

Pharmacological and Anatomical Evidence for an Interaction Between mGluR5- and GABA_A α 1-Containing Receptors in the Discriminative Stimulus Effects of Ethanol

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The discriminative stimulus properties of ethanol are mediated in part by positive modulation of GABA_A receptors. Recent evidence indicates that metabotropic glutamate receptor subtype 5 (mGluR5) activity can influence GABA_A receptor function. Therefore, the purpose of this work was to examine the potential involvement of mGluR5 in the discriminative stimulus effects of ethanol. In rats trained to discriminate ethanol (1 g/kg, intragastric gavage (i.g.)) from water, 2-methyl-6-(phenylethyl)-pyridine (MPEP) (1–50 mg/kg, i.p.) a selective noncompetitive antagonist of the mGluR5 receptor did not produce ethanol-like stimulus properties. However, pretreatment with MPEP (30 mg/kg) reduced the stimulus properties of ethanol as indicated by significant reductions in ethanol-appropriate responding, specifically at 0.5 and 1 g/kg ethanol, and a failure of ethanol test doses (1 and 2 g/kg) to fully substitute for the ethanol training dose. To test whether mGluR5 antagonism altered the GABA_A receptor component of the ethanol stimulus, the ability of MPEP to modulate pentobarbital and diazepam substitution for ethanol was assessed. Pentobarbital substitution (1–10 mg/kg, i.p.) for ethanol was not altered by MPEP pretreatment. However, MPEP pretreatment inhibited the ethanol-like stimulus properties of diazepam (5 mg/kg, i.p.). To examine a potential anatomical basis for these pharmacological findings, expression patterns of mGluR5- and benzodiazepine-sensitive GABA_A α 1-containing receptors were examined by dual-label fluorescent immunohistochemistry with visualization by confocal microscopy. Results indicated that mGluR5- and GABA_A α 1-containing receptors were both coexpressed in limbic brain regions and colocalized on the same cells in specific brain regions including the amygdala, hippocampus, globus pallidus, and ventral pallidum. Together, these findings suggest an interaction between mGluR5- and benzodiazepine-sensitive GABA_A receptors in mediating ethanol discrimination.

Neuropsychopharmacology (2005) 30, 747–757, advance online publication, 17 November 2004; doi:10.1038/sj.npp.1300616

Keywords: ethanol; discriminative stimulus; mGluR5; MPEP; GABA_A; benzodiazepine; diazepam; immunohistochemistry; confocal

INTRODUCTION

The amino-acid glutamate is the primary excitatory transmitter in the mammalian CNS. Fast excitatory actions of glutamate are mediated by ionotropic glutamate receptor (iGluR) *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid, and kainate receptors. Metabotropic glutamate receptors (mGluRs) mediate slower glutamate responses through G-protein coupling to various intracellular signaling cascades that can modulate, or fine-tune, iGluR function (Benquet *et al*, 2002). Eight mGluR

subtypes (mGluR1–8) have been cloned. These receptors have been classified into three groups based on amino-acid sequence similarity, agonist pharmacology, and the signal transduction pathways to which they couple. Group I mGluRs (mGluR1 and 5) upregulate Ca²⁺ cascades, whereas Group II (mGluR2 and 3) and Group III (mGluR4 and 6–8) receptors are both negatively coupled to the cAMP cascade but are distinguished by their differing agonist-antagonist profiles and sequence homologies (Gereau and Conn, 1995).

The availability of selective pharmacological agents has quickly begun to reveal basic functional roles of mGluR5. mGluR5 activation with a selective agonist (CHPG) elevates glutamate release (Pintor *et al*, 2000) and augments NMDA-induced membrane depolarization in striatal slices (Pisani *et al*, 2001). Other evidence indicates that mGluR5 contributes to striatal synaptic plasticity since the mGluR5 selective antagonist, 2-methyl-6-(phenylethyl)-pyridine (MPEP), decreased long-term depression at corticostriatal synapses (Sung *et al*, 2001). Other studies have found

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Received 11 August 2004; revised 7 October 2004; accepted 12 October 2004

Online publication: 13 October 2004 at <http://www.acnp.org/citations/NPP101304040362/default.pdf>

beneficial effects of mGluR5 antagonists in animal models of anxiety, pain, and neurodegeneration (Bordi and Ugolini, 1999; Spooen *et al*, 2001). Moreover, genetic and pharmacological data have started to reveal a role for mGluR5 in addiction. For instance, morphine (10 mg/kg) conditioned reward is inhibited by MPEP (30 mg/kg) (Popik and Wrobel, 2002) but lower doses of MPEP do not alter morphine conditioned place preference (McGeehan and Olive, 2003). Mice lacking the mGluR5 gene do not self-administer cocaine and show no cocaine-induced increase in locomotor activity (Chiamulera *et al*, 2001), indicating a significant role of mGluR5 in the behavioral effects of psychomotor stimulants.

Many of the physiological, biochemical, and behavioral effects of ethanol are known to involve iGluR function (Dodd *et al*, 2000; Mihic, 1999; Aschner *et al*, 2001; Woodward, 1999; Tabakoff and Hoffman, 1993; Littleton *et al*, 2001; Weight *et al*, 1993; Costa *et al*, 2000). In addition to its effects on iGluRs, however, ethanol also modulates general metabotropic receptor activity as evidenced by its ability to reduce basal and stimulated phosphoinositide hydrolysis (Gonzales *et al*, 1986). Low concentrations of ethanol selectively alter neuronal firing rates (Netzeband and Gruol, 1995) and Ca^{2+} levels (Gruol *et al*, 1997) mediated by mGluRs *in vitro*. Chronic exposure to ethanol reduces mGluR1 mRNA levels in cerebellar Purkinje neurons of mice (Simonyi *et al*, 1996) and early withdrawal from ethanol leads to alterations in mGluR-evoked Ca^{2+} signaling in cerebellar neurons (Netzeband *et al*, 2002). Moreover, pharmacologically relevant concentrations of ethanol inhibit glutamate-induced Ca^{2+} -dependent Cl^- currents in *Xenopus* oocytes expressing mGluR5, but have no effect on currents in oocytes expressing mGluR1 (Minami *et al*, 1998), suggesting that ethanol may selectively alter mGluR5 function. In addition, the mGluR5 antagonist MPEP reduces the reinforcing properties of ethanol (Sharko *et al*, 2002), and reduces alcohol relapse to alcohol self-administration in two animal models (Bäckström *et al*, 2004).

