Published in final edited form as:

Alcohol Clin Exp Res. 2009 December ; 33(12): 2134–2140. doi:10.1111/j.1530-0277.2009.01059.x.

Effects of Ethanol on Persistent Activity and Up-States in Excitatory and Inhibitory Neurons in Prefrontal Cortex

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Abstract

Background—Elucidating mechanisms that underlie the neural actions of ethanol is critical for understanding how this drug affects behavior. Increasing evidence suggests that, in addition to mid-brain dopaminergic regions, higher cortical structures play an important role in the pathophysiology associated with alcohol abuse. Previous studies from this laboratory used a novel slice co-culture system to demonstrate that ethanol reduces network-dependent patterns of activity in excitatory pyramidal neurons of the prefrontal cortex. In the present study, we examine the effect of ethanol on the activity of fast-spiking interneurons, a sub-population of neurons critically involved in shaping cortical activity.

Methods—Slices containing the dorsolateral prefrontal cortex were prepared from neonatal C57 mice and maintained in culture. After 2–3 weeks in vitro, whole-cell patch-clamp electrophysiology was used to monitor spontaneous episodes of persistent activity in prelimbic PFC neurons. In some experiments, the use-dependent NMDA receptor blocker, MK801, was included in the pipette recording solution to assess the contribution of NMDA receptors to up-states.

Results—MK801 reduced up-state amplitude and revealed underlying fast EPSPs in excitatory pyramidal neurons while having little effect on these parameters in fast-spiking interneurons. Despite this difference, ethanol (44 mM), significantly reduced up-state duration and up-state area in both pyramidal and fast-spiking interneurons.

Conclusions—These results suggest that ethanol reduces the activity of fast-spiking interneurons due to disruption of network-dependent activity. This would be expected to further impair the ability of PFC networks to carry out their normal function and may contribute to the adverse effects of ethanol on PFC-dependent behaviors.

Introduction

A fundamental question in regard to the development of addictive disorders is how alcohol and other drugs of abuse affect neural circuits that regulate behavior. Over the past twenty of more years, the study of the effects of alcohol and other addictive drugs has largely focused on dopamine rich mid-brain areas involved in natural and drug-induced reward as the initial phase of alcohol consumption is thought to depend upon some level of positive reinforcement that drives further drinking (Vengeliene et al., 2008). It is becoming clear however, that cortical structures that help regulate behavior such as the prefrontal cortex (PFC) are also likely to be important given that excessive drinking often occurs in the face of adverse consequences to the health and well-being of the individual (Bechara, 2005). The functions of the PFC are well known and lesions or damage to this area in experimental animals or humans produces deficits in working memory, decision-making and error

detection (Moghaddam and Homayoun, 2008). These same deficiencies are often observed in individuals suffering from alcohol addiction suggesting that alcohol may induce long-lasting changes in PFC structure or function (Pfefferbaum et al., 2001; Sullivan and Pfefferbaum, 2005). Despite the evidence linking altered PFC function to alcohol abuse, there are relatively few studies that have explored in detail the effects of alcohol on the function of individual PFC neurons.

To address this gap in the understanding of this important issue, we established an *in vitro* slice culture system that preserves many of the features of the intact PFC while allowing a detailed electrophysiological analysis of the cellular effects of alcohol. Using this preparation, we reported that the ability of PFC neurons to engage in complex patterns of persistent activity is compromised by ethanol concentrations associated with mild to moderate behavioral impairment (Tu et al., 2007). Those studies focused exclusively on layer V glutamatergic neurons that provide most of the excitatory output of the PFC. In the present study, we extend those findings and examine the effects of ethanol on local fast-spiking GABAergic interneurons that are critically involved in regulating the excitability of PFC pyramidal output neurons. Fast-spiking (FS) interneurons are particularly interesting since they may express relatively low levels of the N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptor (Angulo et al., 1999; Wang and Gao, 2009). NMDA receptors are thought to be among the more sensitive targets for alcohol in the brain (Jin and Woodward, 2006; Vengeliene et al., 2008) and results from our previous studies suggest that those expressed in PFC pyramidal neurons are particularly sensitive to alcohol (Weitlauf and Woodward, 2008).

Materials and Methods

Animals

All procedures involving animals were conducted under protocols approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina.

