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ETHANOL MODULATES MAMMALIAN CIRCADIAN CLOCK PHASE RESETTING THROUGH EXTRASYNAPTIC GABA RECEPTOR ACTIVATION

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Abstract

Ethanol modulates the actions of multiple neurotransmitter systems, including GABA. However, its enhancing effects on GABA signaling typically are seen only at high concentrations. In contrast, although GABA is a prominent neurotransmitter in the circadian clock of the suprachiasmatic nucleus (SCN), we see ethanol modulation of clock phase resetting at low concentrations (<50 mM). A possible explanation is that ethanol enhances GABAergic signaling in the SCN through activating GABA_A receptors that contain the δ subunit (GABA_{A δ} receptors), which are sensitive to low ethanol concentrations. Therefore, we investigated whether ethanol acts on GABA_{A δ} receptors in the SCN. Here we show that acute application of the GABA_{A δ} receptor antagonist, RO15-4513, to mouse hypothalamic slices containing the SCN prevents ethanol inhibition of nighttime glutamate-induced (photic-like) phase delays of the circadian clock. Diazepam, which enhances activity of GABA_{A γ} receptors, does not modulate these phase shifts. Moreover, we find that RO15-4513 prevents ethanol enhancement of daytime serotonergic (non-photic) phase advances of the circadian clock. Furthermore, diazepam phase-advances the SCN circadian clock when applied alone in the daytime, while ethanol has no effect by itself at that time. These data support the hypothesis that ethanol acts on GABA_{A δ} receptors in the SCN to modulate photic and non-photic circadian clock phase resetting. They also reveal distinct modulatory roles of different GABA_A receptor subtypes in circadian clock phase regulation.

Keywords

suprachiasmatic nucleus; circadian rhythms; GABA; alcohol; benzodiazepine

Alcoholism and alcohol abuse pose major health concerns that are difficult to treat in part due to ethanol's diverse actions throughout the central nervous system. One of the neurotransmitter systems modulated by ethanol is GABA: Ethanol increases GABA release, and enhances the activity of both GABA_A and GABA_B receptors (see (Weiner and Valenzuela, 2006) for review). However, most studies have employed pharmacological concentrations of ethanol to

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study its actions (Mihic et al., 1997; Sigel et al., 1993; Vengeliene et al., 2008). At levels typically associated with social ethanol consumption (< 50 mM), however, the GABAergic effects of ethanol appear to be restricted primarily to a subset of GABA_A receptors that contain the δ subunit (GABA_{A δ} receptors) (Mihic et al., 1997; Wallner et al., 2003; Glykys et al., 2007; Wei et al., 2004; Olsen et al., 2007; Santhakumar et al., 2007). These receptors are thought to be located primarily in extra-synaptic regions, are highly sensitive to GABA, and are believed to tonically inhibit neuronal excitability (Hancher et al., 2005; Wallner et al., 2003; Nusser et al., 1998; Nusser and Mody, 2002).

Our previous research has shown that ethanol modulates phase resetting of the mammalian circadian clock located in the suprachiasmatic nucleus (SCN) at low concentrations both *in vivo* and *in vitro* (Prosser et al., 2008; Ruby et al., 2009; Brager et al., 2009). *In vitro*, ethanol inhibits glutamate-induced phase shifts with an ED₅₀ of about 10 mM, and enhances serotonergic phase shifts with an ED₅₀ of about 20 mM. This latter effect is mimicked by treatments that inhibit glutamate signaling in the SCN, including the TrkB antagonist, K252a. These results are consistent with studies showing that ethanol inhibits brain-derived neurotrophic factor (BDNF) enhancement of NMDA receptor activity in the hippocampus at low concentrations (Kolb et al., 2005). Collectively, these results point to an inhibitory action of ethanol on glutamate signaling in the SCN. However, most SCN neurons are GABAergic (van den Pol, 1993), and GABA plays an important modulatory role in photic and non-photic regulation of SCN timekeeping (Novak et al., 2008). Furthermore, a recent study provides evidence that GABA_{A δ} receptors within the SCN are able to modulate photic phase shifts (Ehlen and Paul, 2009). Based on these recent findings, we investigated whether ethanol enhances GABA signaling in the SCN, and if so, whether this specifically involves GABA_{A δ} receptors. To this end, we compared the effects of the atypical benzodiazepine, RO15-4513, which selectively inhibits GABA_{A δ} receptors, to those of diazepam, which activates the more ubiquitous, synaptically located, GABA_{A γ} receptors (Saxena and MacDonald, 1996; Olsen and Sieghart, 2009; Wallner and Olsen, 2008).