In addition to their reinforcing properties, drugs of abuse produce distinctive subjective (ie discriminative stimulus) effects that are an important determinant of abuse liability (Stolerman, 1992). Using differential reinforcement methods, drug discrimination experiments involve training animals (eg pigeons, mice, rats, gerbils, monkeys, humans) to emit one response (ie a lever press) after being injected with one dose of drug (ie called the training drug or training dose). Thus, reinforcement (food or sucrose) is contingent on selecting the 'correct' or 'drug-appropriate' lever. An alternative response (ie pressing a different lever) results in reinforcement after vehicle injection. Once this discriminated performance is established, other doses of the training drug, or other drugs, are administered to determine if they engender responding on the drug-appropriate lever. The extent to which a novel dose, or novel drug, produces responding on the lever associated with the training drug is called drug 'substitution' or 'generalization.' The interpretation of these results is that a drug that produces high levels of responding on the lever associated with the training drug (ie ethanol) has similar subjective properties and acts by similar neurobiological mechanism(s) (Grant and Colombo, 1993; Kostowski and Bienkowski, 1999). Novel drugs can

also be tested for pharmacological potentiation or antagonism of the discriminative stimulus effects of the training drug by conducting pretreatment experiments (Besheer *et al*, 2003).

The discriminative stimulus effects of ethanol are mediated at least by GABA_A receptors. For example, positive modulators of GABA_A receptors, such as barbiturates, benzodiazepines, and neurosteroids, produce ethanol-like stimulus effects (Ator *et al*, 1993; Barry, 1991; Evans and Balster, 1991; Grant *et al*, 1996, 1997; Jarbe and McMillan, 1983; Shelton and Grant, 2002) in multiple species. MGLuR5 activity has the potential to influence ethanol discrimination based on evidence that mGluR5 can influence GABA neurotransmission (de Novellis *et al*, 2003; Hoffpauir and Gleason, 2002; Diaz-Cabiale *et al*, 2002). For example, the mGluR5 agonist CHPG has been shown to increase GABA levels *in vivo* and antagonism of mGlu5 receptors blocks the CHPG-induced increases in GABA (de Novellis *et al*, 2003). In cultured amacrine cells, GABA-gated currents are enhanced by CHPG administration, an enhancement that is likely due to postsynaptic GABA_A receptors (Hoffpauir and Gleason, 2002). MGLu5 receptors are abundant in limbic brain regions such as the nucleus accumbens, cortex, and hippocampus (Bordi and Ugolini, 1999; Spooen *et al*, 2001; Romano *et al*, 1995), where GABA_A receptors are known to modulate ethanol discrimination. For example, site-specific infusion of GABA_A-positive modulators such as muscimol, pentobarbital, or allopregnanolone in the nucleus accumbens or amygdala substitute fully for systemic ethanol (Besheer *et al*, 2003; Hodge and Cox, 1998; Hodge *et al*, 2001). It is plausible, therefore, that mGlu5 receptors might regulate ethanol discrimination via modulation of GABA_A receptor function.

Thus, the purpose of this study was to determine the potential involvement of mGluR5 in the modulation of the discriminative stimulus effects of ethanol. In rats trained to discriminate (1 g/kg) ethanol from water, MPEP a non-competitive antagonist of the mGlu5 receptor (Gasparini *et al*, 1999) was assessed for its ability to substitute for ethanol and to modulate ethanol discrimination. Further, the ability of MPEP to modulate pentobarbital and diazepam substitution for ethanol was assessed. Finally, to examine the potential anatomical basis for an interaction between mGlu5 and GABA_A receptors, expression patterns of these receptors were examined using dual-label fluorescent immunohistochemistry with confocal visualization.

METHOD

Animals

In all, 12 male Long Evans rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 150–200 g upon arrival to the colony were individually housed in Plexiglas cages. Rats were handled and weighed daily for 2 weeks before lever press training began. Once rats reached a body weight of approximately 300 g, rats were fed 16 g of food daily for the remainder of the 12-month study (weights maintained at approximately 325 g). Water was available continuously in the home cage. The colony room was maintained on a 12-h light/dark cycle and experiments were conducted during the light portion of the cycle. Animals were under continuous

care and monitoring by veterinary staff from the Division of Laboratory Animal Medicine (DLAM) at UNC-Chapel Hill, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). DLAM staff considered all of the rats to be healthy throughout the course of the experiment, indicating that the procedures produced no adverse effects on animal health or well-being. All procedures were also carried out in accordance with the NIH Guide to Care and Use of Laboratory Animals and institutional guidelines.

Apparatus

Operant chambers (Med Associates, Georgia, VT) measuring $31 \times 32 \times 24 \text{ cm}^3$ were used for discrimination sessions. The chambers were located within sound-attenuating cubicles and equipped with an exhaust fan that provided ventilation and masked external sounds. Two response levers were located on the right wall of each chamber. Responses on the levers activated a liquid dispenser centered between the levers that presented fluid in a 0.1 ml dipper cup for 4 s during each operation. A stimulus light was located above each response lever and was activated each time a reinforcer was delivered. The chambers were illuminated by an 8-W light located on the left wall 28 cm above the floor. The chambers were interfaced (Med Associates) to a computer that was programmed to control sessions and record data.

Procedure

Lever press training. Rats were trained to lever press on a fixed ratio 1 (FR1) schedule of reinforcement in overnight sessions. That is, a single lever press on either of the two levers resulted in presentation of 0.1 ml of a liquid sucrose solution (10% (w/v)). After three overnight sessions, 1 h lever press training began. During these sessions, rats were placed in the chamber for 10 min before the house light was illuminated and either the right or the left lever was extended into the chamber and signaled the beginning of the session. The presentation of the right and left lever was alternated each day and the schedule of reinforcement was gradually increased to FR10. That is, once 10 lever presses occurred, the sucrose was presented for 4 s. Rats received equal experience with both levers at each reinforcement schedule. Discrimination training began once responding on the FR10 schedule was stable ($<10\%$ daily variation in the total number of responses).

Discrimination training. Training sessions were conducted 5 days per week (M–F) during which ethanol (1 g/kg) or water was administered by intragastric gavage (i.g.) prior to the start of the 15-min sessions. Immediately following the ethanol or water administration, the rats were placed in the chambers. Similar to lever press training sessions, after 10 min the house light was illuminated and both levers were introduced into the chamber signaling the beginning of the session. Following ethanol administration, completion of 10 responses on the ethanol-appropriate lever resulted in the presentation of the sucrose solution. Similarly, following water administration, completion of 10 responses on the water-appropriate lever resulted in sucrose delivery. During

both the ethanol and water sessions, responses on the inappropriate lever were recorded but produced no programmed consequences. The lever associated with ethanol or water administration was randomly assigned and counterbalanced across animals. Water and ethanol training days varied on a double alternation schedule (W, W, E, E, etc.). The training sessions continued until the percentage of ethanol- and water-appropriate lever press responses emitted prior to the first reinforcer, and during the entire session was $>80\%$ for 10 consecutive days. Once these criteria were met, testing began.

