Tolerance to Sedative/Hypnotic Actions of GABAergic Drugs Correlates With Tolerance to Potentiation of Extrasynaptic Tonic Currents of Alcohol-Dependent Rats

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Abstract

Liang J, Spigelman I, Olsen RW. Tolerance to sedative/hypnotic actions of GABAergic drugs correlates with tolerance to potentiation of extrasynaptic tonic currents of alcohol-dependent rats. J Neurophysiol 102: 224–233, 2009. First published May 6, 2009; doi:10.1152/jn.90484.2008. Alcohol tolerance resulting from chronic administration is well known to be accompanied by cross-tolerance to sedative/anesthetic drugs, especially those acting on the γ-aminobutyric acid type A receptors (GABA_ARs). Rats treated with chronic intermittent ethanol (CIE) show decreased function and altered pharmacology of GABA_ARs in hippocampal neurons, consistent with cell- and location-specific changes in GABA_A subunit composition. We previously observed variably altered sensitivity to GABAergic drugs in vivo and in hippocampal neurons using whole cell patch-clamp recording in brain slices. Here, we examined additional clinical GABAergic drugs to correlate CIE-induced tolerance to potentiation of neuronal GABA_A,R-mediated currents with tolerance of these agents to sedative/anesthetic effects in vivo. Typical of several drug classes and two cell types, in CA1 pyramidal neurons, the benzodiazepine diazepam doubled the total charge transfer (TCT) of miniature postsynaptic inhibitory currents (mIPSCs), whereas it quadrupled the TCT of tonic currents. CIE treatment altered these responses to variable extent, as it did to loss of righting reflex (LORR) induced by these same drugs: 90–95% tolerance to flurazepam, the neuroactive steroid alphaxalone, and ethanol; 30–40% to pentobarbital, etomidate, and the GABA agonist gaboxadol; and no tolerance to propofol. There was a strong correlation between tolerance in the LORR assay and tolerance to enhancement of tonic currents, but not mIPSCs. The striking correlation suggests that the sedative/anesthetic actions of GABAergic drugs may be mediated primarily via the potentiation of extrasynaptic GABA_ARs. This requires the reasonable assumption that the same types of GABA_ARs in other brain regions involved directly in hypnotic drug actions show similar tolerance.

Introduction

Alcohol consumption has profound effects on brain function and behavior. Two million Americans suffer from alcohol withdrawal syndrome (AWS) every year. AWS is a particularly severe manifestation of alcohol abuse, presenting with a variety of symptoms such as hyperexcitability, anxiety, insomnia, agitation, and sometimes seizures (Brower et al. 2001). The disrupted delicate balance between excitatory and inhibitory neurotransmission in the CNS after abruptly stopping chronic consumption of alcohol is believed to underlie the pathophysiology of AWS (De Witte 2004). How neurotransmitter systems are affected by alcohol and how these changes in neurotransmission contribute to AWS are poorly understood.

A major pharmacological target of ethanol (EtOH) is the γ-aminobutyric acid (GABA) inhibitory system (Koob 2004), especially GABA_A receptors (GABA_ARs). These are ligand-gated ion channels formed from a family of 19 related subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3) (Macdonald and Olsen 1994). The variable subunit composition accounts for different sensitivities to GABA, to biological regulatory mechanisms, and to modulatory drugs, such as benzodiazepines, barbiturates, neurosteroids, EtOH, and general anesthetics (Olsen and Homanics 2000; Wallner et al. 2003).

The hippocampus has been associated with sensorimotor processing; ventral hippocampal lesions in rats significantly reduce unconditioned and conditioned defensive behaviors, indicating a role for the ventral hippocampus in modulating anxiety-like behaviors (Bannerman et al. 2003; McNaughton and Gray 2000). The hippocampus is involved in both EtOH and general anesthetic actions on CNS functions and behaviors. EtOH administration alters hippocampal electroencephalogram and neuron firing rates (Grupp 1980; Klemm et al. 1976). Alcohol withdrawal increases electroencephalographic spiking in rat hippocampus (Veatch and Gonzalez 1996). Inhibition of the medial septum or the hippocampus by local injection of a GABA_A agonist, muscimol, increases the potency of general anesthetics to induce a loss of the tail-pinch response and a loss of righting reflex (LORR) (Ma et al. 2002). Other brain areas, including arousal circuits in brain stem and hypothalamus, are also considered important in the action of general anesthetics including GABAergic enhancers, such as etomidate (Rudolph and Möhler 2004).

