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I am submitting herewith a thesis written by Charles Andrew Mangrum entitled “The Effects of Acute Ethanol Treatment on the Suprachiasmatic Nucleus in Adult Male Mice.” I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

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**The Effects of Acute Ethanol Treatment on the
Suprachiasmatic Nucleus in Adult Male Mice**

**A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville**

**Charles Andrew Mangrum
May 2007**

Dedication

To those who have unknowingly directed my life.
They have an intelligence that I shall never possess.

“Two roads diverged in a yellow wood, and I –
I took the one less traveled by,
And that has made all the difference.”
Robert Frost

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Abstract

Light is the primary entraining signal for the mammalian circadian clock located in the suprachiasmatic nucleus (SCN). Light entering the eye leads to release of glutamate directly onto SCN neurons where it binds to *N*-methyl D-aspartate (NMDA) receptors initiating a cascade of cellular processes that ultimately modulates clock phase. SCN neurons show a 24-hour rhythm in neuronal activity that peaks in the middle of the day when isolated in a brain slice preparation. Treatments that phase-shift the SCN clock *in vivo* have been shown similarly to shift this rhythm of neuronal activity *in vitro*. Here, I have investigated the effects of ethanol on circadian rhythms in SCN brain slices.

My experiments have found that acute application of glutamate [1mM] in the early night to mouse SCN brain slices causes a mean phase-delay of 2.7 hours. Co-application of ethanol blocks this phase-delay in a dose dependent manner, with a maximum effect at 20mM. Ethanol could affect the glutamate-initiated pathway through one or more mechanisms including the prevention of glutamate and/or its co-agonists from binding to the NMDA receptor. Experiments, however, show that high levels of glutamate are not able to overcome ethanol's blocking effects. Similarly, excess concentrations of glycine and D-serine, the two potential co-agonist candidates, do not prevent ethanol's block.

Additional experiments showed that high concentrations of glycine applied in the early subjective night caused a phase-advance of 3.4 hours, indicating the presence of glycine receptors in the SCN that are capable of modulating clock phase. Experiments involving strychnine, a glycine receptor antagonist, revealed that when applied alone in

the early subjective night resulted in a phase delay of 2 hours. The mechanism behind strychnine's phase shifting abilities is unknown.

This study has shown that ethanol at a physiologically relevant level has the ability to block glutamate induced phase delays. Though the specific mechanism through which it acts has not been identified, this study suggests ethanol does not interfere with binding of glutamate, nor its co-agonist, to the NMDA receptor. In addition to the ethanol work, my study has shown the phase-shifting effects of glycine and strychnine, two previously unknown phenomena.

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I. Background and Significance

The Suprachiasmatic Nucleus and Circadian Rhythms

In mammals, the suprachiasmatic nucleus (SCN) is located in the hypothalamus superior to the optic chiasm and consists of a group of neurons that make up the primary endogenous clock (Miller et al., 1996; Edery, 2000). The clock is responsible for generating circadian rhythms that occur on an approximate twenty-four hour cycle, with slight timing variances occurring between species. The endogenous clock regulates behavioral and physiological activities so they occur at a time most favorable for survival. The SCN receives both external and internal inputs that modulate its phase. External inputs such as light/dark cycling and temperature change allow the clock to entrain to the external environment, and serve as a means of compensation for the non-exact 24 hour cycle of the circadian clock (Reppert and Weaver, 2000).

Inputs to the SCN primarily occur through three pathways: photic input detected by the eye is carried from the retina directly to the SCN via the retinohypothalamic tract (RHT) (Levine et al., 1991; Moore, 1997); and non-photoc inputs include neuropeptide Y & γ -aminobutyric acid (GABA) signals from the intergeniculate leaflet via the geniculohypothalamic tract (Moore and Card, 1990) and serotonin input from the raphe nuclei (Moore et al., 1978). [See figure 1]

Light-Induced Entrainment and the NMDA Receptor

The photic stimulus is the primary entraining factor of the circadian clock. Light activation of retinal ganglion cells results in release of the neurotransmitter glutamate directly onto SCN neurons, where it binds to *N*-methyl D-aspartate (NMDA) receptors

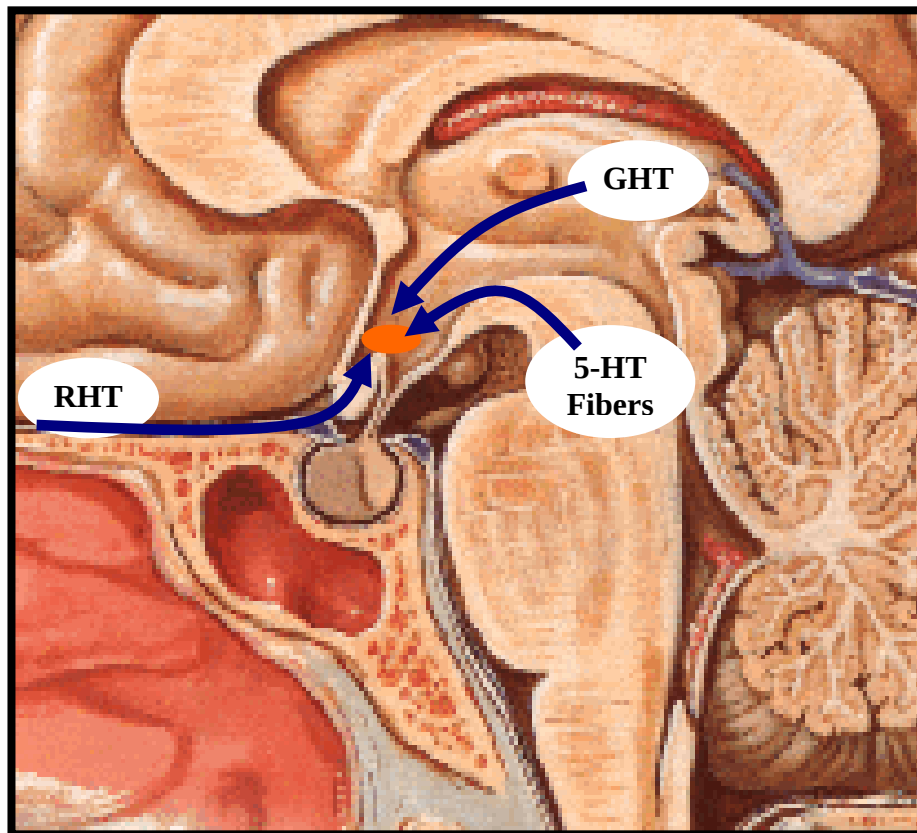
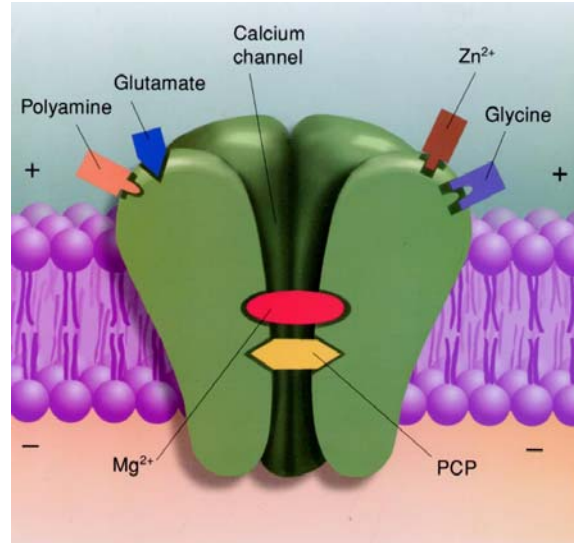


