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Allopregnanolone and Pentobarbital Infused Into the Nucleus Accumbens Substitute for the Discriminative Stimulus Effects of Ethanol

Clyde W. Hodge, Michelle A. Nannini, M. Foster Olive, Stephen P. Kelley, and Kristin K. Mehmert

Background: The discriminative stimulus effects of ethanol are mediated in part by the γ-aminobutyric acid type A ($GABA_A$) receptor system. We have previously shown that microinjections of the competitive $GABA_A$ agonist muscimol in the nucleus accumbens and amygdala fully substitute for the discriminative stimulus effects of systemic ethanol. However, it is not known whether allosteric binding sites on $GABA_A$ receptors located within specific limbic brain regions contribute to the discriminative stimulus effects of ethanol.

Methods: Male Long-Evans rats were trained to discriminate between intraperitoneal injections of ethanol (1 g/kg) and saline under a fixed-ratio 10 schedule of sucrose (10% w/v) reinforcement. Injector guide cannulae, aimed at both the nucleus accumbens core and the hippocampus area CA1, were then implanted to allow site-specific infusion of $GABA_A$-positive modulators.

Results: Infusion of the neurosteroid 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, or 3α-5α-P) in the nucleus accumbens resulted in dose-dependent full substitution for intraperitoneal ethanol (50% effective dose = 0.38 ng/μl per side). Likewise, injection of the barbiturate pentobarbital into the nucleus accumbens also substituted dose-dependently for ethanol (50% effective dose = 1.55 μg/μl per side). However, infusions of either 3α-5α-P or pentobarbital in the hippocampus failed to substitute for ethanol and produced inverted *U*-shaped dose-response curves.

Conclusions: These results demonstrate that allosteric positive modulation of $GABA_A$ receptors in the nucleus accumbens produces full substitution for the stimulus effects of ethanol. This suggests that $GABA_A$ receptors in the nucleus accumbens may play a more influential role in the discriminative stimulus effects of ethanol than those in the hippocampus.

Key Words: Neurosteroid, Allopregnanolone, Pentobarbital, Discriminative Stimulus, Ethanol.

ETHANOL'S EFFECTS ON brain and behavioral processes are mediated, in part, by changes in ionotropic γ-aminobutyric acid type A ($GABA_A$) receptor function (see Grobin et al., 1998). Acute ethanol enhances neuronal Cl^- influx (Mehta and Ticku, 1988; Suzdak et al., 1986) and potentiates GABA-induced (Ticku, 1990) and muscimol-induced (Suzdak et al., 1986) Cl^- influx in various brain regions. These positive actions on $GABA_A$ receptors also seem to influence ethanol's anxiolytic (Liljequist and Engel, 1984), motor (Frye and Breese, 1982), and reinforcing (Hodge et al., 1995) effects.

From the Department of Psychiatry and Bowles Center for Alcohol Studies (CWH), University of North Carolina at Chapel Hill, North Carolina; the Ernest Gallo Clinic and Research Center (MAN, MFO, KKM), University of California at San Francisco; and the Department of Pharmacology and Neuroscience (SPK), Ninewells Hospital, University of Dundee, Scotland, United Kingdom.

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Reprint requests: Clyde W. Hodge, PhD, Department of Psychiatry and Bowles Center, University of North Carolina at Chapel Hill, Thurston-Bowles Bldg., CB 7178, Chapel Hill, NC 27599-7178; Fax: 919-966-5679; E-mail: chodge@med.unc.edu.

