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Cellular and behavioral interactions of gabapentin with alcohol dependence

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Cellular and Behavioral Interactions of Gabapentin with Alcohol Dependence

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Gabapentin is a structural analog of GABA that has anticonvulsant properties. Despite the therapeutic efficacy of gabapentin, its molecular and cellular mechanisms of action are unclear. The GABAergic system in the central nucleus of the amygdala (CeA) plays an important role in regulating voluntary ethanol intake. Here, we investigated the effect of gabapentin on GABAergic transmission in CeA slices, on ethanol intake, and on an anxiety measure using animal models of ethanol dependence. Gabapentin increased the amplitudes of evoked GABA receptor-mediated IPSCs (GABA-IPSCs) in CeA neurons from nondependent rats, but decreased their amplitudes in CeA of ethanol-dependent rats. Gabapentin effects were blocked in the presence of a specific GABA$_B$ receptor antagonist. The sensitivity of the GABA-IPSCs to a GABA$_B$ receptor antagonist and an agonist was decreased after chronic ethanol, suggesting that ethanol-induced neuroadaptations of GABA$_B$ receptors associated with ethanol dependence may account for the differential effects of gabapentin after chronic ethanol. Systemic gabapentin reduced ethanol intake in dependent, but not in nondependent, rats and reversed the anxiogenic-like effects of ethanol abstinence using an acute dependence model. Gabapentin infused directly into the CeA also blocked dependence-induced elevation in operant ethanol responding. Collectively, these findings show that gabapentin reverses behavioral measures of ethanol dependence and, in turn, dependence reverses the effects of gabapentin on CeA neurons, and suggest that gabapentin represents a potential medication for treatment of alcoholism.

Key words: amygdala; ethanol dependence; IPSC; paired-pulse facilitation; anxiety; ethanol-self administration

Introduction

Gabapentin, an amino acid designed as a structural analog of GABA (Sills, 2006), is a novel anticonvulsant drug that came into clinical use as adjunctive therapy in the treatment of human seizures. However, its mechanism of pharmacological action remains unknown. Gabapentin increases (1) the concentration and probably the synthesis of GABA in the brain (Taylor et al., 1998), (2) GABA release from rat striatal brain slices *in vitro* (Gotz et al., 1993), and (3) the nonvesicular release of GABA in rat optic nerves (Kocsis and Honmou, 1994) and rat hippocampal slices (Honmou et al., 1995). Gabapentin also decreases monoaminergic synaptic transmission in rat hippocampus and neocortex by selectively inhibiting Ca^{2+} influx through voltage-operated Ca^{2+} channels (VOCCs) (Fink et al., 2000; Dooley et al., 2002; van Hooft et al., 2002). In addition, gabapentin is an agonist for the endogenously expressed GABA$_B$ gb1a–gb2 heteromers cou-

pled to inhibition of VOCCs in intermediate pituitary melanotrope cell lines and in hippocampal neurons (Bertrand et al., 2001; Ng et al., 2001; van Hooft et al., 2002).

Gabapentin has been used effectively in the treatment of alcohol withdrawal in alcoholics (Bonnet et al., 1999, 2007; Bozikas et al., 2002; Gentry et al., 2002; Voris et al., 2003; Book and Myrick, 2005; Mariani et al., 2006; Myrick et al., 2007) and has a selective action in decreasing the convulsive and anxiety-related signs of ethanol withdrawal in mice (Watson et al., 1997). Gabapentin decreases ethanol-induced anxiety-like behavior in the elevated plus maze, but has no effect on motor coordination or spontaneous locomotor activity in control mice (Watson et al., 1997).

In the present study, we tested the effects of gabapentin on GABAergic synapses in the central amygdala (CeA), a brain region considered pivotal in the behavioral effects of acute and chronic ethanol consumption. We also tested the hypothesis that gabapentin has motivational effects on ethanol self-administration in an animal model of ethanol dependence. Most CeA neurons are GABAergic (Sun and Cassell, 1993; Cassell et al., 1999), either inhibitory neurons with recurrent or feedforward connections or inhibitory projection neurons to brainstem or other downstream regions (e.g., bed nucleus of the stria terminalis). The CeA can be characterized as a "gate" that regulates the flow of information through intra-amygdaloidal circuits, changing the excitability of local and downstream circuitries. Acute

ethanol augments GABAergic transmission in rat CeA neurons through a $GABA_B$ receptor-independent mechanism (Roberto et al., 2003), the latter in contrast to other brain regions [e.g., hippocampus (Wan et al., 1996) and NAcc (Nie et al., 2000)]. In addition, in CeA slices taken from ethanol-dependent rats, acute ethanol increases $GABA_A$ receptor-mediated IPSCs ($GABA_A$-IPSCs) to the same extent as in slices from naive rats, suggesting a lack of tolerance (Roberto et al., 2004a). Here, we show that gabapentin, like ethanol, increases evoked IPSC amplitudes in association with a decrease in paired-pulse facilitation (PPF) of $GABA_A$-IPSCs in CeA neurons from nondependent rats. However, in ethanol-dependent rats, gabapentin had effects opposite to those of ethanol, decreasing IPSCs and increasing PPF in the CeA. Superfusion of a $GABA_B$ receptor antagonist blocked the effect of gabapentin on the amplitude or PPF of the $GABA_A$-IPSCs. Interestingly, the sensitivity of GABA-IPSCs to the $GABA_B$ receptor antagonist and agonist was decreased after chronic ethanol. Systemic administration of gabapentin dose-dependently reduced ethanol intake in dependent, but not in nondependent rats, and local injection of gabapentin into CeA reversed the increased ethanol intake in dependent rats. Gabapentin also suppressed anxiety-like behavior in a model of acute ethanol withdrawal-induced anxiety. These results suggest that gabapentin may regulate ethanol intake and anxiety-related behavior associated with ethanol dependence via synaptic actions in the amygdala.

Materials and Methods
Electrophysiological studies
Slice preparation. We prepared CeA slices as described previously (Roberto et al., 2003, 2004a), from male Wistar rats (200–300 g; 4–7 weeks old) that were anesthetized with halothane (3%) and decapitated. The brains were rapidly removed and placed into ice-cold artificial CSF (aCSF) gassed with 95% O_2 and 5% CO_2. We cut transverse slices 400 μm thick on a Vibratome Series 3000 (Technical Products International), incubated them in an interface configuration for ~30 min, and then completely submerged and continuously superfused (flow rate of 2–4 ml/min) them with warm (31°C) gassed ACSF of the following composition (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 1.5 $MgSO_4 \cdot 7H_2O$, 2.0 $CaCl_2$, 24 $NaHCO_3$, and 10 glucose. The inner chamber had a total volume of 0.8 ml. Drugs were added to the ACSF from stock solutions to obtain known concentrations in the superfusate. At the 2–4 ml/min superfusion rates used, drug concentrations reach 90% of the reservoir concentration within 2 min.

Chronic ethanol treatment. We used the standard ethanol inhalation method of The Scripps Research Institute Alcohol Research Center to induce ethanol dependence in rats (Rogers et al., 1979; Roberto et al., 2004a,b). Briefly, male Wistar rats were housed 2–4 per cage on a 6:00 A.M. to 6:00 P.M. light cycle, with *ad libitum* access to food and water. Rats were exposed to either ethanol vapor or ambient air (sham naive controls). Ethanol-treated rats were continuously exposed to ethanol vapors for 2–4 weeks as described previously (Roberto et al., 2004a,b). We handled sham controls similarly but without exposure to ethanol vapor. On experiment days, the chronic ethanol-treated rats were maintained in the ethanol vapor chamber until preparation of the CeA slices. We made recordings in ethanol-free ACSF from slices of ethanol-dependent rats 2–8 h after cutting the slices, as described previously (Roberto et al., 2004a).

