar results would be obtained for baclofen effects on ethanol-enhanced dopamine levels. The results we achieved with this technique suggest that baclofen is able to attenuate the locomotor stimulant response to ethanol in the absence of an effect on ethanol-induced increases in dialysate dopamine in the NAc, in stark contrast to the effects of baclofen on the effects of other drugs of abuse [56, 57]. One interpretation of this result is that ethanol-induced increases in dopamine to the NAc [48] are dissociated from the effect of ethanol on locomotor activity. However, cautious interpretation is warranted, because microdialysis may not capture a critical aspect of dopamine signaling, namely phasic release. Phasic dopamine release is characterized by large and rapid, yet transient, increases in extracellular dopamine levels, whereas tonic release is characterized by smaller and slower, more prolonged, increases in extracellular dopamine [89–91]. Although microdialysis coupled with HPLC permits a sensitive measurement of dopamine levels, it requires slow perfusion rates and large sample volumes (thereby requiring the protracted sampling time of 15 min used in the current study), and is a highly effective method for measuring basal extracellular dopamine levels and changes in tonic dopamine signaling [90–94]. It may be more difficult to detect increases in dopamine levels elicited by increases in dopamine burst firing [95], and ethanol has previously been found to increase phasic dopamine release [96, 97]. Therefore, it is possible that if the effect of baclofen that is relevant to ethanol stimulation is on phasic dopamine release, this would not be detected under the experimental conditions used in the current study. On the other hand, increases in dopamine induced by ethanol have been previously documented [36, 39, 48, 98] and were found here, so if baclofen affects drug-induced changes in tonic dopamine, that effect should have been detected for ethanol.

Conversely, another possibility is that non-dopaminergic projections to the NAc and/or dopaminergic projections from the VTA to other mesocorticolimbic regions contribute to the modulatory effect of baclofen on the stimulant response to ethanol [54, 99–101]. For instance, electrolytic lesion of the NAc did not attenuate the stimulant response to ethanol in DBA/2J mice, whereas a lesion of the amygdala did [102]. Ethanol has been found to increase extracellular dopamine levels in the amygdala [103], and, using c-Fos immunoreactivity as a marker, neural activation was increased by ethanol in the amygdala of FAST mice to a greater extent than in SLOW mice, an effect which was not seen in the NAc

[104]. Therefore, baclofen-induced alterations in signaling to the amygdala (among other regions) may be another mechanism contributing to the baclofen-induced attenuation of ethanol stimulation in FAST mice.

4.3 Conclusions

Despite these caveats, the results of this experiment are unique and highlight the differences in the neurochemical regulation of mesolimbic dopamine signaling in response to ethanol and other drugs of abuse. Although baclofen was found to significantly attenuate the (tonic) dopamine response to a variety of drugs of abuse, including amphetamine, cocaine, morphine, and nicotine using similar techniques [56, 57], baclofen did not attenuate the tonic dopamine response to ethanol. Whether baclofen may alter the phasic release of dopamine in response to ethanol, or may alter ethanol-induced increases in dopamine in other brain regions, is unknown. However, unlike amphetamine, cocaine, morphine and nicotine [56, 57], these results do not support a GABA_B receptor-dependent regulation of ethanol's actions on the mesolimbic dopamine pathway. Moreover, these data may support a dopamine-independent mechanism by which GABA_B receptor activation attenuates the locomotor stimulant response to ethanol.

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Research Highlights

- !! The GABA_B receptor agonist baclofen attenuates the stimulant response to ethanol.
- !! GABA_B receptor function is linked with ethanol sensitivity only in the striatum.
- !! Baclofen does not reduce the dopamine response to ethanol in the nucleus accumbens.