Chronic intermittent ethanol (CIE) treatment of rats is an established model of alcohol withdrawal and dependence. Rats exposed to intermittent episodes (≥60 doses) of EtOH intoxication and withdrawal (approximating binge-drinking episodes in humans), leads to behavioral hyperexcitability that includes decreased pentyletetrazol-induced seizure threshold and increased anxiety (Becker and Hale 1993; Kokka et al. 1993). Studies in CIE-treated rats revealed various alterations in native GABA_A,R subunit composition and function (Cagetti et al. 2003), including a change in subcellular localization of α4 subunits from extrasynaptic to synaptic sites in hippocampal dentate gyrus granule cells (DGCs) (Liang et al. 2006). CIE rats also show remarkable alterations in the effectiveness of several clinical GABA_A,R modulatory drugs (Cagetti et al.
in the recording chamber. Stock solutions of pentobarbital (10 mM, Sigma) and etomidate (3 mM, Ben Venue Labs, Bedford, OH) were made with distilled water. Stock solutions of tetrodotoxin (TTX, in citrate buffer, 100 μM, Sigma), t(-)-2-amino-5-phosphonoopentanoate (APV, 4 mM, Sigma), and 6-cyano-7-nitroquinoxaline-2,3-dione sodium salt (CNQX, 1 mM) were prepared with distilled water. A stock solution of 5,7,8,9-tetrahydro-5-hydroxy-6H-benzocyclohepten-6-ylidenacetic acid (CGP 54626, 10 mM, Sigma) was made with pure DMSO.

**LORR assay**

The hypnotic effect of etomidate (20 mg/kg, administered intra-peritoneally [ip], in a volume of 10 ml/kg) was determined on saline- and CIE-treated rats (n = 12) using the standard LORR assay (Kakihana et al. 1966) as follows: after drug injection, rats were placed on their backs in a V-shaped trough when they lose consciousness and a timer was started. The duration of LORR ended when the animal was able to flip over three times in 30 s. The durations of LORR are reported as mean (minutes) ± SE. Statistical significance was analyzed with t-test.

**Slice preparation**

Transverse slices (400 μm thick) of dorsal hippocampus were obtained using our standard techniques. Briefly, rats were decapitated under halothane anesthesia and the brains were removed quickly and perfused with cold (~4°C) artificial cerebrospinal fluid (ACSF) composed of (in mM) 125 NaCl, 2.5 KCl, 2 CaCl2, 2 MgCl2, 26 NaHCO3, and 10 d-glucose (Sigma). The ACSF was continuously bubbled with 95% O2-5% CO2 to ensure adequate oxygenation of slices and a pH of 7.4. The brain was glued, at its frontal surface, to a Plexiglas platform and brain slices were prepared with a vibrating blade slicer (Campden Instruments 752M).

**Electrophysiological recording**

Whole cell patch-clamp recordings were obtained at 34 ± 0.5°C from cells located in the CA1 pyramidal or dentate gyrus granule cell layer at a holding potential of 0 mV. Patch electrodes were pulled from thin-wall borosilicate glass pipettes with resistances of 5.5–7.5 MΩ. Patch electrode filling solution contained (in mM): 135 cesium gluconate, 2 MgCl2, 1 CaCl2, 11 ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 HEPE, 2 ATP-K2, 0.2 GTP-7Na2 (pH adjusted to 7.25 with CsOH). GABA2A-R-mediated miniature postsynaptic inhibitory currents (mIPSCs) were pharmacologically isolated by adding 0.5 μM TTX, 40 μM APV, 10 μM CNQX, and 1 μM CGP 54626 to the perfusion. Signals were recorded in voltage-clamp mode with an amplifier (Axoclamp 2B, Molecular Devices, Union City, CA). Whole cell access resistances were in the range of 2.5 to 15 MΩ before electrical compensation by about 70%. During voltage-clamp recordings, access resistance was monitored by measuring the size of the capacitative transient in response to a −5-mV step command and the data were abandoned if changes >20% were encountered. At least 10 min was allowed for equilibration of the pipette solution with the intracellular milieu before commencing mIPSC recordings. Intracellular signal was low-pass filtered at 3 kHz and data were acquired with Digidata 1200B and pClamp 8.2 software (Molecular Devices) at a sampling frequency of 20 kHz.