$GABA_A$ receptor–mediated Cl^- conductance is positively modulated at a GABA recognition site, but also at allosteric sites that bind benzodiazepines, barbiturates, and neuroactive steroids (Peters et al., 1988; Study and Barker, 1981). Accordingly, positive modulators of $GABA_A$ receptors substitute for ethanol in drug discrimination studies. Systemically administered barbiturates substitute for ethanol (Barry, 1991; Barry and Krimmer, 1978; Kline and Young, 1986; Overton, 1977; York and Bush, 1982), and ethanol potentiates stimulus control by pentobarbital (Kline and Young, 1986). Benzodiazepines also substitute for ethanol (Hiltunen and Jarbe, 1986; Kubena and Barry, 1969) and potentiate ethanol discrimination (Jarbe and McMillan, 1983). More recent evidence indicates that various endogenous neuroactive steroids with positive $GABA_A$ receptor–modulating properties, including 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, or 3α-5α-P), also substitute for ethanol (Bienkowski and Kostowski, 1997; Bowen et al., 1999a; Grant et al., 1996, 1997) and potentiate ethanol discrimination (Bowen et al., 1999b). However, because $GABA_A$ receptor systems are distributed throughout the mammalian brain (Pirker et al., 2000; Rabow et al., 1995; Sperk et al., 1997; Wisden et al., 1992),

systemic administration studies cannot determine the extent to which ethanol's discriminative stimulus effects are mediated by receptor activity in specific brain regions.

We recently demonstrated that microinfusions of the direct $GABA_A$ receptor agonist muscimol into the core region of the nucleus accumbens substitute for and potentiate the discriminative stimulus effects of systemically administered ethanol (Hodge and Aiken, 1996; Hodge and Cox, 1998). It is not known, however, whether positive allosteric modulation of $GABA_A$ receptors in the nucleus accumbens contributes to the discriminative stimulus effects of ethanol. Therefore, the purpose of this study was to investigate whether microinjections of positive allosteric $GABA_A$ receptor modulators into the nucleus accumbens or other brain regions would substitute for the stimulus effects of ethanol in drug discrimination procedures.

MATERIALS AND METHODS

Animals

Male Long-Evans rats ($n = 12$; Charles River Laboratories, Wilmington, MA) were individually housed in Plexiglas (Rohm and Haas Co., Philadelphia, PA) cages with water available ad libitum. Body weights were maintained at approximately 310 ± 15 g via food restriction. The colony room was regulated on a 12-hr light/dark cycle with lights on at 6:00 AM. Experiments were conducted during the light portion of the cycle. All rats were weighed and inspected each day for general health. Rats were experimentally and drug naïve at beginning of the study. All animal procedures were conducted according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animals Resources, 1996).

Apparatus

Discrimination sessions were conducted in 31 × 32 × 24 cm operant chambers located within sound-attenuating cubicles with exhaust fans that helped to mask external noise (Med Associates, Georgia, VT). Responses on one of two levers located on the right wall activated a liquid dispenser centered between the two levers that presented fluid in a 0.1-ml dipper for 4 sec during each operation. The operant chambers were interfaced (Med Associates) with a computer (Gateway, San Diego, CA) that was programmed to control sessions and record data. Chambers were illuminated with an 8-W light located on the left wall 28 cm above the dipper.

Drug Discrimination Procedures: Training

Rats were allowed 1 week to adapt to individual housing conditions and daily handling. During this time, food and water were always available. Once target body weights were reached, food was restricted to approximately 16 g/day. Rats were trained to press a single lever on a fixed-ratio 1 (FR 1) schedule of reinforcement that resulted in presentation of 0.1 ml of a liquid sucrose solution (10% w/v). After 3 days, they were then trained to press either the left or the right lever during daily 30-min sessions. The active lever was alternated on a daily basis. Responses on the inactive lever were recorded but produced no programmed consequences. The schedule of reinforcement was gradually increased to FR 10, with only one lever active on any particular session. All animals received an equal history with each lever at each FR value. Once responding was stable (<10% daily variation in the total number of responses), discrimination training was initiated.