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Figure 1. GABA_B receptor function in striatal tissue differs in replicate 1 FAST and SLOW mice, and is increased in the cerebellum by *in vivo* ethanol exposure

(A) In striatal tissue, GABA_B receptor function, as measured by maximum percent stimulation of [³⁵S]GTPγS binding by baclofen ($E_{\max}$) was significantly higher in FAST-1, compared to SLOW-1 mice, but there was no difference between FAST-2 and SLOW-2 mice. ** $p < 0.01$, $n = 7-8$ per line and replicate. (B) *In vivo* treatment with 2 g/kg ethanol, 15-min prior to euthanasia, resulted in a significant increase in baclofen-stimulated $E_{\max}$ in cerebellar tissue ($p = 0.01$); this effect did not differ between the FAST and SLOW lines. $n = 23-24$ per treatment condition. Data are shown as mean $\pm$ S.E.M.

Figure 2. Schematic of NAc microdialysis probe placements for Experiment 2
 Shown are the approximate locations of the active membrane portion of the microdialysis probes, relative to bregma (adapted from [70]).

Figure 3. Baclofen attenuates the locomotor stimulant response to ethanol in FAST mice, but does not significantly alter ethanol-induced increases in dopamine levels in the NAc
(A) Time course of the effect of baclofen on the locomotor stimulant response to ethanol (2 g/kg). **(B)** Both 1.25 and 2.5 mg/kg baclofen significantly attenuated the locomotor stimulant response to ethanol, shown here for the entire 1-h test period (** $p < 0.01$). **(C)** Time course of the effect of baclofen on NAc dopamine levels after saline or 2 g/kg ethanol treatment. Data are quantified as percent change relative to the previous injection (0, 1.25, or 2.5 mg/kg baclofen). **(D)** Average change in dopamine over the 1 h period after ethanol administration. Ethanol significantly increased dialysate dopamine levels [$F_{1,63}=9.1$; $p < 0.01$], but pre-treatment with baclofen (1.25 or 2.5 mg/kg) did not alter this effect. Data are presented as mean $\pm$ S.E.M, $n=10-15$ per baclofen dose and ethanol dose (data are collapsed across replicate, due to the absence of replicate effects). Sal/Bac refers to the injection (0, 1.25, or 2.5 mg/kg baclofen) immediately preceding ethanol administration.

TABLE 1

GABA_B receptor function and maximal percent stimulation of [³⁵S]GTPγS binding by baclofen in FAST and SLOW mice

Region	EC ₅₀ ^a		E _{max} ^b	
	FAST	SLOW	FAST	SLOW
Whole Brain	62.4 μM (52.5–74.1 μM)	61.4 μM (51.4–73.1 μM)	250.2 ± 6.3%	254.1 ± 8.5%
Cerebellum	67.9 μM (59.0–78.2 μM)	69.7 μM (63.1–77.1 μM)	477.0 ± 21.2%	442.7 ± 18.9%
Hippocampus	82.2 μM (70.1–96.4 μM)	80.0 μM (70.5–90.8 μM)	353.6 ± 10.9%	335.0 ± 15.7%
Prefrontal Cortex	93.3 μM (67.1–129.7 μM)	76.6 μM (65.6–89.5 μM)	391.7 ± 19.7%	398.7 ± 14.3%
Striatum	127.6 μM (77.4–209.9 μM)	109.9 μM (87.7–137.7 μM)		
		<i>Replicate 1</i>	238.3 ± 14.8% ^{**}	182.4 ± 7.0%
		<i>Replicate 2</i>	192.4 ± 10.2%	190.5 ± 8.0%
Ventral Midbrain	100.0 μM (77.4–128.8 μM)	75.9 μM (53.2–108.1 μM)	267.4 ± 14.8%	272.1 ± 16.0%

^aMean (95% confidence interval) EC₅₀ for baclofen

^bMean ± S.E.M. maximal percent stimulation of [³⁵S]GTPγS binding above basal binding (100%)

^{**}Significantly different from SLOW-1 (*p* < 0.01)

Note: n's for each region were as follows: Whole Brain = 19 / line; Cerebellum = 28–29 / line; Hippocampus = 15–16 / line; Prefrontal Cortex = 17 / line; Striatum = 15 / line, 7–8 / line and replicate; Ventral Midbrain = 25 / line.