Plasticity of GABA_A receptor-mediated neurotransmission in the nucleus accumbens of alcohol-dependent rats

Jing Liang,1,2 A. Kerstin Lindemeyer,2 Asha Suryanarayanan,1 Edward M. Meyer,1 Vincent N. Marty,1 S. Omar Ahmad,3 Xuesi Max Shao,4 Richard W. Olsen,2 and Igor Spigelman1

1Division of Oral Biology and Medicine, School of Dentistry, University of California, Los Angeles, California; 2Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, California; 3Doisy College of Health Sciences, Saint Louis University, St. Louis, Missouri; and 4Department of Neurobiology, David Geffen School of Medicine, University of California, Los Angeles, California

Submitted 8 August 2013; accepted in final form 2 April 2014

Plasticity of GABA_A receptor-mediated neurotransmission in the nucleus accumbens of alcohol-dependent rats. J Neurophysiol 112: 39–50, 2014. First published April 2, 2014; doi:10.1152/jn.00565.2013.—Chronic alcohol exposure-induced changes in reinforcement mechanisms and motivational state are thought to contribute to the development of cravings and relapse during protracted withdrawal. The nucleus accumbens (NAcc) is a key structure of the mesolimbic dopaminergic reward system and plays an important role in mediating alcohol-seeking behaviors. Here we describe the long-lasting alterations of γ-aminobutyric acid type A receptors (GABA_ARs) of medium spiny neurons (MSNs) in the NAcc after chronic intermittent ethanol (CIE) treatment, a rat model of alcohol dependence. CIE treatment and withdrawal (>40 days) produced decreases in the ethanol and Ro15-4513 potentiation of extrasynaptic GABA_ARs, which mediate the picrotoxin-sensitive tonic current (I_tonic), while potentiation of synaptic receptors, which give rise to miniature inhibitory postsynaptic currents (mIPSCs), was increased. Diazepam sensitivity of both I_tonic and mIPSCs was decreased by CIE treatment. The average magnitude of I_tonic was unchanged, but mIPSC amplitude and frequency decreased and mIPSC rise time increased after CIE treatment. Rise-time histograms revealed decreased frequency of fast-rising mIPSCs after CIE treatment, consistent with possible decreases in somatic GABAergic synapses in MSNs from CIE rats. However, unbiased stereological analysis of NeuN-stained NAcc neurons did not detect any decreases in NAcc volume, neuronal numbers, or neuronal cell body volume. Western blot analysis of surface subunit levels revealed selective decreases in α1 and δ and increases in α4, α5, and γ2 GABA_A subunits after CIE treatment and withdrawal. Similar, but reversible, alterations occurred after a single ethanol dose (5 g/kg). These data reveal CIE-induced long-lasting neuroadaptations in the NAcc GABAergic neurotransmission.

ALCOHOL, one of the oldest drugs, is lawfully consumed in our society for its taste, caloric value, and psychoactive properties. However, excessive alcohol drinking can lead to alcohol dependence and loss of control over alcohol consumption, with serious detrimental health consequences (Room et al. 2005). Alcohol dependence is a chronic relapsing disorder involving cycles of alcohol intoxication and withdrawal. The severity of withdrawal is positively correlated to the number of intoxication and withdrawal cycles, culminating in the alcohol withdrawal syndrome with symptoms that include anxiety, insomnia, agitation, and increased susceptibility to seizures (McKeon et al. 2008). Chronic intermittent alcohol (ethanol, EtOH) exposure is thought to gradually, with each cycle of intoxication and withdrawal, decrease reward signaling while sensitizing the brain stress response system (Koob and Le Moal 2008). The resultant opposing motivational processes are hypothesized to contribute to the development of cravings and relapse. Relapse to alcohol is a critical problem in treating alcoholism, and effective treatments are yet to be found.

The nucleus accumbens (NAcc) is a key neural substrate for the rewarding actions of many drugs of abuse, including alcohol (Koob et al. 1998; Wise 2004). The NAcc is mainly (~95%) composed of GABAergic medium spiny neurons (MSNs) that project massively to the ventral pallidum, substantia nigra, and the ventral tegmental area (VTA) (Chang and Kitai 1985; Nauta et al. 1978). Optogenetic studies have demonstrated that MSNs in the NAcc specifically target GABAergic neurones in the VTA without making direct synaptic contacts with VTA dopamine neurons (Xia et al. 2011). MSNs also form functional GABAergic interconnections within the NAcc (Taverna et al. 2004). MSNs receive extensive glutamatergic inputs from prefrontal cortical areas, hippocampus, thalamus, and basolateral amygdala (Brog et al. 1993; Sesack and Grace 2010). The remaining neurons in the NAcc are subpopulations of local circuit cholinergic and GABAergic interneurons (Bennett and Bolam 1994; de Quitt and Emson 1986; Hussain et al. 1996; Vincent and Johansson 1983; Vincent et al. 1983; Zhou et al. 2002). In addition to the well-described dopaminergic input from the VTA (Beckstead et al. 1979; Dahlström and Fuxe 1964; Fallon and Moore 1978; Swanson 1982), GABAergic neurones from the VTA also send projections to the NAcc (Van Bockstaele and Pickel 1995), thus providing a reciprocal inhibitory loop to this mesolimbic circuit.

In vivo MSNs oscillate between “up” and “down” states (O’Donnell and Grace 1995). The “up” state is brought on by synaptic activation of glutamatergic receptors, which depolarizes the MSN neurons to spike firing threshold. On the basis of recordings in behaving animals it has been suggested that the “up” state is the time when NAcc neurons execute their behaviorally relevant functions (O’Donnell et al. 1999). The patterns of spike discharge are thus sculpted mainly by a combination of glutamatergic excitation, GABAergic inhibition, and modulatory influences of dopamine. Recently, we
found persistent alterations in the intrinsic electrical membrane properties, K\(^+\) currents, and glutamatergic synaptic transmission of MSNs in the NAcc core of rats following chronic intermittent ethanol (CIE) treatment (Marty and Spigelman 2012), a model of alcohol dependence (Spigelman et al. 2013). Here we studied GABA\(_A\) receptor (GABA\(_{AR}\))-mediated currents in NAcc core MSNs in CIE rats and vehicle control (CIV) rats in order to obtain a more comprehensive picture of changes in mesolimbic system signaling in alcohol dependence.