Figure 1: Entrainment inputs to the SCN – the retinohypothalamic tract (RHT) from the retina, geniculohypothalamic tract (GHT) from the intergeniculate leaflet, and serotonin (5-HT) fibers from the raphe nuclei.

(Moore and Lenn, 1972; Levine et al., 1991). NMDA is a membrane bound ionotropic glutamate receptor found throughout the central and peripheral nervous systems [Figure 2A]. This hetero-tetrameric protein is located abundantly in the SCN and can be composed of different combinations of seven subunits classified as: NR1, NR2A-D, and NR3A-B. Dimers of NR1 combine with either NR2 or NR3, the variants being regionally and developmentally specific, to form regulated ion channels (Ciabarra et al., 1995; Das et al., 1998). In the SCN all four isoforms of NR2 have been found; however, NR2B is the most common (O'Hara et al., 2000). NMDA receptors in the SCN form a calcium channel that is regulated by magnesium, which blocks the channel. Magnesium is removed by depolarization of the membrane, along with binding of the agonist, glutamate, and co-agonist, glycine; which then allows for an influx of calcium. Activation of the NMDA receptor is enhanced when the serine residues (Ser890, 896, and 897) of the NR1 subunit and tyrosine of the NR2A (undetermined residue) and NR2B (Tyr1472) subunits are phosphorylated (Wang and Salter, 1994) [Figure 2B].

Serine phosphorylation has traditionally been thought to occur through protein kinase C (Ser890 and 896) and protein kinase A (Ser897) (Tingley et al., 1997); however, recent studies in cerebellar granule cells suggest Akt kinase mediates phosphorylation of Ser897 on the NR1 subunit (Llansola et al., 2004). Phosphorylation of tyrosine residues on the NR2 subunits has been shown in the hippocampus to occur through TrkB (Lin et al., 1998). Research has shown that BDNF regulates both Akt (Sanchez-Perez et al., 2006) and TrkB activation (Lin et al., 1998). Therefore, BDNF likely has a regulatory role in NMDA phosphorylation.

A.



B.

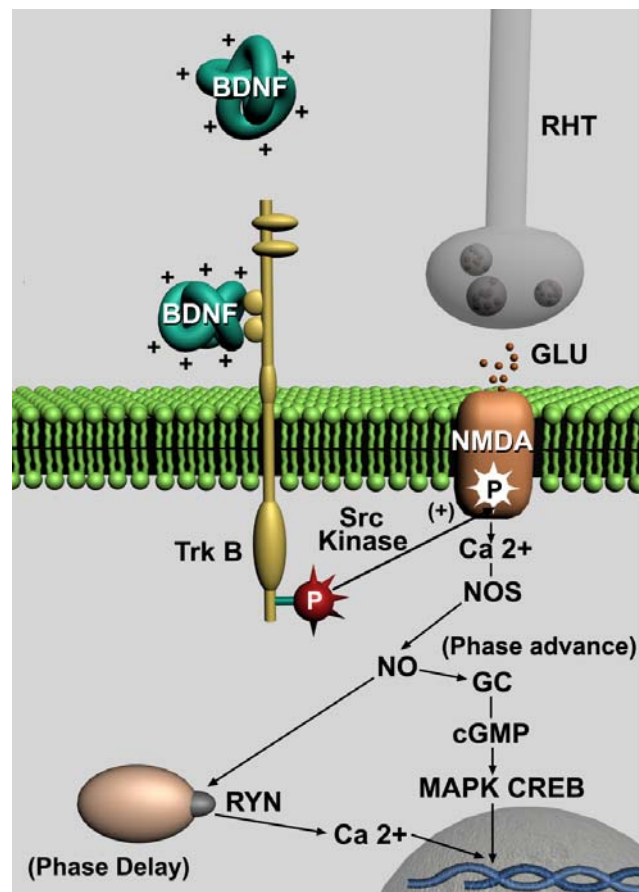


Figure 2: (A) Schematic of NMDA receptor (From Carlson, Neil R. *Physiology Of Behavior*, 9/e published by Allyn and Bacon, Boston, MA. Copyright © 2007 by Pearson Education reprint by permission of the publisher.) (B) Proposed glutamate involved pathway leading to phase shifts of the circadian clock

In the SCN, the increase in intracellular calcium in response to glutamate has been shown to activate nitric oxide synthase (NOS) and result in the production of nitric oxide (NO) (Ding et al., 1994; Ghosh and Greenberg, 1995). NO will initiate one of two cascades depending on the time at which it is present. If present in the early subjective night NO will activate ryanodine receptors which results in the release of Ca^{2+} into the cytosol. Calcium then plays a role in the regulation of clock gene transcription that leads to a delay of the clock's phase. If, however, NO is present in the late subjective night, it allows for the production of cyclic GMP which indirectly activates MAP kinase. When activated, MAP kinase leads to the phosphorylation of the transcriptional factor CREB which can induce gene expression through calcium/cAMP response elements that leads to an advance of the clock's phase (Ding et al., 1997; Obrietan et al., 1998). Initiation of the glutamate signaling, either via light stimulation or direct glutamate application, therefore will result in a phase delay when activated in the early subjective night and a phase advance when activated in the late subjective night.

