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The Mammalian Circadian Clock Exhibits Acute Tolerance to Ethanol

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Abstract

Background—Tolerance to ethanol is observed over a variety of time courses, from minutes to days. Acute tolerance, which develops over 5–60 minutes, has been observed for both behavioral and neurophysiological variables, and may involve changes in signaling through NMDA, GABA, or other receptors. Previous work has shown that both acute and chronic ethanol treatments modulate photic and non-photoc phase resetting of the mammalian circadian clock located in the suprachiasmatic nucleus (SCN). Although not specifically tested, the data thus far do not point to the development of chronic tolerance to the modulatory effects of ethanol. Here we investigated whether acute tolerance the ethanol occurs with respect to *in vitro* phase modulation of the SCN clock.

Methods—Mouse brain slices containing the SCN were pre-treated with ethanol for varying lengths of time, followed by treatment concurrent with either glutamate or the serotonin agonist, 8-hydroxy-DPAT (DPAT). The phase of the SCN circadian clock was assessed the following day through extracellular recordings of SCN neuronal activity. SCN neuronal activity normally peaks during mid-day, and this rhythm can be shifted by treatment with either glutamate or DPAT.

Results—While concurrent treatment of SCN-containing brain slices with ethanol and glutamate blocks glutamate-induced phase delays of the SCN clock, pre-treating the slices with ethanol for ≥ 15 min prevents this inhibition. Likewise, while concurrent treatment with ethanol and DPAT, enhances DPAT-induced phase advances of the SCN clock, pre-treating the slices with ethanol for ≥ 30 min prevents this enhancement.

Conclusions—Both the inhibiting and enhancing effects of ethanol on *in vitro* SCN clock phase resetting show acute tolerance. Additional experiments are needed to determine whether more slowly developing forms of tolerance also occur with respect to the SCN circadian clock.

Keywords

suprachiasmatic nucleus; circadian rhythms; ethanol; glutamate; serotonin; tolerance

INTRODUCTION

Alcoholism is a debilitating disease that has profound personal and societal consequences. Despite many years of research, a full understanding of how ethanol modulates neuronal signaling has not been achieved. One of the many complications associated with studying the effects of alcohol is that they have varying time scales. Tolerance to the behavioral and

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cellular effects to ethanol generally is categorized as being chronic, rapid, or acute. Chronic tolerance develops over the course of several days; rapid tolerance is usually observed 8–24 h after an initial administration of alcohol; acute tolerance occurs 5–60 min following alcohol administration (Khanna et al., 1991; Khanna et al., 1992).

Acute tolerance, sometimes referred to as ‘acute functional tolerance’, is defined as a decrease in the response to ethanol during a single exposure, with ethanol concentration being constant or decreasing at a slower rate (Khanna et al., 2002; Khanna et al., 1992; Erwin et al., 2000; Draski et al., 2001). It has been observed in both behavioral paradigms, such as rotorod performance and sedation after ethanol administration, as well as in electrophysiological paradigms, such as changes in firing rate, channel activity, or neurotransmitter effects after ethanol treatment (Khanna et al., 2002; Gill and Deitrich, 1998; Erwin et al., 2000; Pearson et al., 1997; Allen and Harris, 1987). The propensity to exhibit acute tolerance can vary depending on age, sex, and genetic background (Pearson et al., 1997; Gill and Deitrich, 1998; Kirstein et al., 2002; Silveri and Spear, 2001; Varlinskaya and Spear, 2006; Draski et al., 2001). The cellular basis for acute tolerance is not known, but appears to be distinct from those associated with rapid and chronic tolerance (Khanna et al., 1992; Silveri and Spear, 2001; Draski et al., 2001). However, because acute tolerance appears to be more prominent in animals that drink more alcohol (Silveri and Spear, 2001; Varlinskaya and Spear, 2006; Erwin et al., 2000), a better understanding of its characteristics may lead to treatments that prevent alcoholism and alcohol abuse.