Our data indicate that CIE treatment produces alterations in the subunit composition and function of MSN GABA\(_{AR}\)s as well as alterations in the synaptic release of GABA. Unlike the reversible plasticity of GABA\(_{AR}\)s induced by single EtOH doses, these alterations are long-lasting and are expected to affect information transfer in the mesolimbic system in a predictable manner.

**METHODS**

**Animals.** The Institutional Animal Care and Use Committee approved all animal experiments. Male Sprague-Dawley rats (weighing 190–220 g upon arrival) were housed in the vivarium under a 12:12-h light-dark cycle and had free access to food and water. Rats were randomly assigned to two groups, a control group and a chronic intermittent EtOH (CIE) regimen group. The CIE group for the first 5 doses received 5 g/kg of EtOH (Pharmco Products, Brookfield, CT) as a 25% (wt/vol) solution in water by oral intubation every other day, followed by 55 doses of 6 g/kg of 30% EtOH every day. The control vehicle (CIV) rats received water (20 ml/kg). These treatments were followed by 40–100 days of withdrawal prior to electrophysiological or biochemical experiments. Alternatively, rats were administered a single dose of EtOH (5 g/kg) or water (20 ml/kg) by oral intubation, followed by 1 h to 14 days of withdrawal.

**Electrophysiological recordings.** Transverse brain slices (400 \(\mu\)m thick) containing the NAcc were obtained with standard techniques. Briefly, rats were decapitated under isoflurane anesthesia and brains were quickly removed, trimmed with a razor blade, and glued to the base of a cutting chamber (Leica VT1200S) filled with cold (\(-4^\circ\)C) artificial cerebrospinal fluid (ACSF) composed of (in mM) 125 NaCl, 2.5 KCl, 2 CaCl\(_2\), 2 MgCl\(_2\), 26 NaHCO\(_3\), and 10 D-glucose (Sigma). The ACSF was continuously bubbled with a 95%-5% mixture of O\(_2\) and CO\(_2\) to ensure adequate oxygenation of slices and a pH of 7.4. Patch electrode filling solutions contained (in mM) 135 cesium gluconate, 2 MgCl\(_2\), 1 CaCl\(_2\), 11 ethylene glycol-bis(\(\beta\)-aminoethylether)\(-N,N,N',N'\)-tetraacetic acid (EGTA), 10 HEPES, 2 ATP-K\(_2\), and 0.2 GTP-Na\(_2\); pH adjusted to 7.25 with CsOH. Patch electrode and probe assembly targeting the region of interest were advanced with a four-axis motorized manipulator (MX7600, Siskiyou, Grants Pass, OR) and controller (MC1000e, Siskiyou) with the aid of a dissecting microscope (\(\times7–45\), SZ61, Olympus). Whole cell patch-clamp recordings were obtained with a Multiclamp 700B amplifier and a Digidata 1440A A/D converter (Molecular Devices, Sunnyvale, CA) from cells in the NAcc core region during perfusion (1 ml/min) with oxygenated ACSF at 34 ± 0.5°C. Most cells were putatively identified as MSNs within 30 s of membrane breakthrough by the presence of a characteristic delay in action potential generation with depolarizing current pulses (Marty and Spigelman 2012); those that did not possess these characteristics were discarded. Next, GABA, R-mediated currents were separated pharmacologically by application of TTX (0.5 \(\mu\)M), CNQX (10 \(\mu\)M), APV (40 \(\mu\)M), and CGP54626 (1 \(\mu\)M) in the ACSF. Cells were subsequently voltage-clamped at 0 mV, and recordings began at least 10 min after membrane breakthrough to ensure adequate dialysis by intrapipette contents. EtOH (10–100 mM), diazepam (DZ, 0.3 \(\mu\)M), and Ro15-4513 (0.3 \(\mu\)M) were applied after appropriate dilution in the ACSF.

**Detection and analysis of miniature inhibitory post-synaptic currents and tonic current**

The recordings were low-pass filtered (Clampfit software, Molecular Devices) at 2 kHz and analyzed with the aid of the Mini Analysis Program (Synaptosoft, Fort Lee, NJ). Miniature inhibitory postsynaptic currents (mIPSCs) were detected with threshold criteria of 5-pA amplitude and 20-\(\mu\)C charge transfer. Frequency of mIPSCs was determined from all automatically detected events in a given 100-s recording period. Tonic current magnitudes were obtained from the mean baseline current of a given recording period. For kinetic analysis of mIPSCs, only single events with a stable baseline, sharp rising phase, and exponential decay were chosen. Double and multiple-peak mIPSCs were excluded. The mIPSC kinetics were obtained from analysis of the averaged chosen single events (>120 events/200-s recording period) aligned with half rise time in each cell. Decay time constants were obtained by fitting a double-exponential equation to the falling phase of the averaged mIPSCs of the form \(I(t) = I_1 \times \exp(-\tau_1 t) + I_2 \times \exp(-\tau_2 t)\), where \(I_1\) and \(I_2\) are the amplitudes of the fast and slow decay components and \(\tau_1\) and \(\tau_2\) are their respective decay time constants used to fit the data. To compare decay times, we used a weighted mean decay time constant: \(\tau_{\text{w}} = \frac{\int_0^\infty I(t) \cdot t \cdot \exp(-\tau t) dt}{\int_0^\infty I(t) \cdot \exp(-\tau t) dt}\). Histograms of rise times and other mIPSC kinetics were built with the data analysis and graphing software Origin (V7, OriginLab, Northampton, MA). The histograms were fitted with the multipeaks (Gaussian) fitting tool in Origin.