Training sessions were conducted 5 days per week (Monday through Friday), during which ethanol 1.0 g/kg or saline was administered intraperitoneally (ip) 10 min before the start of 15-min sessions. The animals were placed in the operant chambers, and illumination of the house light signaled the beginning of the session. The lever associated with ethanol or saline administration was assigned randomly and counterbalanced between animals. After ethanol or saline injections, completion of 10 responses on the appropriate lever produced the sucrose solution. Responses on the inappropriate lever were recorded but produced no programmed consequences. There were an equal number of ethanol and saline training days that varied on a double-alternation schedule (ethanol, ethanol, saline, saline, and so on). Training sessions were conducted until the percentage of ethanol- and saline-appropriate lever press responses emitted before the first reinforcer, and during the entire session, exceeded 80% for 10 consecutive days. These criteria allowed no more than two errors before completion of the first FR 10. Once the accuracy criteria were met, injector guide cannulae were surgically implanted to terminate 1 mm dorsal to the nucleus accumbens core and hippocampal area CA1.

Surgery

Rats were anesthetized with pentobarbital (60 mg/kg ip) and placed in a stereotaxic device (David Kopf Instruments, Tujunga, CA) with the incisor bar 3.3 mm below the horizontal plane. Bilateral guide cannulae (26-guage stainless-steel tubing) were implanted to terminate 1 mm dorsal to the nucleus accumbens core and hippocampal area CA1. Cannulae were secured with stainless-steel screws and dental cement. Stylets were placed in the cannulae to prevent obstructions and infections. Stereotaxic coordinates according to the atlas of Paxinos and Watson (1997) for brain sites were nucleus accumbens, +1.5 mm Anterior/Posterior, +3.0 mm Medial/Lateral, and −6.1 mm Dorsal/Ventral at 10° deviations from vertical; and CA1, −4.4 mm Anterior/Posterior, +3.3 mm Medial/Lateral, and −2.2 mm Dorsal/Ventral at 20° deviations from vertical. Behavioral sessions resumed 1 week after surgery to allow for recovery.

Intraperitoneal Ethanol Substitution Testing

Training sessions were resumed after recovery from surgery and continued until performance after ip injections of ethanol and saline again met the accuracy criteria. Once this was accomplished, test sessions were conducted during which an ethanol (0.1–1.5 g/kg ip) substitution curve was determined. Test sessions were identical to training sessions except for the following: they were 2 min in duration, completion of an FR 10 on either lever produced the sucrose solution, and novel doses of ethanol were administered. Test sessions were interspersed randomly with training sessions only if performance during the previous 10 training sessions met the accuracy criteria. If performance during continued training sessions failed to meet the accuracy criteria, testing was delayed until response accuracy was greater than 80% for 10 consecutive days. A minimum of two training sessions were conducted between test sessions. After determination of the ip ethanol substitution curve, microinjection test sessions began.

Site-Specific Microinjection Procedure

Drugs were administered through stainless-steel injectors (33-gauge tubing linked to 26-gauge tubing, Plastics One, Roanoke, VA) that were coupled via PE-20 plastic tubing to two 1.0-μl Hamilton syringes. Syringes were mounted on a Harvard Apparatus (Holliston, MA) microinfusion pump that delivered 0.5 μl per side per minute. Microinjection test sessions were interspersed randomly with training sessions if performance during the previous 10 training sessions met the accuracy criteria. Unanesthetized rats were placed in a plastic tub (27 × 17 × 12 cm) to minimize movement. Stylets were removed, and the cannulae were swabbed with sterile physiologic saline. Bilateral drug injections were performed through 33-gauge stainless-steel hypodermic tubing lowered to 1 mm below the end of the guide cannulae. The pump was operated for 1 min at a flow rate of 0.5 μl per side per minute for a total volume of 0.5 μl per side. Injectors remained in place for 30 additional seconds to allow drug diffusion; ip saline or ethanol was administered immediately, and the rats were placed in the operant chambers. The beginning of test sessions was signaled by illumination of the house light at 10 min after microinjections.

Sham injections were performed in combination with the training dose of ethanol (1.0 g/kg) and saline as a procedural control for possible handling effects. Sham control injections were identical to actual microinjections except that injectors were the same lengths as the guide cannulae to prevent brain penetration and, although the pumps were operated, the syringes were not activated.