Ethanol, Circadian Rhythms, and Glutamate Signaling

The consumption of, and withdrawal from, ethanol has been shown to disrupt normal circadian processes in both humans and research animals. Individuals suffering from alcoholism show higher incidences of insomnia and other related disruptions in the sleep/wake cycle compared to non-alcoholics (Brower, 2001). Similar results showing disruption in the sleep cycles have also been seen in rats chronically exposed to and withdrawn from ethanol (Ehlers and Slawecki, 2000). Additional studies involving research animals chronically exposed to ethanol have shown loss of circadian regulation

of biochemicals such as glucose, potassium, lactic acid (Rajakrishnam et al., 1999), and the hormone corticosterone (Kakihana and Moore, 1976). Ethanol has been shown to significantly lengthen the free-running period of wheel-running in hamsters (Mistlberger and Nadeau, 1991). A more recent study showed that chronic ethanol similarly affected circadian rhythms of wheel running in rats by both lengthening and shortening of the free-running period (Rosenwasser et al., 2005a). Further studies from this group showed that chronic ethanol treatment can block effects of light pulses on circadian rhythms in experimental animals. Rats exposed to a 15 minute light pulse in the subjective late night showed a shortening of free running period that is not seen in rats chronically treated with ethanol (Rosenwasser et al., 2005b). These studies suggest a relationship between ethanol and the circadian clock; however, they do not address its mode of action. It is not known whether ethanol affects the clock itself, an area downstream of the clock or if it acts on entrainment mechanisms upstream of the clock.

In many areas of the central nervous system ethanol has been shown to interfere with glutamate signaling. Acute exposure to ethanol has been shown in some areas of the brain to decrease glutamate binding in the hippocampus and throughout the central nervous system; however, this finding is not consistent in all areas of the brain (Michaelis et al., 1978; Abdollah and Brien, 1995). Glycine binding to NMDA receptors in cerebellar granule cells has also been shown to be limited during acute exposure of ethanol (Hoffman et al., 1989; Rabe and Tabakoff, 1990). Other studies have shown that ethanol can also interfere with phosphorylation of the NMDA receptor possibly through the BDNF involved pathways (Li et al., 2004; Rubin et al., 2004).

Because glutamate signaling is central to the photic input pathway, ethanol could disrupt circadian rhythms by interfering with glutamate signaling in the SCN. A likely mechanism through which ethanol could affect glutamate signaling is either by preventing opening of the NMDA receptor channel or shortening the time it is open (Lima-Landman and Albuquerque, 1989; Lovinger et al., 1989; Weight et al., 1993; Tsai and Coyle, 1998). Ethanol could do this by interfering with binding of glutamate, binding of the co-agonist glycine, or phosphorylation of the receptor. Any of these could inhibit proper activation of the NMDA receptor.

Rationale for This Study

Alcoholism is a disease that greatly affects the lives of both patients and those around them. Consumption of ethanol is known to disrupt normal circadian rhythms, including the sleep-wake cycle. Such disruptions can prevent an individual from functioning normally within society. Although recent research shows a connection between ethanol and circadian rhythms, the exact mechanism(s) is unknown. The purpose of this study is to advance the research on ethanol and the circadian clock by examining the effects of acute ethanol treatment on glutamate signaling in the SCN. Such a study can potentially elucidate some of ethanol's actions within the SCN, which could be used for further research in the treatment of alcoholism.

II. Materials and Methods

Tissue Preparation

Adult (6-12 weeks) male C57bl/J6 mice are housed under 12:12 light-dark conditions with a minimum entrainment period of 1 week prior to experimental use. Coronal brain slices (500 μ M) containing the SCN are prepared and maintained at 37° C and gassed with 95% O₂ / 5% CO₂ in a Hatton-style brain slice chamber, and continuously perfused with Earle's balanced salt solution (EBSS) (pH 7.4) supplemented with glucose, bicarbonate and gentamicin (Prosser, 1998).

Experimental Treatments

Drug treatments diluted in warm, oxygenated medium were bath applied to the brain slices during early subjective night. Immediately prior to drug treatment, perfusion of the standard medium was stopped, the medium was completely removed from the chamber and fresh medium containing the drugs was applied. All treatments were completed under zeitgeber time (ZT), a 24 hour time frame in which hour 0 corresponds to lights-on in the animal colony. Treatments of glutamate were for 10 minutes beginning at ZT 16.0. In experiments involving co-treatment of glutamate and another drug, the co-administered drug was applied alone for 15 minutes (ZT 15.75-16.0), followed by co-application with glutamate for 10 minutes (ZT 16.0-16.17). In all experiments, the drug-treated medium was removed at ZT 16.17 and perfusion of normal medium was restored. Slices were maintained in the chamber through the next day for single-unit recording experiments.

Single Unit Recording Experiments

The day following drug treatment (experimental day 2), spontaneous activity of single SCN neurons was recorded extracellularly with a glass microelectrode filled with 3M NaCl. Individual neurons were recorded for approximately 5 minutes, with 4-7 cells being recorded each hour. The data were later analyzed using a DataWave system to determine average firing rates. The individual firing rates were then used to calculate 2 hour running averages (composed of 8–14 individual firing rates), lagged by 1 hour, to obtain a measure of population neuronal activity. The time of peak neuronal activity was assessed visually by estimating, to the nearest quarter hour, the time of symmetrically highest activity. Phase shifts were calculated as the difference in time-of-peak of untreated slices (control) vs. drug-treated slices. When appropriate, regression analysis of the data was completed using GraphPad Prism (San Diego, CA). Significant differences in time of peak were determined through Student's t-test, where P values less than 0.05 were considered significant.