**Membrane preparation and Western blotting.** NAcc sections were microdissected from individual 400-\(\mu\)m-thick brain sections. Both core and shell regions of the NAcc were included because previous studies did not detect any major differences in the distribution of GABA\(_{AR}\) subunits between these regions (Pirker et al. 2000). Sections were incubated in a small-volume chamber with or without the protein cross-linking reagent bis(sulfosuccinimidyl)suberate (BS\(_3\)) in ACSF at 4°C according to Grosshans et al. (2002). BS\(_3\) is bifunctional and cross-links all proteins exposed to the medium, i.e., cell surface proteins. These large complexes are retained at the top of the gel; thus the band of protein at the identified molecular weight corresponds to that fraction that is intracellular only. The difference between that value and the amount from an equivalent adjacent slice, untreated, and thus total, represents the surface pool. After incubation of NAcc sections in the absence (total) or presence (intracellular) of BS\(_3\), sections were washed three times with Tris wash buffer (pH 7.6) to wash away free BS\(_3\). The washed sections were homogenized in a buffer composed of 1% SDS, 1 mM EDTA, and 10 mM Tris, pH 8.0. Protein content was measured by the Bio-Rad D\(_2\) protein assay system. Protein aliquots (15–40 \(\mu\)g) from samples were separated on 10% SDS-polyacrylamide gel electrophoresis under reducing conditions with the Bio-Rad Mini-PROTEAN 3 Cell system. Proteins were transferred to PVDF membranes (Immun-Blot PVDF membrane, 0.2 mm, Bio-Rad) with Mini-PROTEAN 1 Trans-Blot (Bio-Rad) or the Trans-Blot SD semidyve transfer cell system (Bio-Rad). Blots were probed with anti-peptide \(\alpha\) (proprietary N-terminus synthetic antigen, Novus Biologicals, Littleton, CO), \(\alpha\)2 (aa322–357), \(\alpha\)3 (aa459–467), \(\alpha\)4 (aa379–421), \(\alpha\)5 (aa337–382), \(\gamma\)2 (aa319–366), or \(\delta\) (aa331–430, Santa Cruz Biotechnology) antibodies (all at 1 mg/ml), followed by HRP-conjugated anti-rabbit (Millipore, Billerica, MA), and bands were detected with the Amersham ECL Plus detection kit (GE Healthcare Life Sciences, Piscataway, NJ), apposed to X-ray film (GE Healthcare Life Sciences, Piscataway, NJ), apposed to X-ray film (Fujifilm, Tokyo, Japan). W. Sieghart and colleagues at the Medical University of Vienna, Vienna, Austria kindly provided the \(\alpha\)1, \(\alpha\)2, \(\alpha\)3, \(\alpha\)4, \(\alpha\)5, and \(\gamma\)2 GABA\(_{AR}\) subunit antibodies. These antibodies have been previously extensively verified by various methods including immunoprecipitation, Western blotting, and immunocytochemistry (Mossier et al. 1994; Zezula et al. 1991; Zimprich et al. 1991). A \(\beta\)-actin antibody (aa1–100; Abcam, Cambridge, MA) was used as a loading control in all experiments. Bands from different samples corresponding to the appropriate subunit were analyzed and absorbance
values compared by densitometry using either C.IMAGING analysis systems (Complix, Cranberry Township, PA) or ImageJ (National Institutes of Health, Bethesda, MD). Group differences were evaluated by t-test. P < 0.05 was considered statistically significant.

Histology. CIV (n = 4) and CIE (n = 4) rats were anesthetized with pentobarbital (100 mg/kg ip) and each perfused transcardially first with ~200 ml of cold NaCl (0.8%) solution containing dextrose (0.4%), sucrose (0.8%), CaCl₂ (0.023%), and sodium cacodylate (0.034%) followed by ~300 ml of a fixative solution composed of paraformaldehyde (4%), sucrose (4%), and sodium cacodylate (1.4%). Brains were postfixed overnight in a sodium cacodylate (1.4%) storage buffer. Subsequently, brains were embedded with the Multibrain Technology (NeuroScience Associates, Knoxville, TN) and freeze-sectioned at 60 μm in the coronal plane, every sixth section (360-μm intervals) was processed for NeuN immunohistochemistry to reveal the neuron-specific marker protein and counterstained with Neutral Red, and individual sections were mounted on slides and coverslipped.

Stereology. NeuN-stained cell bodies were quantitatively and qualitatively evaluated for both neuronal number and cell body volume. Estimates were performed ipsilaterally. Brain area was defined anatomically by atlas and the agreement of two investigators (Paxinos and Watson 1998). Every eighth section of NAcc was selected from a random initial sort to ensure random overall sampling. Additionally, the MultiBrain Technology used for the histological procedure ensures that brains embedded in gelatin prior to sectioning possess enough variance to provide a random and unbiased sampling of each of a potential 16 brain sections on each slide (although only 8 were used for this study). The optical fractionator method and the Stereologer software package (Mouton 2000) were used to count NeuN-stained cells. The study used a Nikon Eclipse 80i microscope, linked to a Sony 3CCD Color Digital Video Camera, which operated an Advanced Scientific Instrumentation MS-2000 motorized stage input into a Dell Precision 650 Server and a high-resolution plasma monitor.

The areas of interest were defined with a ×4/1.3 aperture dry lenses, and the stereology was performed at high magnification with ×100/1.4 aperture oil immersion lenses (yielding ×3,600) that allow for clear visualization of the nucleolus and precise definition of the cell walls. When the areas of interest were identified, areas were precisely outlined and checked against an atlas (Paxinos and Watson 1998). The inclusion grid was randomly applied by the software, and high-resolution microscopy was then used to count NeuN-stained cells. Additionally, the nucleator method was performed on each counted neuron to assess cell body volume.

Statistical analysis. The investigators performing the recordings, mIPSC, Western blot, and stereology analysis were blind to the treatment (CIV or CIE) that the rats received. All summary values are presented as means ± SE. Group differences were evaluated by unpaired Student’s t-test, one-way analysis of variance (ANOVA), two-way ANOVA, or two-way repeated-measures (RM) ANOVA, where appropriate. P < 0.05 was considered statistically significant.

RESULTS

CIE-induced switch in EtOH responsiveness from extrasynaptic to synaptic GABAₐRs. After putative identification of NAcc neurons as MSNs in current-clamp recordings, we switched to voltage clamp and recorded GABAₐR currents during pharmacological blockade of ionotropic glutamate receptors, GABAₐRs, and voltage-gated sodium channels. Under these recording conditions GABAₐR currents could be separated into two components: a nondesensitizing tonic current (I头条otonic) and phasic mIPSCs, which are known to be mediated by extrasynaptic and synaptic GABAₐR activation, respectively (Mody and Pearce 2004). Acute application of EtOH (10–100 mM) in MSN recordings from CIV-treated rats preferentially potentiated the picrotoxin-sensitive I头条otonic, whereas the charge transfer (area) of phasic mIPSCs was unaffected by the same concentrations of applied EtOH (Fig. 1). In contrast, in MSN recordings from CIE-treated rats, it was the mIPSC area that was significantly potentiated by EtOH (50–100 mM), whereas the I头条otonic was completely insensitive to potentiation by EtOH (10–100 mM). The increases in mIPSC charge transfer resulted primarily from significant increases in decay time constants (τ₁ and τ₂) without significant increases in mIPSC amplitude (Fig. 1C). There was a trend toward increased frequency of mIPSCs in MSNs from both CIV (~7%, P = 0.13) and CIE (~9%, P = 0.08) rats, but only by 10 mM EtOH. The rise time (10–90%) of mIPSCs was also not significantly affected by acute EtOH (10–100 mM) application in MSNs from CIV or CIE rats (Fig. 1C). In the absence of significant effects of acute EtOH on mIPSC frequency, our data are consistent with CIE-induced increases in responsiveness of synaptic GABAₐRs and decreases in responsiveness of extrasynaptic GABAₐRs to acute EtOH.

