

Using the suprachiasmatic nucleus as a model system to assess tolerance and
withdrawal to alcohol

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Dedication

To my wife and daughter, Hannah & Rosemary Lindsay, for all of their support through my years as a graduate student and for years to come.

Acknowledgements

I began work in the sciences my junior year as an undergraduate in Cynthia Peterson's lab at the university of Tennessee. This opportunity gave me the first step towards defining my career in the biological sciences. Admittedly, I was in over my head as I had no ties to science in any way. With the experience from this two semester introduction to work in a wet lab, I applied to graduate school. I was accepted into the University of Tennessee's biochemistry, cellular, and molecular biology graduate program where I chose to work in Rebecca Prosser's lab. Fortunately for me, I was a natural at performing electrophysiological experiments. Unfortunately for me, I was terrible at making brain dissections. After many words of encouragement from Rebecca and from my graduate colleague, Yuki Yamada, I finally had my first dissection after 40 or so attempts. After numerous presentations, countless draft, many successful and even more unsuccessful experiments in Rebecca's lab, and some choice guidance by my research committee, I am at the end of graduate school with a completed thesis and moving on with my wife, Hannah, and daughter, Rosemary (to whom we affectionately all know as 'Rosie'), to the next chapter in my research career. The support I have received from everyone: Hannah and Rosie, Cynthia Peterson, Rebecca Prosser, my research committee, and my colleagues, has truly fueled my persistence in getting to where I am today. Without all of you I would have been lost. Thank you so much for giving a guy that was in way over his head the means to do what few have done.

Abstract

Alcohol abuse induces many disorders including depression, metabolic syndrome, and sleep disturbances. The strong link between alcohol abuse and sleep problems, along with the close connection between sleep and circadian rhythms, led us to investigate ethanol's effects on the circadian clock. Previous work has shown that acute ethanol blocks photic phase shifts in vivo and glutamatergic phase shifts in vitro. However, neural systems become tolerant to ethanol across different timeframes. Despite both ethanol tolerance and ethanol withdrawal syndrome being listed as criteria for developing alcohol use disorders, little is known about how ethanol tolerance and withdrawal induced hyperexcitability develop and how they differ. Because previous work has shown that acute tolerance to ethanol occurs in the SCN, we tested whether additional forms of tolerance as well as withdrawal induced sensitization develop in the SCN and we identified distinct changes across all of these conditions with respect to N-methyl-D-aspartate (NMDA) receptor related proteins. Our hypotheses were that rapid tolerance, chronic tolerance, and withdrawal induced sensitization occur in the SCN, that these conditions are associated with increased expression of NR2B containing NMDA receptors, and that the extent to which these changes occur differ across tolerance and withdrawal paradigms. We found that rapid tolerance develops in vivo and in vitro with respect to a loss in phase shift inhibition. Additionally, we have discovered chronic tolerance and withdrawal induced sensitization to glutamate also occur in vitro. Furthermore, we compared phosphorylation and total expression of NMDA receptor subunits and NMDA receptor-regulating proteins across the drinking models that induce rapid tolerance, chronic tolerance, or withdrawal. We found no changes during rapid tolerance to ethanol. In contrast, we saw an increase in the ratio of total expression of

NR2B/NR2A during both chronic tolerance and withdrawal from EtOH, an increase in Tyr 1472 phosphorylation during withdrawal, and an increase in mature BDNF and TrkB phosphorylation during withdrawal. Altogether we demonstrated the power of utilizing the SCN for investigating ethanol induced plasticity by identifying rapid tolerance, chronic tolerance, and withdrawal induced sensitization. Additionally we found that the cellular effects of ethanol during these forms of tolerance and withdrawal differ.

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Abbreviations and Symbols

α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	AMPA
Alcohol use disorder	AUD
Analysis of variance	ANOVA
Basal lateral amygdala	BLA
Brain and muscle arylhydrocarbol receptor nuclear translocator like protein 1	Bmal1
Brain derived neurotrophic factor	BDNF
Calcium calmodulin-dependent protein kinase II	CaMKII
Calcium ions	Ca ²⁺
CAMP response element binding protein	CREB
Casein kinase 2	CK2
Chronic intermittent ethanol	CIE
Circadian locomotor output cycles kaput	CLOCK
Constant darkness	DD
Cryptochrome	CRY
Diagnostic statistical manual of mental disorders volume 5	DSM-V
Earle's balanced salt solution	EBSS
Ethanol	EtOH
Excitatory amino acid transporter	EAAT
Gamma-aminobutyric acid	GABA
Gamma-aminobutyric acid A receptor	GABA(A)
Glycogen synthase kinase 3 beta	GSK3 β