At the cellular level, acute tolerance has been associated with several neurotransmitter systems. Acute tolerance to ethanol is seen with respect to inhibition of NMDA- but not AMPA-induced depolarization of sympathetic preganglion neurons (Lin et al., 2003), and to NMDA-induced pressor responses after injections into the medulla (Lin et al., 2006). Similarly, acute tolerance to ethanol is inhibited by NMDA receptor antagonists (Khanna et al., 1992; Khanna et al., 2002) and enhanced by an NMDA receptor agonist (Khanna et al., 2002). On the other hand, acute tolerance to ethanol is also seen with respect to muscimol-induced chloride currents in cerebellar neurons (Allen and Harris, 1987), and acute tolerance is diminished after injections of PCPA (Khanna et al., 2002). Thus, evidence supports the involvement of glutamate, GABA, and serotonin signaling in the development of acute tolerance. Interestingly, these three neurotransmitter systems play critical roles in regulating the phase of the mammalian circadian clock (Gillette et al., 1995; Hastings et al., 1998). Furthermore, we and others have shown that ethanol profoundly disrupts phase regulation of mammalian circadian rhythms. Acute ethanol applied *in vitro* blocks glutamate-induced phase shifts, while it enhances serotonergic phase shifts (Prosser et al., 2008). Acute ethanol also inhibits light-induced phase shifts *in vivo*, which are primarily glutamate-dependent (Ruby et al., 2009). However, because chronic ethanol also inhibits photic phase shifts (Rosenwasser, 2004; Seggio et al., 2007) (Ruby et al., unpublished results), it is unclear whether the circadian system exhibits tolerance to ethanol. To begin addressing this issue, we investigated whether acute tolerance is seen with respect to ethanol effects on *in vitro* glutamatergic and serotonergic phase resetting of the SCN clock.

MATERIALS AND METHODS

Brain slice preparation

Coronal brain slices (500 μ m) containing the SCN were prepared during the daytime from adult, male C57BL/J6 mice, housed in 12:12 LD conditions, as reported previously (Prosser and Gillette, 1989; Prosser et al., 1993; Prosser, 1998). Slices were prepared between Zeitgeber time (ZT) 0–4 (where ZT 0 = lights-on and ZT 12 = lights-off in the donor animal colony). Slices were maintained at the interface of a Hatton-style brain slice chamber (Hatton et al., 1980), where they were perfused continuously with warm (37°C), oxygenated

(95% O₂/5% CO₂), glucose/bicarbonate-supplemented Earle's Balanced Salt Solution (EBSS; Sigma-Aldrich, St. Louis, MO), pH 7.4–7.5. Gentamicin (0.05%) was also added to the perfusion medium.

Experimental protocols

All drugs were prepared in warm, oxygenated EBSS and were bath-applied to the brain slices. At the onset of the drug treatments, perfusion of the standard medium was stopped, the medium was completely removed from the chamber, and fresh medium containing the drugs was applied. Previous experiments have demonstrated that changing the perfusion medium by itself does not affect the phase of the circadian clock.

Drug treatments—1 mM glutamate (Sigma-Aldrich) was applied for 10 minutes beginning at ZT 16 together with 50mM ethanol (diluted from 95% ethanol, AAPER Alcohol). Ethanol administration was initiated at varying times prior to glutamate. In other experiments brain slices were treated at ZT 6 for 10 min with 10 uM (+)-8-hydroxy-2-(di-N-propylamino)tetralin (DPAT; a 5-HT agonist selective for 5HT_{1A/5/7} receptors), together with 50 mM ethanol. Again, ethanol administration was initiated at varying times prior to DPAT. Thus, each brain slice received a single ethanol administration, either alone or combined with glutamate or DPAT. After the drugs were removed, slices were left undisturbed until the following day.