CIE-induced switch in Ro15-4513 responsiveness of extrasynaptic and synaptic GABAₐRs. Previous studies in other brain regions suggested that the EtOH-sensitive extrasynaptic GABAₐRs of CIV rats may be composed of α4β6 subunit combinations (Sundstrom-Poromaa et al. 2002; Wallner et al. 2003), which may be replaced by synaptic apparently EtOH-sensitive α4βγ2 GABAₐRs after CIE treatment (Liang et al. 2007). To provide additional evidence and explore the effect of CIE treatment on responses to Ro15-4513, a partial inverse agonist at the benzodiazepine (BZ) site of α1- and α2-containing GABAₐRs, which was also shown to bind with high affinity at α4-containing GABAₐRs (Knoflach et al. 1996). Importantly, Ro15-4513 has agonist activity at α4βγ2 GABAₐRs but does not modulate α4β3δ GABAₐRs (Brown et al. 2002). Application of Ro15-4513 (0.3 μM) preferentially potentiated I头条otonic (~80%) compared with mIPSCs (~18%) in MSN recordings from CIV rats (Fig. 2, A and B), suggesting a significant contribution of α4βγ2 GABAₐRs to the tonic inhibitory current. In contrast, similar applications of Ro15-4513 in recordings from CIE rat MSNs preferentially potentiated mIPSCs (~40%) compared with I头条otonic (~18%) (Fig. 2, A and B). The potentiation of mIPSCs by Ro15-4513 was mainly due to the slowing of the rise and decay rates of mIPSCs (Fig. 2C). Ro15-4513 application had no effect on the frequency of mIPSCs in MSNs from either CIV or CIE rats (Fig. 2C). These data provided further pharmacological evidence for CIE-induced increases in contribution of α4βγ2 GABAₐRs to synaptic currents.

CIE-induced decreases in diazepam responsiveness of extrasynaptic and synaptic GABAₐRs. To provide additional pharmacological evidence for the specificity of CIE-induced GABAₐR subunit plasticity, we tested mIPSC and I头条otonic responsiveness to acute DZ (0.3 μM) application. Since DZ has no agonist activity at α4 (or α6)-containing GABAₐRs but potentiates other αβγ2 GABAₐRs (Möhler et al. 2000), we expected that increases in α4-containing GABAₐRs would result in diminished potentiation of currents mediated by either synaptic or extrasynaptic GABAₐRs. In NAcc slices from CIV rats, DZ increased the magnitude of I头条otonic and increased the charge transfer of mIPSCs primarily by prolonging their decay, although there was also a small (~5%) but significant increase in mIPSC amplitude (Fig. 3). In contrast, in MSN recordings

J Neurophysiol • doi:10.1152/jn.00565.2013 • www.jn.org

Downloaded from http://jn.physiology.org/ on 2023/06/23 by 12.202.23.3
The composition of GABAARs affects both their pharmacology and synaptic GABAARs. However, alterations in mIPSC frequency with possible alterations in the subunit composition of post-synaptic GABAARs. Subsequent application of picrotoxin (50 μM) reveals the I_{tonic} component. After CIE treatment there is a loss of I_{tonic} potentiation (bottom trace), whereas mIPSCs are visibly potentiated even by 10 mM EtOH.

CIE-induced alterations in mIPSC kinetics. Since the subunit composition of GABAARs affects both their pharmacology and kinetics, we next compared the kinetic properties of mIPSCs and the magnitude of I_{tonic} between MSNs from CIV and CIE rats before application of GABAAR modulators. Table 1 illustrates that the averaged mIPSCs obtained from all recordings in CIV but not CIE rats (Fig. 3C).

CIE-induced decreases in frequency of fast-rising mIPSCs. The large increases in average mIPSC rise time (10–90%) and decreases in mIPSC amplitudes were quite different from our previous experience in hippocampal recordings from CIE rats (Cagetti et al. 2003; Liang et al. 2006). Therefore, we performed separate histogram analyses of mIPSC rise times and amplitudes comparing their distributions between recordings from all CIV and CIE MSNs. A histogram analysis of mIPSC rise times revealed a significant decrease in the proportion of fast-rising events in MSNs from CIE rats compared with CIV rats. These data were consistent with preferential decreases in fast-rising, larger-amplitude mIPSCs in MSNs from CIE rats.
result from possible neurodegenerative changes within the NAcc of CIE rats, we performed unbiased stereological analysis of NeuN-stained NAcc neurons by investigators blinded to the nature of treatments received by the rats. This analysis revealed a lack of any significant changes in NAcc volume, neuronal numbers, or neuronal cell body volume in sections from CIE rats compared with their CIV controls (Fig. 5). However, because of the low power (0.15) of the performed statistical test, these negative findings should be interpreted cautiously.

CIE-induced long-lasting changes in surface GABA<sub>A</sub>R subunit levels. To study the biochemical correlates of functional GABA<sub>A</sub>R changes, we compared Western blots from microdissected NAcc slices of CIE- and CIV-treated rats incubated with or without the membrane-impermeant cross-linking reagent BS<sub>3</sub>. In this way, we were able to identify the intracellular and, indirectly, the cell surface pools of GABA<sub>A</sub>R subunits (Grosshans et al. 2002). Cell surface proteins form high-molecular-weight aggregates with BS<sub>3</sub>, such that they remain at the top of the gel (Grosshans et al. 2002; Liang et al. 2007). However, intracellular proteins are not accessed by the membrane-impermeant reagent and thus can be quantified through Western blot analysis. Subtraction of the intracellular protein from the total protein estimates the cell surface protein content. Such analysis revealed significant decreases in the surface levels of α1 and δ subunits in the NAcc of CIE rats (Fig. 6). In contrast, the surface levels of α4, α5, and γ2 subunits were significantly increased, while those of α2 and α3 GABA<sub>A</sub>R subunits were unchanged in NAcc of rats after CIE treatment and >40 days of withdrawal (Fig. 6).

