Published in final edited form as:

Alcohol Clin Exp Res. 2018 February ; 42(2): 315–328. doi:10.1111/acer.13554.

The mammalian circadian clock exhibits chronic ethanol tolerance and withdrawal-induced glutamate hypersensitivity, accompanied by changes in glutamate and TrkB receptor proteins

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Abstract

Background—Alcohol tolerance and withdrawal-induced effects are criteria for alcohol use disorders listed by the DSM-V. Although tolerance and withdrawal have been studied over many decades, there is still uncertainty regarding mechanistic distinctions that characterize these different forms of ethanol (EtOH)-induced plasticity. Previously, we demonstrated that the suprachiasmatic nucleus (SCN) circadian clock develops both acute and rapid tolerance to EtOH inhibition of glutamate-induced circadian phase shifts. Here, we demonstrate that chronic EtOH tolerance and withdrawal-induced glutamate hypersensitivity occur *in vitro*, and that rapid tolerance, chronic tolerance, and glutamate hypersensitivity have distinct cellular changes.

Methods—We use single unit extracellular electrophysiological recordings to determine if chronic tolerance to EtOH inhibition of glutamatergic phase shifts and withdrawal-induced glutamate hypersensitivity develop in the SCN. We use western blotting to compare phosphorylation state and total expression of NMDA receptor subunits and associated proteins in the SCN after mice were exposed to varying EtOH consumption paradigms.

Results—Chronic tolerance developed after a minimum of 8 days of 4h/day EtOH access, as indicated by a decreased sensitivity to EtOH inhibition of glutamate-induced phase shifts. We also observed an increased sensitivity to glutamate-induced phase shifts in SCN tissue following withdrawal. We demonstrated an increase in the ratio of NR2B:NR2A NMDA receptor subunit expression after 21 days, but not after 10 days of EtOH drinking. This increase persisted during EtOH withdrawal, along with an increase in NR2B Y1472 phosphorylation, mature BDNF and phosphorylated TrkB.

Conclusions—These results demonstrate that multiple tolerance forms and withdrawal-induced glutamate hypersensitivity occur in the SCN and that these different forms of EtOH-induced plasticity are accompanied by distinct changes in cellular physiology. Importantly, this study further demonstrates the power of using the SCN as a model system to investigate EtOH induced plasticity.

Introduction

Alcohol use disorders (AUD) develop after long periods of ethanol (EtOH) consumption. Investigations of the cellular processes associated with AUD are complicated because the effects of EtOH change as one progresses from short-term to long-term drinking. This transition is clinically relevant, however, as it likely contributes to development of AUD (Hagman and Cohn 2011; Costin and Miles 2014). The acute effects of EtOH include inhibition of glutamate signaling and enhancement of GABA signaling (Roberto et al 2004; Proctor et al 2006; Möykkynen and Korpi 2012). Extended EtOH exposure leads to changes in synaptic localization, total expression, and phosphorylation of receptor proteins (Roberto et al 2004; Clapp et al 2010; Wu et al 2010). Although these effects are seen throughout the brain, the specific changes appear to differ regionally. Further complicating the picture, varying EtOH exposure paradigms have been used to investigate EtOH-induced changes in different brain areas, including the suprachiasmatic nucleus (SCN) (Prosser et al 2008; Brager et al 2010; Ruby et al 2009; Seggio et al 2009; Prosser and Glass 2009; Lindsay et al 2014; Rosenwasser et al 2014).

A critical component of the transition from social drinking to EtOH abuse is thought to be a decrease in sensitivity to EtOH-induced effects, termed tolerance. Three major forms of EtOH tolerance have been described that differ based on time after initial exposure: acute tolerance (observed 5–15 minutes after drinking), rapid tolerance (observed 8–24 hours after drinking), and chronic tolerance (develops after days/weeks of EtOH exposure). For example, acute tolerance is seen with respect to ataxia and motor impairment (Gill and Deitrich, 1998; Ramirez et al 2011), rapid tolerance with hypothermic and hypnotic effects (Gilliam 1989; Silveri and Spear 1999), and chronic tolerance with sleep and loss of righting reflex (Dyr and Taracha 2012; Daut et al 2015). Although the mechanisms underlying these different forms of tolerance are still under investigation, many cellular changes have been noted. With tolerance in general, glutamate and γ -aminobutyric acid (GABA) systems adapt to increase and decrease their baseline signaling properties, respectively. Acute tolerance has been linked to increased expression of N-methyl-D-aspartate (NMDA) receptor NR2A and NR2B subunits in the hippocampus and cortex (Roh et al 2011). Transgenic studies also suggest a role for $\alpha 1$ subunits of GABA receptors in acute tolerance (Werner et al 2009). Finally, changes in expression and phosphorylation of key residues of NMDA and GABA receptor subunits have been demonstrated during chronic tolerance (Wu et al 2010; Kroener et al, 2012; Wang et al 2011; Liang et al 2007; Lindemeyer et al 2014). EtOH withdrawal following chronic exposure is typically characterized by insomnia, psychomotor agitation, hallucinations, and delirium tremens (Schuckit 2014). At the cellular level, withdrawal is accompanied by changes in both glutamate and GABA signaling (Clapp et al 2010; Carlson et al 2016).

In summary, the cellular mechanisms associated with acute, rapid, and chronic EtOH tolerance and EtOH withdrawal appear to differ (Radcliffe et al 2006; Roberto et al 2006; Clapp et al 2010; Lindsay et al 2014; Tran et al 2015). However, rarely have the cellular mechanisms underlying these different forms of EtOH-induced plasticity been assessed in a single study. Thus our goal was to assess functional and protein changes in the SCN across multiple forms of EtOH tolerance and EtOH withdrawal.

We use the SCN circadian clock as a model system to investigate EtOH. The SCN circadian clock is the primary endogenous oscillator regulating daily rhythms in behavior and physiology in mammals. External cues such as light phase-shift the circadian clock in order to maintain proper synchrony with the environment. AUD is often accompanied by disrupted circadian rhythms, including sleep, and these disruptions may contribute to drinking relapse (Gillin et al 1994; Brower 2003).