Alcohol liquid diet exposure. During ethanol liquid diet exposure, the sole source of nutrition available to rats in the home cage was the ethanol or control liquid diet, although water was still available to all rats *ad libitum*. One liter of ethanol liquid diet contained 3 g of vitamins (MP Biomedicals), 5 g of salt (MP Biomedicals), 92 ml of 95% v/v ethanol, 711 ml of Boost (high-protein chocolate-flavored nutritional energy drink), and 197 ml of water; 1 L of control liquid diet was similar except that it contained 126 g of sucrose (isocalorically matched to ethanol liquid diet;

Sigma-Aldrich) instead of 95% v/v ethanol. Control diet availability for nondependent rats was yoked to intakes of ethanol liquid diet by dependent rats on the previous day, and standardized for body weights. At these concentrations, rats derived 41% of their caloric intake from ethanol/sucrose.

BAL and body weight. We determined blood alcohol levels (BALs) of the ethanol-dependent animals from tail-blood samples taken three times per week. Control animals were also routinely sampled to control for possible effects of handling. The mean BAL of all ethanol-dependent animals was 176.2 ± 11 mg/dl ($n = 33$). The mean body weight of ethanol-dependent animals was 245 ± 12 g ($n = 24$), compared with a mean body weight of 249 ± 20 g ($n = 18$) for sham control animals. We conducted all procedures in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Electrophysiology. We recorded from CeA neurons with sharp micropipettes (3 M KCl) using discontinuous voltage- or current-clamp mode. In voltage-clamp mode, we used a switching frequency of 3–5 kHz and, on a separate oscilloscope, continuously monitored electrode settling time and capacitance neutralization at the headstage. We held most neurons near their resting membrane potential (RMP). Data were acquired with an Axoclamp-2A preamplifier (Molecular Devices) and stored for later analysis using pClamp software (Molecular Devices). We evoked pharmacologically isolated GABA-IPSCs by stimulating locally within the CeA through a bipolar stimulating electrode while superfusing the slices with the glutamate receptor blockers 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 mM) and DL-2-amino-5-phosphonovalerate (APV; 30 μM). In some cells, we also applied 1 μM (2S)-3[[(1S)-1-(3,4-dichlorophenyl)-ethyl]amino-2-hydroxy-propyl](phenylmethyl)phosphinic acid hydrochloride [CGP 55845A (CGP); a $GABA_B$ receptor antagonist] to further isolate $GABA_A$ receptor-mediated IPSCs. At the end of the recording, to confirm the GABAergic nature of the IPSC, we often superfused (1 μM) CGP 55845A and 30 μM bicuculline (or 50 μM picrotoxin); these antagonists completely blocked the IPSCs (see Fig. 1B). Initially, we evoked IPSCs during superfusion of gabapentin (10–75 μM; ~0.09–0.64 μg/ml) and we used the maximal concentration (50 μM; ~0.43 μg/ml) of gabapentin in all subsequent electrophysiological experiments. To determine the response parameters for each cell, we performed an input–output (I–O) protocol (at least three times for each experimental condition: baseline, drug, and wash). A range of incrementally adjusted currents was applied (typically between 50 and 250 mA; 0.125 Hz), starting at the threshold current required to elicit an IPSC up to the voltage required to elicit the maximum amplitude. We maintained the stimulus strengths throughout the entire duration of the experiment. We normalized three stimulus intensities of equal steps (threshold, half-maximal, and maximal) as 1–3×. Hyperpolarizing and depolarizing current steps (200 pA increments, 750 ms duration) were also applied to generate voltage–current (V–I) curves. We quantified the evoked IPSC amplitudes and V–I responses by using Clampfit software (Molecular Devices).

We examined PPF in each neuron using 50 ms interstimulus intervals (Roberto et al., 2004a). The stimulus strength was adjusted such that the amplitude of the first IPSC was 50% of maximal, determined from the I–O relationship. We calculated the paired-pulse ratio (average of three sequential paired-pulse IPSCs evoked at half-maximal intensity stimuli, 30 ms intervals between sets of paired pulse stimuli) as the second IPSC amplitude over that of the first IPSC amplitude. All measures were taken before gabapentin or ethanol superfusion (control), during their superfusion (5–10 min), and after washout (20–30 min). We normalized all values to percentage of control and expressed them as mean ± SEM. Data were analyzed with a between-subjects ANOVA or within-subjects ANOVA with repeated measures and, when appropriate, with the Newman–Keuls *post hoc* test with $p < 0.05$ considered statistically significant. In some cases, we used a Student's paired or unpaired t test for individual means comparisons.

Drugs. CGP 55845A was a gift from Novartis Pharma. We purchased D-AP5, CNQX, picrotoxin, baclofen, and bicuculline from Sigma, gabapentin from ChemPacific, and ethanol from Remet.

Behavioral studies
Effects of systemic gabapentin on ethanol intake. Male Wistar rats (250–300 g at the start of training) were initially trained to self-administer 10%

(w/v) ethanol in daily 30 min operant sessions via the use of a saccharin fading procedure (Funk et al., 2006). Once operant responding stabilized, we divided rats into two groups matched for ethanol responding and exposed them to either ethanol vapor (dependent group; $n = 14$) or ambient air (nondependent group; $n = 14$) for a period of at least 4 weeks. Tail blood samples were collected several times per week, and BALs were maintained in a range of 150–200 mg%. Doses of gabapentin tested in the present study are comparable with those tested in human studies of alcohol-drinking behavior and craving [300–1200 mg daily (Myrick et al., 2007), which produces a plasma level of ~40 μM/ml (Dierking et al., 2004)], and these doses are defined by high tolerability, few side effects, and low dropout rates in subjects (for review, see Pappagallo, 2003). We tested five doses (0, 5, 10, 30, and 120 mg/kg, i.p.) of gabapentin in a within-subjects Latin-square design; two tests occurred per week. On test days, rats were removed from vapor chambers, and blood was collected immediately from tails for BAL determination. Rats were injected with gabapentin 90 min later, returned to the home cage for a 30 min wait period, and then tested for operant ethanol self-administration 2 h after termination of vapor inhalation. After testing, rats were immediately returned to vapor chambers. We analyzed data with two-way (vapor history x gabapentin dose) mixed-design ANOVAs and, when appropriate, with the Student–Newman–Keuls *post hoc* test, with $p < 0.05$ considered statistically significant.

Effects of intra-CeA gabapentin on ethanol intake. We initially trained Male Wistar rats to self-administer 10% (w/v) ethanol in daily 30 min operant sessions in the manner described above. Once operant responding stabilized, rats were divided into groups matched for ethanol responding and exposed to either ethanol-containing liquid diet (dependent group; $n = 9$) or a calorically matched control liquid diet (nondependent group; $n = 8$) for a period of ~14 weeks. We collected tail bloods at various time points during the dark cycle; rats consumed quantities of ethanol liquid diet sufficient to achieve BALs between 192.10 ± 18.26 mg% and 233.67 ± 13.93 mg%. During this 14 week period, we tested rats twice per week at 6 h withdrawal for operant ethanol responding. After establishment of stable operant responding across test days in dependent and nondependent rats, all rats underwent stereotaxic surgery and were implanted bilaterally with cannulas aimed at the CeA. Subsequently, rats underwent a series of "sham" infusions to habituate to the procedure and were then infused with four NPY doses in a Latin-square design plus twice with aCSF. Those NPY data are part of another study not presented here, but operant response data after vehicle infusion during the gabapentin Latin-square design did not significantly differ from operant response data after vehicle infusion before the NPY Latin square. After a washout period of 14 d, we tested rats for the effects of gabapentin (20 μg) or vehicle infused directly into the CeA on operant ethanol intake. These gabapentin/vehicle infusions were administered in a within-subjects Latin-square design. On operant test days, we removed liquid diet bottles and recorded intakes 2 h before the start of the dark cycle (8:00 A.M.). Six hours later (4 h into the dark cycle), rats were infused with gabapentin and, after a 30 min wait period, tested for operant alcohol responding. After all experimental procedures, cannula placements, and patency were histologically verified. We analyzed data with two-way (vapor history by gabapentin dose) mixed-design ANOVAs and, when appropriate, with Student–Newman–Keuls *post hoc t* tests, with $p < 0.05$ considered statistically significant.