Single-dose EtOH-induced reversible changes in surface GABA<sub>A</sub>R subunit levels. To explore the mechanisms of EtOH-induced GABA<sub>A</sub>R plasticity, we studied the time dependence of changes in the surface levels of select GABA<sub>A</sub>R subunits in NAcc slices of CIE- and CIV-treated rats incubated with or without the membrane-impermeant cross-linking reagent BS<sub>3</sub>. In this way, we were able to identify the intracellular and, indirectly, the cell surface pools of GABA<sub>A</sub>R subunits (Liang et al. 2007). Analyses of NAcc Western blots revealed a clear increase in the surface θ subunit protein fraction at 1 h after EtOH intoxication (Fig. 7). These increases occurred at the expense of the cell surface α4 subunit content because total content was unchanged at 1 h after EtOH intoxication (not shown). At 2 days after EtOH there was a large increase in the surface α4 subunit protein, which gradually returned to control levels at 14 days (Fig. 7). In contrast, intracellular and total α1 subunit content were unchanged at 1 h after EtOH, but at 2 days after single-dose EtOH both intracellular and surface content were significantly reduced, consistent with the persistent decrease in α1 seen in CIE, with recovery to control levels.

J Neurophysiol • doi:10.1152/jn.00565.2013 • www.jn.org
Fig. 3. Decreased potentiation of NAcc GABA<sub>A</sub>R-mediated tonic and phasic currents by diazepam (DZ) after CIE treatment and >40 days of withdrawal. A: examples of individual NAcc neuron recordings from CIV-treated (top) and CIE-treated (bottom) rats. Note the loss of DZ (0.3 μM) potentiation of both I<sub>tonic</sub> and mIPSCs from CIE rats. B: mIPSCs (top) and I<sub>tonic</sub> (bottom) are potentiated by DZ in CIV but not CIE rats [mIPSC area: treatment: F(1,25) = 14.6, P < 0.001; [DZ]: F(1,25) = 41.5, P < 0.001; treatment × [DZ] interaction: F(1,25) = 14.6, P < 0.001; I<sub>tonic</sub>: treatment: F(1,25) = 60.6, P < 0.001; [DZ]: F(1,25) = 69.6, P < 0.001; treatment × [DZ] interaction: F(1,25) = 60.6, P < 0.001]. C: summary graphs of DZ-induced changes in various mIPSC kinetic parameters. Amplitude: treatment: F(1,25) = 5.47, P = 0.029; [DZ]: F(1,25) = 4.26, P = 0.051; treatment × [DZ] interaction: F(1,25) = 5.47, P = 0.029. Decay τ<sub>r1</sub>: treatment: F(1,25) = 9.3, P = 0.006; [DZ]: F(1,25) = 42.4, P < 0.001; treatment × [DZ] interaction: F(1,25) = 9.3, P = 0.006. Decay τ<sub>r2</sub>: treatment: F(1,25) = 0.62, P = 0.44; [DZ]: F(1,25) = 5.43, P = 0.038; treatment × [DZ] interaction: F(1,25) = 4.4, P = 0.048; [DZ]: F(1,25) = 4.9, P = 0.038; treatment × [DZ] interaction: F(1,25) = 4.4, P = 0.048. Each point represents a mean ± SE value from 6 or 7 neurons (2–4 rats/group). **P < 0.001, *P < 0.05 between CIV and CIE groups; ††P < 0.001, †P < 0.01, §P < 0.05 vs. pre-DZ baseline value (2-way ANOVA with Holm-Sidak post hoc).

by 7 days (Fig. 7). We also observed that the surface α<sub>5</sub> subunit protein at the 1 h time point was unchanged, followed by a large increase at 2 days after EtOH and recovery to control levels at the 14 day time point (Fig. 7).

The decreases in surface α<sub>4</sub> subunit levels observed at 1 h after EtOH intoxication are consistent with rapid internalization of α<sub>4</sub>β<sub>6</sub> extrasynaptic GABA<sub>A</sub>Rs, which are sensitive to low [EtOH] (Gonzalez et al. 2012; Liang et al. 2007; Shen et al. 2011; Sundstrom-Poromaa et al. 2002; Wallner et al. 2003).

Therefore, we also measured the alterations in δ subunit protein, a substantial fraction of which normally coassembles with the α<sub>4</sub> subunits to form extrasynaptic or persynaptic receptors (Jia et al. 2005; Liang et al. 2006; Sun et al. 2004; Wei et al. 2003, 2004). At 1 h after EtOH the cell surface δ subunit levels decreased to 37% of their vehicle controls (Fig. 8). At 2 days after EtOH, the surface δ subunit fraction remained decreased to 24% of control values, with return to control levels measured at 14 days after single-dose EtOH (Fig. 8).

Previous studies have indicated that when hippocampal δ subunit levels are diminished by EtOH treatment in vivo or in vitro, the most likely partner for the transcriptionally upregulated α<sub>4</sub> subunit is the γ<sub>2</sub> subunit (Liang et al. 2007; Shen et al. 2011). Therefore, we measured the changes in γ<sub>2</sub> subunit levels in the NAcc after EtOH intoxication. At 1 h after EtOH, we could not detect significant changes in the intracellular or total γ<sub>2</sub> subunit fractions (Fig. 8). However, at 2 days after EtOH administration there was an apparent large increase (100%) in the cell surface γ<sub>2</sub> subunit fractions (which did not reach statistical significance because of the high variability in this data set from both groups of rats), with return to control levels measured at 14 days (Fig. 8).