Hours	h
Mature BDNF	mBDNF
Medium excitatory post-synaptic current	mEPSC
Minute	min
Mitogen activated protein kinases	MAPK
N-methyl D-aspartate	NMDA
Neuronal cell adhesion molecule-1	NCAM-1
NMDA receptor subunit 1	NR1
NMDA receptor subunit 2A	NR2A
NMDA receptor subunit 2B	NR2B
NMDA receptor subunit 2C	NR2C
Period	PER
Polysialic acid	PSA
Polyvinylidenedifluoride	PVDF
Post-synaptic density-95	PSD-95
Postsynaptic density-95 [PSD-95]/Discs large/zona occludens-1	PDZ
Potassium ions	K ⁺
Protein kinase A	PKA
Protein kinase C	PKC
Radioimmunoprecipitation assay	RIPA
Receptor-like protein tyrosine phosphatase α	RPTP α
Serine 1303	S1303
Serine 1480	S1480

Serotonin	5-HT
Sodium ions	Na ⁺
Striatal enriched tyrosine receptor phosphatase-33	STEP-33
Striatal enriched tyrosine receptor phosphatase-61	STEP-61
Suprachiasmatic nucleus	SCN
Tissue plasminogen activator	tPA
Transcription/translation feedback loop	TTFL
Tris-buffered saline containing 20% tween	TBST
Tropomyosin receptor kinase B	TrkB
Tyrosine 420	Y420
Tyrosine 706	Y706
Tyrosine 1472	Y1472
Threonine 286	T286
Threonine 305	T305
Urokinase plasminogen activator	uPA
Ventral tegmental area	VTA
Zeitgeber time	ZT

Chapter 1

General Introduction

Background

Alcohol causes a wide range of changes in the physiology, cell signaling, and genetic regulation of many brain regions. Prolonged use of alcohol can lead to alcohol use disorder (AUD). The diagnostic statistical manual of mental disorders volume 5 (DSM-V) suggests that the two primary components of AUD are drug seeking and drug dependence (APA, 2013). Focusing on the latter, the criteria describing dependence includes seeking alcohol even when aversive to health, cravings, developing tolerance, and experiencing symptoms of withdrawal after sudden abstinence (Hagman and Cohn 2011; APA, 2013). The neurocircuitry mediating the effects of alcohol relevant to the development of addictive behavior include the prefrontal cortex, amygdala, nucleus accumbens, hypothalamus, thalamus, and ventral tegmental area (Noori et al 2012). However, ethanol (EtOH) modifies signals in many other regions of the brain that have been used to investigate EtOH related phenomena. In this research we focused on the suprachiasmatic nucleus (SCN) in the lower hypothalamus, as a model system for studying EtOH tolerance and withdrawal.

The SCN houses the endogenously driven primary oscillator that regulates the timing of circadian rhythms over 24 hours. The firing rate of neurons in the SCN exhibits a circadian rhythm that increases and peaks during the day and decreases at night. This oscillation in neuronal firing is one of the primary output signals from the SCN that coordinates the timing of behavior and physiology throughout the body (Colwell 2011). The rhythm of neuronal action potentials in the SCN is controlled by a feedback mechanism involving key clock genes known as the transcription/translation feedback loop (TTFL; Hastings et al 2008). The TTFL allows for synchronizing or entraining the

timing of endogenous rhythms to external environmental cues like light. In a properly functioning clock the internal rhythms, including the SCN neuronal activity rhythm, maintain synchrony with the external environment in a process known as entrainment. Entrainment ensures the correct timing of physiological processes like sleep, blood pressure, and feeding patterns (Saper et al 2005; Dominguez-Rodriguez et al 2010; Schibler et al 2003). Acute disruptions in clock function can occur when changing time zones or when working shift schedules. Continuous circadian disruptions over long periods can lead to problems such as metabolic syndrome and certain forms of cancer, and are even linked to Alzheimer's disease (Maury et al 2010; Takahashi et al 2008; van Someran et al 1996).

Anatomically the SCN is divided into two regions, the ventral core and dorsal shell. The ventral core receives glutamatergic inputs from the optic tract. The ventral core also receives neuropeptide Y and gamma aminobutyric acid (GABA) inputs from the intergeniculate leaflet and serotonergic and GABAergic inputs from the dorsal raphe nucleus (Moore and Silver 2008). The core then communicates these signals to the shell, however the circuitry involved in ventral to dorsal communication is still under investigation. After receiving inputs from the core, the dorsal shell sends circadian outputs mainly to the hypothalamus which then relays these signals to other brain regions (Abrahamson and Moore 2001).

We and others have demonstrated that EtOH consumption can disrupt the SCN clock, including altering its endogenous period and inhibiting normal phase regulation (Brager et al 2010; Brager et al 2011; Ruby et al 2009; Rosenwasser et al 2005; McCulley et al 2013; Prosser et al 2003; McElroy et al 2009; Prosser and Glass 2009; Lindsay et al

2014). At least some of these effects are due to actions specifically within the SCN, since EtOH applied to the SCN via microdialysis or applied in vitro inhibits phase resetting (Prosser 2003; Prosser et al 2008; Prosser and Glass 2009; Brager et al 2010; Ruby et al 2009). Conversely, other effects appear to involve actions outside the SCN (Pickard et al 1996; Graff et al 2006; Prosser et al 2008). The combined effects of EtOH on the circadian system impair proper adjustments to changes in environmental cycles, and could contribute to the circadian disruptions seen in alcoholics (Rouhani et al 1990; Rajakrishnan et al 1999; Kubota et al 2002; Rosenwasser et al 2005). This is supported by multiple reports showing disruptions in physiological rhythms in alcoholic patients (Adinoff et al 1991; Röjdmarm et al 1993; Kühlwein et al 2003). Given that poor sleep quality is a common cause of relapse drinking (Gillin et al 1994; Brower, 2001; Brower 2003) and disrupted circadian rhythms can contribute to impaired sleep (Dijk et al 2000; Crowley 2011; Ditisheim et al 2013), a better understanding of how EtOH affects the circadian clock is important.