Stereotaxic surgeries. We surgically implanted cannulas using aseptic procedures as described previously (Funk et al., 2006). We determined the stereotaxic coordinates (anteroposterior, −2.6; mediolateral, ±4.2; dorsoventral, −5.2 relative to bregma from skull surface) according to Paxinos and Watson (1986). We implanted rats bilaterally with two cannulas aimed at the CeA. The injection cannula (33 gauge) extended 1.0 mm beyond the tip of the guide cannula (26 gauge) when inserted (components from Plastics One). Before further manipulation, we monitored the rats during 7 d of recovery to determine that the animal had resumed normal activity such as mobility, feeding, and drinking.

Microinfusions. We used a Harvard 33 microinfusion pump for all drug infusions (rate of 0.25 μl/min for 2 min), and left the injection cannula in the guide cannula for 1 additional minute to allow for adequate diffusion of the solution. We delivered infusions to the cannula via

polyethylene tubing (PE 20) that was connected to a Hamilton 10 μl syringe. After infusions, we immediately placed rats in operant boxes; sessions started 30 min after the end of infusions.

Gabapentin effects on anxiety-like behavior produced by bolus injection of acute ethanol. Acute bolus injections of ethanol produce elevations in anxiety-like behavior after BALs return to zero, and this elevated anxiety-like behavior has been validated as a measure of acute withdrawal (Lal et al., 1991; Jung et al., 2000). We injected male Wistar rats with saline or 10% (w/v) ethanol (3 g/kg, i.p.). Ten hours later, we placed the rats in the center of an elevated plus maze and allowed them to explore the closed and open arms of the apparatus for a 5 min period. Previous experiments (Morse et al., 2000) showed that BALs are undetectable 10 h after bolus injection of 3 g/kg ethanol. Thirty minutes before anxiety testing, separate groups of rats were injected with one of several doses of gabapentin (0, 30, 60, or 120 mg/kg, i.p; $n = 8$–10 per dose). We analyzed data (percentage of time spent in open arms of plus maze) with two-way (injection solution × gabapentin vs vehicle) between-subjects ANOVAs and, when appropriate, with the Student–Newman–Keuls *post hoc* test, with $p < 0.05$ considered statistically significant.

Results

Electrophysiological studies

Gabapentin enhances GABAergic synaptic transmission in CeA neurons from nondependent rats

We recorded from a total of 140 CeA neurons (mean RMP, −76 ± 1.9 mV). Gabapentin (10–75 μM) did not significantly alter RMP, input resistance, or spike amplitudes in CeA neurons (data not shown). Superfusion (10 min) of 10–75 μM gabapentin dose-dependently increased the amplitude of GABA-IPSCs (Fig. 1A) (mean increase over all three stimulus strengths). This gabapentin–IPSC interaction in CeA had an apparent EC_{50} value of 27 μM (Fig. 1A). The 50 μM concentration of gabapentin produced a maximal increase in the mean amplitude of evoked GABA-IPSCs (by 40%) measured over all stimulus strengths (Fig. 1B) ($n = 8$). Therefore, the 50 μM concentration was used in all subsequent electrophysiological experiments. This augmentation of GABA-IPSCs was blocked or occluded, at least in part (compare Fig. 4B), by 1 μM CGP, a $GABA_B$ receptor antagonist (Fig. 1B) ($n = 5$). Subsequent superfusion of CGP 55845A, together with 30 μM bicuculline (Fig. 1B) or 50 μM picrotoxin (data not shown) ($n = 3$), completely blocked the IPSCs. To test whether this synaptic effect could derive from a presynaptic site of action, we examined PPF of the GABA-IPSCs. Generally, changes in PPF are inversely related to the probability of transmitter release (Andreasen and Hablitz, 1994; Roberto et al., 2003). Gabapentin significantly ($p < 0.05$) decreased PPF of GABA-IPSCs ($n = 8$) (Fig. 1C), suggesting increased GABA release. However, in the presence of CGP 55845A, gabapentin did not decrease the PPF ratio ($n = 5$) (Fig. 1C), suggesting that gabapentin enhances GABAergic transmission in CeA via a presynaptic $GABA_B$ receptor-related mechanism.

Because gabapentin, like ethanol (Roberto et al., 2003), increased IPSC amplitudes in CeA neurons, we superfused gabapentin alone and then coapplied ethanol with gabapentin onto CeA slices from naive rats to investigate the possibility of a common mechanism (Fig. 2A). In the presence of 50 μM gabapentin, 44 mM ethanol, a maximally effective concentration (Roberto et al., 2003, 2004a) still increased GABA-IPSCs (Fig. 2A) and further decreased PPF (Fig. 2B).

Gabapentin decreases GABA-IPSCs in CeA neurons from ethanol-dependent rats

We previously reported an increased baseline GABAergic transmission and a persistent acute ethanol-induced increase of GABA release in CeA neurons of ethanol-dependent rats (Roberto et al.,
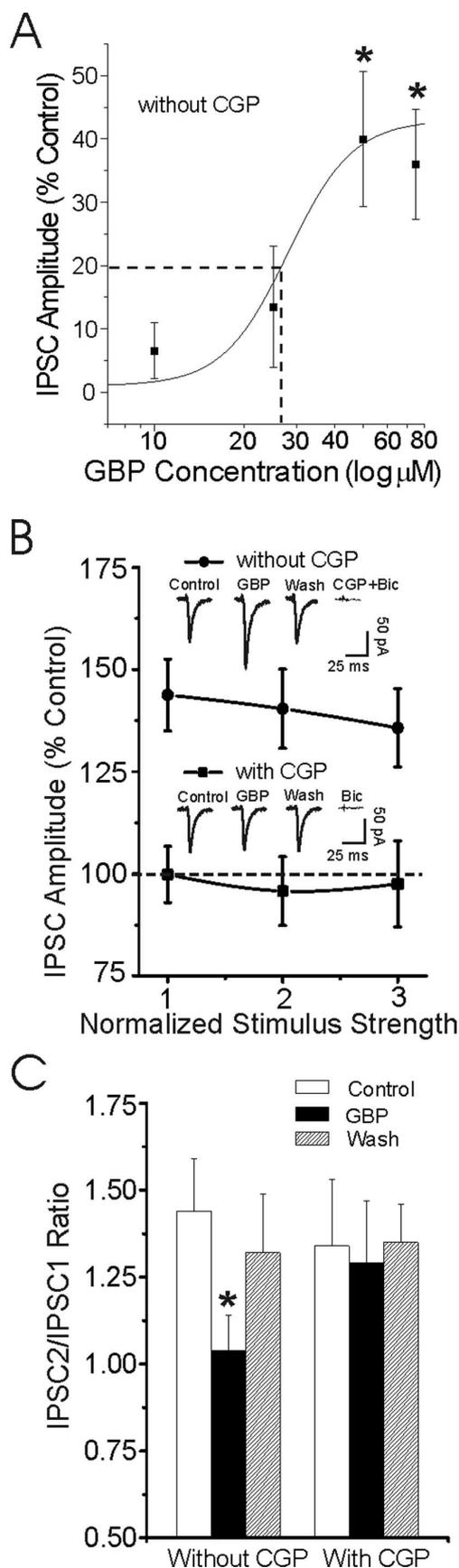
Figure 1. Gabapentin dose-dependently increases basal GABAergic transmission in the CeA from nondependent rats through a GABA$_B$ receptor-related mechanism. **A**, Concentration–response relationship for gabapentin (10, 25, 50, and 75 μM) enhancement of mean IPSC amplitudes in CeA neurons, expressed as a percentage of control (number of cells for the 4 doses: 3, 5, 8, and 4 respectively). The logistic curve, plotted by Origin Software (Microcal), using $y = (A1 - A2)/(1 + (x/xo) \times p + A2)$, gives an apparent EC$_{50}$ value (dashed line) of 27 μM gabapentin for IPSC enhancement. Parameters of the logistic curve were set at: upper asymptote fixed at 140% and lower at 100%. Rate was fixed at 4.0, with "center" unfixed. Error bars indicate SEM. *$p < 0.05$. **B**, Gabapentin (50 μM) superfusion for 10 min significantly ($p < 0.05$) increased the mean amplitudes of evoked GABA-IPSCs without (solid circles; $n = 8$), but not with (solid squares; $n = 5$) coapplication of 1 μM CGP, a GABA$_B$ receptor antagonist. Note that CGP completely blocked the gabapentin-induced enhancement of IPSCs. Inset, Representative recordings of evoked IPSCs. Superfusion of GABA$_A$ and GABA$_B$ receptor blockers completely blocked these IPSCs. **C**, Gabapentin (50 μM) significantly (*$p < 0.05$) decreased the mean PPF of IPSCs only in the absence of CGP. The mean ($\pm$SEM) of the paired-pulse ratio is expressed as IPSC2/IPSC1.