Testing procedures. Test sessions were identical to the training sessions, except that they were 2 min in duration, and completion of an FR10 on either lever resulted in sucrose delivery. Reinforcement was delivered during test sessions to assess the possible effects of test treatments on overall response rates. These test sessions were interspersed with training sessions only if performance during the previous five training sessions met the accuracy criteria. If the criteria were not met, training continued until response accuracy was $>80\%$ for 5 consecutive days, consequently, not all rats were used in each test condition.

Substitution Testing

Ethanol substitution. In four different test sessions, various ethanol doses (0.1, 0.5, 1, and 2 g/kg, i.g.) were administered to determine an ethanol substitution curve. Rats ($n=12$) received each ethanol dose in a random order.

MPEP substitution. To test whether MPEP would substitute for ethanol, rats ($n=6$) were administered MPEP (0, 1, 10, 30, and 50 mg/kg, i.p.) 20 min before receiving a water gavage and were then placed in the chambers. Each MPEP dose was administered once in random order.

Ethanol substitution with MPEP pretreatment. In order to evaluate the effects of MPEP pretreatment on the stimulus effects of multiple ethanol doses, rats ($n=8$) were administered MPEP (30 mg/kg, i.p.) or saline 20 min before ethanol (0.1, 0.5, 1, and 2 g/kg, i.g.). Rats were tested once on each pretreatment combination and the combinations were tested randomly.

GABA_A-positive modulator substitution with MPEP pretreatment. Saline or MPEP (30 mg/kg, i.p.) was administered 20 min before pentobarbital injection (1, 3, and 10 mg/kg, i.p.). Rats ($n=10$) were tested once on each pretreatment combination and the combinations were tested randomly. After pentobarbital testing, saline or MPEP (30 mg/kg, i.p.) was administered 20 min before diazepam injection (1, 3, and 5 mg/kg, i.p.). Rats ($n=11$) were tested once on each pretreatment combination and the combinations were tested randomly. Gavage was withheld during these tests.

Drugs

Ethanol (95% (w/v)) was diluted in distilled water to a concentration of 20% (v/v) and was administered i.g. in

various volumes to obtain doses of 0.1, 0.5, 1, and 2 g/kg. A corresponding volume of water to the 1 g/kg ethanol training dose was used. Pentobarbital (Sigma-Aldrich, St Louis, MO) was dissolved in saline, and injected i.p. at a volume of 1 ml/kg. MPEP (Sigma) was also dissolved in saline by gently heating the solution and injected i.p. at a volume of 1 ml/kg, except for the 50 mg/kg dose, which was injected at a volume of 2 ml/kg. Diazepam (Sigma) in a suspension of 1% carboxymethylcellulose was injected i.p. at a volume of 2 ml/kg.

Data Analysis

Response accuracy was expressed as the percentage of ethanol-appropriate lever presses upon delivery of the first reinforcer. Response rate (responses/min) was analyzed for the entire session and provided an index of locomotor ability. Complete substitution for the ethanol stimulus was defined as >80% choice of the ethanol lever upon completion of the first FR10 during test sessions. If an animal did not complete an FR10 during these test sessions, then that animal was not included in the response accuracy analysis, but was included in the response rate analysis. The left-most panels of each figure show the percentage of ethanol-appropriate lever responding on completion of the first FR10 and response rate during the ethanol and water session that preceded testing of that drug combination. One or two-way repeated measures analysis of variance (ANOVA) were used to analyze response accuracy and response rate data. Planned comparisons were made within each pretreatment condition (eg each diazepam dose compared to lowest diazepam dose in the saline pretreatment condition), and between the pretreatment conditions at each test drug dose (eg responses after saline and MPEP compared at each diazepam dose). ED₅₀ values for the dose effects were determined by log-dose probit analysis when possible, and paired *t*-tests were used to compare the ED₅₀'s for the ethanol, pentobarbital, and diazepam tests.

Immunohistochemistry

At the conclusion of the ethanol discrimination study, the animals (*n* = 10) were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. The brains were removed from the skull and placed in the same fixative solution for at least 24 h before being washed with PBS and sliced on a vibratome into 40 µm sections. Primary antibodies used in the study were a rabbit polyclonal antibody raised against mGluR5 (NeuroMics, Northfield, MN; dilution 1:300) and a guinea-pig polyclonal antibody raised against GABA_A α1-subunit (courtesy of Dr Jean-Marc Fritschy; dilution 1:300). The fluorescent-coupled secondary antibodies were Alexa Fluor[®] 488 goat anti-rabbit IgG (1:200 dilution) for mGluR5 and Alexa Fluor[®] 594 goat anti-guinea pig IgG (1:200 dilution) for GABA_A α1 (Molecular Probes, Eugene, OR, USA). Visualization was performed using a Zeiss 410 laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Images were saved in tagged image format and exported to Adobe Photoshop CS (Adobe Systems, Mountain View, CA) for analysis.

RESULTS

Ethanol Substitution

The left-most panels of Figure 1a and b show the percentage of ethanol-appropriate lever responding on completion of the first FR10 and response rate during the ethanol and water session that preceded testing of the ethanol dose response. Responding on the ethanol-appropriate lever during the ethanol session was approximately 96% and less than 2% on the water session. The percentage of ethanol-appropriate responses increased significantly as a function of ethanol test dose ($F(3,47) = 52.31$, $p < 0.001$), indicating that the procedures established reliable stimulus control. Both the 1 and 2 g/kg test doses of ethanol substituted fully for the 1 g/kg training dose (Figure 1a), with an ED₅₀ value of 0.56 g/kg (± 0.06 SEM). The training dose (1 g/kg) did not affect response rate (Figure 1b). However, a significant reduction in lever press responding was observed ($F(3,47) = 5.85$, $p = 0.003$), with the highest dose (2 g/kg) suppressing responding relative to the lowest dose tested.

MPEP Substitution

As shown in Figure 2a, MPEP did not substitute for the stimulus effects of 1 g/kg ethanol ($F(4,20) = 1.58$, $p = 0.22$).