Light- and glutamate-induced phase resetting in the SCN clock requires NMDA receptor activation (Ding et al 1997; Mintz et al 1999; Wang et al 2008). Numerous studies have shown that EtOH disrupts the ability of the SCN clock to respond to synchronizing cues such as light. For example, EtOH administration alters the circadian clock free-running period in vivo, and inhibits photic phase resetting in rodents acutely in vivo (Prosser and Glass 2009; Ruby et al 2009), acutely in vitro (Prosser et al 2008; Prosser and Glass 2009), and chronically in vivo (Rosenwasser et al 2005; Ruby et al 2009; Seggio et al 2009; Brager et al 2010). Because EtOH is known to disrupt NMDA receptor function across a variety of exposure paradigms, we chose NMDA receptors as our primary focus for assessing EtOH-induced cellular changes. Previously we showed that acute and rapid tolerance to EtOH occur in the SCN in mice (Prosser and Glass 2009; Lindsay et al 2014). Here we expand upon these studies, showing that chronic tolerance and withdrawal-induced glutamate hypersensitivity also develop in the SCN in vitro, and these EtOH-induced changes are each accompanied by distinct changes in specific parameters of NMDA receptor expression and closely associated signaling mechanisms.

Materials and methods

Mice

Adult (~8 week old) C57BL/6 adult male mice were obtained from Envigo (Indianapolis, IN). Mice were individually housed in polycarbonate cages under a 12:12 light/dark (LD) cycle, with food and water available *ad libitum* unless otherwise noted. The experiments followed the National Institution of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Tennessee Knoxville Institutional Animal Care and Use Committee. In all experiments, mice were allowed access to either water or 15% EtOH from drip-resistant sipping tubes (Med Associates, Inc., St. Albans, VT).

Brain slice preparation, and drinking schedules

The different drinking paradigms used in this study are illustrated in Figure 1). The rapid tolerance drinking paradigm was performed as described in Lindsay et al 2014. Briefly, mice had their water bottle replaced with one containing a 15% EtOH/water solution for a single 12 h lights-off period. At lights-on following EtOH access, the volume of EtOH consumed and the weight of the mouse was recorded. Afterwards, 500um coronal brain slices containing the SCN were prepared for electrophysiological recording or for western blot assays. Brain slices were maintained at 37°C in an interface style brain slice chamber and perfused with Earle's balanced salt solution (EBSS), pH 7.4, supplemented with 95% O₂/5% CO₂, sodium bicarbonate, glucose and gentamicin.

We used a 4h/day drinking protocol to investigate chronic tolerance. This involved exchanging the water bottles with ones containing a 15% EtOH/water solution beginning one hour prior to lights off and continuing through 3 hours after lights off, for a total of 4 hours. EtOH and water consumption and body weight of each mouse was measured daily. The 4h EtOH access protocol continued for 10–21 days depending on the experiment. At lights-on following the last day of drinking, coronal brain slices containing the SCN were prepared and maintained as described above. We used this drinking protocol because it generated EtOH drinking patterns that were very consistent across different mice and across multiple days within individual mice.

For EtOH withdrawal, mice underwent the chronic drinking protocol for up to 21 days followed by 2–6 days with no EtOH access (food and water available ad-libitum). At lights-on following the withdrawal period, coronal brain slices were prepared and maintained as described above.

Drug treatment

During the first night after brain slice preparation, SCN tissue received bath application of drugs dissolved in EBSS at Zeitgeber time (ZT) 16 (where ZT 0 = lights-on and ZT 12 = lights-off in the animal colony). Drug treatments were performed by stopping the perfusion and replacing perfusion media in the chamber with EBSS containing the drug treatment for 10 minutes. Afterwards, the solution was removed and the perfusion was resumed. Drug treatments contained a combination of glutamate (Sigma Aldrich, St. Louis, MO) and EtOH (Decon Laboratories, Inc., King of Prussia, PA). SCN tissue used for western blot assays, which did not receive in vitro drug treatments, were collected at ZT 16 and stored at -80°C until protein extraction.

Electrophysiological recordings

Extracellular single-unit recordings commenced the morning of the second day in vitro. Recordings were performed using glass microelectrodes filled with 5M NaCl as in previous studies (Prosser et al 2008; Prosser and Glass 2009; Lindsay et al 2014). Each cell was recorded for 5 minutes, with at least 5 cells recorded per hour over the course of a 10h period. Recordings and firing rate analysis were done using DataWave software (Loveland, CO). Only cells with a signal to noise ratio of greater than 2:1 were analyzed. The firing rates of individual neurons were combined into 2 h running averages, and the peak in neuronal activity was assessed visually as the time of symmetrically highest activity calculated to the nearest quarter hour (Prosser 2003; Prosser et al 2008; Prosser and Glass 2009).

Protein extraction

SCN tissue samples were thawed, placed in RIPA lysis buffer containing 1mM Tris pH 8.8, 5mM SDC, 5mM SDS, 1% triton, protease inhibitor cocktail (Thermo Scientific, Waltham, MA), and phosphatase inhibitor cocktail (Thermo Scientific), and lysed using 15 sec sonication pulses. The supernatant was separated from the pellet using centrifugation at 13000 rpm at 4°C . Protein concentrations were determined using a Bradford assay. The supernatant was then loaded into a 7% poly-acrylamide gel and electrophoresis was

performed. Proteins were transferred to a polyvinylidene fluoride (PVDF) FL immobilon membrane (EMD Millipore, Billerica, MA) using the Transblot turbo system (Bio-Rad Laboratories, Hercules, CA).

Western blot assays

Following protein extraction, western blots were performed as reported previously (Lindsay et al 2014). Briefly, PVDF membranes were blocked with 5% BSA in Tris buffer saline with 20% tween 20 (TBST) then incubated with primary antibodies for 2 hours at room temperature or overnight at 4°C. Primary antibodies used were: α -phospho-Y1472 (1:1000, EMD Millipore), α -phospho-S1480 (1:1000, AbCam, Cambridge, United Kingdom), α -NR2B (1:1000, EMD Millipore), α -NR1 (1:1000, AbCam), α -NR2A (1:1000, EMD Millipore), α -PSD-95 (1:2000, EMD Millipore), α -BDNF (detects both mature and pro BDNF; 1:200, Santa Cruz Biotechnology, Dallas, TX), α -TrkB (detects both full length and truncated TrkB; 1:200, Santa Cruz), α -phospho-tropomyosin receptor kinase A Y680 (binds correspondingly to Y706 of TrkB; 1:200, Santa Cruz), and α -Actin HRP conjugate (Santa Cruz Biotechnology, Dallas, TX). After rinsing, blots were exposed to secondary antibodies of donkey α -Rabbit (Li-COR, Lincoln, NE), or donkey α -Goat (Li-COR) conjugated to infrared dye (LI-COR). Blots were allowed to incubate for an hour with secondary antibodies. Protein bands were detected using the Imagestudio program. When assessing the ratio of NR2B to NR2A protein expression, the same blot was stripped and re-probed for each protein so that comparisons between relative levels of NR2B and NR2A were made within each blot.