Single-unit recordings and data analysis

Single-unit recordings commenced near the beginning of day 2 *in vitro* (between ZT 22 and ZT 1). The procedure has been described previously (Prosser et al., 1993; Prosser, 1998). Briefly, the spontaneous activity of single SCN neurons was recorded extracellularly using glass microelectrodes. Each neuron was recorded for 5 min, and the data stored for later determination of firing rate using a DataWave system (Berthoud, CO). Typically, 4–7 cells were recorded during each hour. These individual firing rates were then used to calculate 2 h running averages, lagged by 1 h ($\pm$ SEM), to obtain a measure of population neuronal activity. As in previous studies (Prosser et al., 1993; Prosser, 1998), the time of peak neuronal activity was assessed visually by estimating, to the nearest quarter hour, the time of symmetrically highest activity. For example, if the two highest 2 h means are equal, then the time of peak is estimated to be halfway between them. Phase shifts were calculated as the difference in time-of-peak of untreated slices vs. drug-treated slices. Using these methods, the consistency of the results obtained for each experimental manipulation is such that differences in phase of as little as one hour are often statistically significant with few ($n=2$ to 3) replicates (e.g., (Prosser, 2003; Prosser et al., 2006)).

Statistical Analysis

Differences in the time of peak neuronal activity were assessed using one-way ANOVA, followed by post-hoc (Dunnett's) test. In all cases, the level of significance was set at $p<0.05$.

RESULTS

Ethanol inhibition of glutamate-induced phase shifts exhibits acute tolerance

We have shown previously that 1 mM glutamate applied at ZT 16 phase delays the SCN neuronal activity rhythm by about 3 h, and these phase delays are completely blocked by co-treatment with EtOH (Prosser et al., 2008). Here we show (Fig 1) that when EtOH (50mM) is applied to SCN brain slices beginning 15 min prior to glutamate/EtOH co-treatment the glutamate-induced phase delays are no longer inhibited. A shorter (5 min) ethanol

pretreatment is not sufficient to prevent the inhibition, while longer pretreatments (up to at least 3 h) remain unable of inhibiting the glutamate-induced phase delay. Thus, *in vitro* glutamate-induced phase shifts exhibit acute tolerance to EtOH that takes between 5 and 15 min to fully develop. These results are summarized in Fig 2.

Ethanol enhancement of DPAT-induced phase shifts exhibits acute tolerance

We have also shown previously that 10 μ M DPAT applied to SCN brain slices at ZT 6 induces 3 h phase advances of the neuronal activity rhythm that are enhanced by approximately 50% by co-treatment with EtOH (Prosser et al., 2008). Here we show (Fig 3) that applying EtOH (50mM) to SCN brain slices beginning 15 min prior to DPAT/EtOH co-treatment still results in enhanced phase advances. However, after pretreating slices with EtOH for 30 min or longer (up to at least 3 h) no increases in the magnitude of the DPAT-induced phase advances are seen. Thus, *in vitro* serotonergic phase shifts also exhibit acute tolerance to EtOH, which in this case takes between 15 and 30min to fully develop. These results are summarized in Fig 4.

DISCUSSION

Acute tolerance to ethanol, which generally develops over the course of 5–60 min, has been observed in a variety of *in vivo* and *in vitro* paradigms (Khanna et al., 2002; Gill and Deitrich, 1998; Erwin et al., 2000; Pearson et al., 1997; Allen and Harris, 1987). Although the cellular basis for acute tolerance is not known, its appearance has been associated with a propensity to drink more alcohol (Silveri and Spear, 2001; Varlinskaya and Spear, 2006; Erwin et al., 2000). Thus, a more thorough understanding of acute tolerance may provide clues to the motivation to drink alcohol.