2003, 2004a), suggesting a lack of tolerance to the acute ethanol effects on GABA release. Therefore, we tested the effects of gabapentin on basal GABAergic transmission after chronic ethanol exposure. In direct contrast to the CeA of nondependent rats, gabapentin significantly ($p < 0.05$) decreased the mean GABA-IPSC amplitude (to 65% of control; $n = 7$) (Fig. 3A) in the CeA of dependent rats. Subsequent superfusion of CGP 55845A and bicuculline completely blocked these IPSCs. Furthermore, baseline PPF of GABA-IPSCs was significantly ($p < 0.05$) less in neurons from dependent rats compared with that of nondependent rats, suggesting that the probability of basal GABA release was augmented after chronic ethanol (Fig. 3B) (Roberto et al., 2004a). However, gabapentin significantly ($p < 0.05$) increased PPF of GABA-IPSCs ($n = 6$) (Fig. 3B), suggesting that the drug decreased GABA release in CeA from dependent rats. This decrease of GABA-IPSCs was also blocked by CGP 55845A (to 96 $\pm$ 5%, mean across three stimulus intensities, $p > 0.05$; $n = 4$) (data not shown). Furthermore, acute ethanol (44 mM) significantly ($p < 0.05$) decreased the PPF of GABA-IPSCs (Fig. 3B) (Roberto et al., 2004a), indicating both increased GABA release and a lack of tolerance for this acute ethanol effect. To test the ethanol–gabapentin interactions in CeA of dependent rats, the same protocol of Figure 2B was repeated, where gabapentin was first superfused alone and then coapplied with ethanol. In the presence of gabapentin, acute ethanol (44 mM) significantly ($p < 0.05$; $n = 7$) overcame the depressive effect of gabapentin (Fig. 3C).

To determine whether GABA$_B$ receptors regulate evoked GABAergic transmission per se, we superfused 1 μM CGP 55845A. CGP 55845A significantly ($p < 0.05$; $n = 13$) increased the mean amplitude of evoked IPSCs (by 12 $\pm$ 5%, averaged over all stimulus intensities) in CeA from naive rats (Fig. 4A). This increase in the IPSC amplitude was associated with a significant ($p < 0.05$) decrease in PPF, suggesting a tonic activation of presynaptic GABA$_B$ receptors in naive rats (Fig. 4C). In contrast, in CeA from ethanol-dependent rats, CGP 55845A did not alter the mean evoked IPSCs (98 $\pm$ 4%, averaged over all stimulus intensities; $n = 10$) (Fig. 4A) and did not affect mean PPF (Fig. 4C). Figure 4B shows the time course for CGP 55845A effects on IPSCs evoked at half-maximal stimulus intensity from both naive and ethanol-dependent rats. CGP 55845A significantly increased the amplitude of IPSCs only in CeA of naive rats.

We next examined whether chronic ethanol treatment (CET) affects the sensitivity of CeA synapses to baclofen, a GABA$_B$ agonist. Baclofen (10 μM) markedly depressed evoked GABA-IPSC amplitudes in neurons of naive rats (to 38% of control; $n = 11$; averaged over all stimulus intensities) (Fig. 4D), with recovery during washout (20 min). The baclofen-induced inhibition of GABA-IPSCs was significantly ($p < 0.001$) reduced (to 86% of control; $n = 10$) in neurons of CET rats (Fig. 4D). In addition, in

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CET neurons ($n = 7$), baclofen-induced depression was associated with a smaller increase of the PPF ratio of GABA$_A$-IPSCs compared with that in neurons of naive rats ($n = 6$) (Fig. 4E,F).

Behavioral studies

Effects of systemic gabapentin on ethanol intake

An animal model of self-administration in dependent rats has been established and validated (Roberts et al., 1996; O'Dell et al., 2004). Figure 5A illustrates operant responding for ethanol by dependent and nondependent rats 2 h after termination of ethanol vapor inhalation and 30 min after intraperitoneal injection of five doses of gabapentin. A two-way mixed-design ANOVA yielded a significant interaction effect of ethanol vapor history (between-subjects factor) and gabapentin dose (within-subjects factor) on operant ethanol responding ($F_{(4,92)} = 3.61$; $p < 0.01$) and ethanol intake (grams per kilogram; $F_{(4,92)} = 3.79$, $p < 0.01$). Chronic ethanol vapor exposure increased ethanol intake (grams per kilogram) in dependent rats relative to nondependent rats ($p < 0.05$), and that effect was attenuated by the three highest doses (10, 30, and 120 mg/kg) of gabapentin ($p > 0.05$ vs nondependent controls). The two highest doses of gabapentin (30 and 120 mg/kg) also significantly suppressed ethanol intake (grams per kilogram) by dependent animals relative to vehicle ($p < 0.01$ in both cases). We obtained similar results with operant ethanol responses. No dose of gabapentin altered ethanol lever presses (grams per kilogram) by nondependent rats (Fig. 5A), nor did any dose of gabapentin alter water responding by any group of rats (Fig. 5B) ($p > 0.05$ in all cases). Data for two dependent rats were excluded because those rats failed to exhibit reliable and elevated operant ethanol responding after chronic ethanol vapor exposure, and data for a third rat was excluded because the Geigy Extreme test revealed one of its datum to be an extreme outlier on a gabapentin test day.