Organotypic slice co-cultures

Slice co-cultures were prepared as previously described (Tu et al., 2007). Briefly, postnatal day 1–3 C57 mouse pups were sacrificed and brains were placed in ice-cold, sucrose-substituted Ringers solution containing (in mM) 200 sucrose, 1.9 KCl, 6 MgCl₂, 0.5 CaCl₂, 10 glucose, 0.4 ascorbic acid, 25 HEPES (titrated to pH 7.3 with KOH) bubbled with 95% O₂/5% CO₂. Individual coronal sections containing dorsolateral prefrontal cortex were cut at a thickness of 400 μm using a Leica VT-1000 vibratome. Slices were oriented adjacent to one another on a Millipore millicell insert in a 6 well culture dish. Each well contained 1 ml of serum-containing media consisting of 50% basal medium Eagle, 25% Earle's balanced salt solution, 25% heat-inactivated horse serum with 33 mM glucose, 25 mM HEPES, 170 μM streptomycin and 0.235 mM Glutamax. After 3 days *in vitro* (DIV), the media was changed to one containing reduced amounts of horse serum (5%). After 14 DIV, 5-fluoro-2-deoxyuridine (4 μM) was added to prevent glial overgrowth. Cell culture dishes were kept in a humidified incubator equilibrated with 5% CO₂ and were maintained for 15–40 days with media changes every 2–3 days.

Electrophysiology

On the day of recording, the section of the Millipore membrane containing a slice co-culture was carefully removed from the insert and was submerged in a recording chamber. The slice co-culture was perfused at approximately 2 ml/min with artificial cerebrospinal fluid (ACSF) containing, (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 1.3 MgCl₂, 2 CaCl₂, 0.4 ascorbic acid and 10 glucose. ACSF solutions were maintained at a temperature

of 32–33°C using a flow-through solution heater and heated recording chamber under thermistor-control of a TC-344B temperature controller (Warner Instruments, Hamden, CT). All solutions were continuously bubbled with a mixture of 95% O₂/5% CO₂. Patch pipettes (1.5 mm × 1.1 mm; 2–3 MΩ) were filled with an internal recording solution containing (in mM): 130 K-gluconate, 10 KCl, 2 MgCl₂, 1 EGTA, 10 HEPES, 2 Na₂ATP, and 0.3 NaGTP, pH 7.2. Whole-cell patch-clamp recordings were made from visually identified deep-layer pyramidal cells from the prelimbic region of the PFC using an Olympus BX51W microscope with infrared video imaging and Dodt gradient contrast optics. Gigaohm seals were obtained in voltage-clamp mode using an Axoclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) and following breakthrough and determination of input resistance, the amplifier was switched to current-clamp mode. Pipette access resistance (5–15 MΩ) was monitored throughout the experiment and cells showing a significant deviation in access resistance were not used for analysis. Data were acquired at 10 kHz using an ITC-16 interface (Instrutech, Port Washington, NY) controlled by AxographX software running on a Macintosh G4 computer.

Data Analysis

Data were analyzed for statistical significance using standard t-test and 1-way Anova with Prism 4 software (GraphPad Software, San Diego, CA). A significance level was set at $p < 0.05$ for all analyses.

Results

Prefrontal cortical neurons in a slice co-culture preparation display spontaneous persistent activity patterns characterized as alternating episodes of depolarized upstates with action potential firing and quiescent, hyperpolarized down-states. Each set of recordings shown in Figure 1 is from a different culture and in each case, the activity of two neurons was simultaneously monitored using dual whole-cell, patch-clamp electrophysiology. The neuron type was determined by cell morphology, current-evoked firing patterns, action potential half-width, input resistance measurements and in FS neurons, the presence of fast synaptic EPSCs. As shown in the three different examples, transitions between down- and up-states in recorded neurons are highly synchronized regardless of cell type. In contrast, action potential firing that occurred during the up-state was not synchronized as previously reported (Seamans et al., 2003). Although some pairs of neurons were synaptically coupled, these were too few to permit further analysis.

Seamans et al. (2003) demonstrated that NMDA receptors play an important role in the sustained depolarization that occurs in pyramidal neurons during up-states. In addition, findings from several studies suggest that fast-spiking (FS) interneurons may lack appreciable NMDA receptor-mediated currents (Angulo et al., 1999; Wang and Gao, 2009). Because both pyramidal and FS neurons engage in simultaneous episodes of up-states in the slice cultures, we recorded from either pyramidal or FS interneurons in the absence and presence of an NMDA blocker to further investigate the role of NMDA receptors in up-states. When bath applied, NMDA antagonists disrupt network activity and up-states in slice cultures (Seamans et al., 2003) thus preventing the study of spontaneous or evoked persistent activity. To avoid this confound, we included the NMDA channel blocker MK801 (2 mM) in the recording pipette and monitored up-state parameters in individual neurons. As shown in Figure 2, inclusion of MK801 in the recording pipette induced a qualitative change in up-state characteristics in pyramidal neurons while having little effect on FS interneurons. Specifically, in pyramidal neurons, MK801 revealed the presence of fast individual EPSPs that were normally hidden during the sustained depolarization of the up-state (Figure 2A, right panel). In most FS neurons tested, MK801 had little effect on up-state characteristics and individual fast EPSPs were observed both with and without MK801 (Figure 2A, left