EXPERIMENTAL PROCEDURES

Brain slice preparation

Coronal brain slices (500 μ m) containing the SCN were prepared during the daytime from adult, male C57BL/6 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—Brain slices were treated for 10 minutes beginning at ZT 16 with various combinations of glutamate (1 mM; Sigma-Aldrich), ethanol (20mM; diluted from 95% ethanol, AAPER Alcohol), RO15-4513, or diazepam (10 μ M). In other experiments brain slices were

treated at ZT 6 for 10 min with various combinations of (+)8-hydroxy-2-(di-N-propylamino) tetralin (10 μ M; DPAT; a 5-HT agonist selective for 5HT_{1A/5/7} receptors), ethanol (100mM), RO15-4513, or diazepam. 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 4). 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 ANOVA, followed by Dunnett's test. In all cases, the level of significance was set at $p<0.05$.

RESULTS

Ethanol inhibition of glutamate-induced phase shifts is dose-dependently blocked by RO15-4513

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 20 mM ethanol ($ED_{50} \approx 10$ mM) (Prosser et al., 2008). Here we show (Fig. 1A) that when we applied the GABA_{A δ} receptor antagonist, RO15-4513, together with glutamate and ethanol, it completely reversed the inhibition by ethanol. The reversal effect of RO15-4513 was dose-dependent, with an ED_{50} of about 75nM (Fig. 1B). RO15-4513 applied by itself had no phase-shifting effect, nor did it modulate glutamate-induced phase shifts when applied in the absence of ethanol. These results, which support ethanol acting by stimulating GABA_{A δ} receptors, are summarized in Fig. 2.

Ethanol inhibition of glutamate-induced phase shifts is not blocked by diazepam

Although the previous results support ethanol acting to enhance signaling of GABA_{A δ} receptors, they do not exclude ethanol acting on the more common, benzodiazepine-sensitive, GABA_{A γ} receptors. To address this issue we investigated the effects of diazepam. If ethanol activates GABA_{A γ} as well as GABA_{A δ} receptors in the SCN, then other compounds that selectively activate GABA_{A γ} receptors should reproduce at least some of the actions of ethanol. However, when we applied diazepam to SCN brain slices it did not block glutamate-induced phase shifts (Fig. 3). Diazepam applied alone at this time did not induce a phase shift, nor did it alter ethanol inhibition of glutamate-induced phase shifts (Fig. 3). Together, these data indicate that ethanol does not enhance GABA_{A γ} receptor activity in the SCN, at least at the low concentration used in these experiments.

Ethanol enhancement of serotonergic phase shifts is dose-dependently blocked by RO15-4513

We have also previously shown 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 100 mM ethanol ($ED_{50} \approx 20$ mM) (Prosser et al., 2008). As shown in Fig. 4A, when RO15-4513 was co-applied with DPAT and ethanol, the ethanol-induced enhancement did not occur. Instead, we observed the normal 3 h phase advance normally induced by DPAT when applied alone. This effect of RO15-4513 was dose-dependent, with an ED_{50} of 30–40 nM (Fig. 4B). Furthermore, treatment with RO15-4513 alone had no effect, nor did it have any effect when combined with either glutamate or ethanol. These results, which again support ethanol acting on GABA_{A δ} receptors, are summarized in Fig. 5.