Statistical methods

All statistical analyses were performed using SigmaPlot. Individual mice were considered experimental units for electrophysiological recordings. Tissue from a single mouse (or pooled samples when necessary) was the experimental unit for western blots. Post-hoc Holm-Sidak analysis using t-test or ANOVA was used for paired comparisons or among multiple comparisons for each experiment, respectively. Significance was considered when $P < 0.05$.

Results

EtOH consumption across multiple treatments

Mice allowed to consume EtOH for a single 12 h lights-off period had an average EtOH consumption of 10.62 ± 0.32 g/kg, $n=112$. Mice given 4 h of daily access to EtOH for 10 days had an average daily EtOH consumption of 3.36 ± 0.23 g/kg/day, $n=93$. Mice given 4 h of daily access to EtOH for 21 days had an average daily EtOH consumption of 2.52 ± 0.24 g/kg/day, $n=29$. Because all experiments assessed SCN tissue that had been maintained in vitro for a minimum of 4h, we did not measure blood alcohol levels. However, for these three groups of mice, based on previous reports of BAC levels after comparable amounts of EtOH consumption or EtOH injections, these mice may have experienced peak BACs of 0.11–0.16 (Kardayan et al 2013; Crabbe et al 2017).

Chronic tolerance to EtOH occurs in the SCN

In brain slices prepared from EtOH naïve mice, firing rates normally peak around ZT6 (Figure 2A). The mean time of peak in control slices occurred at 6.1 ± 0.30 n=3, which is consistent with previous research (e.g., Prosser et al 2008). To begin assessing the development of EtOH tolerance, we investigated the ability of EtOH to inhibit phase shifts induced by 1 mM glutamate, as we have used in previous studies (Prosser, et al., 2008; Cooper et al., 2017). This concentration of glutamate induces maximal phase shifts of the circadian clock when applied for 10 min to SCN-containing brain slices (e.g., Cooper et al., 2017). As illustrated in data from individual experiments (Figures 2B and C) and summarized in Figure 2G, glutamate (1mM) applied at ZT16 to SCN slices from EtOH naïve mice induced a phase delay of ~3 hours that was significantly inhibited by co-application of 20 mM EtOH (One-way ANOVA $F_{7,17.416}$ $p < 0.001$; glutamate treatment in EtOH naïve mice = -3.34 ± 0.21 h delay n=4 vs glutamate + 20mM EtOH = -0.32 ± 0.24 h delay, n=3; post-hoc analysis: $t_7=7.377$ $p < 0.001$).

The next set of experiments investigated the effects of glutamate $\pm$ EtOH treatments applied to brain slices prepared from mice that had undergone 4 h/day EtOH consumption for 10 days. ANOVA analysis indicated there were significant differences in the time of peak neuronal activity across treatments compared to the time of peak in EtOH naïve brain slices receiving no treatment (One-way ANOVA n=3 $F_{7,17.416}$ $p < 0.001$). Post-hoc analysis determined that brain slices from mice that had undergone 4 h/day EtOH for 10 days exhibited no difference in the time of peak neuronal activity compared to brain slices prepared from EtOH naïve mice (phase delay after 10 day EtOH with no treatment = -0.67 ± 0.37 h, n=3, vs EtOH naïve control = -0.0 ± 0.30 n=3; post-hoc analysis: $t_7=1.374$ $p=0.81$; Figure 2G). Applying 1mM glutamate alone at ZT16 to brain slices from these mice induced a significant phase delay relative to brain slices from EtOH naïve mice (phase delay after 10-day EtOH 1mM glutamate = -2.84 ± 0.21 h, n=3 vs EtOH naïve control = 0.00 ± 0.30 h, n=3; post-hoc analysis: $t_7=5.847$ $p < 0.001$; Figure 2G), consistent with the effects of glutamate seen in brain slices from EtOH naïve mice. However, as seen when comparing Figure 2F to Figure 2C, co-applying 20mM EtOH with 1mM glutamate to brain slices prepared from these mice did not inhibit the glutamate-induced phase shifts (phase delay after 10day EtOH 1mM glutamate with 20mM EtOH = -2.82 ± 0.45 h, n=3 vs EtOH naïve control; post-hoc analysis: $t_7=5.804$ $p < 0.001$). However, increasing the concentration of EtOH co-applied with 1mM glutamate to 200mM completely blocked the glutamate-induced phase shifts in brain slices prepared from the 10-day EtOH drinking mice (phase delay = -0.32 ± 0.30 h vs EtOH naïve control; post-hoc analysis: $t_7=0.653$ $p=0.89$; Figure 2G). Thus 4h/day consumption of EtOH for 10 days decreased the sensitivity of in vitro glutamate-induced phase resetting to EtOH inhibition, consistent with the development of chronic tolerance. To more carefully assess the change in EtOH sensitivity that occurs with this drinking paradigm, we tested the ability of an intermediate concentration of EtOH (150mM) to inhibit glutamate-induced phase shifts. As seen in Figure 2G, 150mM EtOH was also unable to inhibit glutamate-induced phase shifts in brain slices prepared from these mice (phase delay after 10-day EtOH with 150mM EtOH = -1.82 ± 0.58 h, n=3 vs EtOH naïve control; post-hoc analysis: $t_7=3.743$ $p=0.023$; Figure 2G).

Next we investigated how many days of EtOH drinking are needed to induce chronic tolerance. As shown in Figure 2H 20mM EtOH continued to inhibit glutamate-induced phase shifts in brain slices after mice had undergone up to 7 days of chronic drinking, but not in brain slices from mice that had consumed EtOH for 8 or more days. One-way ANOVA analysis showed a significant effect of days of EtOH consumption (one-way ANOVA: $n=3$ $F_{11}=8.582$ $p<0.001$; phase delay after 7 days of 4h/day EtOH = -0.65 ± 0.25 h vs EtOH naïve control = 0.00 ± 0.30 h $n=3$, post-hoc analysis: $t_{11}=1.210$ $p=0.89$, $n=3$; 8 days EtOH -2.48 ± 0.29 h vs EtOH naïve control, post-hoc analysis: $t_{11}=4.624$ $p=0.001$; 9 days EtOH -2.78 ± 0.30 h vs EtOH naïve control, post-hoc analysis: $t_{11}=5.167$ $p<0.001$; 10 days EtOH -2.32 ± 0.71 h vs EtOH naïve control, post-hoc analysis: $t_{11}=4.314$ $p=0.002$). Thus, at least 8 days of drinking EtOH is needed to generate chronic tolerance as assessed under these conditions.