**DISCUSSION**

Alcohol-seeking behaviors, craving, and relapse to drinking after withdrawal are most likely dictated by long-term changes in neuronal excitability and synaptic plasticity of the brain.
reward and stress systems (Koob and Le Moal 2008). Here we demonstrate profound plasticity of GABAergic inhibitory neurotransmission in the MSNs of NAcc, a key neural substrate for the rewarding actions of many drugs of abuse, including alcohol. This plasticity involves both presynaptic and postsynaptic elements. The observed decreases in the basal frequency of GABAergic mIPSCs from CIE rats are consistent with decreases in vesicular GABA release or decreases in the number of active GABA release sites (Korn and Faber 1991). Histograms of mIPSC rise time histograms were also consistent with preferential decreases in frequencies of mIPSCs with fast rise times and larger amplitudes. Decreases in the number of GABA release sites could result from the degeneration of GABAergic neurons and their associated synaptic terminals on the NAcc MSNs. Fast-spiking (FS) GABAergic interneurons preferentially form synapses on the cell bodies of MSNs, while the GABAergic MSNs innervate mainly the dendritic regions of neighboring MSNs (Koos et al. 2004; Tepper et al. 2008). Unbiased stereological analysis of neurons in the NAcc failed to detect any decreases in neuronal numbers or their volumes that would indicate neurodegenerative changes in the NAcc of CIE rats. One possibility is that neurodegenerative changes may be limited to only a small population of FS interneurons, which constitute <5% of neurons in the NAcc and therefore are not detectable by our stereological analysis. Future studies focusing on parvalbumin-positive FS interneurons might be useful in that regard. Another possibility is that neurodegenerative changes are limited only to GABAergic neurons with cell...
bodies outside the NAcc, such as the reciprocal GABAergic pathway from the VTA (Van Bockstaele and Pickel 1995). However, recent optogenetic studies revealed that this projection, at least in mice, forms synapses almost exclusively on a very small population of cholinergic interneurons in the NAcc (Brown et al. 2012), which was not examined in our recordings. Alternatively, decreases in GABA release in the NAcc of CIE-treated rats may be mediated by active mechanisms that do not involve neurodegeneration-induced decreases in the numbers of GABAergic synaptic release sites.

Long-lasting alterations in the MSNs of CIE rats also involved postsynaptic GABA$_A$R function. The pharmacological responsiveness of postsynaptic GABA$_A$Rs was altered such that the sensitivity of $I_{\text{tonic}}$, mediated by extrasynaptic GABA$_A$Rs, to EtOH, Ro15-4513, and DZ was greatly reduced. In contrast, sensitivity of mIPSCs in MSNs from CIE rats to both EtOH and Ro15-4513 was enhanced, while DZ sensitivity was lost. Notably, acute EtOH application did not alter action potential-independent mIPSC frequency in CIV or CIE rats, in contrast to observations in central amygdala, where EtOH application does increase mIPSC frequency (Nie et al. 2009; Roberto et al. 2003). Therefore EtOH-induced potentiation of mIPSCs in NAcc MSNs is mediated by postsynaptic mechanisms rather than by increased presynaptic release of GABA. We previously reported analogous pharmacological changes in recordings from CA1 pyramidal and dentate granule neurons from CIE rats (Liang et al. 2006, 2007). Such changes in MSNs of CIE rats are consistent with cell location-specific alterations in the relative abundance and subunit composition of GABA$_A$Rs. Thus we hypothesized that decreases in $\alpha_1$ and $\alpha_5$ subunit sensitivity to EtOH and Ro15-4513 involve decreased abundance of extrasynaptic $\alpha_1/\beta_2/\gamma_2$ GABA$_A$Rs, while decreases in DZ sensitivity were hypothesized to involve decreases in $\alpha_1/\beta_2/\gamma_2$ GABA$_A$Rs and increases in $\alpha_4$-containing GABA$_A$Rs at both synaptic and extrasynaptic sites.

The use of Western blot techniques permitted us to relate changes in the function of GABA$_A$Rs to changes in the relative surface levels of individual GABA$_A$R subunits. Consistent with the above hypothesis, we show significant decreases in surface

---

**Fig. 6.** Altered surface GABA$_A$R subunit levels in the NAcc of CIE rats. A: examples of gels from the microdissected NAcc region incubated with ACSF (Tot) or with the bis(sulfosuccinimidyl)suberate (BS$_3$) cross-linking reagent (Int). BS$_3$-linked cell surface proteins are present as high-molecular-weight aggregates that do not reliably enter the gel. In contrast, gel migration of the intracellular protein β-actin is unaffected. After each “Int” band intensity is normalized to its β-actin control, the difference between that value and the amount from an equivalent “Tot” adjacent slice represents the surface pool. B: summary graph of changes in cell surface GABA$_A$R subunits after CIE treatment relative to CIV controls (dashed line). Data are means ± SE from CIV or CIE treatments (n = 4 – 6 rats/treatment). *P < 0.05 (unpaired t-test) compared with CIV controls.

---

**Fig. 7.** Reversible changes in $\alpha_1$, $\alpha_4$, and $\alpha_5$ subunit proteins in microdissected NAcc after single EtOH dosing: summary graph of changes in cell surface $\alpha_1$, $\alpha_4$, and $\alpha_5$ subunit content at various time points after single-dose EtOH (5 g/kg, gavage) relative to vehicle-treated controls (dashed line). Data are means ± SE from vehicle or single-dose EtOH treatments (n = 4 or 5 rats/treatment). *P < 0.05 (unpaired t-test) compared with vehicle-treated controls.
levels of α1 and δ subunits, unaltered α2 and α3 subunit levels, and significant increases in α4, α5, and γ2 subunit levels in the NAcc of CIE rats. These changes are remarkably similar to those previously observed in the hippocampus of CIE rats (Cagetti et al. 2003; Liang et al. 2007). Analogous alterations in the α1, α4, and γ2 subunit levels were reported in the basolateral amygdala of rats after CIE treatment and withdrawal (Diaz et al. 2011). One important exception is the increase in α5 subunit levels in the NAcc, which was not observed in the hippocampus of CIE rats. Previous studies revealed an important role of α5 subunits in mediating \( I_{\text{tonic}} \) in murine striatal MSNs (Ade et al. 2008; Janssen et al. 2009). These studies also revealed, using transgenic mice, that δ subunits are not necessary for the activation of \( I_{\text{tonic}} \) by α5-containing GABA\(_A\)Rs (Janssen et al. 2009). Taken together, these observations suggest that CIE treatment leads to reductions in extrasynaptic (EtOH sensitive, DZ insensitive) α4β3δ and reductions in both synaptic and extrasynaptic (EtOH insensitive, DZ sensitive) α1β2γ2 GABA\(_A\)Rs. Compensation for the loss of these inhibitory receptor subunit combinations is likely achieved by upregulation of α4β2γ2 and α5β2γ2 GABA\(_A\)Rs at synaptic and extrasynaptic locations, respectively. Extrasynaptic insertion of α4β2γ2 likely involves protein kinase C (PKC) phosphorylation of α4 subunits (Abramian et al. 2010), while insertion and clustering of α5β2γ2 is likely facilitated by the actin-binding protein radixin (Loebrich et al. 2006). The specific role of γ2 subunits in the membrane insertion, clustering, and localization to synaptic or extrasynaptic sites of CIE-induced α4β2γ2 and α5β2γ2 GABA\(_A\)Rs remains to be determined.