**Data analysis**

All recordings were low-pass filtered off-line (Clampfit software) at 2 kHz with a digital Gaussian filter (Clampfit software, Molecular Devices). The mIPSCs were detected and analyzed as described previously (Cagetti et al. 2003) using the MiniAnalysis program (version 5.4; Synaptosoft, Decatur, GA) with threshold criteria of:

- **Preparation of drugs**
  Stock solutions of diazepam (1 mM, Sigma) and alphaxalone (3 mM, Sigma) and propofol (10 mM, Abbott Laboratories, North Chicago, IL) were made with pure dimethyl sulfoxide (DMSO, Fisher Scientific). The final concentration of DMSO did not exceed 42 μM
amplitude 5 pA and area 20 femtocoulombs (fC). Frequency of mIPSCs was determined from all automatically detected events in a given 100-s recording period. For kinetic analysis, only single-event mIPSCs with a stable baseline, sharp rising phase, and exponential decay were chosen during visual inspection of the recording trace. Double- and multiple-peak mIPSCs were excluded. The mIPSC kinetics were obtained from analysis of the averaged envelope of chosen single events (>120 events per 100-s recording period) aligned with half rise time in each cell. The mIPSC area was calculated as the integral of the averaged envelope (in fC). The total charge transfer of mIPSCs is the sum of the areas under all mIPSCs with respect to the baseline (Fig. 1B) in a 100-s recording period. All comparisons of group differences in mIPSC kinetics and tonic currents were made using two-way repeated-measures ANOVA with SAS software (v9, SAS Institute, Cary, NC). Statistical differences of the drug concentration dependences of responses between saline- and CIE-treated groups were tested with the slope parameters within the repeated-measures ANOVA model. The investigator performing the recordings and mIPSC analysis was blinded to the treatment (saline or CIE) the rats received.

RESULTS

Whole cell voltage-clamp recordings were performed on CA1 neurons or DGCs in hippocampal slices in the presence of TTX (0.5 μM), APV (40 μM), CNQX (10 μM), and CGP 54262 (1 μM). Under these conditions (in the absence of applied GABA), we were able to record both GABA_A_R-mediated persistent tonic currents and mIPSCs before and during application of drugs. We studied the CIE-induced changes in extrasynaptic GABA_A_R function by analyzing tonic currents and synaptic GABA_A_R function by analyzing mIPSCs.

To determine the relative contributions of synaptic and extrasynaptic GABA_A_Rs in neuronal inhibitory responses to sedative/anesthetic drugs, we first analyzed the synaptic and tonic currents in response to diazepam (DZ, 3 μM) followed by picrotoxin application in CA1 neurons from naïve rats (Fig. 1). DZ produced large increases in the holding current (I_hold), which was blocked by application of picrotoxin (50 μM), such that I_hold gradually dropped down to below control baseline (Fig. 1A). The GABA_A_R-mediated tonic current (I_tonic) was calculated as the difference between the pre-DZ I_hold and I_picro (Fig. 1A). Analysis revealed that for any given control recording period, the total charge transfer (TCT) of I_tonic greatly exceeded the TCT of mIPSCs (compare 8,000 to 333 fC; Fig. 1, B and C). We further calculated the TCT changes of mIPSCs and I_tonic in 100-s sections during diazepam and picrotoxin application. After DZ application the mIPSC TCT was increased from 8.73 ± 0.004 to 16.47 ± 0.012 pC, equivalent to 197.5 ± 21.1% of control, a nearly twofold increase (e.g., Fig. 1B). By contrast, the I_tonic TCT was increased from 2,020 ± 116 to 8,740 ± 375 pC after DZ application, equivalent to 440.5 ± 40.3% of control, greater than a fourfold increase (e.g., Fig. 1C). These results suggest that in hippocampal CA1 neurons, under the conditions used, tonic currents contribute the bulk of inhibitory charge transfer before drug application and DZ potentiates tonic currents to a greater extent than mIPSCs. This observation is somewhat surprising in light of the general assumption that synaptic GABA_A_Rs are the main

Extrasynaptic GABA_A_Rs produce significant inhibitory current that is modulated by benzodiazepines and other modulators such as sedative/anesthetic drugs.

FIG. 1. Differential contributions of synaptic and extrasynaptic γ-aminobutyric acid type A receptor (GABA_A_R)–mediated currents to neuronal inhibition are revealed by total charge transfer (TCT). A: CA1 neuron recording from a control rat before and during sequential perfusion of diazepam (DZ, 3 μM) and picrotoxin. The GABA_A_R-mediated control tonic current (I_tonic) was calculated as the difference between the pre-diazepam (DZ) holding current (I_hold) and I_picro. B and C: representative traces showing the calculation of TCT carried by miniature postsynaptic inhibitory currents (mIPSCs) and I_tonic before and after DZ. Note the much larger TCT contributed by I_tonic compared with the TCT of mIPSCs. Also note the larger DZ potentiation of I_tonic TCT compared with mIPSC TCT.
target of benzodiazepines. Other allosteric modulators with sedative/anesthetic actions were likewise found to enhance extrasynaptic tonic GABA<sub>A</sub>R currents more than synaptic currents, as shown in the following text. This was found in both CA1 pyramidal neurons and in DGCs.