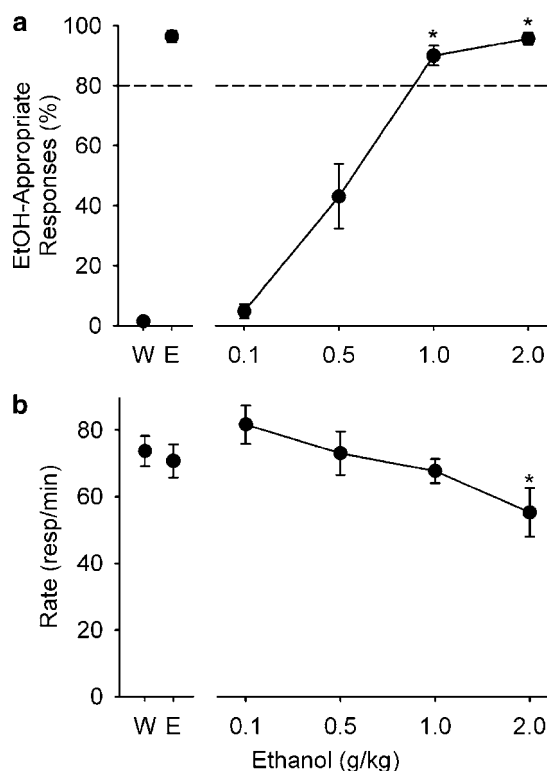


Figure 1 (a) Mean (\pm SEM) percentage of ethanol-appropriate responding upon completion of the first FR10 at each ethanol dose tested (*n* = 12). (b) Mean (\pm SEM) test session response rate at each ethanol dose tested. Data points to the left of the x-axis break represent performance on the water (W) and ethanol (E) training session prior to testing. Data points to the right of the x-axis break represent test session performance following IG ethanol administration. The horizontal dashed line (>80%) represents full substitution for the discriminative stimulus effects of ethanol (1 g/kg, ig.). *Significant difference from 0.1 g/kg ethanol (Tukey's, $p < 0.05$).

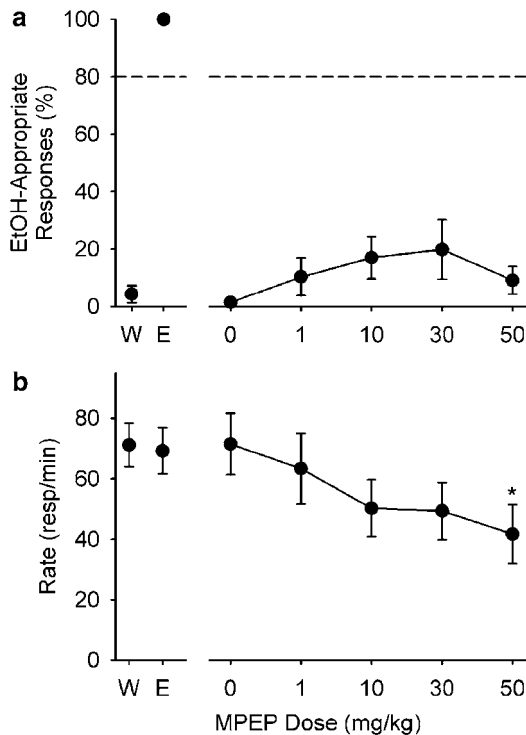


Figure 2 (a) Mean (\pm SEM) percentage of ethanol-appropriate responding upon completion of the first FR10 at each MPEP dose tested ($n=6$). (b) Mean (\pm SEM) test session response rate at each MPEP dose tested. Data points to the left of the x-axis break represent performance on the water (W) and ethanol (E) training session prior to testing. Data points to the right of the x-axis break represent test session performance following i.p. MPEP administration. The horizontal dashed line ($>80\%$) represents full substitution for the discriminative stimulus effects of ethanol (1 g/kg, i.g.). *Significant difference from saline (Tukey's, $p<0.05$).

All MPEP doses tested failed to substitute for ethanol. The highest MPEP dose tested (50 mg/kg) reduced response rate relative to saline ($F(4,20) = 2.91$, $p = 0.047$).

Ethanol Substitution with MPEP Pretreatment

Figure 3a shows the ethanol dose–response curve following saline and MPEP pretreatment. MPEP pretreatment at the 1 and 2 g/kg ethanol doses includes seven animals due to the death of one animal before testing was completed. Similar to the previous ethanol dose–response curve presented in Figure 1a, dose-dependent substitution for the ethanol training dose (1 g/kg) was observed. This dose-dependent substitution was inhibited by MPEP pretreatment. The two-way repeated measures ANOVA revealed a main effect of ethanol dose ($F(3,21) = 20.57$, $p<0.001$). After saline pretreatment, ethanol-appropriate responding was greater at every ethanol dose (0.5, 1, and 2 g/kg) relative to the 0.1 g/kg ethanol dose, p 's <0.03 . MPEP pretreatment significantly altered this pattern of responding, as only the highest ethanol dose (2 g/kg) produced significantly greater ethanol-appropriate responding than the 0.1 g/kg ethanol dose, $p = 0.002$. There was a significant main effect of pretreatment injection ($F(1,7) = 15.37$, $p = 0.005$). MPEP pretreatment significantly reduced ethanol-appropriate responding at the 0.5 and 1 g/kg ethanol doses, p 's <0.001 . The ethanol

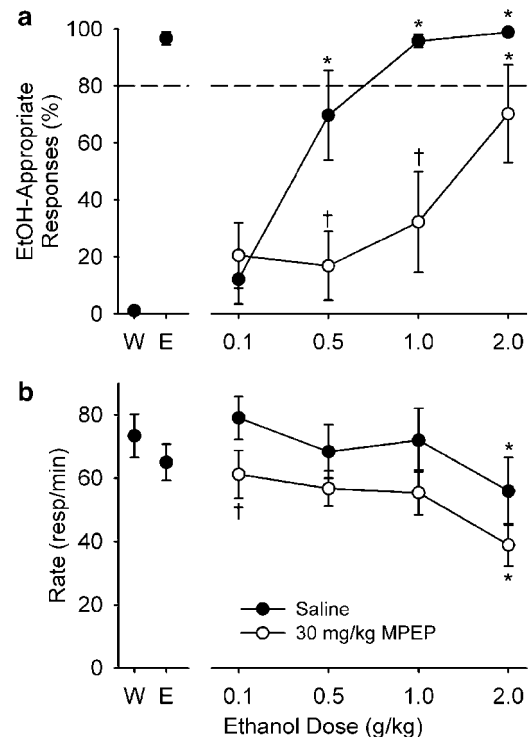


Figure 3 (a) Mean (\pm SEM) percentage of ethanol-appropriate responding upon completion of the first FR10 after saline or MPEP pretreatment at each ethanol dose ($n=8$). (b) Mean (\pm SEM) test session response rate after saline or MPEP pretreatment at each ethanol dose tested. Data points to the left of the x-axis break represent performance on the water (W) and ethanol (E) training session prior to testing. The horizontal dashed line ($>80\%$) represents full substitution for the discriminative stimulus effects of ethanol (1 g/kg, i.g.). *Significantly different from the respective 0.1 g/kg ethanol dose within pretreatment condition. †Significant difference between saline and MPEP pretreatment (Tukey's, $p<0.05$).

dose by pretreatment injection interaction was also significant, ($F(3,19) = 4.62$, $p = 0.01$). The mean $ED_{50} \pm$ SEM for ethanol substitution was 0.39 ± 0.08 g/kg following saline pretreatment and 1.33 ± 0.19 g/kg following MPEP pretreatment. This 3.4-fold rightward shift in the ethanol dose–response curve was significant, ($t(3) = 5.4$, $p = 0.01$).