We also investigated the effects of intra-CeA gabapentin on ethanol intake (grams per kilogram) by dependent and nondependent rats 6 h after removal of ethanol liquid diet and 30 min after intra-CeA infusion of two doses (0, 20 μg) of gabapentin. There was a significant interaction effect of ethanol vapor history and gabapentin versus vehicle on operant ethanol responding ($F_{(1,10)} = 5.99$; $p = 0.034$) and ethanol intake (grams per kilogram; $F_{(1,10)} = 6.32$, $p = 0.031$, two-way mixed-design ANOVAs). Chronic exposure to ethanol liquid diet increased ethanol intake (grams per kilogram) in dependent rats relative to nondependent rats ($p < 0.05$), and this elevated ethanol intake by dependent animals was blocked by intra-CeA infusion of 20 μg of gabapentin. We obtained similar results with operant ethanol responses. Operant water responding was not affected by liquid diet history or gabapentin infusion (data not shown) ($p > 0.05$ in all cases). Data for five rats (three dependent and two nondependent) were excluded because those rats were confirmed to have one or both cannulas placed inaccurately in the CeA. Gabapentin did not alter water responding by any group of rats ($p > 0.05$).
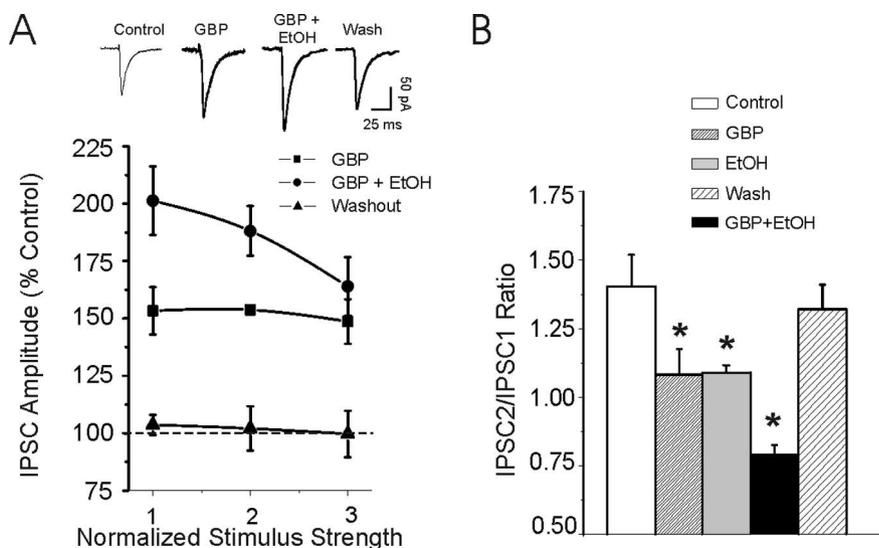

Figure 2. Gabapentin (50 μM) and ethanol additively augment IPSC amplitudes in CeA from nondependent rats. **A**, Pooled data from five CeA neurons show that ethanol (44 mM) coapplied with gabapentin further increased GABA-IPSC amplitudes, suggesting that gabapentin and ethanol may use different intracellular mechanisms to augment GABA-IPSCs. **B**, Gabapentin (50 μM) significantly ($p < 0.05$; $n = 7$) decreased the mean paired-pulse ratio, and ethanol further significantly ($p < 0.05$; $n = 7$) decreased it, with recovery on washout. Ethanol alone also significantly (*$p < 0.05$) decreased PPF of GABA-IPSCs ($n = 6$) as we have shown previously (Roberto et al., 2003). Error bars indicate SEM.

Effects of gabapentin on anxiety-like behavior produced by an acute bolus injection of ethanol

Acute bolus ethanol injection significantly decreased the percentage of time spent on open arms of the elevated plus maze 10 h after injection (after BALs returned to zero) relative to saline-injected controls (Fig. 6) ($t_{(14)} = 2.25$; $p < 0.05$). Gabapentin dose-dependently increased the percentage of time spent in the open arms of the plus maze by ethanol-injected rats ($F_{(3,27)} = 3.33$; $p < 0.05$). The highest dose of gabapentin (120 mg/kg) significantly attenuated the ethanol-induced decrease in the percentage of time spent on the open arms of the elevated plus maze ($p < 0.01$). Gabapentin did not affect the percentage of time spent in open arms of the elevated plus maze by ethanol-naive animals. Table 1 displays time spent in the open and closed arms during the 5 min plus-maze test. A separate two-way ANOVA indicated that animals injected with ethanol exhibited fewer closed arm entries during the plus-maze test relative to saline-injected controls ($F_{(1,54)} = 6.04$; $p = 0.017$) (data not shown). There was no effect of gabapentin nor was there an interaction effect on closed arm entries.

Discussion

Our previous *in vitro* and *in vivo* findings showed that ethanol dependence is associated with increased GABAergic transmission in the CeA (Roberto et al., 2003, 2004a). Gabapentin is an analog of GABA with anticonvulsant properties, and it has been used to treat ethanol withdrawal in humans (Book and Myrick, 2005; Mariani et al., 2006; Bonnet et al., 2007; Myrick et al., 2007). However, little is known of its mechanism of action. The observation that gabapentin increases GABA release in brain slices led us to evaluate the effects of gabapentin on GABAergic transmission in rat CeA slices, and to explore the behavioral effects of gabapentin on anxiety and drinking in nondependent and dependent rats. Our electrophysiological findings show that gabapentin has strikingly different effects in nondependent and ethanol-dependent rats, both cellularly and pharmacologically. In nondependent rats, gabapentin facilitated GABAergic transmission in
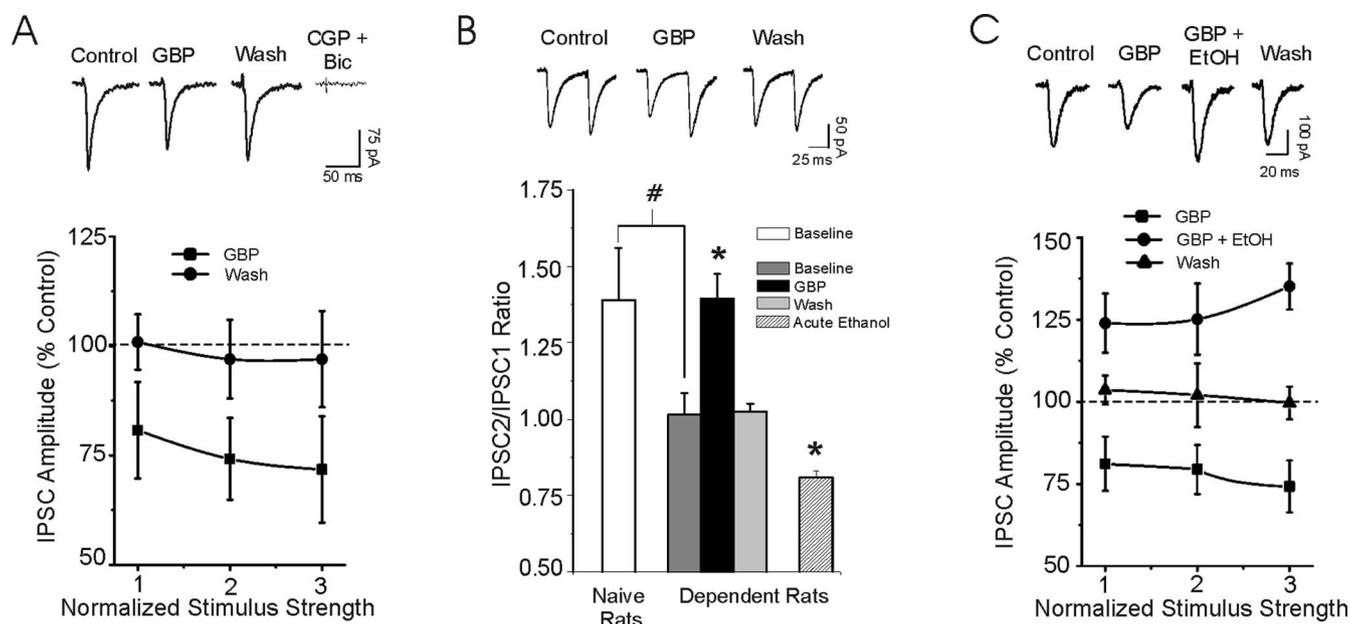
Figure 3. Gabapentin (50 μM) reduces IPSC amplitudes in CeA neurons from ethanol-dependent rats. ***A***, Top, Representative evoked IPSCs from a CeA neuron of ethanol-dependent rat recorded under various treatment conditions. Superfusion of CGP 55845A and bicuculline completely blocked these IPSCs. Bottom, In contrast to slices from naive controls (see Fig. 1*A*), application of gabapentin for 10 min to slices from ethanol-dependent rats decreased IPSC amplitudes with recovery on washout. CGP 55845A was not present in the bathing solution. ***B***, Top, Representative traces of PPF of IPSCs recorded under various treatment conditions from a dependent rat. Gabapentin increased PPF. Bottom, Pooled data showing that gabapentin (50 μM) significantly (*$p < 0.05$) increased PPF of GABA-IPSCs ($n = 6$), with recovery on washout. In dependent rats, ethanol alone significantly (*$p < 0.05$) decreased PPF of GABA-IPSCs ($n = 8$) (Roberto et al., 2004a). As reported previously (Roberto et al., 2004a), in dependent rats, baseline PPF of IPSCs is significantly ($^{\#}p < 0.05$) lower compared with that in nondependent rats. ***C***, Top, Evoked IPSCs in CeA neurons from an ethanol-dependent rat recorded under various treatment conditions. Bottom, Pooled data of ethanol–gabapentin interactions. Acute ethanol overcame the depressive effect of gabapentin and significantly ($p < 0.05$; $n = 7$) increased the mean amplitude of GABA-IPSPCs in CeA neurons, with recovery on washout. Error bars indicate SEM.