III. Results

Glutamate-Induced Phase Delays

SCN neuronal activity in control (non-treated) slices exhibits a peak near the middle of the subjective day, with a mean time ($\pm$ SEM) = ZT 5.5 $\pm$ 0.5 (n=3) [Figure 3]. Application of 1mM glutamate for 10 minutes at ZT 16.0 leads to a mean phase delay of 2.7 hours, with a neuronal activity peaking at ZT 8.2 $\pm$ 0.5 (n=3) [Figure 3].

Ethanol Blocks Glutamate-Induced Phase Delays in a Dose-Dependent Manner

As shown in Figure 3, co-application of 20mM ethanol with glutamate at ZT 16 completely blocks the 2.7 hour delay in peak firing rate observed when glutamate is applied alone [mean time of peak is ZT 5.9 $\pm$ 0.3 (n=3)]. This inhibition by ethanol is dose dependent, with complete blocking occurring with 20mM ethanol and an EC₅₀ of 10.41mM [Figure 4]. Ethanol [20mM] applied alone had little effect on the time of peak neuronal activity when compared to control experiments. The mean peak firing time in these experiments occurred at ZT 6.0 $\pm$ 0.2 (n=3). The results of these experiments are summarized in Figure 5.

Excess Glutamate Does Not Prevent Inhibition by Ethanol

To begin investigating the mechanism through which ethanol inhibits the glutamate phase delays, I first tested whether ethanol blocks glutamate binding to its receptor. To do this I tested whether excess glutamate [50mM] could overcome the inhibition induced by a half-max concentration of ethanol [10mM] [mean time of peak = ZT 7.4 $\pm$ 0.7 (n=3)]. The results showed that excess glutamate was unable to overcome

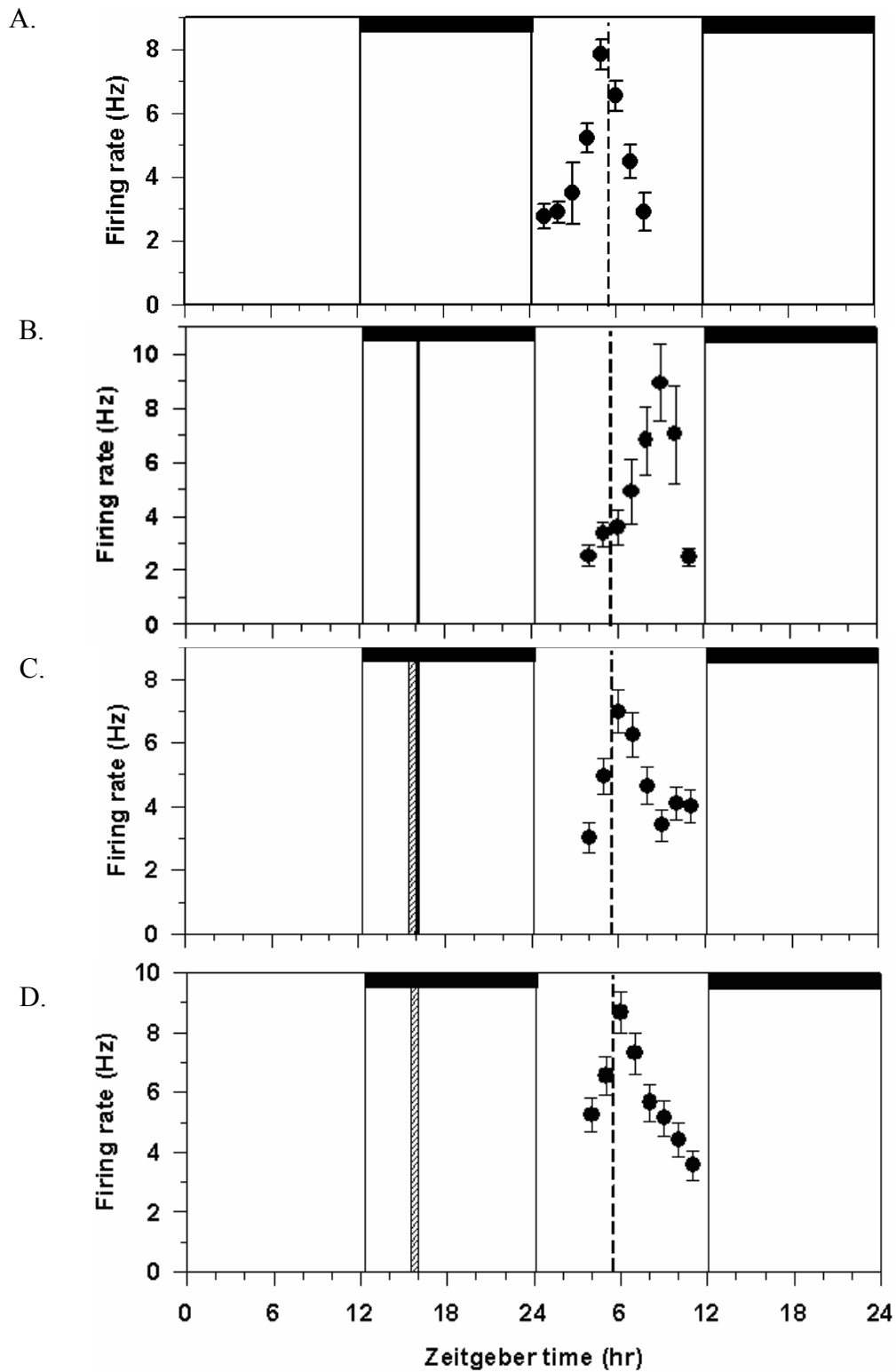


Figure 3: Firing rates of SCN neurons under various drug treatments. Dark horizontal bars represent subjective night, vertical lines show time of drug treatment, and dashed line represents average time of peak firing for control experiments. (A) Control (B) 1mM Glu (C) 1mM Glu + 20mM EtOH (D) 20mM EtOH