As shown in Figure 3b, response rate was dose dependently reduced by ethanol ($F(3,21) = 6.38$, $p = 0.003$), with significant rate reductions observed at the highest ethanol dose (2 g/kg) relative to the lowest dose (0.1 g/kg) after saline and MPEP pretreatment, p 's <0.02 . The main effect of pretreatment injection was also significant ($F(1,7) = 7.58$, $p = 0.03$), but no significant interaction was observed. At the lowest ethanol dose tested (0.1 g/kg), response rate was significantly lower after MPEP pretreatment, $p = 0.02$. MPEP did not induce a rate reduction at any other ethanol dose. Taken together, MPEP pretreatment reduced ethanol-appropriate responding, at doses that were not affected by response rate reductions (0.5 and 1 g/kg).

Pentobarbital Substitution for Ethanol with MPEP Pretreatment

Pentobarbital dose dependently substituted for ethanol as shown in Figure 4a. Two rats did not complete a first FR

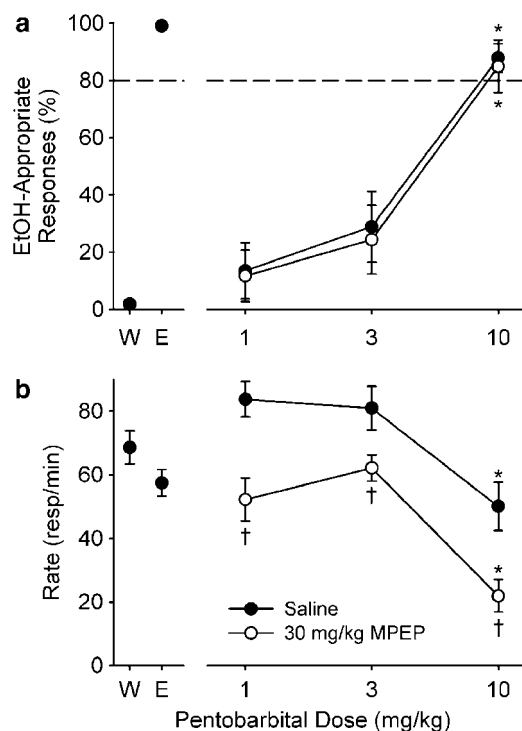


Figure 4 (a) Mean (±SEM) percentage of ethanol-appropriate responding upon completion of the first FR10 after saline or MPEP pretreatment at each pentobarbital dose tested ($n=10$). (b) Mean (±SEM) test session response rate after saline or MPEP pretreatment at each pentobarbital dose tested. Data points to the left of the x-axis break represent performance on the water (W) and ethanol (E) training session prior to testing. The horizontal dashed line (>80%) represents full substitution for the discriminative stimulus effects of ethanol (1 g/kg, i.g.). *Significantly different from the respective 1 mg/kg pentobarbital dose within pretreatment condition. †Significant difference between saline and MPEP pretreatment (Tukey's, $p<0.05$).

when MPEP was administered before the highest pentobarbital dose (10 mg/kg). Consequently, those animals are not included in the response accuracy analysis (Figure 4a), but are included in the response rate analyses (Figure 4b). The two-way repeated measures ANOVA revealed a main effect of pentobarbital dose ($F(2,18)=23.13$, $p<0.001$), with the highest pentobarbital dose (10 mg/kg) producing significantly greater responding on the ethanol-appropriate lever than the lowest dose (1 mg/kg) after saline and MPEP pretreatment, p 's <0.002 . Pentobarbital substitution was not altered by MPEP pretreatment, as confirmed by the lack of a significant main effect of pretreatment injection ($F<1$). The pentobarbital dose by pretreatment injection interaction was also not significant ($F<1$). The mean ± SEM ED_{50} for pentobarbital substitution was 5.26 ± 0.81 mg/kg following saline pretreatment and 4.89 ± 0.85 mg/kg following MPEP pretreatment.

Response rate was significantly altered by pentobarbital dose ($F(2,18)=17.05$, $p<0.001$), with a significant reduction observed at the 10 mg/kg dose after saline and MPEP pretreatment, p 's <0.001 (Figure 4b). Response rate was also reduced by MPEP pretreatment ($F(1,9)=15.46$, $p=0.003$), and at each pentobarbital dose MPEP decreased responding relative to saline pretreatment, p 's <0.03 . The pentobarbital dose by pretreatment injection interaction was not significant ($F<1$).

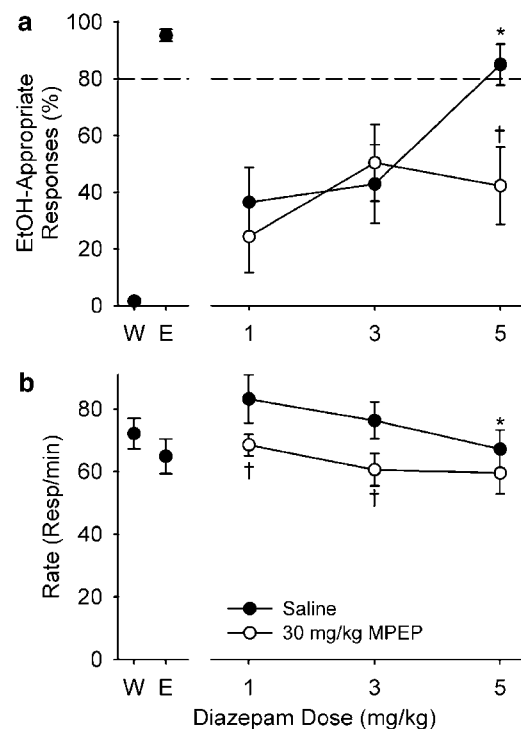


Figure 5 (a) Mean (±SEM) percentage of ethanol-appropriate responding upon completion of the first FR10 after saline or MPEP pretreatment at each diazepam dose tested ($n=11$). (b) Mean (±SEM) test session response rate after saline or MPEP pretreatment at each diazepam dose tested. Data points to the left of the x-axis break represent performance on the water (W) and ethanol (E) training session prior to testing. The horizontal dashed line (>80%) represents full substitution for the discriminative stimulus effects of ethanol (1 g/kg, i.g.). *Significantly different from the respective 1 mg/kg diazepam dose within pretreatment condition. †Significant difference between saline and MPEP pretreatment (Tukey's, $p<0.05$).