Increased glutamate sensitivity in the SCN after 2 days of withdrawal from EtOH

Next we investigated the effects of EtOH withdrawal on the SCN circadian clock. In this case, we assessed changes in the sensitivity of the SCN circadian clock to glutamate induced phase shifts. First we generated a dose response curve for glutamate-induced phase delays in brain slices from EtOH naïve mice (e.g., Cooper et al 2017). There was an abrupt shift from no phase-shifting response with 1uM glutamate to a maximum ~3h phase delay with 10uM glutamate (Figure 3A). In contrast, 1uM glutamate applied to SCN brain slices from mice that had undergone EtOH drinking for 21 days followed by 2 days of withdrawal induced significant phase shifts of about 2.5h (one-way ANOVA: $n=3$ $F_9=28.642$ $p<0.001$; withdrawal 1uM glutamate -2.32 ± 0.36 h $n=3$ vs 0uM EtOH naïve -0.0 ± 0.30 h $n=3$, post-hoc analysis: $t_9=5.842$ $p<0.001$; vs EtOH naïve 1uM glutamate $=0.25\pm0.6$ h, $n=3$ $t_9=6.473$ $p<0.001$) (Figure 3A). A similar phase-shift was induced by 10uM glutamate applied to SCN tissue under these conditions, indicating that EtOH withdrawal leads to a change in glutamate sensitivity and not a change in the amplitude of the phase-shifting response.

To assess whether this change in glutamate sensitivity was the result of EtOH withdrawal or if it was due to the prior EtOH consumption, we investigated the phase-shifting effects of glutamate applied to brain slices prepared from mice having undergone 21 days of EtOH consumption without subsequent EtOH withdrawal. As summarized in Figure 3A, neither 1uM nor 10uM glutamate was able to induce phase shifts in these brain slices, while a higher concentration of glutamate (100uM) was sufficient to induce a phase delay (one-way ANOVA: $n=3$ $F_9=28.642$ $p<0.001$; 1uM glutamate 21days 4h/day EtOH exposed mice = 0.18 ± 0.08 h $n=3$ vs 0uM EtOH naïve, post-hoc analysis: $t_9=0.462$ $p=1.00$; 10uM glutamate 21days 4h/day EtOH exposed mice = 0.017 ± 0.08 h, $n=3$ vs 0uM EtOH naïve, post-hoc analysis: $t_9=0.042$ $p=1.00$; 100uM glutamate 21days 4h/day EtOH = -3.40 ± 0.14 h vs 0uM EtOH naïve, post-hoc analysis: $t_9=8.574$ $p<0.001$). Thus, immediately prior to EtOH withdrawal, the SCN clock appears to be slightly less sensitive to glutamate-induced phase shifts, and glutamate hypersensitivity develops during the period of EtOH withdrawal.

To clarify the duration of EtOH consumption needed to generate the withdrawal-associated glutamate hypersensitivity, we varied the number of days of EtOH consumption prior to the 2 days of withdrawal. As summarized in Figure 3B, withdrawal-induced glutamate

hypersensitivity was seen after 17 or 21 days of chronic EtOH, but not after 10 or 14 days (one-way ANOVA: $n=3$ $F=8.397$ $p=0.003$; 1uM glutamate 17day 4h/day EtOH with 2d withdrawal = -2.40 ± 0.50 $n=3$ vs no EtOH control, post-hoc analysis: $t_4=4.057$ $p=0.009$; 1uM glutamate 21day 4h/day EtOH with 2d withdrawal = -2.32 ± 0.36 , $n=3$ vs no EtOH control, post-hoc analysis: $t_4=3.917$ $p=0.009$; 1uM glutamate 14day 4h/day = -0.32 ± 0.36 $n=3$ vs no EtOH control, post-hoc analysis: $t_4=0.254$ $p=0.81$; 1uM glutamate 10day 4h/day EtOH with 2d withdrawal = -0.32 ± 0.36 $n=3$ vs no EtOH control, post-hoc analysis: $t_4=0.535$ $p=0.84$). Additionally, we clarified the duration of glutamate hypersensitivity after withdrawal. Withdrawal-induced glutamate hypersensitivity was still apparent after 4 days but not after 6 days of EtOH withdrawal (one-way ANOVA: $n=3$ $F_4=28.967$ $p<0.001$; 1uM glutamate 21day 4h/day EtOH with 4d withdrawal = -2.65 ± 0.25 $n=3$ vs no EtOH control, post-hoc analysis: $t_4=7.186$ $p<0.001$; 1uM glutamate 21day 4h/day EtOH with 6d withdrawal = 0.017 ± 0.08 $n=3$ vs no EtOH control, post-hoc analysis: $t_4=0.045$ $p=0.97$; Figure 3C).

EtOH-induced changes in NR2B:NR2A ratio, NR2B phosphorylation, and NMDA signaling modulators

Previously, we determined that total expression of the NMDA receptor subunit subtype 2B (NR2B) and phosphorylation of the NR2B tyrosine 1472 (Y1472) residue do not change in the SCN during rapid tolerance (Lindsay et al 2014). Here we expanded our investigation of potential changes in NMDA receptors and related proteins in response to varying EtOH exposure and withdrawal paradigms. We performed western blot assays on SCN tissue from EtOH naïve mice, and from mice receiving 1 night (12h) of drinking, 10 days or 21 days of 4-hr/day drinking, and 21 days of drinking followed by 2 days of withdrawal. As seen in Figure 4, no significant changes were observed with respect to total NR2A or NR2B expression in SCN tissue across the 5 conditions (one-way ANOVA NR2A: $n=6$ $F_4=2.115$ $p=0.100$; one-way ANOVA NR2B: $n=7-11$ $F_4=1.945$ $p=0.119$). However, the ratio of NR2B:NR2A expression was significantly higher after 21 days of drinking and it remained higher after 2 days of withdrawal when compared to SCN tissue from naïve mice (one-way ANOVA: $n=6$ $F_4=3.987$ $p=0.012$; 21d: 3.67 ± 0.84 $n=6$, post-hoc analysis: $t_4=3.504$ $p=0.017$; withdrawal 3.45 ± 0.57 $n=6$, post-hoc analysis: $t_4=3.232$, $p=0.030$; Figure 4C). We also assessed total expression of the NMDA NR1 subunit, however no changes were observed (one-way ANOVA: $n=4$ $F_4=0.552$ $p=0.70$; 1 ± 0.00 control, 1.21 ± 0.17 overnight, 1.09 ± 0.15 10 day, 1.34 ± 0.29 21 day, 1.36 ± 0.28 withdrawal; data not shown).