**Determination of CIE-induced tolerance to various allosteric modulators of extrasynaptic and synaptic GABA<sub>A</sub>R-mediated responses in hippocampal CA1 neurons and DGCs**

We previously observed CIE-induced tolerance to modulation of GABA<sub>A</sub>R-mediated responses in CA1 neurons by benzodiazepines, neuroactive steroids, and gaboxadol, also known as THIP (Cagetti et al. 2003; Liang et al. 2004). In this study we extended the list of sedative/anesthetic tested and also examined modulation of GABA<sub>A</sub>R-mediated responses in DGCs. Further, we examined the effect of CIE on the in vivo actions of these drugs using the LORR assay for any agents we did not previously test.

**CIE-induced tolerance to diazepam, alphaxalone, and propofol in DGCs**

We studied the effects of the benzodiazepine DZ and the neuroactive steroid anesthetic, alphaxalone (alphax), application on DGCs. DZ (3 μM) potentiated the <i>I</i><sub>hold</sub> in DGC by 184% and the mIPSC area by 107% (Fig. 2, A and C). After CIE treatment, the effects of DZ on DGC <i>I</i><sub>hold</sub> (enhanced 15%) and on mIPSCs (enhanced 34%) were both dramatically decreased, as we previously also observed in CA1 neurons (Cagetti et al. 2003). The mIPSC area was reduced in CIE-treated rats in the absence of drugs, due to a more rapid decay rate, as we reported earlier for DGCs (Liang et al. 2006) and CA1 neurons (Cagetti et al. 2003). Alphaxalone concentration-dependently enhanced the <i>I</i><sub>hold</sub> by 37% at 0.3 μM and by 96% at 3 μM in DGCs from saline-treated rats (Fig. 2, B and D). Alphaxalone also increased the mIPSC area by 38% at 0.3 μM and 64% at 3 μM. After CIE treatment, the effect of alphaxalone on DGC <i>I</i><sub>hold</sub> was greatly attenuated, although its effect on mIPSCs remained intact. Thus <i>I</i><sub>hold</sub> was enhanced by 21 and 46% with 0.3 and 3 μM alphaxalone, respectively (Fig. 2D). The mIPSC area increased by 41 and 83% with 0.3 and 3 μM alphaxalone, respectively. The data summary shows that the concentration-dependent enhancement by alphaxalone on <i>I</i><sub>hold</sub> after CIE treatment was significantly reduced from that of the saline group (Fig. 2D, bottom), whereas there was no difference, or only a slight increase, in the concentration-dependent enhancement in mIPSC area of these two groups (Fig. 2D, top). No desensitization in the <i>I</i><sub>hold</sub> was observed in the presence of alphaxalone in neurons from either saline- or CIE-treated rats.

Finally, we tested the intravenous anesthetic propofol for modulation of GABA<sub>A</sub>R-mediated responses in DGCs and possible alteration by CIE. This drug enhanced both extrasynaptic...
aptic and synaptic GABA<sub>A</sub>R-mediated currents and did not show tolerance following CIE treatment (Fig. 2E). Propofol concentration-dependently enhanced the I<sub>hold</sub> by 16, 160, and 195% and increased the mIPSC area by 40, 59, and 80% at 3, 10, and 30 μM propofol, respectively, in neurons (Fig. 2E) from saline-treated rats. After CIE treatment, the effect of propofol on DGC I<sub>hold</sub> was slightly increased in DGCs. I<sub>hold</sub> was enhanced by 30, 196, and 231% and the area of mIPSC increased by 67, 97, and 139% with 3, 10, and 30 μM propofol, respectively (Fig. 2E).

**CIE-induced partial tolerance in extrasynaptic GABA<sub>A</sub>R-mediated tonic current in response to etomidate and pentobarbital, but not synaptic responses, whereas propofol showed no tolerance in enhancing both currents in CA1 neurons**

In hippocampal CA1 neurons from saline-treated rats, the GABAergic anesthetic etomidate concentration-dependently enhanced the I<sub>hold</sub> by 68, 154, and 241% at 3, 10, and 30 μM etomidate, respectively, in neurons (Fig. 3, A and B) from saline-treated rats. Etomidate also increased the mIPSC area by 61, 91, and 110% with concentrations of 3, 10, and 30 μM, respectively. After CIE treatment, the effect of etomidate on hippocampal CA1 neurons I<sub>hold</sub> was enhanced by 41, 100, and 193% with 3, 10, and 30 μM etomidate, respectively (Fig. 3, A and B). In contrast, etomidate produced a greater response of mIPSC area than that in neurons from CIE-treated rats. The areas of mIPSC were increased by 88, 156, and 215% with 3, 10, and 30 μM etomidate, respectively. No desensitization was observed in the presence of etomidate in either saline- or CIE-treated rats. These data suggest that the sensitivity of extrasynaptic GABA<sub>A</sub>Rs to etomidate was reduced, whereas the sensitivity of synaptic GABA<sub>A</sub>Rs was increased in CIE-treated rats.