Drugs and Dosing

Drug solutions were prepared immediately before injection. For systemic administration, ethanol (95% w/v) was diluted in saline (0.9%) to a concentration of 20% v/v and was administered ip in varied volumes to obtain doses of 0.1, 0.5, 1.0, and 1.5 g/kg. Corresponding volumes of saline (0.9%) were also administered. For central administration, 3α-5α-P was dissolved in (2-hydroxypropyl)-β-cyclodextrin (45% w/v) in sterile deionized water. Pentobarbital was dissolved in sterile deionized water. All drugs and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

3α-5α-P and pentobarbital were tested for ethanol substitution after administration in the hippocampus (CA1) or nucleus accumbens core. Both drugs were tested first in the hippocampus and then in the nucleus accumbens. Drug (dose) order was as follows: hippocampus [3α-5α-P (1.0, 0.33, 2.0, and 0.55 ng/μl) and pentobarbital (0.5, 5.0, and 20.0 μg/μl)] and nucleus accumbens [3α-5α-P (0.33, 0.1, and 0.55 ng/μl) and pentobarbital (0.5 and 5.0 μg/μl)]. The effects of each dose were determined once in each animal.

Histology

Once the experiment was completed, the rats were deeply anesthetized with pentobarbital and then perfused transcardially with sodium phosphate buffer solution (pH 7.5) followed by 10% formalin. Brains were removed and stored in a solution of 10% formalin/30% sucrose for at least 7 days. The brains were then sliced into 30-μm sections and stained with cresyl violet. Cannulae placement was verified with a standard light microscope (Bausch and Lomb, Rochester, NY). The data were compiled from only the bilateral injections determined to be within the target brain regions.

Data Analysis

Accuracy of responses was expressed as a percentage of total ethanol-appropriate lever presses during the entire session. Response rate (responses per minute) was analyzed for the entire session as a measure of possible nonspecific effects on behavior. Group averages of ethanol and saline training 5 days immediately before the onset of testing represented control performance for effects of ip ethanol. Sham injection performance was used as the control for microinjection data. Complete substitution for the ethanol stimulus was defined as >80% choice of the ethanol lever during the entire session, whereas partial substitution was defined as between 40% and 80% ethanol-lever responding. Response rates were analyzed for statistical differences with repeated-measures ANOVA with Tukey's post hoc comparison. The 50% effective dose (ED_{50}) values were determined by log-dose probit analysis. Data were used only from animals determined histologically to have bilateral injectors in target brain regions and in which performance during training sessions continued to meet the accuracy criteria.

RESULTS

Histology

Histological examination of coronal brain sections showed that the injectors were bilaterally located in the targeted brain areas of nine rats. The range of injector locations in each brain region studied is shown in Fig. 1. Injectors in the nucleus accumbens were located in the nucleus accumbens core or at the core/shell border. Hippocampus injections were in the medial dorsal region near CA1. Data are presented only from animals that received bilateral microinjections in the specified brain areas.

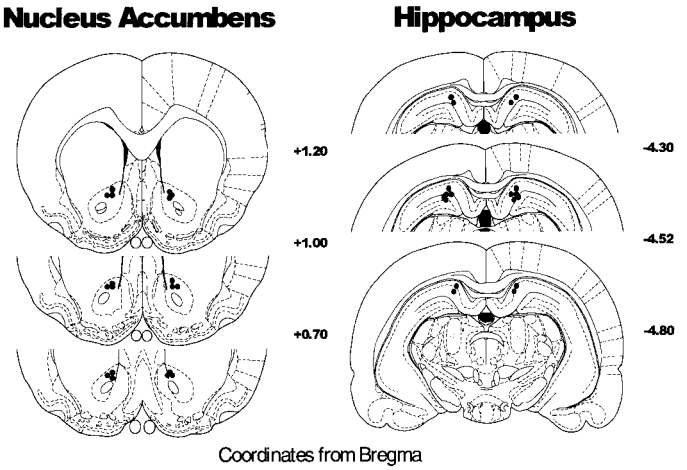

Fig. 1. Representation of rat coronal brain sections showing histological localization of injectors. The black dots in each section indicate each individual injector placement. Data were used only from animals that received bilateral injections in the target brain regions. Figures are adapted from the atlas of Paxinos and Watson (1997). Numbers beside each section represent the plane of the section in millimeters from the bregma. Reprinted with permission.