Next we investigated changes in NR2B subunit phosphorylation. Relative to EtOH naïve SCN tissue and SCN tissue after overnight EtOH consumption, we found a significant increase in NR2B Y1472 phosphorylation in SCN tissue from mice with 21 days of chronic drinking followed by 2 days of withdrawal, but no significant changes in other groups (one-way ANOVA: $n=3-8$ $F_4=3.507$ $p=0.018$; withdrawal: 4.24 ± 1.23 $n=8$, vs control, post-hoc analysis: $t_4=3.23$, $p=0.030$; vs overnight 1.02 ± 0.33 $n=8$; post-hoc analysis: $t_4=3.214$ $p=0.028$ Figure 5A). We also assessed the amount of NR2B serine 1480 (S1480) phosphorylation across these same experimental conditions, however no significant changes were noted (one-way ANOVA: $n=4$ $F_4=0.570$ $p=0.69$; 1 ± 0.00 control, 1.56 ± 0.39 overnight, 1.07 ± 0.23 10 day, 1.14 ± 0.38 21 day, 1.03 ± 0.33 withdrawal; $F_4=0.570$, data not shown).

Phosphorylation of NMDA receptors has been linked to activation of TrkB receptors. Specifically, binding of mature brain-derived neurotrophic factor (mBDNF) to TrkB receptors induces autophosphorylation, which leads to increased NR2B Y1472 phosphorylation (Mizuno et al 2003). Therefore, to investigate potential upstream mechanisms leading to changes in phosphorylation of NR2B Y1472 in the SCN during tolerance and withdrawal, we investigated changes in proBDNF and mBDNF expression, truncated and full length TrkB expression, and TrkB phosphorylation in SCN tissue. We found a significant increase in mBDNF total expression during withdrawal compared to tissue from EtOH naïve controls (one-way ANOVA: $n=6$ $F_4=3.037$ $p=0.036$; withdrawal 2.07 ± 0.34 , $n=6$, post-hoc analysis: $t_4=3.104$, $p=0.046$; Figure 5C). Furthermore, an increase in pTrkB at Tyr 706 occurred during withdrawal compared to all other conditions (one-way ANOVA: $n=4$ $F_4=4.286$ $p=0.011$; withdrawal 2.55 ± 0.50 , $n=4$; vs control post-hoc analysis: $t_4=3.401$, $p=0.039$; vs overnight 1.13 ± 0.168 , post-hoc analysis: $t_4=3.114$, $p=0.049$; vs 10 day 1.04 ± 0.393 , post-hoc analysis: $t_4=3.312$, $p=0.042$; vs 21 day 1.08 ± 0.29 , post-hoc analysis: $t_4=3.231$, $p=0.044$; Figure 5d). No significant changes were noted for expression of proBDNF (one-way ANOVA: $n=6$ $F_4=0.185$ $p=0.94$; 1 ± 0.00 control, 1.04 ± 0.16 overnight, 1.14 ± 0.16 10 day, 1.10 ± 0.17 21 day, 1.03 ± 0.10 withdrawal; data not shown), truncated TrkB (one-way ANOVA: $n=4$ $F_4=0.211$ $p=0.93$; 1 ± 0.00 control, 0.95 ± 0.07 overnight, 0.80 ± 0.24 10 day, 0.84 ± 0.24 21 day, 0.95 ± 0.22 withdrawal; data not shown), or full length TrkB (one-way ANOVA: $n=4$ $F_4=0.640$ $p=0.64$; 1 ± 0.00 control, 0.91 ± 0.14 overnight, 1.40 ± 0.45 10 day, 1.01 ± 0.28 21 day, 1.34 ± 0.29 withdrawal; data not shown).

Lastly, with respect to assessing changes in protein expression, we investigated two additional proteins whose expression has been linked to changes in glutamate signaling: the postsynaptic density scaffolding protein, PSD-95 and the tyrosine kinase, Fyn (Roche et al 2001; Prybylowski et al 2005). However, we found no significant differences in either PSD-95 (one-way ANOVA: $n=7$ $F_4=1.398$ $p=0.26$; 1 ± 0.00 control, 1.49 ± 0.27 overnight, 1.99 ± 0.44 10 day, 1.01 ± 0.28 21 day, 1.34 ± 0.29 withdrawal; data not shown) or Fyn (one-way ANOVA: $n=6$ $F_4=1.432$ $p=0.25$; 1 ± 0.00 control, 1.08 ± 0.10 overnight, 0.99 ± 0.13 10 day, 1.56 ± 0.24 21 day, 1.88 ± 0.46 withdrawal; data not shown) in the SCN across all experimental conditions.

Discussion

Time course of chronic tolerance and withdrawal

Previously we demonstrated the SCN develops rapid tolerance after mice are given access to EtOH for a single night, and this tolerance is characterized by a decrease in EtOH inhibition of photic (in vivo) and glutamate-induced (in vitro) circadian clock phase resetting (Lindsay et al 2014). Here we extend these findings to show that the SCN also develops chronic tolerance to EtOH and withdrawal-induced glutamate hypersensitivity. Chronic tolerance is characterized by a 10-fold right-ward shift in the dose response curve for EtOH inhibition of glutamate induced phase delays compared to that seen in SCN tissue from EtOH-naïve mice. This shift in sensitivity is similar to that seen previously with rapid tolerance (Lindsay et al 2014). Conversely, EtOH withdrawal leads to a 10-fold left-ward shift in the dose response curve for glutamate induced phase delays. Together with our previous demonstration of

acute EtOH tolerance in the SCN in vitro (Prosser and Glass 2009), these results illustrate that the SCN circadian clock exhibits a high degree of EtOH-induced plasticity in its phase-resetting properties. Given that EtOH disrupts a variety of circadian rhythms, including hormone secretion and sleep parameters, which may increase the propensity for relapse drinking (Brower 2003; Kühlwein et al 2003) it will be important to determine the cellular mechanisms underlying EtOH-induced plasticity in the SCN.