Similar results were obtained with the general anesthetic pentobarbital. Pentobarbital concentration-dependently enhanced I<sub>hold</sub> by 141, 331, and 459% at 10, 30, and 100 μM pentobarbital in hippocampal CA1 neurons from saline-treated rats and this was reduced to 94, 254, and 327% by the same three concentrations after CIE. Compared with the result from saline-treated rats, the concentration-dependent enhancement of I<sub>hold</sub> in response to pentobarbital was reduced (Fig. 3C). Pentobarbital also produced an increase of mIPSC area by 39, 58, and 75% with concentrations of 10, 30, and 100 μM, respectively (Fig. 3C). After CIE, the mIPSC area was increased by 74, 123, and 165% with 10, 30, and 100 μM pentobarbital, respectively. In neurons from CIE-treated rats, pentobarbital produced a greater concentration-dependent response of mIPSC area than that in saline-treated rats (Fig. 3C). No desensitization was observed in the presence of pentobarbital in either saline- or CIE-treated rats. These data suggest that the sensitivity of extrasynaptic GABA<sub>A</sub>Rs to pentobarbital...
is reduced, whereas the sensitivity of synaptic GABA\textsubscript{A}Rs is increased in CIE-treated rats.

We also studied the effects of the general anesthetic propofol on hippocampal CA1 neurons. Propofol concentration-dependently enhanced \( I_{\text{hold}} \) by 17, 164, and 190% at 3, 10, and 30 \( \mu \text{M} \) propofol, respectively, in CA1 neurons from saline-treated rats. Propofol also increased mIPSC area by 22, 36, and 50% with the same three concentrations. After CIE treatment, a similar concentration-dependent response of \( I_{\text{hold}} \) to propofol was observed, increasing by 15, 163, and 197% with 3, 10, and 30 \( \mu \text{M} \) propofol, respectively. However, the mIPSC area was increased by 45, 88, and 93% by the same concentrations of propofol in CA1 neurons from CIE-treated rats, proportionately a greater effect than that in saline-treated rats (Fig. 3D).

No desensitization was observed in the presence of propofol in either saline- or CIE-treated rats. These data suggest that CIE treatment does not reduce the sensitivities of synaptic and extrasynaptic GABA\textsubscript{A}Rs in hippocampal CA1 neurons to propofol. The effects of CIE treatment on the responses in postsynaptic and extrasynaptic tonic currents to propofol are similar between CA1 neurons and DGCs (data not shown).

The effects of CIE on etomidate, pentobarbital, and propofol modulation of GABA\textsubscript{A}Rs in CA1 neurons with respect to other quantitative parameters of the mIPSCs were also determined (Fig. 4). Major changes in decay kinetics and amplitude of mIPSCs are consistent with the data for mIPSC area (Fig. 3), as reported previously for diazepam, alphaxalone, EtOH, and a few other drugs (Cagetti et al. 2003; Liang et al. 2004, 2006). Minor changes were found in the mIPSC frequency and rise time (Fig. 4).

FIG. 4. Effects of etomidate, pentobarbital, and propofol on kinetics of mIPSCs from CA1 neurons in saline- and CIE-treated rats. Summary graphs of changes in frequency, rise time (10–90%), amplitude, decay \( \tau_1 \), and decay \( \tau_2 \). Open circles represent effects of etomidate \((n = 7, 2 \text{ rats})\), pentobarbital \((n = 6, 3 \text{ rats})\), and propofol \((n = 6, 3 \text{ rats})\) on neurons from saline-treated rats. Black triangles represent effect of etomidate \((n = 7 \text{ cells}, 2 \text{ rats})\), pentobarbital \((n = 6, 3 \text{ rats})\), and propofol \((n = 8, 3 \text{ rats})\) on neurons from CIE-treated rats. * and † are as described in the legend of Fig. 2.