Diazepam Substitution for Ethanol with MPEP Pretreatment

Diazepam dose dependently substituted for ethanol as shown in Figure 5a. One animal died before completion of the tests, and accordingly, the 1 mg/kg diazepam dose following MPEP pretreatment includes data from 10 animals. The two-way repeated measures ANOVA revealed a significant main effect of diazepam dose ($F(2,20)=4.94$, $p=0.02$). After saline pretreatment, significantly greater responding on the ethanol-appropriate lever at the highest diazepam dose (5 mg/kg) relative to the 1 mg/kg diazepam dose was evident, $p=0.002$. This effect was prevented by MPEP pretreatment. The main effect of pretreatment injection was not significant; however, the diazepam dose by pretreatment injection interaction was significant ($F(2,19)=3.72$, $p=0.04$). MPEP pretreatment reduced ethanol-appropriate responding at the 5 mg/kg diazepam dose relative to saline pretreatment, $p=0.01$. The mean ± SEM ED_{50} for diazepam substitution was 2.23 ± 0.21 mg/kg and was 2.03 ± 0.26 mg/kg following MPEP pretreatment. Given that MPEP pretreatment reduced diazepam substitution, the ED_{50} following MPEP pretreatment was determined from the data of five animals.

Figure 5b illustrates response rate for the diazepam substitution tests. There was a significant main effect of

diazepam dose ($F(2,20) = 4.69$, $p = 0.02$), with a response reduction observed after saline pretreatment at the highest diazepam dose (5 mg/kg) relative to the lowest diazepam dose (1 mg/kg), $p = 0.006$. MPEP pretreatment also significantly reduced response rate ($F(1,10) = 11.14$, $p = 0.007$); a significant reduction was observed at the 1 and 3 mg/kg diazepam doses. The diazepam dose by pretreatment drug interaction was not significant. Thus, MPEP pretreatment significantly reduced ethanol-appropriate responses at the 5 mg/kg diazepam dose. A response rate reduction was observed at this dose after saline pretreatment; however, MPEP pretreatment did not enhance that response rate reduction.

Immunohistochemistry with Confocal Microscopy

In order to determine if there is an anatomical basis for the pharmacological interaction between BZ-sensitive GABA_A receptors and mGlu5 receptors, dual-label fluorescent immunohistochemistry with confocal microscopy was used to examine both the individual and joint expression patterns of mGluR5 and GABA_A receptors expressing the $\alpha 1$ molecular subunit, which constitutes the majority of BZ-sensitive GABA_A receptors (Mohler *et al*, 1997). Previous work has shown that GABA_A $\alpha 1$ receptors and mGlu5 receptors are both expressed in brain regions known to modulate ethanol discrimination such as the nucleus accumbens, amygdala, and hippocampus (Romano *et al*, 1995; Hodge and Cox, 1998; Pirker *et al*, 2000; Schwarzer *et al*, 2001; Besheer *et al*, 2003). In the shell and core (Figure 6a) of the nucleus accumbens, relatively few cell bodies showed GABA_A $\alpha 1$ immunoreactivity, consistent with Härtig *et al*, (1995). Several GABA_A $\alpha 1$ -labeled processes were evident with some processes visibly extending into the ventral pallidum. Consistent with the existing literature, diffuse mGluR5 staining was observed throughout the striatum including the core (Figure 6b) and shell regions of the nucleus accumbens. In the basolateral amygdala, moderate mGluR5 and GABA_A $\alpha 1$ immunoreactivity was found with labeled cell bodies and processes. In the CA1, CA2, CA3, and dentate gyrus of the hippocampus, intense mGluR5 staining of cell bodies and processes was evident (Figure 6e). GABA_A $\alpha 1$ staining was also found on the cell bodies and processes in the same regions of the hippocampal formation (Figure 6d). The pallidal regions (ventral pallidum and globus pallidus) were also examined given that mGluR5 and GABA_A $\alpha 1$ are expressed in those regions (Lu *et al*, 1999; Pirker *et al*, 2000; Schwarzer *et al*, 2001; Poisik *et al*, 2003) and that activation of mGluR5 in the nucleus accumbens has been shown to increase GABA levels in the ventral pallidum (Diaz-Cabiale *et al*, 2002), suggesting a potential interaction between the two receptor systems in this brain area. In the present work, intense staining of both GABA_A $\alpha 1$ and mGluR5 was found on cell bodies and processes in the ventral pallidum (Figure 6g and h) and the globus pallidus (Figure 6j and k).

The coexpression of mGluR5 and GABA_A $\alpha 1$ on neurons within these brain regions has not been examined previously. As illustrated in Figure 6f, i and l, mGluR5 and GABA_A $\alpha 1$ were coexpressed on cells in all of the limbic

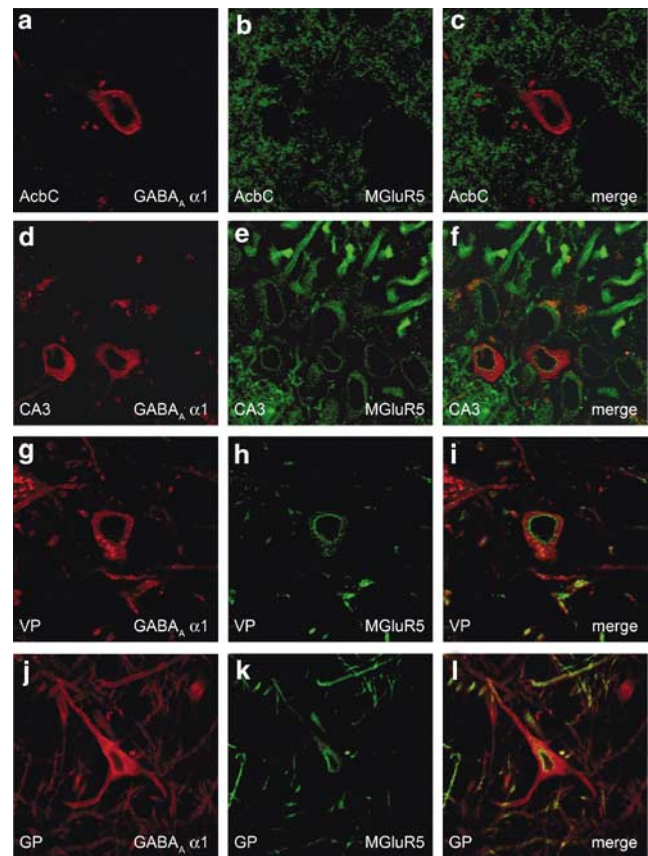


Figure 6 Confocal images from a representative rat showing the $\alpha 1$ -subunit of the GABA_A receptor and mGluR5 in the core of the nucleus accumbens (AcbC; a–c), the CA3 region of the hippocampus (CA3; d–f), the ventral pallidum (VP; g–i), and the globus pallidus (GP; j–l). GABA_A $\alpha 1$ labeling appears in red, and mGluR5 labeling appears in green. Images a–i taken at $\times 63$ magnification; images j–l taken at $\times 40$ magnification.

brain regions examined, except for the nucleus accumbens (Figure 6c). Interestingly, the coexpression pattern was relatively consistent across the other brain regions. That is, intense mGluR5 immunoreactivity appeared to be on the nuclear membrane, whereas intense GABA_A $\alpha 1$ staining was localized on cell surface membrane receptors and processes.