the CeA, but did not affect ethanol intake. However, in dependent rats, gabapentin decreased GABAergic transmission in the CeA and reduced excessive ethanol intake. Furthermore, gabapentin suppressed the anxiogenic-like effects of withdrawal from an acute alcohol injection.

It has been reported that gabapentin enhances GABA release from rat striatal brain slices (Gotz et al., 1993) and nonvesicular release of GABA in both rat optic nerves (Kocsis and Honmou, 1994) and rat hippocampal slices (Honmou et al., 1995). Here we find that gabapentin, like ethanol, increases GABAergic transmission in CeA slices from nondependent rats and decreases PPF of IPSCs, suggesting that its action is mediated, at least in part, by an increased release of GABA. However, in the CeA slices from nondependent rats, coapplication of ethanol and gabapentin additively increase GABA-IPSC amplitudes, suggesting that gabapentin and ethanol use different intracellular mechanisms to augment GABA-IPSCs in naive CeA. The gabapentin-induced augmentation of GABA-IPSCs was inhibited or occluded (at least in part) by a GABA$_B$ receptor antagonist, suggesting an important role for these receptors in the effects of gabapentin. Note, however, that GABA$_B$ receptors are not required for the enhancement of IPSCs by acute ethanol in the CeA, as we showed previously (Roberto et al., 2003).

A series of studies in other laboratories have shown that gabapentin effects involve GABA$_B$ receptors, at least in part (Xiong and Stringer, 1997; Stringer and Lorenzo, 1999; Parker et al., 2004). Gabapentin acts as an agonist at GABA$_B$ receptors coupled to VOCCs in mouse cultured neurons (Bertrand et al., 2001; van Hooft et al., 2002). Nevertheless, despite this compelling evidence, conventional binding studies failed to demonstrate a direct interaction between gabapentin and GABA$_B$ receptors (Lanneau et al., 2001; Jensen et al., 2002). Gabapentin also may bind

the α-2-δ type-1 subunit of voltage-gated calcium channels, as has been reported in the spinal dorsal horn (Xiao et al., 2007). Further studies will be required to determine the exact molecular mechanisms underlying the effects of gabapentin and ethanol on GABA release at CeA synapses.

Another factor that may influence the wide range of gabapentin effects in different brain regions in slice preparations may be the wide range of gabapentin concentrations used between studies. The doses (10–75 μM) of gabapentin used in the present study are similar to those tested in other cellular studies such as in rat entorhinal slices (10–100 μM) (Cunningham et al., 2004; Brown and Randall, 2005) and rat trigeminal nucleus (30 μM) (Maneuf et al., 2001). In contrast, low concentrations of gabapentin (<5 μM) are effective in DRG (Yang et al., 2005) and high doses are required in cultured neurons (1000 μM) (Cheng et al., 2006).

We previously found increased GABA "tone" in CeA after chronic ethanol exposure (Roberto et al., 2004a) and lack of tolerance for the acute ethanol effect (Roberto et al., 2004a). In the present study, gabapentin decreased GABA-IPSC amplitudes in CeA from ethanol-dependent rats by decreasing GABA release, "normalizing" the chronic ethanol-induced effect. However, ethanol still augmented the GABA-IPSCs even in the presence of gabapentin, further suggesting that these drugs may produce their effects via different mechanisms. Nonetheless, the gabapentin-induced decrease in GABAergic transmission in dependent rats might account for the attenuation of excessive ethanol intake. We speculate that during the development of ethanol dependence, neuroadaptive changes occur in the GABAergic system, including presynaptic GABA$_B$R function (e.g., a reduced sensitivity and/or downregulation of GABA$_B$ receptors). Our findings with CGP 55845A and baclofen support this hypothesis

and suggest that alteration in $GABA_B$ receptor function could be responsible for the differential effects of gabapentin in naive versus ethanol dependent rats. Although $GABA_B$ receptors may not be involved in the acute ethanol effect in CeA neurons, they could be involved in the increased GABAergic tone and the gabapentin-induced depression of GABA-IPSC amplitudes in CeA after chronic ethanol. Because there is evidence that $GABA_B$ receptors modulate ethanol action at both electrophysiological (Wan et al., 1996; Peris et al., 1997; Siggins et al., 2005; Weiner and Valenzuela, 2006) and behavioral levels (Castelli et al., 2005; Czachowski et al., 2006; Maccioni et al., 2007), future studies will assess possible changes in the expression of $GABA_B$ receptor subunit proteins and/or mRNA in CeA.

In the present study, systemic gabapentin injection dose-dependently blocked the anxiogenic-like effects associated with "hangover" after acute administration of a high ethanol dose, but did not affect anxiety-like behavior in ethanol-naive animals. Acute exposure to a high dose of alcohol reliably produces increases in anxiety-like behavior (Zhang et al., 2007) and a negative emotional state as measured by brain stimulation reward thresholds (Schulteis and Liu, 2006). These effects are amplified after multiple ethanol exposures (Schulteis and Liu, 2006; Zhang et al., 2007), suggesting a role for negative emotional states during early-stage alcohol use and in the subsequent transition to addiction. A similar correspondence has been observed in studies of opioid withdrawal where both rat and human studies have validated that acute withdrawal after single doses of drug mimics much of the pharmacology of acute withdrawal after chronic drugs (Stitzer et al., 1991; Schulteis et al., 2003).