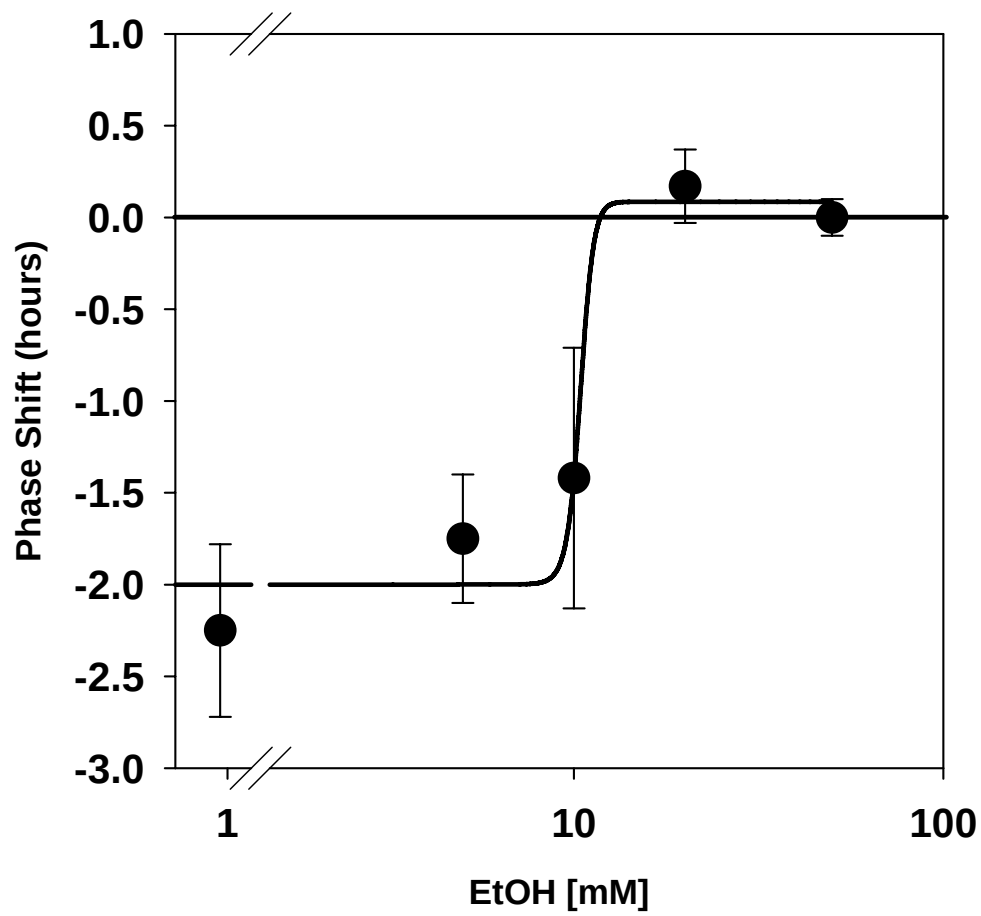


Figure 4: Dose response curve showing ethanol's blocking effects on glutamate-induced phase delays. $EC_{50}=10.4$. Plotted are the mean ($\pm$ SEM) phase shifts induced by 1mM glutamate applied with increasing concentrations of ethanol, together with the non-linear regression for the data.

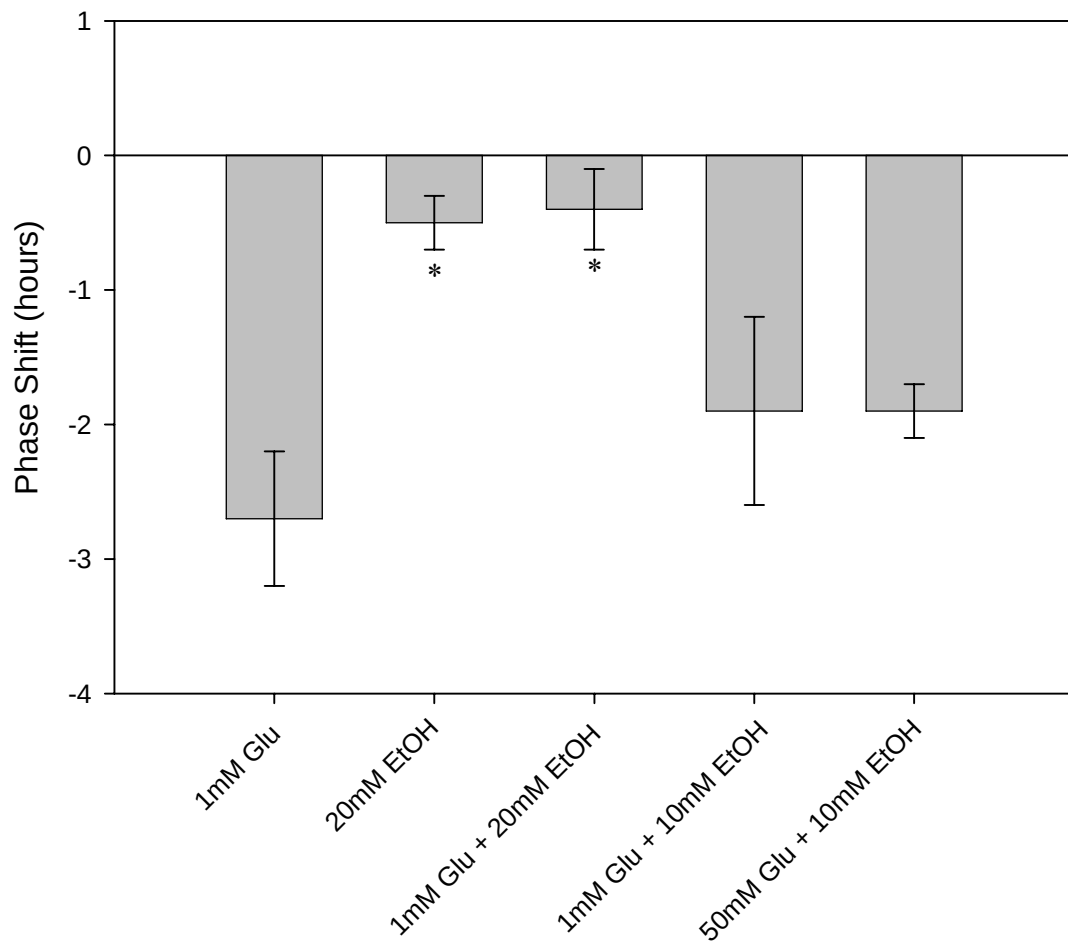


Figure 5: Summary of glutamate and ethanol experiments. Plotted are the mean ($\pm$ SEM) phase shifts induced by the treatments indicated. *Significant difference compared to Glu treatment alone ($P < 0.05$). There is no significant difference compared to control experiments.

the inhibition induced by 10mM ethanol [mean time of peak = ZT 7.4 ± 0.2 (n=2)].