panel). To quantify the effect of MK801, we determined up-state amplitude and up-state area for both pyramidal neurons and FS neurons recorded with and without MK801 in the pipette. As shown in Figure 2B, the net mean amplitude of spontaneous up-states in pyramidal neurons was ~5 mV higher than that of FS neurons. Intracellular MK801 significantly reduced upstate amplitude in pyramidal neurons to the level of FS neurons while having no effect on up-state amplitude in FS neurons. Up-state area was also measured as an indicator of total charge transfer during the sustained depolarization of the up-state. Since the duration of an up-state varies between cultures, area for each up-state was normalized by dividing it by its duration. In pyramidal neurons, normalized up-state area in pyramidal neurons was higher than that of FS neurons despite the higher firing frequency of FS neurons. As with up-state amplitude intracellular MK801 significantly reduced normalized up-state area in pyramidal neurons while having no effect on FS neurons (Figure 2B). These data are consistent with the idea that NMDA receptors make a significant contribution to up-states in pyramidal neurons while having less impact in FS interneurons.

In a previous study, we showed that ethanol (25–100 mM) inhibited spontaneous and evoked periods of persistent activity of glutamatergic pyramidal neurons in slice co-cultures (Tu et al., 2007). To extend these findings, we measured spontaneous episodes of up-states in both pyramidal and FS neurons in slice co-cultures before, during, and after exposure of slice co-cultures to 44 mM ethanol, a concentration equivalent to approximately 0.2% blood alcohol level. A total of over 900 up-state episodes were measured and for each neuron, multiple episodes were averaged to given a mean value for each measurement period. Table 1 shows the mean value ($\pm$ SEM) for each up-state parameter measured under the three different conditions for pyramidal and FS interneurons. Ethanol had no significant effect on down-state membrane potential in either pyramidal or FS neurons. Ethanol also had little effect on averaged up-state membrane potential in either cell type as net up-state amplitude (V_{mUp} minus V_{mDown}) was not altered by ethanol. In contrast to these results, ethanol significantly reduced the average duration of up-states in both pyramidal and FS neurons. For example, Figure 3A shows the effect of 44 mM ethanol on the duration of individual up-states during a simultaneous recording of a pyramidal neuron and a FS interneuron from the same culture. Figures 3B and 3C summarize the effects of ethanol for all pyramidal and FS neurons tested and illustrate the consistent effect of ethanol on up-state duration. Note that although the average duration of up-states in pyramidal neurons was numerically higher than that of FS neurons, this reflects the fact that different cultures were used across these studies. In experiments where both pyramidal and FS neurons were recorded simultaneously in the same culture, up-state durations between pyramidal and FS neurons were essentially identical (see dual recordings presented in Figures 1 and 3A).

Unlike ethanol's consistent decrease of up-state duration, its effect on spiking was variable showing a trend towards an increase in pyramidal neurons and a decrease in FS neurons (Table 1). As expected due to its effect on up-state duration, average upstate area was also reduced by 44 mM ethanol (Table 1). However, when area was normalized to the duration of each individual episode, no differences were found between control, ethanol and washout conditions (Table 1). During washout of the ethanol solution, some neurons showed evidence of a rebound in up-state duration and this reached statistical significance in FS neurons (Figure 3C). A similar trend was observed for cell spiking cell and this was significant for pyramidal but not fast-spiking neurons.