Diazepam phase advances the SCN clock when applied during the subjective day

To investigate whether ethanol also activates GABA_{A γ} receptors in the SCN, we again utilized the GABA_{A γ} receptor agonist, diazepam. Unlike what was seen with ethanol, however, diazepam applied alone to SCN brain slices at ZT 6 induced a 3 h phase advance (Fig. 5). These results precluded any further investigation into the effects of diazepam in combination with DPAT and ethanol.

DISCUSSION

Ethanol modulation of GABA signaling is a well documented phenomenon. For example, ethanol increases GABA_A chloride currents and GABA-induced membrane hyperpolarization (Hancher et al., 2005; Wallner et al., 2003; Carta et al., 2004; Nusser and Mody, 2002; Sigel et al., 1993; Borghese et al., 2006). Treatments that increase GABAergic tone enhance ethanol actions, while those that decrease GABAergic activity inhibit ethanol actions (Weiner and Valenzuela, 2006; Vengeliene et al., 2008). More controversial issues regarding these GABA-related effects are, which GABA receptor subtypes are modulated by ethanol, and more specifically, which of these receptors are linked to the disruptive effects of ethanol abuse on the circadian timing system.

GABA_A receptors are pentameric, generally composed of 2 α 's, 2 β 's, and either a γ or δ subunit (Saxena and MacDonald, 1996; Olsen and Sieghart, 2009). The majority of studies investigating ethanol actions on GABA receptors have used concentrations greater than 50 mM (Mihic et al., 1997; Sigel et al., 1993; Vengeliene et al., 2008; Weiner and Valenzuela, 2006), which is higher than levels attained during normal social consumption (Olsen et al., 2007). Lower concentrations of ethanol consistently enhance the activity only of GABA_{A δ} receptors (Mihic et al., 1997; Wei et al., 2004; Sigel et al., 1993), although not all studies have confirmed these results (Borghese et al., 2006; Borghese and Harris, 2007; Botta et al., 2007). In addition to their greater sensitivity to ethanol, GABA_{A δ} receptors have several interesting properties: They are highly sensitive to GABA (Wallner et al., 2003); are found primarily in extra-synaptic regions (Nusser et al., 1998); and are insensitive to most benzodiazepines (Saxena and MacDonald, 1996; Olsen and Sieghart, 2009; Choi et al., 2008). However, the atypical benzodiazepine, RO15-4513, inhibits ethanol enhancement of GABA_{A δ} receptor activity (Olsen and Sieghart, 2009). Our demonstrations that ethanol affects photic and non-photic phase modulation of the SCN circadian clock at low concentrations (Prosser et al., 2008; Ruby et al., 2009; Brager et al., 2009), that these effects are inhibited by RO15-4513, and that they are not mimicked by diazepam, are consistent with ethanol acting on GABA_{A δ} receptors.

GABA is one of the primary neurotransmitters associated with the SCN circadian clock. Most, if not all, SCN neurons are GABAergic (Decavel and van den Pol, 1990), and daily GABA pulses can synchronize SCN pacemaker neurons in dispersed cell culture (Liu and Reppert,

2000). A variety of GABA receptor subunits are expressed in the SCN (Gao et al., 1995; Naum et al., 2001; O'Hara et al., 1995; Belenky et al., 2003), and GABA receptors are seen in pre-, post- and extra-synaptic locations (Belenky et al., 2003). Pharmacological studies have provided evidence for the presence of both δ - and δ -subunit-containing GABA receptors in the SCN (Kawahara et al., 1993; Shimura et al., 1996).

Injections of the general GABA agonist, muscimol, into the SCN *in vivo* inhibits photic phase shifts at night (Mintz et al., 2002; Gillespie et al., 1996; Gillespie et al., 1997) and blocks light-induced increases in c-fos immunoreactivity (Gillespie et al., 1999) and *Period 1* and *2* gene expression in the SCN (Ehlen et al., 2008). Conversely, muscimol induces daytime phase advances when applied to the SCN either *in vivo* (Mintz et al., 2002; Gamble et al., 2003) or *in vitro* (Tominaga et al., 1994) and decreases daytime *Period 1* expression in the SCN (Ehlen et al., 2008). Similar, although not identical, results have been obtained with diazepam (Ralph and Menaker, 1986; Ralph and Menaker, 1985; Ralph and Menaker, 1989; Colwell et al., 1993), but these studies all involved peripheral injections, which could be acting outside the SCN (Della Maggiore and Ralph, 1999). This possibility is reinforced by the results we have presented here, which match those obtained with muscimol.