Prior to exploring changes at the cellular level, we sought to clarify the duration of EtOH consumption necessary to develop chronic tolerance and withdrawal-induced glutamate hypersensitivity in the SCN. Using the 4-h/day drinking paradigm, we find that mice need to consume EtOH for at least 8 days to develop chronic tolerance, consistent with other investigations (Broadwater et al 2011). In contrast, mice need more than 14 days of EtOH consumption to subsequently exhibit withdrawal-induced glutamate hypersensitivity. Previous research suggests that the duration of drinking necessary to induce withdrawal hypersensitivity varies across different EtOH exposure paradigms (after 4 days of vapor inhalation in Olive and Becker 2008 vs 1 – 2 weeks of drinking in Roberto et al 2004 and Wang et al 2010). Importantly, we found that chronic EtOH drinking without withdrawal does not cause glutamate hypersensitivity. Together, the difference time courses of EtOH-induced changes support the hypothesis that distinct cellular adaptations underlie their development.

Prior results indicate that under a constant no-choice EtOH drinking protocol, the SCN circadian clock does not exhibit chronic tolerance with respect to EtOH inhibition of photic phase shifts in vivo (i.e., photic phase shifts continue to be inhibited throughout the duration of EtOH consumption) in both hamsters and mice (Ruby et al 2009; Brager et al 2010). Our data showing chronic tolerance assessed in vitro raise questions regarding the underlying differences. In Brager et al (2010), mice consumed higher amounts of EtOH (20g/kg/day vs. 3.36 ± 0.23 g/kg/day in the current study), and drinking was not restricted to a 4 h period each day, while we used what is considered a “binge-drinking” like paradigm (Crabbe et al 2017). It should be noted, however, that relative to the total time EtOH was available in these two studies (24 h/day vs. 4 h/day) EtOH consumption was quite comparable. This equality is likely not that simple, however, because mice tend to maximize their EtOH consumption during early night (e.g., Brager et al 2011). A separate in vivo study found that 21 days of voluntary EtOH consumption induces no change in the size of photic phase shifts (suggestive of chronic tolerance), while 21 days of forced EtOH consumption leads to smaller photic phase shifts (Seggio et al 2009). Another study from the same lab (Rosenwasser et al 2014) observed reduced effects of EtOH on circadian period after sustained high EtOH intake in rats, which is consistent with chronic tolerance, but this effect is seen in a sex- and strain-dependent fashion. Thus, the specific EtOH exposure paradigm and animal model used are critical variables to consider when investigating chronic EtOH tolerance in the SCN.

Likewise, in vivo experiments investigating EtOH withdrawal-induced changes in circadian phase shifts have shown mixed results. For instance, enhanced photic phase shifts are seen during EtOH withdrawal in hamsters (Ruby et al, 2009), while withdrawal does not alter photic phase shifts in mice (Brager et al 2010). In contrast, free-running circadian

periodicity after withdrawal from 12 days of EtOH exposure is altered in mice, suggesting that EtOH continues to affect clock-regulated mechanisms during withdrawal (Logan et al 2012). Here we demonstrate that withdrawal-induced glutamate hypersensitivity occurs in the SCN *in vitro* after mice consume EtOH for 17 days but not 14 days. A careful assessment of photic phase resetting sensitivity using a range of light intensities *in vivo* following a 4 h/day drinking paradigm could clarify the critical variables that either lead to or prevent glutamate hypersensitivity during EtOH withdrawal.

Another important difference is that the previous studies investigated photic phase-resetting *in vivo* vs. the *in vitro* glutamate-induced phase resetting used here. Because EtOH affects many brain areas that directly or indirectly modulate circadian clock functioning, the lack of tolerance seen *in vivo* could reflect changes in EtOH actions across multiple regions that counteract or obscure changes occurring in the SCN. For example, the SCN receives serotonin (5-HT) input from the dorsal raphe nucleus that inhibits photic phase shifts (Bradbury et al 1997). Previously, we showed that acute application of EtOH to SCN tissue enhances serotonergic phase shifts *in vitro* (Prosser et al 2008), while EtOH inhibits serotonergic phase shifts when administered peripherally *in vivo*, which points to extra-SCN sites of action (Ruby et al., 2009). Another possible explanation for the differences between *in vivo* and *in vitro* results could stem from bath-applied glutamate acting more broadly within the SCN relative to synaptically released glutamate. This possibility could be explored in future experiments using *in vitro* optic chiasm stimulation-induced release of glutamate in the SCN rather than bath-applied glutamate. The down-side of such experiments, of course, is that it is harder to titrate the amount of glutamate released by the optic chiasm stimulation.

Hyperexcitation during EtOH withdrawal has been reported across many brain regions. This effect was described by Finn and Crabbe (1997), who suggested that adaptations that initially develop in order to offset the effects of EtOH are left unopposed during withdrawal, thus leaving neurons in a hyperactive state. Withdrawal-induced hyperexcitability is thought to contribute to the synaptic remodeling observed in the orbital frontal cortex (Nimitvilai et al 2016). Evidence of withdrawal-induced hyperexcitation is also seen in the basal lateral amygdala (BLA), as indicated by shortened paired-pulse ratio intervals and increased miniature excitatory post-synaptic current (mEPSC) amplitude and frequency (Läck et al 2007). The differences in mEPSCs seen in the BLA during chronic drinking do not persist after 24 h of withdrawal (Läck et al 2007), consistent with our results showing differences between chronic EtOH and EtOH withdrawal conditions. Thus withdrawal-induced glutamate hypersensitivity in the SCN is consistent with hyperexcitable states found in other brain regions.

Chronic EtOH consumption increases the NR2B to NR2A ratio in the SCN

To begin assessing the cellular changes underlying the EtOH-induced plasticities, we focused on NMDA receptors, which are critically involved in photic phase resetting in the SCN (Ding et al 1997; Mintz et al 1999; Wang et al 2008). We found a significant increase in the NR2B:NR2A ratio in the SCN after 21 days of drinking that is not seen with shorter durations of EtOH consumption, and this increase is maintained during EtOH withdrawal.