Results of these experiments are summarized in Figure 5.

Effects of excess Glycine on Inhibition by Ethanol

In the next set of experiments I investigated whether ethanol inhibits glutamate induced phase delays by competing with glycine for binding to NMDA receptors. Work completed by Ito *et al* (1991) found that a 1nM glycine was sufficient to enhance NMDA activity in SCN neurons. Therefore the brain slices in my experiment were treated with 10nM glycine, a 10 fold increase compared to that of the Ito group, along with ethanol [20mM] and glutamate [1mM]. Treatment with this concentration of glycine did not block ethanol's inhibition of glutamate-induced phase delays [mean time of peak = ZT 6.2 ± 0.5 (n=5)]. Additionally, 10nM glycine alone had no effect on the time of peak activity [mean time of peak = ZT 5.5 ± 0.7 (n=2)]. I could not try blocking the effects of ethanol with higher doses of glycine, because higher concentrations of glycine (10uM), when applied alone, induced significant phase advances, with peak firing time occurring at ZT 3.4 ± 1.7 (n=4). I sought to determine whether these phase advances by glycine are the result of activating glycine receptors by testing whether the glycine receptor antagonist, strychnine, could block these phase advances. However, strychnine alone [100-500nM] induced phase delays of approximately 2 hours [mean time of peak = ZT 7.3 ± 0.3 (n=3)]. Experiments with lower concentrations of strychnine were not conducted because strychnine is no longer effective in inhibiting glycine receptor activation in the SCN at concentrations below 100nM (Ito *et al*, 1991). The results of these experiments are summarized in figure 6.

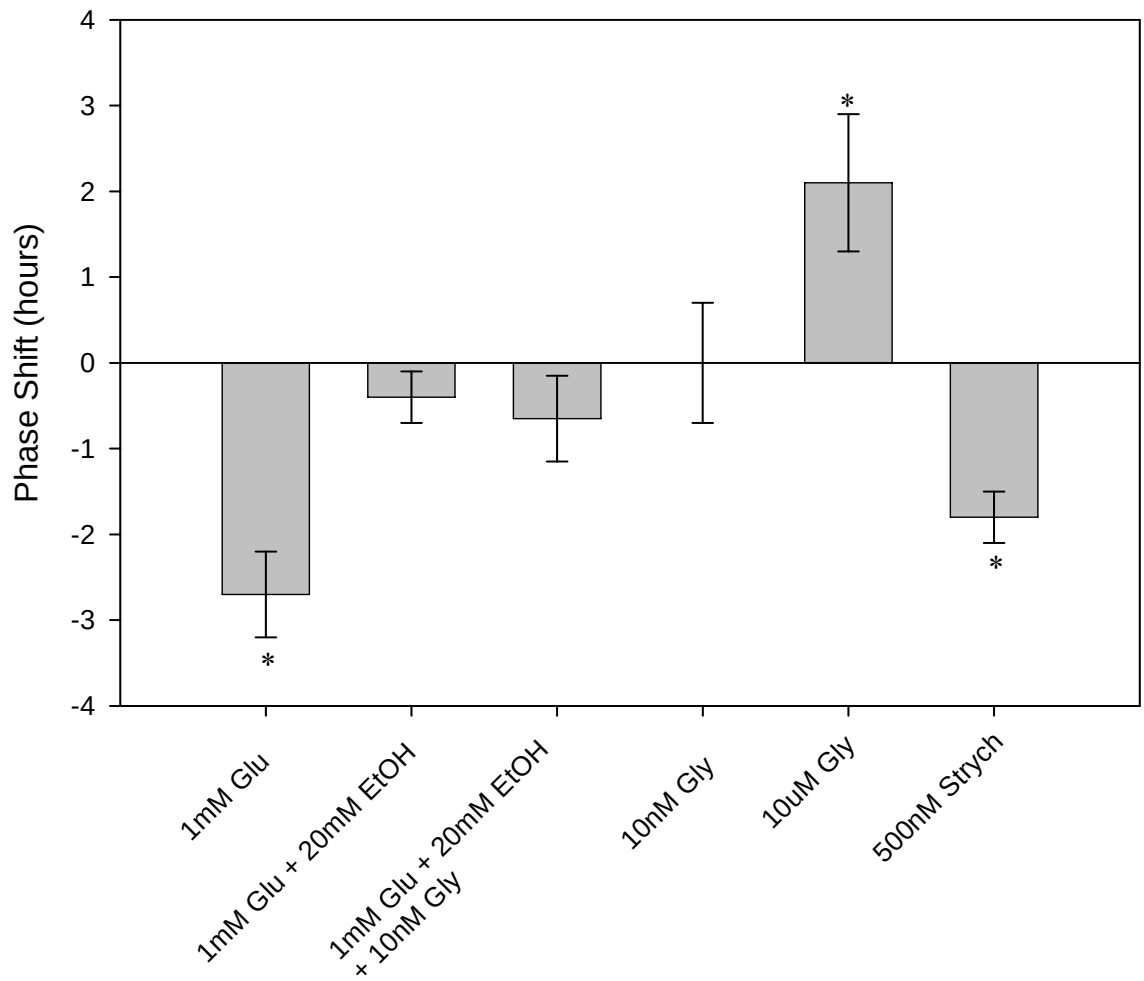


Figure 6: Summary of glycine experiments. Plotted are the mean ($\pm$ SEM) phase shifts induced by the treatments indicated. Glu and Glu+EtOH results are included for reference. *Significant difference compared to control experiments ($P < 0.05$).