Acquisition and ip Ethanol Substitution

During the initial training days, the percentage of responses on the ethanol lever occurred at approximately chance levels on the two-lever task (i.e., 40–50%). After 52 training days, the behavior of all rats reached the accuracy criteria of greater than 80% responding on the ethanol lever after ethanol injection and less than 20% responding on the ethanol lever after saline injection for 10 consecutive days.

Performance of all rats during control conditions and postsurgery ethanol substitution test sessions is shown in Fig. 2. The percentage of ethanol-lever responding (Fig. 2A) was approximately 95% during ethanol control sessions and <5% during saline control sessions; this indicates that the procedures established reliable stimulus control. The behavior of all individual animals demonstrated dose-dependent substitution of ethanol. Whereas the 0.1 g/kg dose of ethanol had no effect and the 0.5 g/kg dose produced partial substitution, both the 1.0 and 1.5 g/kg doses of ethanol substituted fully for the training dose and were significantly greater than saline [1.0 g/kg, $F(1,17) = 349.68$, $p < 0.001$; 1.5 g/kg, $F(1,17) = 4032.80$, $p < 0.001$]. The ED_{50} for ethanol substitution was 0.69 g/kg ($\pm$0.07 g/kg). Responses rates during substitution test sessions at 0.1 ($q = 4.24$, $p < 0.05$), 0.5 [$F(1,17) = 7.31$, $p < 0.05$], and 1.0 ($q = 3.30$, $p < 0.05$) g/kg doses of ethanol were significantly higher than during training sessions (Fig. 2B). Response rates at 1.5 g/kg ethanol during substitution tests were not different from saline (Fig. 2B).

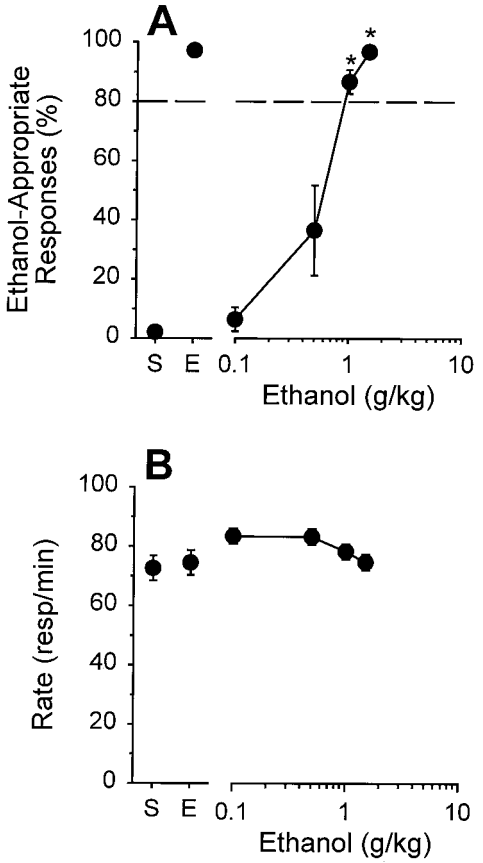

Fig. 2. Mean ± SEM percentage of ethanol-appropriate responses (A) and mean ± SEM total session response rate (B) plotted as a function of ethanol dosage. Data points to the left of the *x* axis break represent performance during the last saline (S) or ethanol (E; EtOH) training session before the start of the test sessions. Data points to the right of the *x* axis break represent test session performance after ip ethanol administration. Training and test sessions began 10 min after ip ethanol administration. The horizontal dashed line (>80%) represents full substitution for the discriminative stimulus effects of ethanol (1.0 g/kg ip). All points represent the mean performance of nine animals. $^{*}p < 0.001$ versus saline.