We previously tested the duration of LORR induced by a number of GABAergic drugs such as EtOH, flurazepam, al-
ANOVA. Percentage change in 14–17 rats tested by pentobarbital, 7 rats tested by propofol, 9–11 rats tested by LORR induced by pentobarbital and propofol was adapted from our previous and not phasic, GABAAR-mediated currents. The idea of ability of hippocampal neurons by potentiation of the tonic, 2004) exert most of their inhibitory effects on intrinsic excit- has been shown that the general anesthetics propofol, thiopen- and no tolerance (propofol) show corresponding degrees of partial tolerance (pentobarbital, etomidate, and gaboxadol), and GABAergic drugs with which CIE treatment produces a large degree of tolerance for LORR (EtOH, alfaxalone, flurazepam), partial tolerance (pentobarbital, etomidate, and gaboxadol), and no tolerance (propofol) show corresponding degrees of reduced potentiation of GABAAR-mediated tonic currents, but not synaptic currents (Table 1). These data show an excellent correlation between alterations of duration of LORR and the changes of \( I_{\text{hold}} \) in response to these drugs in hippocampal neurons after CIE treatment (Fig. 5).

**DISCUSSION**

**Effects of sedative/anesthetic GABAergic drugs on neurons, and thus possibly on LORR, are primarily mediated via enhancement of extrasynaptic GABAARs**

The purpose of these studies was to explore the role of GABAAR-mediated synaptic and extrasynaptic currents in responding to sedative/anesthetic drugs after CIE treatment. It has been shown that the general anesthetics propofol, thiopental (Bieda and MacIver 2004), and isoflurane (Caraíscos et al. 2004) exert most of their inhibitory effects on intrinsic excitability of hippocampal neurons by potentiation of the tonic, and not phasic, GABAAR-mediated currents. The idea of extrasynaptic GABAARs as major drug targets has been extended to the action of neurosteroids (Stell et al. 2003) and EtOH (Hanchar et al. 2005; Jia et al. 2007; Liang et al. 2006; Sundstrom-Poromaa et al. 2002; Wei et al. 2004).

We first evaluated the relative contribution of synaptic and extrasynaptic total charge transfer changes before and after sedative/anesthetic drug application in normal untreated rat hippocampal slices. Our results show, for the benzodiazepine (diazepam) action on CA1 neurons, that the GABAAR-mediated tonic current measured under our conditions contributes more of the inhibitory charge transfer both before and after the drug application and that the drug increases the tonic current charge transfer to a greater extent than the synaptic. A similar effect was found for seven representative GABAergic sedative/hypnotic/anesthetics and for two types of cells: CA1 pyramidal neurons and DGCs. Due to the need for long recording periods where several consecutive concentrations of drugs were applied, we were unable to apply picrotoxin for all the recordings such that the absolute \( I_{\text{tonic}} \) values could not be obtained in the current studies. The total charge transfer in a given cell in vivo depends on a number of variables including overall cellular and circuit activity and, especially, the extracellular GABA concentration, affecting parameters relevant to membrane physiology. Thus our observations in slices in vitro may not apply in vivo. Also, total charge transfer can be used to represent the function of tonic currents, but does not represent all the facets of synaptic currents, so despite the correlation in Fig. 5 between tolerance to drug-induced LORR and modulation of tonic currents but not synaptic currents, this does not totally exclude the contribution of synaptic current changes to the phenomenon of drug tolerance.

Nevertheless, we observed that CIE treatment induced full or partial loss of the \( I_{\text{hold}} \) responsiveness to most of these drugs. Propofol is an unexplained exception. These findings are con-

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**TABLE 1. CIE-induced changes in sedative/anesthetic drug effects**

<table>
<thead>
<tr>
<th>Drug</th>
<th>CA1 Neuron Response</th>
<th>DGC Response</th>
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<tbody>
<tr>
<td></td>
<td>Percentage change from control response</td>
<td></td>
</tr>
<tr>
<td>LORR</td>
<td>( m_{\text{PS}} )</td>
<td>( I_{\text{hold}} )</td>
</tr>
<tr>
<td>Benzodiazepine</td>
<td>(-90 \pm 3)</td>
<td>(-90 \pm 1)</td>
</tr>
<tr>
<td>Alfaxalone</td>
<td>(-92 \pm 1)</td>
<td>(-84 \pm 2)</td>
</tr>
<tr>
<td>Propofol</td>
<td>(-2 \pm 3)</td>
<td>(-4 \pm 3)</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>(-31 \pm 5)</td>
<td>(-24 \pm 7)</td>
</tr>
<tr>
<td>Etomidate</td>
<td>(-32 \pm 3)</td>
<td>(-48 \pm 4)</td>
</tr>
<tr>
<td>EtOH</td>
<td>(-91 \pm 3)</td>
<td>(+302 \pm 2)</td>
</tr>
<tr>
<td>Gadoxadol</td>
<td>(-36 \pm 6)</td>
<td>(+5172 \pm 14)</td>
</tr>
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Percentage change in LORR is defined as: \(|\text{Duration of LORR induced by a drug in CIE rat} - \text{duration of LORR induced by the same drug in vehicle rat}|/\text{Duration of LORR induced by the same drug in vehicle rat}| \times 100\%. LORR induced by pentobarbital and propofol was adapted from our previous reports (Cagetti et al. 2003). Each number represents a mean averaged from 14–17 rats tested by pentobarbital, 7 rats tested by propofol, 9–11 rats tested by etomidate, and 12–14 rats tested by EtOH. Statistical difference was determined by ANOVA. Percentage change in \( I_{\text{hold}} \) is defined as \( (\Delta I_{\text{hold}})/\text{vehicle } I_{\text{hold}} \times 100\%. (1) \) Benzodiazepine: flurazepam was assayed for LORR; diazepam was assayed for slices.
consistent with our hypothesis that alterations in the composition and localization of α1, α4, γ2, and δ subunits after CIE treatment are responsible for the changes in GABAergic function and pharmacological sensitivity to sedative/anesthetic drugs. The findings also provide additional support for the idea that tonic currents play a major role in the physiological response to sedative/anesthetic drugs because we observed a striking correlation in the extent of CIE-induced tolerance to the soporific action of these drugs and the extent of CIE-induced tolerance to modulation of extrasynaptic GABAergic-mediated tonic currents (Fig. 5) but not synaptic currents (Table 1).