Systemic gabapentin injection also dose-dependently suppressed increases in operant ethanol responding produced by withdrawal from chronic ethanol vapor in dependent rats, but did not alter ethanol self-administration in nondependent animals. This lack of gabapentin effect in nondependent rats is not likely attributable to a floor effect on operant responding for ethanol, because baseline responding of those animals for water was far lower than baseline responding for ethanol, and also because past findings from our group have presented substantially lower baseline operant ethanol responding in nondependent animals than those seen here (Valdez et al., 2002; Funk et al., 2006, 2007). The doses of systemically administered gabap-
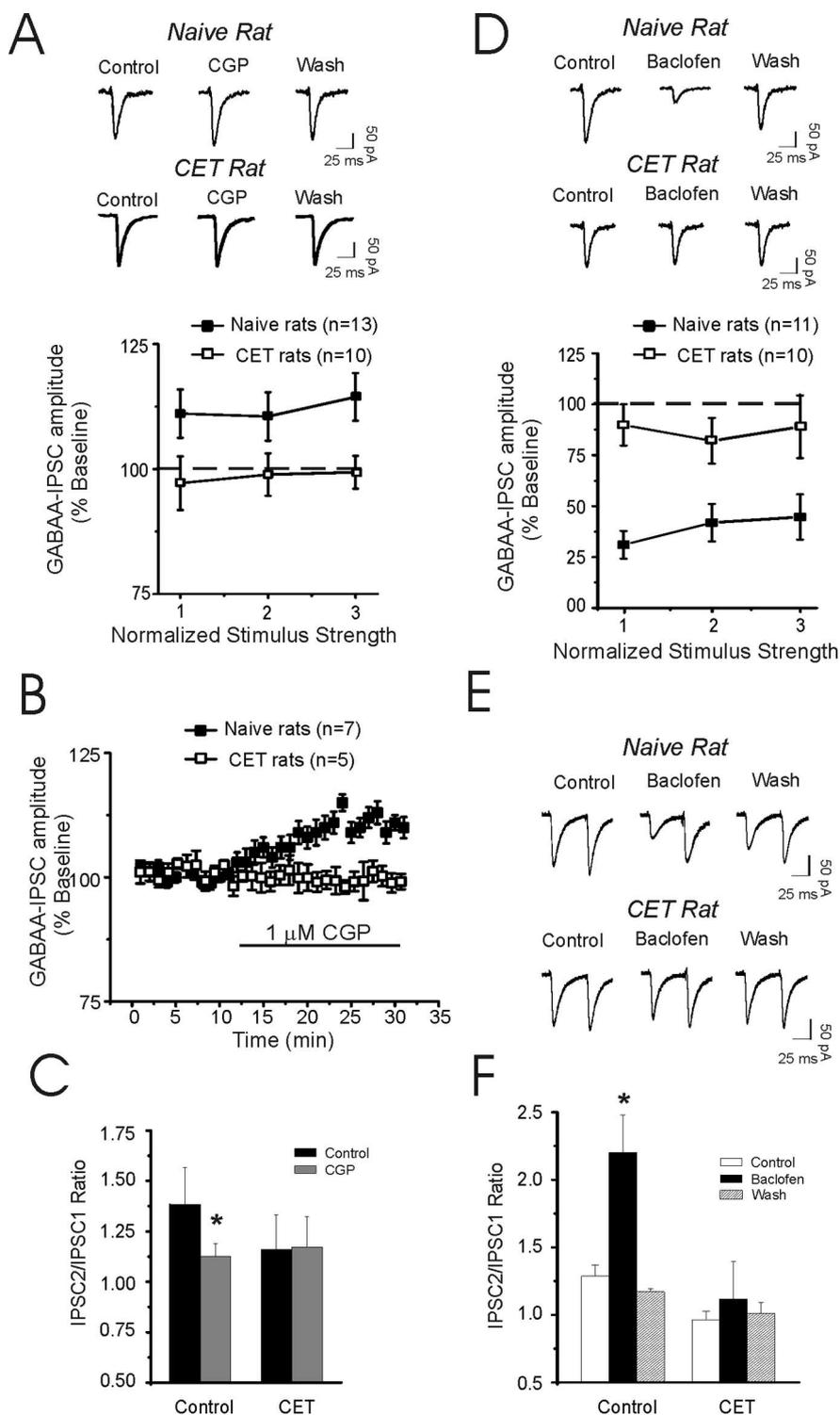

Figure 4. Effects of CGP and baclofen on IPSC amplitude in CeA. **A**, I–O curve from CeA neurons of naive and ethanol-dependent rats. CGP 55845A (1 μM) in the presence of APV and CNQX significantly ($p < 0.05$; $n = 13$) increased mean IPSC sizes (averaged over all stimulus intensities) in naive animals. **B**, Time course of mean IPSCs evoked by half maximal stimulus intensity recorded from CeA neurons from naive ($n = 7$) and ethanol-dependent ($n = 5$) rats. **C**, CGP 55845A also significantly (*$p < 0.05$) decreased the mean PPF of IPSCs, but only in CeA from naive rats. **D**, The $GABA_B$ receptor agonist baclofen (10 μM) is less effective in CeA neurons taken from CET rats compared with naive rats. Superfusion of baclofen for 10 min markedly ($p < 0.001$) decreased the GABA-IPSC amplitudes in neurons from naive rats. In neurons from CET rats, the effects of baclofen were significantly ($p < 0.05$) reduced, suggesting a decrease in $GABA_B$ receptor sensitivity after CET. **E**, Representative traces of a paired-pulse study (at 50 ms interstimulus intervals) of IPSCs in CeA neuron from naive (top) and CET (right) rats (bottom). **F**, Pooled data of mean PPF ratios in neurons of naive and CET rats. Baclofen significantly (*$p < 0.05$) increased PPF ratio of IPSCs in naive ($n = 7$) rats, but not in CET ($n = 6$) rats. Error bars indicate SEM.
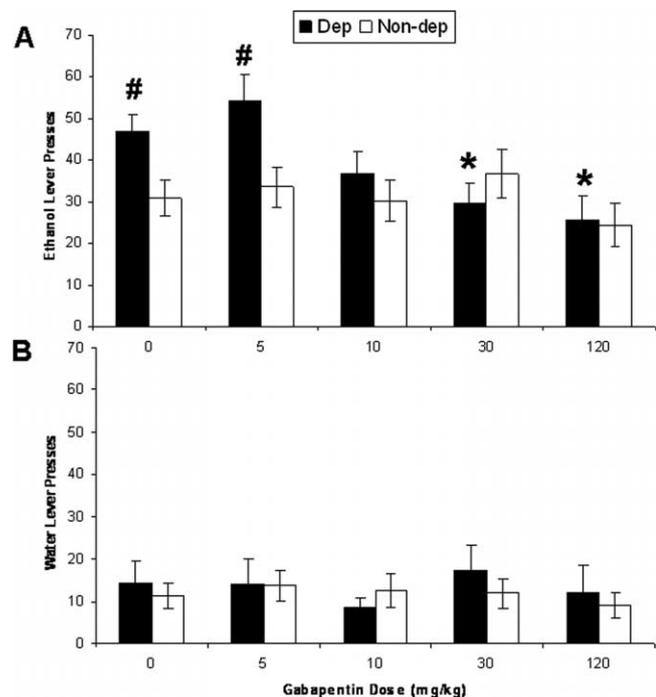
Figure 5. Gabapentin decreases ethanol intake. *A*, The data reflect mean (±SEM) "ethanol lever presses" or "operant ethanol responses" (grams per kilogram) by rats either exposed to chronic ethanol vapor (dependent rats; black bars; $n = 11$) or not exposed to ethanol vapor (nondependent rats; white bars; $n = 14$) after pretreatment with gabapentin. Rats were tested for 30 min operant ethanol self-administration 2 h after removal from vapor chambers and 30 min after injection of various doses (0, 5, 10, 30, or 120 mg/kg, i.p.; all rats injected with all doses of gabapentin in a Latin-square design) of gabapentin. Dependent rats consumed significantly more ethanol than nondependent rats, an effect that was blocked by the three highest doses (10, 30, and 120 mg/kg) of gabapentin. The two highest doses (30 and 120 mg/kg) of gabapentin suppressed ethanol intake in dependent rats relative to saline injection. No dose of gabapentin affected ethanol intake by nondependent rats. *$p < 0.05$, significant difference from vehicle (0 mg/kg gabapentin) in the same group of rats; #$p < 0.05$, significant difference from nondependent rats injected with the same gabapentin dose. *B*, Gabapentin did not alter water responding by any group of rats (Fig. 6B) ($p > 0.05$ in all cases).