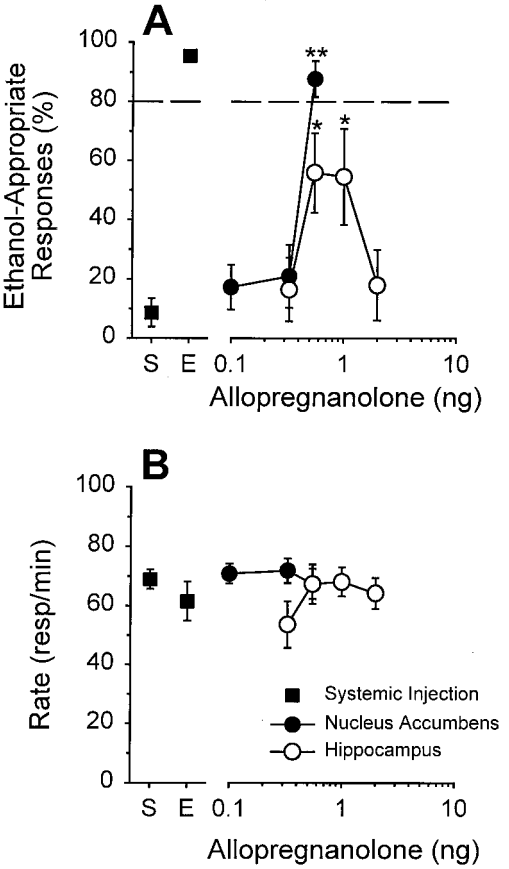

Fig. 3. Mean ± SEM percentage of ethanol-appropriate responses (A) and mean ± SEM total session response rate (B) plotted as a function of 3α-5α-P dosage. Rats received 3α-5α-P injections in the nucleus accumbens (●, $n = 9$) or dorsal CA1 region of the hippocampus (○, $n = 9$). Data points to the left of the *x* axis break represent control saline (S) or ethanol (E) ip performance combined with sham microinjections. The horizontal dashed line (>80%) represents full substitution for the discriminative stimulus effects of ethanol (1.0 g/kg ip). Drugs and doses were administered in a randomized order. $^{*}p < 0.05$ versus saline. $^{**}p < 0.001$ versus saline.

3α-5α-P Infusion Into the Hippocampus or Nucleus Accumbens

As seen in Fig. 3A, the neurosteroid 3α-5α-P dose-dependently substituted for 1.0 g/kg ip ethanol when infused into the nucleus accumbens. Doses of 0.1 and 0.33 ng/μl had no effect, whereas a 0.55 ng/μl dose fully substituted for ethanol [$F(1,15) = 42.84, p < 0.001$ versus saline]. The ED_{50} value for ethanol substitution when 3α-5α-P was infused into the nucleus accumbens was 0.38 ± 0.11 ng/μl. The response rate was not significantly altered by 3α-5α-P infusion into the nucleus accumbens.

Full substitution for ethanol was not observed when 3α-5α-P was infused into the CA1 region of the hippocampus. Instead, an inverted *U*-shaped curve resulted, with the low (0.33 ng/μl) and high (2.0 ng/μl) doses being without effect and midrange doses of 0.55 and 1.0 ng/μl partially substituting for ethanol [$F(1,17) = 14.94, p < 0.05$ and $F(1,17) = 9.35, p < 0.05$ versus saline, respectively]. Response rates during 3α-5α-P infusion into the CA1 at all doses were not significantly different from saline controls.