Our results provide the first evidence that the circadian clock system exhibits acute tolerance to ethanol. In particular, they demonstrate that the *in vitro* phase resetting pathways activated by glutamate and serotonin develop tolerance that occurs with similar, albeit not identical, time courses. Acute tolerance to ethanol inhibition of glutamate-induced phase shifts takes between 5 and 15 min to develop, while that for ethanol enhancement of serotonergic phase shifts takes between 15 and 30 min to develop. Whether this difference is due to the different times of treatment (night vs. day) or whether it is due to the different neurotransmitter signaling processes (or a combination of both) is not known. However, our previous work indicates that, rather than having a direct action on serotonin receptors, the modulatory effects of ethanol on serotonergic phase resetting in the SCN involve inhibition of glutamatergic signaling, since the effects of ethanol on serotonergic phase shifts are mimicked by various treatments that block NMDA signaling in the SCN (Prosser et al., 2008). However, we cannot rule out involvement of other neurotransmitters, such as GABA.

Most studies report that acute tolerance develops over the course of 10–50 min (Khanna et al., 1992; Pearson et al., 1997; Gill and Deitrich, 1998; Khanna et al., 2002; Erwin et al., 2000; Silveri and Spear, 2001; Lin et al., 2006), although some indicate that tolerance is seen within 5 min of ethanol application (Varlinskaya and Spear, 2006; Allen and Harris, 1987). However, between-study differences in *in vivo* and *in vitro* preparations, methods of ethanol administration, and ethanol concentrations can make such comparisons difficult. Focusing specifically on the effects of *in vitro* ethanol, Lin et al (Lin et al., 2003) found that tolerance to ethanol (100 mM) inhibition of NMDA-induced depolarization of sympathetic pre-ganglion neurons is seen in a subset of neurons after 40–50 min. Conversely, Pearson et al. (1997) found that acute tolerance to ethanol inhibition of cerebellar neuronal activity occurs after 5–10 min of repeated ethanol pressure injections. Similarly, Allen and Harris (1987), using a combination of *in vivo* and *in vitro* administrations, found that tolerance to ethanol (10–50 mM) enhancement of muscimol-induced chloride currents in cerebellar neurons

starts 5 min post-ethanol administration. Interestingly, in this latter study, acute tolerance was still seen at 60 min but not 24 h after acute ethanol administration. Our findings that acute tolerance is still seen after ethanol pre-treatments of at least 3 h is consistent with this time course (although we did not extend the pre-treatment duration beyond 3 h).

There is evidence that the circadian system does not develop chronic tolerance to ethanol. For example, disruptions in circadian rhythms and sleep persist in humans after chronic alcohol consumption (Rosenwasser, 2001; Danel and Touitou, 2004; Spanagel et al., 2005), and often continue long after alcohol withdrawal (Brower, 2001; Roehrs and Roth, 2001; Landolt and Gillin, 2001). Also, chronic ethanol administration in mice and hamsters continues to inhibit photic phase shifts (Rosenwasser, 2004; Seggio et al., 2007) (unpublished data) much like what has been seen after acute ethanol (Ruby et al., 2009).

At this point it is unclear why chronic tolerance to ethanol has not been seen in association with the circadian system. It is possible that this is due to the relative insensitivity to ethanol exhibited by hamsters and C57 mice and/or brain levels of ethanol not reaching sufficiently high levels after *in vivo* administration. However, in these experiments we observed tolerance to ethanol in brain slices prepared from C57 mice. Also, when it has been monitored *in vivo* (Ruby et al., 2009, Ruby et al., unpublished data) ethanol concentrations in the SCN have reached levels comparable to those used in these experiments. A more likely possibility is that the (high) intensity of the phase-shifting stimuli used in the *in vivo* studies may have masked the expression of any tolerance that may have developed. It is also possible that a combination of neuronal changes occur in response to chronic ethanol *in vivo* that compensate for each other, leading to little or no overall change in the phase shifting response to light pulses. While glutamate is the dominant neurotransmitter associated with photic phase-shifting responses, peptides such as neuropeptide Y, vasoactive intestinal peptide, pituitary adenylate cyclase-associated peptide, and gastrin-related peptide all have been shown to modulate the effects of glutamate (Reghunandanan et al., 1993; Aida et al., 2002; Yannielli and Harrington, 2004; Kallingal and Mintz, 2006), as have both serotonin and GABA (Mintz et al., 2002; Yannielli and Harrington, 2004). Therefore, it will be important to investigate the acute and chronic effects of ethanol on each of these signaling systems within the SCN in order to fully understand the mechanisms through which ethanol affects circadian clock phase modulation. Investigating chronic tolerance in an *in vitro* preparation may also help identify changes in individual neurotransmitter systems.