Figure 6. Gabapentin reverses ethanol-induced increases in anxiety-like behavior produced by acute bolus injection of ethanol. The data reflect the mean (±SEM) percentage time spent in the open arms of an elevated plus maze during a 5 min test. Control rats spent ∼30% of the time in the open arms, and values that fall below controls reflect an increase in anxiety-like behavior because those animals spent less time in the open arms. We injected rats with either 10% w/v ethanol (3 g/kg, i.p.) or an equivalent volume of saline. Ten hours later, we placed rats in the center of the plus maze and recorded the time spent in the open and closed arms. Thirty minutes before plus-maze testing, separate groups of rats were injected with various doses of gabapentin (0, 30, 60, or 120 mg/kg, i.p.; $n = 8–10$ per dose). Ethanol produced an increase in anxiety-like behavior that was dose-dependently reversed by pretreatment with gabapentin, whereas gabapentin did not affect anxiety-like behavior in saline-injected controls. *$p < 0.05$ relative to saline control at same gabapentin dose; #$p < 0.01$ relative to ethanol-treated animals injected with vehicle.

entin that effectively reversed dependence-induced increases in anxiety-like behavior and ethanol self-administration in the present investigation are comparable with the range of gabapentin doses effective in treating other behavioral sequelae in rats, such as convulsive (Freitas et al., 2006) and nonconvulsive (Williams et al., 2006) seizures.

The role of the CeA in the systemic effects of gabapentin is strengthened by our findings that gabapentin infused directly into the CeA blocked dependence-induced increases in ethanol intake during withdrawal from an ethanol liquid diet. There was also a nonsignificant tendency for intra-CeA gabapentin to increase ethanol intake by nondependent rats, consistent with the opposite electrophysiological profiles of gabapentin in ethanol-dependent versus ethanol-naive rats. It is worth reiterating that rats in the CeA local injection study were exposed to chronic ethanol liquid diet, whereas rats in the systemic study were exposed to chronic intermittent ethanol vapor. However, past findings from our lab indicate that these two procedures produce similar BALs in rats, and that exposed rats reliably exhibit somatic and motivational symptoms associated with alcohol dependence during withdrawal (Gilpin et al., unpublished findings).

Together, these results suggest that activation of GABA systems in the CeA may selectively suppress ethanol self-administration by reversing a high-anxiety state that drives exces-
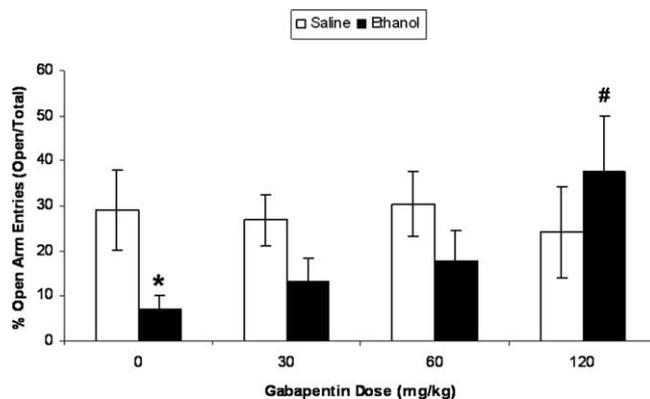
sive drinking in dependent animals. These findings also suggest an important gabapentin–ethanol interaction on GABAergic transmission in CeA that markedly adapts during development of ethanol dependence. The mechanism of gabapentin influence on naive CeA may be associated with its action on GABA$_B$ receptors, although other mechanisms may be involved. CeA neurons are predominantly GABAergic (Sun and Cassell, 1993; Cassell et al., 1999), either local inhibitory interneurons or inhibitory projection neurons that target brain-stem or other nuclei, such as bed nucleus of the stria terminalis. The fine-tuning of the GABAergic inhibitory system in the CeA is thus a prerequisite for controlling both local and output neurons projecting to these downstream nuclei. Because GABAergic drugs are typically robust anxiolytics (Kalueff and Nutt, 2006), it may seem paradoxical that gabapentin [and other anxiolytics such as nociceptin (Roberto and Siggins, 2006) and cannabinoids (unpublished data)] would depress GABAergic transmission in a brain region known to be involved in stress-related behavior. However, because gabapentin selectively suppresses dependence-induced increases in ethanol intake and also opposes ethanol actions in CeA neurons from ethanol-dependent rats, it may functionally influence neuronal responsivity of the inhibitory CeA gating that regulates information flow through the local intra-amygdaloidal circuits (e.g., by disinhibition of CeA), leading to an increased inhibition of the downstream regions.

The elimination of alcohol from blood after high-dose exposure (acute or chronic) produces increases in anxiety-like behavior in rodents, and that state is likely an important motivational factor in the compulsive ethanol-seeking behavior associated with ethanol dependence (Koob, 2006). Gabapentin, via modulation of GABAergic neurotransmission, may abolish this high-anxiety state and thus normalize ethanol intake. Although the acute withdrawal time point used to explore the behavioral effects of gabapentin may not have direct relevance to the human clinical situation (humans do not tend to relapse during acute with-

drawal), the withdrawal model effectively predicted the clinical efficacy of acamprosate (De Witte et al., 2005). Thus, our data may provide a similar rationale for the use of gabapentin as a potential treatment for certain components of the alcohol addiction cycle (Lee and Rodgers, 1990; Le et al., 1999; Anton et al., 2006; Heilig and Egli, 2006).

Table 1. Time spent ± SEM in the open and closed arms of the elevated plus maze and closed arm entries ± SEM by rats injected intraperitoneally with one of two doses (0.0 or 3.0 g/kg) of 10% w/v ethanol

Ethanol dose	Gabapentin dose (mg/kg)	n	Time spent in open arms (s)	Time spent in closed arms (s)	Closed arm entries
0.0 g/kg	0	8	35.70 ± 12.02	101.30 ± 18.26	26.00 ± 3.78
	30	7	42.60 ± 9.51	112.90 ± 7.61	23.71 ± 1.95
	60	8	54.75 ± 12.77	126.38 ± 5.53	23.13 ± 3.07
	120	8	29.00 ± 12.47	148.75 ± 6.44	19.88 ± 8.46
3.0 g/kg*	0	8	14.10 ± 6.39	189.00 ± 20.39	19.75 ± 3.85
	30	7	24.30 ± 10.97	148.80 ± 34.33	20.14 ± 2.68
	60	8	31.38 ± 11.45	164.63 ± 25.46	9.63 ± 2.35
	120	8	43.00 ± 16.54	130.00 ± 27.53	13.25 ± 4.86

The plus-maze test was administered 10 h after ethanol injection, and 30 min after intraperitoneal injection with one of four gabapentin doses (0, 30, 60, or 120 mg/kg). The plus-maze test lasted 300 s in total duration.

*$p < 0.05$ significant main effect of ethanol on closed arm entries.

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