This major finding demonstrates that CIE treatment alters the sensitivities of extrasynaptic GABAergicRs in parallel to the altered in vivo sensitivity to the sedative/anesthetic drugs. Although the hippocampus may contribute to the general state of excitability, arousal, and sedative drug action, we do not suggest it mediates these actions. Rather, we suggest, and think it reasonably likely, that similar plasticity occurs in extrasynaptic GABAergicRs in other brain regions critical for sedative drug action—thus the effects of sedative/anesthetic GABAergic drugs on LORR are primarily mediated via enhancement of extrasynaptic GABAergicRs. Further, their role in drug-reward circuits deserves more attention. Without pushing the concept too far, it is fair to note that these GABAergicR subtypes are also likely to play a role in endogenous sleep circuits and mechanisms.

CIE-induced changes in extrasynaptic GABA$_{1}R$ composition and localization

A comparison of αβδ and αβγ2 GABAergicRs studied in expression systems revealed that the δ-containing GABAergicRs have a greater current enhancement in response to etomidate, pentobarbital, propofol, and neuroactive steroids, such as tetrahydrodeoxy-corticosterone and alphaxalone, than that of the γ2-containing GABAergicRs (Brown et al. 2002; Wallner et al. 2003; Wohlfarth et al. 2002). Hippocampal DGs express the α4/δ combination primarily in perisynaptic and extrasynaptic membrane sites (Wei et al. 2003). The δ-containing GABAergicRs have been suggested to generate tonic inhibition, to prevent seizures and to serve as preferential targets for neuroactive steroids (Stell et al. 2003; Wohlfarth et al. 2002). The preferential partnering of δ with α4 and α6 but not α1 is consistent with the preferential action of the nonbenzodiazepine sedatives on these receptors relative to α1, 2, 3, and 5 subunits, whose preferential partner is the benzodiazepine-sensitive γ2 and is consistent with maintained sensitivity to pentobarbital and etomidate in the α1 knockout mice (Blednov et al. 2003). On the other hand, under our conditions, benzodiazepine-sensitive GABAergicRs carry some of the tonic current in hippocampal neurons and CIE treatment reduces this drug action, apparently correlated with reduced levels of the α1 subunit. In CIE-treated rats, δ and α1 subunit protein levels persistently decrease in the hippocampus, whereas γ2 and α4 proteins increase (Cagetti et al. 2003; Liang et al. 2006). In the current study, the loss of alphaxalone potentiation of tonic currents in hippocampal DGs indicates a functional down-regulation of δ-containing GABAergicRs in CIE-treated rats resembling the situation in δ subunit knockout mice (Mihalek et al. 1999; Spigelman et al. 2003), as well as GABA$_{1}R$ α4 subunit knockout mice (Liang et al. 2008). We suggest that the sedative/anesthetic actions of the nonbenzodiazepines are preferentially mediated by α4βδ-containing extrasynaptic GABAergicRs. The precise mechanisms of CIE-induced alterations in GABAergic subunit composition and localization remain to be determined. Our studies on the plasticity induced by a single large dose of EtOH are aimed at further understanding mechanisms of dependence (Liang et al. 2007).