Effects of D-Serine on Inhibition by Ethanol

Although glycine has been thought to be the endogenous co-agonist at the NMDA receptor, more recent research has shown that in at least some brain regions, including the hypothalamus, D-serine is a more effective co-agonist (Mothet et al., 2000). Thus, I tested whether exogenous D-serine could prevent ethanol's inhibition of glutamate-induced phase delays. When 10nM D-serine was applied with 20mM ethanol and 1mM glutamate, a mean peak time of $ZT = 6.0 \pm 0.7$ (n=2) was observed. Additionally, D-serine [100uM] showed no effect on the phase when applied alone [mean time of peak = $ZT 6.2 \pm 0.2$ (n=3)] or on glutamate-induced phase shifts when applied with 1mM glutamate [mean time of peak = $ZT 9$ (n=1)] [see Figure 7]. Thus, exogenous D-serine was unable to overcome the inhibition by ethanol.

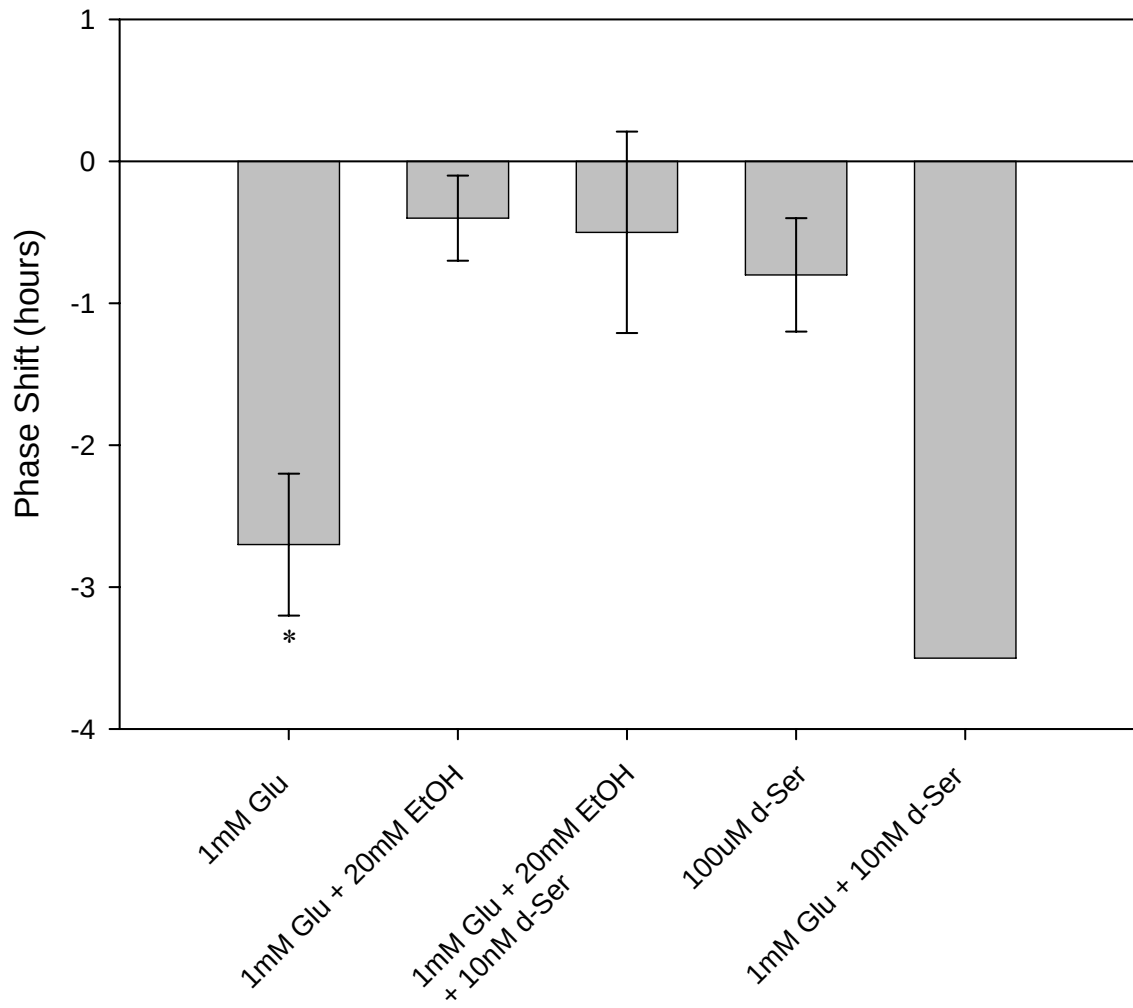


Figure 7: Summary of D-serine experiments. Plotted are the mean ($\pm$ SEM) phase shifts induced by the treatments indicated. Glu and Glu+EtOH results are included for reference. *Significant difference compared to control (non-treated) experiments ($P < 0.05$)

IV. Discussion and Future Directions

Ethanol Blocks Glutamate-Induced Phase Delays

The goal of this research project was to determine the effects of ethanol on glutamate-induced phase shifts of the circadian clock. I have found that acute *in vitro* treatments of ethanol block early night glutamate-induced phase delays in a dose dependent manner. The maximum blocking effect occurs at an ethanol concentration of 20mM, an amount equal to 0.92% blood alcohol content. The effective concentration from my study is a 2-10 fold decrease from that of other ethanol studies (Reynolds and Brien, 1994; Gruol et al., 1997). When applied alone, ethanol has no effect on the clock's phase. The results of this experiment, along with those involving glutamate and ethanol, indicate that ethanol's affect is on the glutamate signaling pathway. It is possible, however, that ethanol may also affect other components of the clock, but no such effect was observed from this study.

The limited number of studies looking at ethanol and circadian rhythms have shown that ethanol can alter the rhythms. These studies showed that chronic consumption of ethanol altered the length of the free-running period in both hamsters and rats, but failed to show a direct connection to the circadian clock. Therefore the results could be due to ethanol working on the clock itself or by altering physiological factors outside the SCN. The study conducted by Rosenwasser, Fecteau, and Logan in 2005, showed that chronic ethanol consumption blocked light pulse induced shortening of the free-running period in rats. Although their study did not examine the mechanism behind this observation, they proposed several possible effects of ethanol, including having an acute antagonist effect on the NMDA receptor.

My study showed direct effects of acute ethanol on the circadian clock in the SCN, specifically that it interferes with the photic entraining mechanism of the clock by blocking glutamate-induced phase delays. This data is consistent with a proposed explanation for the results observed in the Rosenwasser *et al* study. However, other mechanisms could also explain their results.