In summary, we show that the SCN circadian clock exhibits acute tolerance to ethanol modulation of glutamatergic and serotonergic phase resetting when isolated *in vitro*. The tolerance develops over time courses of between 5 and 30 minutes, and continues through at least 3 h of ethanol application. Future studies can extend these findings to determine whether the circadian clock also develops more long-term forms of compensation, and investigate the signaling systems associated with these changes.

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Figure 1.

Acute tolerance to ethanol inhibition of glutamate-induced phase delays. Shown are the 2 h means $\pm$ SEM of SCN neuronal activity obtained in individual experiments. (A) Control experiment with no drug treatment showing normal time of peak activity around ZT 6; (B) Glutamate treatment at ZT 16 induces a 3 h phase delay, so neuronal activity peaks around ZT 9; (C) Ethanol co-treatment prevents the glutamate-induced phase delay; (D) Pre-application of ethanol for 15 min prior to glutamate/ethanol co-treatment produces the normal 3 h phase delay, demonstrating acute tolerance. Horizontal bars: time of lights-off in the animal colony; vertical bars: time of drug treatment(s); dotted line: mean time-of-peak in control experiments. Arrows: time of peak neuronal activity.

Figure 2.

Summary of phase-shifting experiments with glutamate and ethanol. Shown are the mean ($\pm$ SEM) phase delays induced by different treatments. Glutamate applied at ZT 16 induces phase delays of about 2.5 h that are inhibited by co-application of ethanol. A 5 min pretreatment with ethanol continues to inhibit glutamate-induced phase delays, while longer durations of ethanol pretreatment no longer have any inhibitory effect. Ethanol applied alone has no effect. One-way ANOVA indicated a significant effect of treatment ($F_{18} = 88.9$)

*Post-hoc (Dunnett's) test indicates $p < 0.001$ vs. control experiments.

Figure 3.

Acute tolerance to ethanol enhancement of DPAT-induced phase advances. Shown are the 2 h means $\pm$ SEM of SCN neuronal activity obtained in individual experiments. (A) DPAT treatment at ZT 6 induces a 3 h phase advance, so neuronal activity peaks around ZT 3; (B) Ethanol co-treatment increases the DPAT-induced phase advance to about 4.5 h; (C) Pre-application of ethanol for 15 min prior to DPAT/ethanol co-treatment still increases the phase advance to about 4.5 h; (D) Pre-application of ethanol for 30 min prior to DPAT/ethanol co-treatment produces the normal 3 h phase delay, demonstrating acute tolerance. See Fig 1 for details.

Figure 4.

Summary of phase-shifting experiments with glutamate and DPAT. Shown are the mean ($\pm$ SEM) phase delays induced by different treatments. DPAT applied at ZT 6 induces phase advances of about 3 h that are increased to 4.5 h by co-application of ethanol. A 15 min pretreatment with ethanol continues to enhance DPAT-induced phase advances, while longer durations of ethanol pretreatment no longer have any enhancing effect. Ethanol applied alone has no effect. One-way ANOVA indicated a significant effect of treatment ($F_{15} = 35.7$) *Post-hoc (Dunnett's) test indicates $p < 0.001$ vs. DPAT treatment experiments.