Discussion

The major finding of the present study is that ethanol, at behaviorally relevant concentrations, profoundly reduces up-state activity in both pyramidal and FS interneurons of the prefrontal cortex. This occurs despite significant differences in the sensitivity of these

neurons to intracellularly applied MK801, a potent use-dependent blocker of NMDA receptors (Tovar and Westbrook, 1999). In a previous study, we showed that ethanol inhibited spontaneous and evoked up-states in deep-layer cortical pyramidal neurons maintained in PFC:Hippocampal:Ventral Tegmental Area slice co-cultures (Tu et al., 2007). In that study, up-state duration was the most sensitive indicator of ethanol's effect on persistent activity and showed significant inhibition at ethanol concentrations of 25 mM, near the legal limit for intoxication in most countries. At higher concentrations, ethanol also had effects of up-state amplitude and spiking although these effects were more variable. In the present study using PFC:PFC co-cultures, 44 mM ethanol had little effect on up-state amplitude but significantly inhibited up-state duration by 40–50% in both pyramidal and FS interneurons. Total up-state spike activity was not significantly affected in the present study although this measure showed considerable variability (Table 1). Taken together, these results suggest that ethanol's primary effect on persistent activity is to reduce the duration of up-states while modulatory inputs provided by other regions may influence its effects on up-state amplitude and spiking.

Seamans et al. (2003) showed that in slice co-cultures, PFC up-states are initiated by a brief EPSP/IPSP sequence that requires both AMPA and GABA_A receptors. Isolating these currents with pharmacological blockers abolishes persistent activity so determining their ethanol sensitivity in active slice co-cultures is problematic. To more directly assess this question, we used acute slices and directly examined the effect of acute ethanol on glutamatergic and GABAergic synaptic responses in deep-layer PFC neurons. In that study, ethanol, up to 66 mM, had only modest effects on spontaneous or stimulus-evoked synaptic AMPA and GABA_A currents while higher concentrations (88 mM) slightly depressed these responses (Weitlauf and Woodward, 2008). A similar lack of effect of ethanol was noted for spontaneous AMPA and GABA_A-mediated events. These results suggest that at ethanol concentrations associated with moderate intoxication (22–44 mM), AMPA and GABA_A receptors may not be the primary site of action for ethanol's effects on persistent activity in slice co-cultures. One caveat to note is that those recordings were obtained from glutamatergic pyramidal neurons recorded in acute slices of rat PFC while those in the present study were carried out using mouse PFC neurons.

Following initiation of an up-state, membrane depolarization reduces the magnesium block of NMDA receptors allowing these currents to make a major contribution to the relatively constant level of depolarization observed during up-states in glutamatergic pyramidal neurons (Seamans et al., 2003; Tu et al., 2007). However, unlike AMPA or GABA_A-mediated responses, NMDA receptors in deep-layer PFC neurons are sensitive to ethanol and significant inhibition is observed at concentrations as low as 22 mM (Weitlauf and Woodward, 2008). Of interest in this regard is the finding that adult PFC pyramidal neurons contain significant amounts of the NR2B subunit that is thought to be one of the more ethanol sensitive NMDA receptors (Jin and Woodward, 2006; Kash et al., 2008; Wang et al., 2008).

In the present study, the effect of ethanol on up-state characteristics was similar when compared between pyramidal neurons and FS neurons. However, findings from several studies show that PFC FS neurons lack significant NMDA receptor-mediated responses suggesting that up-states in these neurons should be less affected by ethanol. We found that in slice co-cultures, up-state amplitude and area in most FS neurons were unaffected by intracellular MK801 suggesting that these neurons express relatively few NMDA receptors. In contrast, MK801 reduced average up-state amplitude in pyramidal neurons and allowed individual EPSPs during the up-state to be detected. These findings generally confirm the idea that the slow kinetics of NMDA receptors may bridge the periods between successive fast AMPA-mediated EPSPs and provide a more uniform membrane depolarization during

up-states (Seamans et al., 2003). In computational models of membrane bistability that contain recurrent connections between excitatory and inhibitory neurons, reducing the strength of NMDA currents dramatically shortens the duration of up-states (Durstewitz and Seamans, 2006; Wang, 1999; Wolf et al., 2005). This prediction has been verified experimentally in vitro as bath application of low concentrations of competitive NMDA antagonists to slice co-cultures dramatically reduces up-state duration (Seamans et al., 2003; Tu et al., 2007). Thus, although FS neurons appear to lack appreciable NMDA receptors, up-state duration in slice cultures is reduced in these neurons due to the overall inhibitory effect of ethanol on network activity. Although these findings suggest that ethanol reduces persistent activity via an action on NMDA receptors, unlike MK801, ethanol had little effect on upstate amplitude. This may reflect differences in the efficacy of these compounds as NMDA antagonists as ethanol acts as a weak allosteric modulator of NMDA activity while MK801 is an irreversible channel blocker. This may also reflect other as yet identified mechanisms of action of ethanol on prefrontal cortical neuron activity.

In summary, these results of this study demonstrate that ethanol, at concentrations achieved during voluntary drinking, has profound effects on up-state dynamics in both pyramidal and FS interneurons in the prefrontal cortex. These effects are likely to have a dramatic effect on the ability of the PFC to perform its normal function.