Pentobarbital Infusion Into the Hippocampus or Nucleus Accumbens

As seen in Fig. 4A, pentobarbital dose-dependently substituted for 1.0 g/kg ip ethanol when infused into the nucleus accumbens. The 0.5 μg/μl dose had no effect, whereas a 5 μg/μl dose fully substituted for ethanol [$F(1,15) = 13.38, p < 0.05$ versus saline]. The ED_{50} for ethanol substitution when pentobarbital was infused into the nucleus accumbens was 1.55 ± 0.10 μg/μl. Response rates during pentobarbital infusion into the nucleus accumbens at both doses tested were not significantly different from saline controls.

Full substitution for ethanol was not observed when pentobarbital was infused into the CA1 region of the hip-

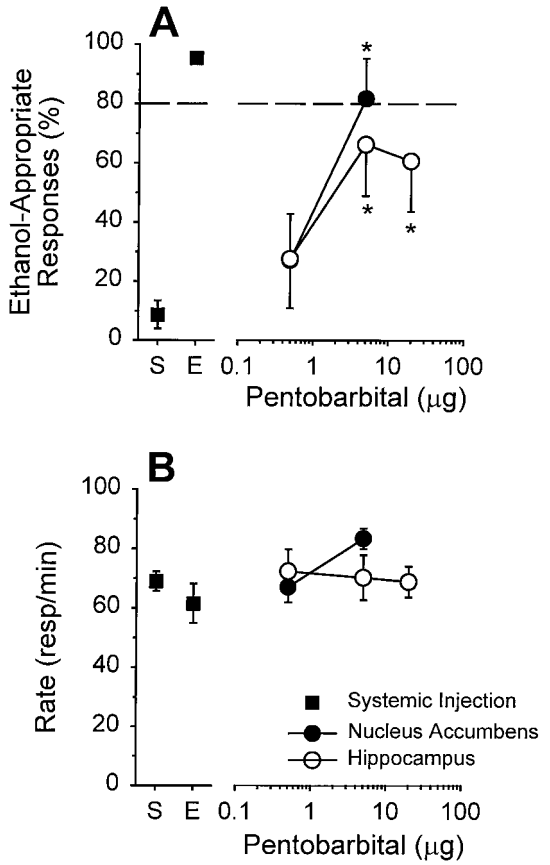

Fig. 4. Mean ± SEM percentage of ethanol-appropriate responses (A) and mean ± SEM total session response rate (B) plotted as a function of pentobarbital dosage. Rats received pentobarbital injections in the nucleus accumbens (●, $n = 9$) or dorsal CA1 region of the hippocampus (○, $n = 9$). Data points to the left of the *x* axis break represent control saline (S) or ethanol (E) ip performance combined with sham microinjections. The horizontal dashed line (>80%) represents full substitution for the discriminative stimulus effects of ethanol (1.0 g/kg ip). Drugs and doses were administered in a randomized order. *$p < 0.05$ versus saline.

pocampus. The low (0.5 $\mu g/\mu l$) dose was without effect, whereas higher doses of 5 and 20 $\mu g/\mu l$ partially substituted for ethanol [$F(1,15) = 5.70$, $p = 0.05$ and $F(1,15) = 10.03$, $p < 0.05$ versus saline, respectively]. Response rates after pentobarbital infusion into the CA1 at all three doses were not significantly different from saline controls.

DISCUSSION

The main finding of this study is that infusion of positive allosteric $GABA_A$ receptor modulators into the nucleus accumbens produces full substitution for the discriminative stimulus effects of systemically administered ethanol. These data extend other findings that showed that systemic administration of positive modulators of $GABA_A$ receptors, such as barbiturates (Barry and Krimmer, 1978; Kline and Young, 1986; Overton, 1977; York and Bush, 1982) and endogenous neuroactive steroids (Bienkowski and Kostowski, 1997; Bowen et al., 1999a; Grant et al., 1996, 1997), substitutes fully for the discriminative stimulus effects of systemically administered ethanol. Results from this study demonstrate that site-specific infusion of pentobarbital or 3α-5α-P produces discriminative stimulus effects that correspond to those of systemic ethanol. Moreover, the results of this study agree with previous findings, which demonstrated that the direct $GABA_A$ agonist muscimol substitutes fully for systemic ethanol when infused in the nucleus accumbens (Hodge and Aiken, 1996; Hodge and Cox, 1998) or amygdala (Hodge and Cox, 1998). Together, this evidence indicates that stimulation of $GABA_A$ receptors in the nucleus accumbens by direct or allosteric modulation is sufficient to produce discriminative stimulus effects that correspond to those of systemic ethanol.