Therefore, this change in NMDA receptor subunit expression is induced during chronic EtOH consumption but is not necessary for the expression of chronic tolerance, which is seen as early as after 10 days of drinking. However, the increase in NR2B:NR2A expression could be necessary for withdrawal-induced glutamate hypersensitivity, a possibility that will need to be explored in subsequent studies.

Although we did not see a change in total NR2B subunit expression during either chronic EtOH or withdrawal, other EtOH induced changes could affect NMDA receptor function. For example, chronic drinking increases membrane localization of NR2B subunits in many regions (Qiang et al 2007). Moreover, EtOH withdrawal can increase lateral movement of NR2B containing NMDA receptors to extra-synaptic zones (Clapp et al 2010). Changes in receptor localization across these drinking paradigms is another possibility worth exploring.

Increased phosphorylation of Y1472 occurs during EtOH withdrawal

In addition to changes in receptor subunit composition, NMDA receptor signaling is modulated by phosphorylation of specific residues. For example, phosphorylation of NR2B Y1472 potentiates NMDA receptor currents and increases synaptic recruitment of NMDA receptors (Goebel et al 2005). We found that during EtOH withdrawal, but not during EtOH consumption, there is a significant increase in phosphorylation of NR2B Y1472 in the SCN. Thus, this change appears to be induced by the cessation of EtOH consumption, and is consistent with the glutamate hypersensitivity we observed for the SCN circadian clock. Increases in Y1472 phosphorylation have previously been seen after chronic EtOH in the rat striatum and in the rat hippocampus (Wang et al 2010, Wu et al 2010). Both studies also found no change in total expression of NR2A and NR2B proteins, consistent with our findings. Moving forward, it would be interesting to assess whether pharmacological inhibition of Y1472 phosphorylation during withdrawal reduces or prevents the development glutamate hypersensitivity in the SCN.

Phosphorylation of Y1472 has been shown to increase membrane localization of NR2B-containing NMDA receptors and maintain NR2B subunit interactions with the scaffolding protein PSD-95 (Roche et al 2001; Prybylowski et al 2005). Although we did not see any changes in PSD-95 expression, others likewise have shown increased Y1472 phosphorylation during withdrawal without a change in PSD-95 expression (Clapp et al 2010). Yet there is evidence PSD-95 is involved in EtOH effects, as increased clustering of PSD-95 and its colocalization with NR2B containing NMDA receptors occur during chronic EtOH (Carpenter-Hyland et al 2004; Clapp et al 2010). Colocalization assays may help determine whether there are changes in PSD-95 interactions with NR2B in the SCN during EtOH withdrawal.

Increases in mBDNF and TrkB phosphorylation occur during withdrawal

One mechanism through which Y1472 becomes phosphorylated is via TrkB signaling. Binding of mBDNF to TrkB induces autophosphorylation of the receptor, allows activation of downstream signals, and promotes neuronal survivability. Our results show increases in mBDNF expression and TrkB phosphorylation during withdrawal. There are mixed reports in the literature regarding EtOH effects on mBDNF, including decreases in BDNF binding to

TrkB in the hippocampus (Kolb et al 2005) and increases in expression of BDNF, TrkB, and downstream kinases in the striatum (Logrip et al 2008). These differences suggest that the changes are region specific, with our results consistent with this conclusion. The increased TrkB phosphorylation we observe during withdrawal runs counter to results from some previous studies (Pandey et al 2008; Nona et al 2013). Thus this change may also be specific to the SCN. Nevertheless, these results are consistent with the increase in NR2B Y1472 phosphorylation we observe in the SCN following EtOH withdrawal.

Cellular mechanisms underlying rapid and chronic tolerance in the SCN are still unknown

Clearly there is a great deal we still do not know about the cellular changes occurring in the SCN in response to EtOH, especially those associated with rapid tolerance and chronic tolerance after short durations of EtOH. It is possible that other proteins contribute to these effects. One possible candidate is a change in α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. For example, Beckley et al demonstrate that AMPA receptor expression in the nucleus accumbens increases in response to a single 4-hour binge exposure to EtOH (2016). Another likely possibility is that EtOH enhances signaling by GABA(A) receptors. Chronic EtOH exposure alters GABA subunit composition (Liang et al 2007; Lindemeyer et al 2014), and we have shown that at least some effects of EtOH in the SCN involve activation of δ -subunit-containing GABA(A) receptors (McElroy et al 2009). Given the complexity of EtOH effects in the brain, investigating both AMPA and GABA(A) receptors will likely clarify the cellular mechanisms underlying EtOH induced plasticity in the SCN.

Conclusions

To our knowledge, these are the first data carefully delineating timing differences in the development of rapid tolerance, chronic tolerance, and EtOH withdrawal plasticity. Together with our previous studies, we have shown that multiple forms of EtOH-induced plasticity occur in the SCN in response to different EtOH exposure paradigms, and that each likely involves distinct cellular mechanisms that develop over different timeframes. We observed an increase in the NR2B:NR2A ratio after chronic EtOH that is still seen during EtOH withdrawal, when it additionally is accompanied by an increase in NR2B Y1472 phosphorylation, TrkB phosphorylation, and mBDNF. Some or all of these changes could be necessary for the EtOH tolerance and glutamate hypersensitivity that develop. A visual representation of these changes is shown in Figure 6. Because both EtOH tolerance and withdrawal can lead to increases in EtOH consumption (Bell et al 2001; Das et al 2016), a better understanding of the distinct cellular processes underlying each of these conditions may improve treatments to alleviate or prevent development of alcohol dependence. The data from this study also demonstrate the power of using the SCN circadian clock to investigate nuanced differences in EtOH effects in the brain. Importantly, continuing this line of investigation should enhance our understanding of how alcohol abuse leads to disruptions in circadian rhythms and sleep.

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Figure 2.