Previous studies demonstrated that extrasynaptic α5β2 GABA\(_A\)Rs in the hippocampus are DZ sensitive; this sensitivity is eliminated by the introduction of δ or α4 subunits and reduced by substitution of γ2 with γ1 or γ3 subunits (reviewed in Möhler et al. 2000). The loss of DZ sensitivity of \( I_{\text{tonic}} \) in MSNs from CIE rats is consistent with the observed increases in α4, α5, and γ2 subunit levels in the NAcc of CIE rats and their likely assembly into α4β2γ2 or α5β2γ2 GABA\(_A\)Rs. It remains to be determined whether the protein levels of γ1 or γ3 subunits are increased in NAcc of CIE rats. However, increases in γ1 subunit mRNA have previously been reported in the cortex of chronic EtOH-treated rats (Devaud et al. 1995), as well as in the hippocampus of CIE rats (Cagetti et al. 2003). Interestingly, recent studies have demonstrated that replacing α1 with α2 and γ1 with γ2 at engineered GABAergic synapses results in slower mIPSC kinetics and produces a more diffuse clustering of GABA\(_A\)Rs containing these subunits (Dixon et al. 2014). Analogous subunit “switches” at MSN synapses after CIE treatment might contribute to the observed increases in rise time of mIPSCs, as an alternative to the proposed decrements in FS interneuron-MSN GABA release sites.

The lack of overall changes in the magnitude of \( I_{\text{tonic}} \) was surprising, compared with the large decreases observed in the hippocampus (Liang et al. 2007), but also consistent with the compensatory increases in α5 subunit content in the NAcc of CIE rats. The present study’s demonstration of preferential EtOH sensitivity of \( I_{\text{tonic}} \) in MSN neurons from CIV rats is also consistent with the demonstration that selective reduction of the GABA\(_A\)_\(\delta\)-subunit expression in the NAcc of rats, with virus-mediated RNAi techniques, reduces EtOH self-administration and preference and decreases the reinforcing effects of EtOH (Rewal et al. 2009, 2012). Selective reduction of the GABA\(_A\)_\(\delta\)-subunit expression in NAcc also decreases EtOH intake (Nie et al. 2011). It is noteworthy that the decreases in low-to-moderate voluntary EtOH intake were selective for RNAi injections in the medial shell of the NAcc and not in the core (Nie et al. 2011; Rewal et al. 2009, 2012). In contrast, the NAcc core has been implicated in the reinstatement of conditioned alcohol seeking (Chaudhri et al. 2008) and in the aversion- and punishment-resistant aspects of alcohol dependence (Seif et al. 2013). The core of the NAcc was also implicated in locomotor sensitization to EtOH, which resulted in decreased NMDA receptor-dependent synaptic plasticity selectively in the core but not the shell, and increased EtOH consumption in mice (Abrahao et al. 2013). Further studies would be needed to determine the relative contributions of NAcc shell and core regions to the large increases in EtOH consumption observed after CIE treatment and withdrawal (Rimondini et al. 2002, 2003; Valdez et al. 2002).

In MSNs at their resting (“down”) state, similar to hippocampal interneurons (Song et al. 2011), the GABA\(_A\)_\(\delta\) current reversal potential is depolarizing, making baseline \( I_{\text{tonic}} \) excitatory. This was elegantly demonstrated in loose cell-attached recordings from striatal MSNs during activation of cortical glutamatergic inputs (Ade et al. 2008). In these recordings, excitatory synaptic activation of single action potentials in MSNs was reduced during application of L655,703, an α5 subunit GABA\(_A\)-preparing modulatory inverse agonist ligand that preferentially antagonizes \( I_{\text{tonic}} \) in MSNs (Ade et al. 2008). Increasing \( I_{\text{tonic}} \) conductance in hippocampal interneurons was shown to enhance shunting-mediated inhibition, eventually overpowering excitation (Song et al. 2011). Analogously, acute EtOH administration should decrease excitability of MSNs in CIV rats via enhancement of extrasynaptic GABA\(_A\)-mediated shunting inhibition. In contrast, the observed “switch” of EtOH sensitivity from \( I_{\text{tonic}} \) to synaptic currents in CIE rats suggests that only the shunting inhibition of phasic synaptic currents would be responsive to potentiation by acute EtOH in these alcohol-dependent rats. Analysis of time-dependent alterations in the surface levels of select GABA\(_A\)_\(\delta\) subunits in the NAcc after single doses of EtOH revealed increased internalization of α4 and δ subunits at 1 h after EtOH administration, without changes in α1 or γ2 subunits. These data provided further support for the internalization of extrasynaptic EtOH-sensitive α4β3δ GABA\(_A\)Rs as an early event in the cycles of intoxication and withdrawal that eventually result in the persistent GABA\(_A\)_\(\delta\) alterations of CIE rats (Liang et al. 2007; Shen et al. 2011). Recent studies in the hippocampus revealed that this internalization is detectable within 5 min of EtOH (3 g/kg ip) administration and involves increased association of δ subunit with clathrin adaptor proteins AP2-μ2 (Gonzalez et al. 2012). Increased phosphorylation of the β3 subunit was also observed but was not directly associated with the AP2 machinery. Since phosphorylation of the α4 subunit dictates its insertion into the membrane, rather than its endocytosis (Abramian et al. 2010; Werner et al. 2011), the precise mechanism of EtOH-induced α4β3δ GABA\(_A\)R internalization remains to be elucidated.