Substitution for ethanol by $GABA_A$-positive modulators varied as a function of brain region. When infused into the nucleus accumbens, both 3α-5α-P and pentobarbital substituted fully for ethanol. However, when infused into the hippocampus, both compounds produced only partial substitution. Because both brain regions have high levels of $GABA_A$ receptors (Persohn et al., 1992; Pirker et al., 2000; Rabow et al., 1995; Sperk et al., 1997; Wisden et al., 1992) that are sensitive to the effects of ethanol (Crews et al., 1996), this finding suggests that $GABA_A$ receptors in the hippocampus may not be essential for discriminative stimulus effects of ethanol. By contrast, infusions of the noncompetitive NMDA antagonist MK-801 into the CA1 substitute fully for ethanol; this indicates that the actions of ethanol at NMDA receptors in the region participate in its discriminative stimulus effects (Hodge and Cox, 1998).

Differential brain region involvement in GABA-mediated ethanol discrimination may reflect differential expression and function of $GABA_A$ receptor subunits. The $GABA_A$ receptor chloride ion channel complex is thought to be a heteropentameric protein composed of subunits that have been classified into five major families: α_1 to α_6, β_1 to β_3, γ_1 to γ_3, ϵ, and δ (Luddens et al., 1995; MacDonald and Olsen, 1994). Expression and assembly of $GABA_A$ receptor subunit subtypes varies across brain regions [see Rabow et al. (1995) and Sieghart (1995) for reviews], and evidence suggests that neurosteroids and barbiturates may bind to separate modulatory sites on the $GABA_A$ receptor (MacDonald and Olsen, 1994; Sieghart, 1992). In *Xenopus* oocytes expressing various combinations of $GABA_A$ receptor subunits, the presence of the γ_2 subunit decreased the efficacy of 3α-5α-P in receptors composed of the $\alpha_3\beta_1\gamma_2$ subunits (Shingai et al., 1991). Of particular relevance to this study, γ_2 subunits of $GABA_A$ receptors are considerably less abundant in the nucleus accumbens as compared with the CA1 region of the hippocampus (Rabow et al., 1995), and this could help clarify why 3α-5α-P exhibited greater efficacy in the discriminative stimulus effects of ethanol when infused in the nucleus accumbens.

The two $GABA_A$-positive modulators substituted for etha-

nol with differential potency when infused in the nucleus accumbens. The ED_{50} for ethanol substitution by 3α-5α-P (0.38 ± 0.11 ng/μl) was approximately 4000-fold lower than the ED_{50} for substitution by pentobarbital (1.55 ± 0.10 μg/μl). These results are consistent with previous studies that showed that neurosteroids are extremely potent and positively modulate $GABA_A$ receptors at low nanomolar concentrations (Majewska et al., 1986; Morrow et al., 1987), whereas higher (i.e., micromolar) concentrations of pentobarbital are needed to positively modulate $GABA_A$ receptor function (Akaike et al., 1985; MacDonald et al., 1989).

In conclusion, results from this study indicate that nucleus accumbens infusions of the $GABA_A$-receptor-positive modulators pentobarbital or 3α-5α-P substitute fully for the discriminative stimulus effects of ethanol. This suggests that site-specific activation of $GABA_A$ receptors in the nucleus accumbens may be an important determinant of the discriminative stimulus effects of systemically administered ethanol. Moreover, activation of $GABA_A$ receptors in the hippocampus (CA1) may not be critical for the stimulus effects of ethanol.

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