DISCUSSION

The discriminative stimulus effects of ethanol are mediated in part by positive modulation of GABA_A receptor activity (Ator *et al*, 1993; Grant and Lovinger, 1995; Hodge and Cox, 1998; Kostowski and Bienkowski, 1999; Hodge *et al*, 2001; Shelton and Grant, 2002). Evidence indicates that mGlu5 receptors can modulate GABA_A receptor function (Awad *et al*, 2000; Hoffpauir and Gleason, 2002) and extracellular GABA levels. Moreover, ethanol inhibits glutamate-induced Ca^{2+} -dependent Cl^{-} currents in *Xenopus* oocytes expressing mGluR5 (Minami *et al*, 1998). Together, these findings suggest that the GABA_A receptor component of ethanol's stimulus effects may be modulated by mGluR5 activity.

The purpose of this work was to examine the involvement of mGlu5 receptors in the discriminative stimulus properties of ethanol. Long Evans rats were trained by differential

reinforcement to discriminate ethanol (1 g/kg, i.g.) from water vehicle using standard two-lever operant drug discrimination procedures (Hodge and Cox, 1998; Hodge *et al*, 2001; Besheer *et al*, 2003). The discrimination training procedures established reliable stimulus control as ethanol dose dependently substituted for the training dose of ethanol (1 g/kg). Administration of the selective mGluR5 antagonist MPEP (0–50 mg/kg) did not produce ethanol-like stimulus effects when administered alone, as indicated by low ethanol-appropriate responding. The highest dose of MPEP (50 mg/kg) significantly decreased response rate, which indicates that the compound was behaviorally active in the dose range tested and supports previous studies that found locomotor suppression by high doses of MPEP (Spooren *et al*, 2000). These data demonstrate that antagonism of mGluR5 function alone was not sufficient to produce discriminative stimulus properties that correspond to ethanol (1 g/kg).

A primary finding of the present study is that mGluR5 antagonism inhibited the discriminative stimulus properties of ethanol. Under control conditions, test doses of ethanol (1 and 2 g/kg) substituted fully (ie >80% ethanol-lever selection) for the discriminative stimulus properties of the ethanol (1 g/kg) training dose. Following MPEP (30 mg/kg) pretreatment, these test doses failed to fully substitute for the training dose. In addition, administration of MPEP prior to test doses of ethanol shifted the ethanol substitution curve more than three-fold to the right. This MPEP-induced shift in the ED₅₀ for ethanol substitution was attributable to significant reductions in ethanol-appropriate responding following ethanol (0.5 and 1 g/kg) test doses. Reductions in response rate were seen only after combination of MPEP with the highest dose of ethanol (2 g/kg) tested, indicating that the reduction in the discriminative stimulus properties of ethanol was not associated with locomotor impairment. These results suggest that the discriminative stimulus effects of ethanol may be mediated in part by mGluR5 function.

In order to determine if the mGluR5 antagonist reduced ethanol discrimination by altering the GABA_A receptor component of the ethanol stimulus, the effect of MPEP pretreatment on pentobarbital and diazepam substitution for ethanol (1 g/kg) was examined. Consistent with previous reports, pentobarbital (Ator *et al*, 1993; Grant *et al*, 1996; Hodge *et al*, 2001; Shelton and Grant, 2002) and diazepam (Jarbe and McMillan, 1983; Ator *et al*, 1993; Kostowski and Bienkowski, 1999; Green-Jordan and Grant, 2000; Shelton and Grant, 2002) dose dependently substituted for the stimulus effects of ethanol. MPEP pretreatment did not alter pentobarbital (1–10 mg/kg) substitution for ethanol. However, MPEP enhanced the response rate-reducing effects of pentobarbital, indicating that the mGluR5 antagonist was behaviorally active and able to modulate the motor effects of pentobarbital. Although a higher MPEP dose would most likely further enhance the locomotor depressant effects of pentobarbital, rendering effects on ethanol-appropriate responding difficult to interpret, it remains possible that a lower MPEP dose may have altered pentobarbital substitution. Conversely, the ethanol-like stimulus properties of diazepam (5 mg/kg) were significantly inhibited by 30 mg/kg MPEP pretreatment. The ED₅₀ for ethanol substitution by diazepam was not altered by MPEP administration. This

was due to the selective inhibition of ethanol-appropriate responding following only the highest dose of diazepam (5 mg/kg) tested. MPEP did not alter response rate when coadministered with diazepam, indicating that inhibition of the ethanol-like stimulus effects of diazepam was not associated with motor impairment. Both pentobarbital and diazepam are positive modulators of GABA_A receptors, and consequently share a similar mechanism of action. That is, both compounds potentiate the action of GABA at the GABA_A receptor (Study and Barker, 1981; Steinbach and Akk, 2001; Skerrett and Macdonald, 1984). However, pentobarbital can also directly gate the GABA_A receptor in the absence of GABA (Serafini *et al*, 2000; Akk and Steinbach, 2000; Bormann, 1988). Another difference between the two compounds is that benzodiazepines, like diazepam, have specific binding sites on GABA_A receptors (see later), while a specific binding site(s) for the barbiturates, like pentobarbital, has not yet been determined (Serafini *et al*, 2000). Further, barbiturates have pharmacological actions on other ligand-gated ion channel receptors such as neuronal nicotinic acetylcholine receptors and inhibitory glycine receptors (for a review see Krasowski and Harrison, 1999). Thus, the lack of an effect of MPEP pretreatment (30 mg/kg) on pentobarbital substitution for ethanol could be due to nonspecific effects of pentobarbital. These findings suggest that the mGluR5 antagonist MPEP inhibited the discriminative stimulus effects of ethanol specifically by reducing, or otherwise interfering with, the component of ethanol discrimination that is mediated by benzodiazepine-sensitive GABA_A receptors.

Evidence of an interaction between GABA_A and mGlu5 receptors has been reported (Hoffpauir and Gleason, 2002). In that study, the activity of GABA_A receptors in retinal amacrine cells was enhanced by mGluR5 activation, as evidenced by an increase in GABA-gated currents after administration of CHPG, an mGluR5 agonist. The mGlu5 receptor has also been shown to influence GABA levels. For example, using microdialysis techniques, de Novellis *et al*, (2003) found that activation of mGluR5 by perfusion of CHPG through the dialysis probe significantly enhanced extracellular levels of GABA in the periaqueductal gray of rats. Similarly, intra-accumbens administration of CHPG increased GABA levels in the ventral pallidum (Diaz-Cabiale *et al*, 2002). In both studies, MPEP pretreatment blocked the agonist-induced increases in GABA levels. Clearly, mGlu5 receptors can influence GABA functioning, a major mediating factor in the discriminative stimulus effects of ethanol. The MPEP-induced reduction in the stimulus properties of ethanol, and the ethanol-like properties of diazepam, may have been a result of altered GABAergic functioning by MPEP pretreatment. Given that pentobarbital substitution for ethanol was not affected, BZ-sensitive GABA_A receptors may have been differentially affected by MPEP-induced alterations. To our knowledge, interaction between BZ-sensitive GABA_A and mGlu5 receptors has not been examined previously.