More recently, gaboxadol, a GABA agonist that selectively activates GABA_{A δ} receptors, (Weiner and Valenzuela, 2006; Olsen and Sieghart, 2009), was shown to inhibit light- and NMDA-induced phase shifts in hamsters *in vivo*, but not to induce daytime phase shifts on its own (Ehlen and Paul, 2009). These results are consistent with our findings that ethanol blocks glutamate-induced phase shifts *in vitro*, but does not phase-shift the clock when applied by itself in the daytime. In contrast, we found that diazepam, which acts on GABA_{A γ} receptors but not GABA_{A δ} receptors, does not inhibit glutamate-induced phase shifts, but does phase-shift the SCN clock when applied alone in the day. Together, these data indicate that the GABAergic effects of ethanol in the SCN (at least at relatively low concentrations) are restricted to activating GABA_{A δ} receptors, with little or no apparent stimulation of GABA_{A γ} receptors. While we did not extend our investigation into late-night glutamate-induced phase advances, our studies on *in vitro* ethanol effects thus far (Prosser et al., 2008) have shown few differences between early and late night experiments.

An important point raised by these data is that there is a clear difference in the effects on the SCN circadian clock of activating GABA_{A γ} receptors, which generate phasic inhibition, vs. GABA_{A δ} receptors, which tonically inhibit neuronal activity. Activation of the former receptors can phase-shift the SCN clock, while stimulating the latter receptors only appears capable of modulating the phase resetting effects of other treatments. One explanation for this is that only synaptically located receptors may have access to the protein complexes that must necessarily be modulated to phase-shift the clock. In contrast, their distance from the synaptic protein complexes may prevent extra-synaptic receptors from initiating phase resetting processes, while still allowing them to enhance or diminish the phase-shifting effects initiated by synaptic receptors. Previous studies have also focused on the question of distinct roles of phasic vs. tonic GABA currents in modulating rate-coded sensory information, neuronal networking, and neuronal excitability (Semyanov et al., 2004; Farrant and Nusser, 2005). Whether such differences are important in regulating SCN circadian clock phase resetting is at the present time unknown.

Our data do not exclude potential actions of ethanol on other neurotransmitter systems in the SCN. In addition to GABA, ethanol is known to modulate a variety of neurotransmitters that regulate SCN function, including glutamate, serotonin, and acetylcholine (Vengeliene et al., 2008). Our previous results demonstrate that, in addition to inhibiting glutamate-induced phase shifts, ethanol enhances serotonergic phase shifts *in vitro* (Prosser et al., 2008). This enhancing effect is mimicked by a variety of treatments, including glutamate antagonists and

a TrkB antagonist (Prosser et al., 2008), all previously shown to inhibit glutamate signaling in the SCN (Ding et al., 1994; Michel et al., 2006). These data are, therefore, consistent with ethanol inhibiting glutamate signaling in the SCN. Consistent with this theory, low concentrations of ethanol have been shown to inhibit BDNF/TrkB -induced increases in glutamate signaling in the hippocampus (Kolb et al., 2005). Thus, it is feasible that ethanol affects the SCN circadian clock through modulating multiple neurotransmitter systems involved in photic and non-photoc phase regulation.

In conclusion, ethanol at low concentrations modulates glutamatergic and serotonergic phase shifts of the SCN circadian clock *in vitro* at least in part through activating GABA_{Aδ} receptors. These actions of ethanol within the SCN are distinct from those of the benzodiazepine, diazepam. Diazepam, which enhances GABA_{Aγ} receptor activity, phase-advances the SCN circadian clock when applied alone during the daytime, and does not inhibit nighttime phase delays induced by glutamate. The difference between these two GABAergic effects may be due to the synaptic vs. extra-synaptic locations of the receptors in the SCN and/or their ability to directly access the cellular processes associated with circadian clock phase resetting.