In contrast with α4 and δ subunits, decreases in surface α1 subunits were not observed at 1 h after EtOH, but maximal decreases were seen at 2 days after EtOH, returning to control levels by 7 days after EtOH (Fig. 7). Similar delayed decreases were previously observed in the CA1 and dentate gyrus regions.
of the hippocampus after a single EtOH dose (Liang et al. 2007), and numerous investigators have reported long-lasting decreases in α1 subunit expression after chronic EtOH treatment in different brain regions (Cagetti et al. 2003; Devaud et al. 1995; Dizá et al. 2011; Mhatre andTicku 1992; Morrow et al. 1990). Initial EtOH-induced decreases in surface α1-containing GABAARs appear to involve selective increases in PKCγ activity, which in turn increases internalization of α1 GABAARs (Kumar et al. 2010), while activation of PKA has been shown to enhance GABAAR plasticity (Pandey et al. 2008; Sakharkar et al. 2012). Previously, we demonstrated enhancement of GABAARs (Kumar et al. 2010), while activation of PKA has been shown to enhance GABAAR plasticity (Pandey et al. 2008; Sakharkar et al. 2012). Interestingly, chronic EtOH administration is associated with region-specific changes in BDNF expression (Davis 2008), and BDNF-mediated second messenger pathways in the dorsolateral striatum have been implicated in control of voluntary EtOH intake (Logrip et al. 2009).

At 2 days after single-dose EtOH, surface α4 and α5 subunit levels in the NAcc were significantly increased (Fig. 7) along with increases in surface γ2 levels (Fig. 8). Similar changes in α4 and γ2, but not α5, subunit levels were previously reported for the CA1 and dentate gyrus regions of the hippocampus in rats after single-dose EtOH (Liang et al. 2007). Therefore, the increases in α5 subunit levels appear to be specific to the NAcc. The mechanism of the transcriptionally driven increases in α4 was demonstrated to involve EtOH-induced activation of the heat shock immediate-early gene pathway elements to enhance the expression of Gabra4 and several other alcohol-responsive genes (Pignataro et al. 2007). Furthermore, selective PKCγ activation was shown to be required for EtOH-induced increases in membrane surface expression of α4 subunits in cultured cerebral cortical neurons (Werner et al. 2011). Presently it is unknown whether similar transcriptional and posttranscriptional mechanisms underlie the EtOH-induced increases in α5 and γ2 subunit levels in the NAcc.

At 2 days after single-dose EtOH the alterations in GABAAR surface subunit levels were remarkably similar to those observed in CIE rats, yet by 1–2 wk after the single EtOH dose surface levels of all examined subunits returned to control levels, whereas the increases were persistent in the NAcc of CIE rats (compare Figs. 6, 7, and 8). Numerous studies have shown that CIE treatment is particularly effective at producing long-lasting alterations in physiology and behavior; however, the mechanisms behind the persistence of CIE-induced alterations are only now beginning to be examined. There is evidence that epigenetic mechanisms may participate in EtOH-induced GABAAR plasticity (Pandey et al. 2008; Sakharkar et al. 2012). It would be of interest to examine whether similar epigenetic alterations may be present after CIE treatment in rats or in the brains of alcoholics.

Our present data point to a decrement in the presynaptic release and postsynaptic GABAAR receptor function in the NAcc of CIE rats. Previously, we demonstrated enhancement of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)-mediated glutamatergic neurotransmission in MSNs of CIE rats (Marty and Spigelman 2012). This potentiation of glutamatergic inputs should enhance the integration of dendritic excitability and increase the probability of action potential initiation at the soma, in effect increasing the likelihood of an “up” state, when NAcc neurons execute their behaviorally relevant functions (O’Donnell et al. 1999). The observed decrements in GABAergic neurotransmission should further contribute to the increased probability of an “up” state and also alter spike timing in favor of increased frequency of discharge, particularly given the faster spike repolarization and larger afterhyperpolarization also observed in the MSNs of CIE rats (Marty and Spigelman 2012). Action potential discharge during the “up” state constitutes the pertinent physiological coding event required for the induction of spike time-dependent plasticity (STDP), an activity-dependent long-term synaptic plasticity, that can bidirectionally modulate the strength of glutamatergic and GABAergic synaptic inputs in MSNs and interneurons (reviewed in Fino and Venance 2011). Moreover, GABAergic inhibition was recently demonstrated to govern the polarity of STDP, because blockade of GABAARs was able to completely reverse the temporal order of plasticity at corticostriatal synapses in rats and mice, thus establishing a central role for GABAergic circuits in shaping STDP (Paille et al. 2013). The combined effect of the various alterations in MSNs of CIE rats is expected to greatly increase information transfer from areas such as the prelimbic cortex, hippocampus, and amygdala to the MSN GABAergic projections areas such as the ventral pallidum and the VTA. It is noteworthy that analogous decrements in GABAergic neurotransmission (Otaka et al. 2013), potentiated glutamatergic neurotransmission (Conrad et al. 2008), and enhanced action potential afterhyperpolarization (Mu et al. 2010) were observed after cocaine withdrawal, suggesting shared neurobiological mechanisms of dependence between alcohol and other drugs of abuse.

In summary, we demonstrated persistent decrements in GABAergic neurotransmission in NAcc core MSNs of rats during withdrawal from CIE treatment, a model of alcohol dependence. These include decreased synaptic release of GABA and alterations in GABAAR subunit composition, function, and pharmacological sensitivity to ligands, including EtOH. Together with the previously observed enhancement in glutamatergic neurotransmission (Marty and Spigelman 2012) and the modulatory influences of dopamine (see companion article, Liang et al. 2014) these alterations should result in greater signal throughput from MSNs in the NAcc core to their projection areas. Experiments using a combination of optogenetic and operant behavioral techniques could reveal the extent to which these CIE-induced alterations contribute to the compulsive and excessive EtOH intake in alcohol dependence.

ACKNOWLEDGMENTS

We thank Prof. Werner Sieghart (Medical University of Vienna, Vienna, Austria) for the generous supply of GABAAR subunit antibodies. We also thank Drs. Felix Schweitzer and Thomas Otis (Department of Neurobiology, UCLA) for help with data analysis and interpretation.

GRANTS

This work was supported by National Institute on Alcohol Abuse and Alcoholism Grants AA-016100 (I. Spigelman), AA-07680 (R. W. Olsen), AA-017991 (J. Liang), and AA-021037 (E. M. Meyer).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

J Neurophysiol • doi:10.1152/jn.00565.2013 • www.jn.org
AUTHOR CONTRIBUTIONS


REFERENCES