Chronic tolerance to ethanol (EtOH) inhibition of glutamate-induced phase delays. (A-F) Shown are the 2-hour mean $\pm$ SEM of suprachiasmatic nucleus neuronal activity obtained in individual experiments. (A) Extracellular electrophysiological recordings in SCN tissue from an EtOH naïve mouse with no drug treatment shows a peak at Zeitgeber time (ZT) 6. (B) A 10 min bath application of 1mM glutamate (Glu) at ZT16 to SCN tissue from an EtOH naïve mouse induced a 2 hour phase delay. (C) Co-application of 20mM EtOH with 1mM glutamate blocked the glutamate induced phase delay in SCN tissue from an EtOH naïve mouse, indicating that the Glu-induced phase delay was inhibited. (D) In a control

experiment with SCN tissue from a mouse given daily 4 h EtOH access for 10 days, neuronal activity peaked at ZT6, similar to that shown in **A**. **(E)** A 10 min bath application of 1mM Glu at ZT16 to SCN tissue from an EtOH exposed mouse induced ~ a 2.5 hour phase delay, similar to that shown in **B**. **(F)** Co-application of 20mM EtOH and 1mM Glu did not inhibit the Glu induced phase shift in SCN slices from a mouse that had undergone the chronic EtOH protocol for 10 days. 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. **(G)** Shown are the mean $\pm$ SEM phase shifts induced by glutamate and EtOH treatments applied to brain slices of EtOH naïve mice and mice after 10 days of the chronic EtOH protocol. In SCN slices from EtOH naïve mice, Glu induced significant phase delays that were blocked by 20 mM EtOH. In SCN tissue from chronic EtOH mice, Glu induced significant delays that were not blocked by either 20 or 150 mM EtOH, but were blocked by 200 mM EtOH. (n=3 for each treatment). **(H)** Plotted are the mean ($\pm$ SEM) phase shifts induced by 1mM Glu + 20mM EtOH applied to brain slices prepared from mice given access to 15% EtOH for varying numbers of days. 20mM EtOH was able to completely inhibit Glu induced phase shifts after mice had been exposed to 0–7 days of chronic EtOH, no longer inhibited Glu-induced phase shifts after mice had been given access to EtOH for 8 or more days. One-way ANOVA indicated a significant effect of treatment, *Post-hoc (Holm-Sidak) test indicates $p < 0.05$ versus control experiments. *** $p < 0.001$

Figure 3.

SCN circadian clock sensitization to glutamate phase resetting after EtOH withdrawal. (A) Plotted are the mean ($\pm$ SEM) phase delays induced by 0 μ M, 1 μ M, and 10 μ M glutamate in SCN tissue from EtOH naïve mice (white bars, $n=3$), mice given access to EtOH (4 h/day) for 21 days (gray bars, $n=3$), and mice given access to EtOH for 21 days followed by two days of EtOH withdrawal (orange bars, $n=3$). ***Post-hoc (Holm-Sidak) test indicates $p < 0.001$ versus tissue from control EtOH naïve mice. (B) Plotted are the mean ($\pm$ SEM) phase delays induced by 1 μ M glutamate treatment applied to SCN tissue from mice that had undergone 2 days of EtOH withdrawal after varying numbers of days of chronic drinking.

**Post-hoc (Holm-Sidak) test indicates $p < 0.01$ versus tissue from control EtOH naïve mice. (C) Plotted are the mean ($\pm$ SEM) phase delays induced by 1uM glutamate treatment applied to SCN tissue from mice that had undergone varying durations of EtOH withdrawal after 21 days of chronic drinking. **Post-hoc (Holm-Sidak) test indicates $p < 0.001$ versus 0uM glutamate EtOH naïve control.

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Figure 4.

Protein expression of NMDA NR2B and NR2A subunits in the SCN after rapid, chronic 10 days, chronic 21 days, and EtOH withdrawal procedures. (A) Above, plotted are the mean band densities ($\pm$ SEM) for NR2A normalized to control tissue and relative to actin ($n=7$ for all conditions). Below are representative images for NR2A (170 kDa) and actin (42 kDa) bands across all treatments. (B) Above, plotted are the mean band densities ($\pm$ SEM) for NR2B normalized to control tissue relative to actin ($n=7$ for chronic 21 days group; $n=11$ for all other groups). Below are representative images for NR2B (180 kDa) and actin bands across all treatments, respectively. (C) Plotted are the mean ratios of band densities for NR2B to NR2A normalized to control tissue and relative to actin ($n=7$ for all conditions). The same blot was stripped and re-probed for either NR2B or NR2A so comparisons of NR2B:NR2A ratios were made within each experimental replicate. *Post-hoc (Holm-Sidak) test indicates $p<0.05$ versus control experiments.

Figure 5.

Protein expression for phosphorylated and total NR2B, mBDNF, and phosphorylated TrkB in the SCN after rapid, chronic 10 days, chronic 21 days, and EtOH withdrawal procedures. (A) Plotted are mean band densities ($\pm$ SEM) for phosphorylation of the Y1472 residue of NR2B normalized to control tissue and relative to actin across different conditions (n=3 for chronic 21 days, n=8 for other conditions). Below are representative images for phospho-NR2B and actin bands across all treatments, respectively. *Post-hoc (Holm-Sidak) test indicates $p < 0.05$ versus control experiments. (B) Plotted are the mean band densities ($\pm$ SEM) for the ratio of Y1472 phospho-NR2B to NR2B expression normalized to control

tissue and relative to actin (n=6 for all conditions). **(C)** Above, plotted are mean band densities ($\pm$ SEM) for mBDNF protein expression normalized to control tissue and relative to actin across all conditions (n=6 for all conditions). Below are representative images for mBDNF (14 kDa) and actin bands across all treatments, respectively. *Post-hoc (Holm-Sidak) test indicates $p<0.05$ versus control experiments. **(D)** Above, plotted are mean band densities ($\pm$ SEM) for phosphorylation of the Y706 residue of the TrkB receptor normalized to control tissue and relative to actin across different conditions (n=3 for chronic 21 days; n=8 for other conditions). Below are representative images for phospho-TrkB (140 kDa) and actin bands across all treatments, respectively. **Post-hoc (Holm-Sidak) test indicates $p<0.05$ versus all conditions.

Figure 6.

Model depicting cellular changes associated with chronic EtOH exposure and EtOH withdrawal occurring in the SCN. After 21 days (but not 10 days) of 4h/day chronic EtOH access, there is an increase in the ratio of NR2B to NR2A NMDA subunits. This change is not necessary for tolerance, which is seen after 10 days of chronic EtOH, so other changes must also be occurring. However, it may be a prerequisite of EtOH withdrawal induced plasticity. During withdrawal, the increase in NR2B:NR2A ratio persists in addition to increases in Y1472 phosphorylation of NR2B subunit. Upstream, increases in mBDNF expression and Y706 phosphorylation of TrkB receptors are also seen. One possibility is that increased TrkB activity promotes NR2B phosphorylation via the Src family kinase, Fyn. These changes may be responsible for the enhanced glutamate sensitivity seen in the SCN after EtOH withdrawal.