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Abbreviations

BDNF, brain-derived neurotrophic factor; DPAT, (+)8-hydroxy-2-(di-N-propylamino) tetralin; EBSS, Earle's Balanced Salt Solution; GABA_{Aδ} receptors, GABA_A receptors containing the δ subunit; GABA_{Aγ} receptors, GABA_A receptors containing the γ subunit; SCN, suprachiasmatic nucleus; ZT, Zeitgeber time.

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Figure 1.

Ethanol inhibition of glutamate-induced phase delays is reversed by RO15-4513. **A.** Shown are the 2 h means $\pm$ SEM of SCN neuronal activity obtained in a control experiment and in experiments where slices were treated at ZT 16 on the first day *in vitro* with the compounds indicated. In the control experiment, neuronal activity peaks near ZT 6 on the second day *in vitro*. Neuronal activity peaked approximately 3 h later in brain slices that were treated with glutamate (1mM) at ZT 16, indicating the SCN clock had been phase-delayed by 3 h. Co-application of 20mM ethanol treatment with glutamate to SCN brain slices blocked the phase delay. Treating slices with RO15-4513 + glutamate + ethanol reinstated the glutamate-induced phase delay. Horizontal bars: time of lights-off in the animal colony; vertical bars: time of drug treatment; dotted line: mean time-of-peak in control experiments. **B.** Dose response curve for RO15-4513 reversal of ethanol inhibition. Shown are the mean phase shifts ($\pm$ SEM) induced by glutamate applied alone, glutamate + ethanol, or glutamate + ethanol + RO15-4513.

Figure 2.

Summary of phase-shifting experiments with glutamate. Shown are the mean ($\pm$ SEM) phase shifts induced by different treatments applied to SCN brain slices at ZT 16. Glutamate applied at ZT 16 induced phase delays of about 2.5 h that were inhibited by ethanol. RO15-4513 reversed that inhibition, but had no effect when applied alone or with either glutamate or ethanol. * $p < 0.05$ vs. control experiments.

Figure 3.

Summary of phase-shifting experiments with diazepam. Shown are the mean ($\pm$ SEM) phase shifts induced by different treatments applied to SCN brain slices at ZT 16. Diazepam did not reverse the ethanol inhibition of glutamate-induced phase delays. Diazepam also had no effect when applied alone or in combination with glutamate. * $p < 0.05$ vs. control experiments.

Figure 4.

Ethanol enhancement of DPAT-induced phase advances is reversed by RO15-4513. **A.** Shown are the 2 h means $\pm$ SEM of SCN neuronal activity obtained in experiments where slices were treated at ZT 6 on the first day *in vitro* with the compounds indicated. DPAT alone induced a 3 h phase advance. Ethanol co-treatment increased the phase advance to 4.5 h. Addition of RO15-4513 to the DPAT + ethanol treatment restored the normal 3 h phase advance. **B.** Dose response curve for RO15-4513 reversal of ethanol effect. Shown are the mean phase shifts ($\pm$ SEM) induced by DPAT applied alone, DPAT + ethanol, or DPAT + ethanol + RO15-4513. See Fig. 1 legend for details.

Figure 5.

Summary of phase-shifting experiments with DPAT. Shown are the mean ($\pm$ SEM) phase shifts induced by different treatments applied to SCN brain slices at ZT 6. DPAT applied at ZT 6 induced phase advances of 3 h that were increased to 4.5 h by ethanol. RO15-4513 reversed this effect. RO15-4513 has no effect when applied alone or with either DPAT or ethanol. Diazepam induced a 2.5 h phase advance when applied to SCN brain slices alone. * $p < 0.05$ vs. control experiments; ** $p < 0.05$ vs. DPAT treatment.