Acknowledgments

The authors thank S. Kroener for helpful discussions during preparation of the manuscript. These studies were supported by funds from P50AA10761 and T32AA07474.

Support: P50AA10761 and T32AA07474.

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Figure 1.

Spontaneous up-states in pyramidal (Pyr) and fast-spiking (FS) neurons in slice cultures of prefrontal cortex. Each panel shows an example of a spontaneous upstate recorded simultaneously from a pair of neurons using the current-clamp mode of whole-cell patch-clamp electrophysiology. Different cultures were used for each pair of neurons shown. Note simultaneous beginning and end of up-state in each pair of neurons and asynchronous firing of action potentials. Scale bars y-axis; 10 mV, x-axis, 2 seconds.

Figure 2.

Effect of the NMDA antagonist MK801 on up-states in pyramidal and fast-spiking interneurons in slice cultures of prefrontal cortex. A) Upper traces show firing pattern of FS (left) and pyramidal neuron (right) during current injection. Middle and bottom panels show effect of intracellular MK801 (2 mM) on up-states during the initial period of recording and 15 minutes following breakthrough. Note appearance of fast EPSPs in pyramidal neuron with exposure to MK801. Scale bars y-axis; 10 mV; x-axis; 2 seconds). Recordings were done in different cultures. Panel B shows effect on intracellular MK801 on up-state amplitude and normalized area in pyramidal and fast-spiking neurons. Data are mean ($\pm$ SEM, N=6–14 for pyramidal, 5–10 for FS neurons). Symbol (*): value significantly

different from corresponding control value (unpaired t-test, $p < 0.05$). (##): value significantly different from that of pyramidal neurons (unpaired t-test, $p < 0.05$).

Figure 3.

Effect of ethanol on up-state duration in pyramidal and fast-spiking interneurons in slice cultures of prefrontal cortex. A) Representative example of the effect of 44 mM ethanol (horizontal line) on duration of individual spontaneous up-states in pyramidal (open circle) and fast-spiking interneuron (closed circle). Neurons were recorded from the same culture simultaneously using dual, whole-cell patch-clamp electrophysiology. B, C) Summary of effect of ethanol on up-state duration in pyramidal (B) and fast-spiking interneuron (C). Each symbol represents up-state duration for an individual neuron recorded before, during and after exposure to 44 mM ethanol. The mean value for each group is represented by the horizontal bar (N=14 for pyramidal, 10 for fast-spiking neurons). Symbol (*): value

significantly different from corresponding control value (one-way Anova, $p < 0.05$; Dunnett's post-hoc test).

Table 1

Effects of 44 mM ethanol on up-state parameters in pyramidal and fast-spiking neurons in PFC co-cultures.

Vm _{down}	Vm _{up}	ΔVm	Duration	Spikes	Area	Area/Duration
(mV)	(mV)	mV	(sec)	(number)	(mV.sec)	(mV.sec sec-1)
<i>Pyramidal Neurons (N=14)</i>						
<i>Control</i>						
-68.24 (0.97)	-52.38 (1.41)	16.05 (0.96)	5.65 (0.49)	8.66 (2.48)	76.72 (5.74)	14.24 (0.96)
<i>EtoH</i>						
-68.44 (1.43)	-50.65 (1.01)	17.79 (1.50)	2.72* (0.23)	14.31 (3.24)	41.92* (4.62)	16.54 (1.99)
<i>Washout</i>						
-66.49 (1.97)	-48.14 (1.44)	18.35 (1.35)	5.01 (0.61)	37.48* (8.83)	81.11 (9.40)	17.75 (1.69)
<i>FS Neurons (N=10)</i>						
<i>Control</i>						
-65.05 (0.88)	-54.15 (1.40)	10.90 (1.14)	2.93 (0.25)	28.27 (14.71)	34.17 (6.63)	10.82 (1.30)
<i>EtoH</i>						
-65.05 (1.56)	-54.83 (1.88)	10.22 (1.22)	1.67* (0.14)	15.83 (6.61)	17.08* (2.62)	9.53 (1.22)
<i>Washout</i>						
-64.19 (1.68)	-52.06 (2.06)	12.13 (1.31)	3.70* (0.32)	59.08 (30.70)	39.89 (6.76)	10.40 (1.10)

Values are mean (±SEM). Symbol (*) indicates value significantly different from corresponding control value (repeated measures Anova, p≤0.05; Dunnett's post-hoc test).