The results presented in this project are also consistent with a behavioral study conducted by Ruby and Glass (2006). Their study found that acute late-night intraperitoneal injections of ethanol (0.5-2.0g/kg) inhibited light-induced phase advances in Syrian hamsters in a dose dependent manner (Ruby et al., 2006). *In vivo* pharmacokinetics from their study found a maximum level of 51mM ethanol in the SCN 40 minutes after injection of 2.0g/kg ethanol in the peritoneal. Although our *in vitro* study and their *in vivo* study investigated the effects of ethanol at different times, the combined results demonstrate that ethanol can act directly within the SCN to block photic phase shifts.

The study conducted by Mistlberger and Nadeau (1991) found that chronic ethanol consumption by Syrian hamsters had no effect on the clock's phase entrainment to the light-dark cycle. Their experiment, however, involved chronic free-will drinking of the ethanol. Therefore the difference in their results and that of the Ruby and Glass could be explained by compensation from chronic ethanol consumption.

Ethanol does not Block Glutamate or Co-agonist Binding

The dose dependent effects of ethanol indicate a possible competitive inhibition of the binding of the agonist and/or co-agonist to the NMDA receptor. However, the fact

that ethanol continues to fully block the phase shifts in the presence of an excess amount of glutamate suggests that ethanol is not competitively inhibiting glutamate from binding to the NMDA receptor. Additionally, neither excess glycine nor excess D-serine was able to overcome ethanol's blocking effects. These results suggest that the binding of the co-agonist is not being competitively inhibited by ethanol. If such an inhibition existed one would expect a full or partial recovery from the ethanol block.

It is possible that ethanol simultaneously blocks the binding of both the agonist and co-agonist and that an excess of both would be necessary to overcome ethanol's block. Such a situation, however, would be unlikely because it would require ethanol to interfere at separate and dissimilar locations of the NMDA receptor (see Figure 2a). Also, it is possible that the concentration of excess glycine was too low to out compete the ethanol. Higher concentrations were not used in order to prevent unwanted activation of glycine receptors. If, however, ethanol was competing with glycine, it would seem that any excess amount of glycine would show at minimum a partial recovery of the phase-delay.

Glycine and Strychnine Phase Shift the SCN Clock

Early night treatments of 10uM glycine produced a phase advance of slightly over 3 hours. Although glycine has been shown to be rhythmically released in the SCN (Shinohara et al., 1998), this study provides the first evidence of glycine receptor activation altering the circadian clock. The mechanisms and purpose of these receptors are unknown. A study conducted by Ito *et al* (1991) suggested that glycine may serve a dual role in SCN neurons by modulating the NDMA at low concentrations, while at high

concentrations functioning as an inhibitory neurotransmitter by activation of glycine receptors. The results of my project support such a role for glycine in the circadian clock. When present at low concentrations, glycine could serve as the co-agonist for NMDA activation, but when higher concentrations are present in the early night it could activate glycine receptors that then, via an unknown mechanism, lead to a phase advance. This advance was shown to oppose the glutamate-induced delay. These opposite shifts in the phase canceled one another out and resulted in a peak of neuronal activity consistent with that of control experiments. Thus, glycine likely has a regulatory function in the SCN clock. This hypothesis could be tested with a series of electrophysiological experiments similar to those described in this project.

Interestingly, it was found that strychnine delayed the clock's phase by approximately 2 hours when applied in the early night. Strychnine has been viewed as a specific antagonist for glycine receptors (Curtis et al., 1971). If such is the case, then the results from the strychnine experiments would support the hypothesis that glycine receptors play a role in modulating the clock phase. However, it is now believed that strychnine is not as specific as originally thought. Studies have shown strychnine to function as an NMDA open channel blocker (Bertolino and Vicini, 1988) and also a nicotinic acetylcholine antagonist (Hiroaki et al., 1998). In the SCN strychnine has been shown to attenuate inhibitory effects of both NMDA and NPY (Schmahl and Bohmer, 1997). Thus, strychnine could be working via an unknown mechanism independent of glycine receptors to alter clock phase.

Future Directions

This project has served as one of the beginning steps into the research of how ethanol affects circadian rhythms. It has eliminated key parts of the glutamate signaling pathway as sites of action for ethanol; however, many other sites remain to be studied. It is important to find if ethanol has an effect on phosphorylation of the NMDA receptor or the pathway that leads to its phosphorylation. If ethanol is found to prevent NMDA phosphorylation by interrupting the pathway, it would be important to localize where ethanol's effects occur. Additionally, it is necessary to find if ethanol alters intracellular nitric oxide levels. Performing these experiments would further narrow and even possibly identify the specific site of action for ethanol.

To show if ethanol affects phase-advances the same as the delays, similar experiments should be conducted showing its effects on late-night glutamate treatments. Such studies have been done *in vivo* in hamsters and show similar blocking effects on light-induced phase advances as is seen in my project with phase-delays (Ruby et al., 2006). Based on this study, one would expect similar results with late-night treatments of tissue slices. If ethanol is shown to block phase advances in a similar fashion as it does with phase delays, this would suggest ethanol is affecting the glutamate signaling pathway upstream from the point where the pathway splits to generate either a phase-delay or a phase-advance.

Finally, for a comprehensive understanding of how ethanol affects the clock it would be important to study the non-photoc entraining pathways of the circadian clock. Neurons extending from raphe nuclei release serotonin directly onto the SCN where it can affect the clock's phase either directly or by inhibiting the photic entrainment

pathway (Prosser, 2000; Rea and Pickard, 2000). Research has shown chronic and possibly acute ethanol exposure increases serotonergic functioning (LeMarquand et al., 1994). It is possible therefore that ethanol may enhance serotonin's effects, both in shifting clock phase on its own and blocking photic induced shifts. Similar to serotonin, neuropeptide Y from the intergeniculate leaflet can block light-induced phase shifts (Biello et al., 1997). Chronic ethanol treatments have been shown to reduce general neuropeptide concentrations in the SCN (Madeira et al., 1997), which would suggest that ethanol might interfere with NPY's ability to phase-shift the clock or to block photic-induced phase shifts. Studies should be conducted to see if a similar effect is observed with acute treatments and if these treatments, chronic or acute, affect the clock's phase.

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Vita

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