CIE-induced changes in synaptic GABA$_{1}R$ composition and localization

In the present and previous studies (Liang et al. 2004, 2006), we observed that the responsiveness of GABA$_{1}R$-mediated mIPSCs to several sedative/anesthetic drugs such as pentobarbital, propofol, etomidate, gaboxadol, and EtOH were enhanced after CIE treatment (Table 1). Synaptic GABAergicRs have been identified to be mainly composed of α1β2γ2 subunits (reviewed in Farrant and Nusser 2005; Moss and Smart 2001). The α1β2γ2 comprise roughly 40% of all GABAergicRs in the mammalian brain. The α1-containing GABAergicRs have a distinct pharmacology, including high sensitivity to the modulatory effects of the benzodiazepine agonists, such as diazepam and zolpidem. In contrast, the α4-containing GABAergicRs have been localized primarily at extrasynaptic sites (Sun et al. 2004; Wei et al. 2003) and they are insensitive to the modulatory effects of diazepam and zolpidem, but are enhanced by brexarenol and the benzodiazepine negative modulator Ro15-4513 (Cagetti et al. 2003). The α4δ-containing GABAergicRs are sensitive to low [EtOH] (Wallner et al. 2006; but see Borgbøe et al. 2006). The α1- and α4-containing GABAergicRs exhibit considerable pharmacological differences between the synaptic and extrasynaptic GABAergicRs (Bai et al. 2001; Farrant and Nusser 2005). Once these two distinct populations of receptors are modified, e.g., in their localization in synaptic and extrasynaptic sites or their subunit composition, by any mechanism (e.g., CIE treatment or status epilepticus), they lead to changes in the relative pharmacological sensitivities of the synaptic or extrasynaptic GABAergicRs-mediated currents to drugs (Brooks-Kayal et al. 1998; Cagetti et al. 2003; Liang et al. 2004, 2006). This includes the relative benzodiazepine and anesthetic affinities. We have demonstrated previously by electron microscopy that α4-containing GABAergicRs location changes from extra- and perisynaptic to central synaptic sites in the molecular layer of the dentate gyrus after CIE treatment and withdrawal (Liang et al. 2006). Our current data provide further pharmacological evidence that α4-containing GABAergicRs “switch” to synaptic locations where they contribute to the enhanced responsiveness of GABAergic synaptic currents to the sedative/anesthetic drugs after CIE treatment. How these responses to chronic EtOH are developed in the GABAergic α4 subunit knockout mouse (Liang et al. 2008) should be interesting.

Relationship of CIE-induced changes to alcoholism

The rat CIE model of alcohol dependence mimics that of human dependence in that heightened and persistent withdrawal signs are induced by our regimen of chronic ethanol administration. The in vivo behavioral changes in CIE-treated rats, which include decreased seizure thresholds (Kokka et al. 1993), heightened anxiety (Liang et al. 2006), and tolerance to sedative/anesthetic drugs (Cagetti et al. 2003; this study), are
much like the symptoms observed in human alcoholics (Brower et al. 2001; Roehrs and Roth 2001). However, the high dosage of ethanol and 60 repeated episodes of severe intoxication and mini-withdrawal are a rather severe and unusual treatment, although we claim not inappropriate as a model. We have demonstrated that there is little or no measurable pathology in the CIE rats of both nervous system and liver (Olsen, unpublished data). The results reported here suggest that in hippocampal CA1 neurons and DGCs, CIE treatment induces functional and pharmacological changes in GABAARs, consistent with “switches” of α1 subunits to α4 subunits and a down-regulation of δ-containing GABAARs. The GABAAR subunit changes may underlie the in vivo behavioral changes. These remarkable plasticity changes are likely to be produced to some extent even by less severe alcohol regimens. Thus our results suggest that alcoholic hyperexcitability is partly due to decreases in both synaptic and extrasynaptic GABAAR-mediated tonic inhibition, an important regulator of neuronal excitability. The decreased potentiation of inhibitory GABAergic tonic currents resulting from subunit changes after CIE treatment may underlie the tolerance to the soporific action of sedative/anesthetics. We further propose a tentative conclusion that one of the most difficult aspects of alcohol dependence to treat clinically is the insomnia because traditional antianxiety aids and EtOH lose their effectiveness as sedative/hypnotics (Brower et al. 2001; Roehrs and Roth 2001). GABAergic sleep aids that are active on those GABAAR subtypes found in CIE-treated rats may retain their usefulness for alcoholic insomnia. By contrast, synaptic GABAARs are unlikely to be major contributors to sedative/anesthetic actions. It will be important to assess the profile of traditional GABAergic anxiolytics and anticonvulsants in the alcoholic rat model for possible cross-tolerance and ineffectiveness with symptoms of alcohol withdrawal including heightened anxiety and seizures. These results advance our understanding of the GABAergic mechanisms that underlie behaviors such as anxiety and hyperexcitability that accompany alcohol consumption and withdrawal and may lead to improved therapies.

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