A majority of BZ-sensitive GABA_A receptors with high affinity for diazepam include the $\alpha 1\beta 2\gamma 2$ -subunit combination. The $\alpha 2\beta 3\gamma 2$ - and $\alpha 3\beta 3\gamma 2$ -subunit combinations also express high affinity for diazepam but are less abundant throughout the brain (McKernan and Whiting, 1996). The

BZ-binding site is generally thought to exist on the α -subunit (Fuchs *et al*, 1988; Stephenson *et al*, 1990). The GABA_A α 1-subunit is distributed in brain regions known to modulate ethanol discrimination (eg nucleus accumbens, amygdala, hippocampus; Pirker *et al*, 2000), as is the mGlu5 receptor (Romano *et al*, 1995), and these distribution patterns were also found in the immunohistochemical studies of the present work. Further, we found that mGluR5 and GABA_A α 1 were coexpressed on cell bodies in all of the limbic brain regions examined, with the exception of the nucleus accumbens. The lack of coexpression in the nucleus accumbens may be due to the relatively few GABA_A α 1 immunoreactive cell bodies, and the diffuse nature of the mGluR5 staining in that brain region. Regardless, the consistent pattern of coexpression on cells suggests that mGluR5- and GABA_A α 1-containing receptors might interact at the brain regional level.

We also observed coexpression of mGlu5 and GABA_A α 1 receptors on the same cells in brain regions that modulate the discriminative stimulus effects of ethanol such as the amygdala and hippocampus (Hodge and Cox, 1998; Hodge *et al*, 2001; Besheer *et al*, 2003). Representative cells from the hippocampus (CA3) are shown in Figure 6 (panel f). In those cells showing coexpression, intense mGluR5 immunoreactivity appeared to be on the nuclear membrane, whereas GABA_A α 1 was localized on the cell surface, or plasma membrane. This pattern of mGluR5 nuclear membrane distribution is consistent with O'Malley *et al* (2003). In that study, using confocal microscopy mGluR5 was colocalized with lamin B2, a nuclear membrane marker. Accordingly, mGlu5 receptors expressed on nuclear membranes can produce sustained oscillatory Ca²⁺ responses (O'Malley *et al*, 2003), which indicates that these receptors can modulate intracellular signaling. This suggests that mGlu5 receptors might modulate GABA_A receptor function on specific cells via alterations in Ca²⁺-linked second messenger systems such as PKC (eg Hodge *et al*, 1999), which is a possible basis for the pharmacological interaction between mGlu5 and GABA_A receptors seen in the present work.

In addition to finding mGluR5 and GABA_A α 1 coexpression in brain regions known to modulate ethanol discrimination, the same cellular coexpression pattern was also observed in the ventral pallidum and the globus pallidus. Interestingly, GABA_A α 1 receptors in the ventral pallidum are known to mediate ethanol self-administration (June *et al*, 2003), but the role of pallidal regions in ethanol discrimination has not been examined. In the present study, both the ventral pallidum and globus pallidus showed intense immunoreactivity for the GABA_A α 1-subunit, which is consistent with previous findings that showed prominent expression of the α 1-subunit along with β 2- and γ 2-subunits (Schwarzer *et al*, 2001), and suggests that these brain regions mediate pharmacological sensitivity to diazepam. Thus, the mGluR5 antagonist tested in the present study may have inhibited both ethanol discrimination, and diazepam substitution for ethanol, by modulating GABA_A receptor function within pallidal regions.

Other evidence suggests that mGlu5 and GABA_A receptors may interactively modulate neural circuits among brain regions. For example, a majority of projection neurons from the nucleus accumbens to the ventral pallidum express

mGluR5 (Lu *et al*, 1999), suggesting that GABAergic projections from the accumbens can be modulated by mGluR5 activity. Indeed, infusion of the mGluR5 agonist CHPG into the nucleus accumbens increased GABA levels in the ventral pallidum (Diaz-Cabiale *et al*, 2002). The nucleus accumbens plays a major role in mediating the discriminative stimulus effects of ethanol. GABA_A-positive modulators such as muscimol and allopregnanolone, microinjected into the nucleus accumbens produce ethanol-like stimulus properties (Hodge and Cox, 1998; Hodge *et al*, 2001; Besheer *et al*, 2003). Therefore, there exists the possibility that the discriminative stimulus properties of ethanol are mediated in part by GABAergic projection neurons from the nucleus accumbens to the pallidal regions. Moreover, antagonism of mGluR5 in this pathway may have inhibited ethanol discrimination and diazepam substitution for ethanol by altering GABA neurotransmission in the ventral pallidum. Clearly, the potential role of nucleus accumbens projections and the cellular interactions between mGluR5 and GABA_A α 1 in modulating the stimulus effects of ethanol will need to be further examined. However, given the behavioral and immunohistochemical findings of the present work and the existing literature, the foundation exists to predict an interaction between mGluR5 and GABA_A α 1 receptors within limbic circuits.

In conclusion, drugs of abuse produce distinctive subjective effects in humans that are often described as 'high' or 'euphoria'. These subjective effects play a major role in the onset and maintenance of drug-taking behaviors (Stolerman, 1992). Thus, examination of the discriminative stimulus effects of drugs is essential for the effective development of therapeutics designed to aide in drug abuse-related disorders. Indeed, MPEP pretreatment has been found to reduce the acute reinforcing effects of ethanol (Sharko *et al*, 2002) and relapse to ethanol-seeking behavior (Bäckström *et al*, 2004), suggesting that targeting the mGlu5 receptor system may be a potential therapeutic strategy for the treatment of alcohol-related disorders. The findings from the present work suggest a specific behavioral mechanism by which MPEP may reduce ethanol reinforcement and relapse. That is, MPEP appears to reduce the subjective (ie discriminative stimulus) properties of ethanol, which may contribute to reductions in chronic drinking or excessive consumption during relapse. Moreover, the present data indicate that MPEP does not substitute for the stimulus effects of ethanol, indicating minimal coabuse liability. Further examination of potential interactions between ethanol's physiological and behavioral effects and the mGlu5 receptor system may indeed reveal the mGluR5 system to be a novel and effective therapeutic target in the treatment of alcoholism.

ACKNOWLEDGEMENTS

This work was supported by Grants AA09981 and AA011605 from the National Institute on Alcohol Abuse and Alcoholism to CWH. We would like to thank Dr Jean-Marc Fritschy for the gift of the GABA_A α 1 antibody, Drs John J Lemasters and Jae Sung Kim from the Bowles Center for Alcohol Studies Imaging core, and Mary Beth Wilkie for technical assistance.

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