As mentioned above, the development of alcohol tolerance is one symptom of alcohol dependence. Many labs have investigated the mechanisms of tolerance in hopes of discovering effective treatments for, or ways to prevent, alcohol dependence. The most heavily studied forms of tolerance, known as acute, rapid, and chronic tolerance, differ with respect to how quickly they develop after initial alcohol consumption. In experiments using mammalian models, acute, rapid, and chronic tolerances develop within minutes, to hours, to days of alcohol consumption, respectively. The timing of these forms of tolerance is consistent with that seen in humans (Bierness and Vogel-Spott 1984; Hiltunen and Jarbe 1990; Bennett et al 1993). Experimentally, the different timing of

development for these forms of tolerance makes them easily distinguishable. Although the cellular mechanisms associated with these tolerance types are thought to differ (Khanna et al 1991a; Grobin et al., 1998; Roberto et al., 2006), there is little direct evidence of this based on within-experiment comparisons. Identifying differences in the mechanisms of tolerance could clarify the processes through which dependence to alcohol develops.

Alcohol withdrawal-induced hyperexcitability is another characteristic of alcohol dependence. Research has shown that sudden abstinence after long-term EtOH use leads to a variety of changes in neuronal signaling that are distinct from those seen during tolerance. For instance, withdrawal from EtOH increases presynaptic glutamate release in many brain regions (Christian et al., 2013, Faingold et al., 2000, Kash et al., 2009 and Roberto et al., 2004). Other studies have shown changes in pharmacological properties of GABA receptors following EtOH withdrawal (Veatch and Becker 2005; Kumar et al 2009; Linsenhardt & Boehm 2010). Although EtOH-induced changes in glutamate and GABA have been studied extensively using a wide variety of experimental paradigms, it is noteworthy that few studies have compared the changes in neuronal signaling associated with chronic EtOH exposure and EtOH withdrawal in a single experiment.

Similar to other brain regions, we have shown that acute tolerance to EtOH inhibition of glutamate-induced phase resetting develops in the SCN *in vitro* (Prosser and Glass 2009). Conversely, evidence suggests that chronic tolerance to EtOH does not develop with respect to inhibition of *in vivo* photic phase resetting (Brager et al 2010). However, results from another study suggest that the lack of chronic tolerance seen in

those experiments may have been due to using forced EtOH consumption versus free-choice EtOH drinking (Seggio et al 2009). With respect to the effects of EtOH withdrawal on circadian rhythms, enhanced photic phase shifts are seen during withdrawal in hamsters (Ruby et al, 2009), while EtOH withdrawal does not alter photic phase shifts in mice (Brager et al, 2010). On the other hand mice exhibit altered free-running circadian periodicity after withdrawal from 12 days of EtOH exposure (Logan et al 2012). In light of these variable results, we have investigated whether multiple forms of EtOH tolerance and withdrawal-induced glutamate sensitization occur in the SCN, using in vitro glutamate-induced circadian rhythm phase shifts as our primary experimental output. Moreover, we used multiple drinking paradigms to directly compare the different forms of EtOH-induced plasticity. At a functional level, we assessed changes in ionotropic glutamate (N-methyl-D-aspartate; NMDA) and GABA(A) receptor expression and phosphorylation in response to different EtOH consumption paradigms.

Our approach is unique in that we are comparing the cellular changes that occur during rapid and chronic tolerance and during withdrawal-induced glutamate sensitization in a single model system. Our goals are two-fold: to clarify the cellular mechanisms through which EtOH affects the SCN circadian clock, and to better understand the cellular changes that occur during and after EtOH consumption. Given the epidemiological evidence linking alcohol tolerance and withdrawal symptoms to the development of alcohol abuse and relapse, the data from these experiments could be used to develop novel cellular targets for pharmacological intervention of AUD.

Circadian rhythms and the suprachiasmatic nucleus

The transcriptional/translational feedback loop

The circadian clock in the mammalian suprachiasmatic nucleus (SCN) synchronizes daily endogenous rhythms to external environmental cues. The mammalian circadian oscillator includes a transcriptional/translational feedback loop (TTFL) that functions over the 24 hour circadian day while maintaining synchrony with external timing cues (Figure 1.1a and b). At sunrise, the transcription factors CLOCK and Bmal1 dimerize and activate transcription of two families of genes, *Period (Per)* and *Cryptochrome (Cry*; Reppert and Weaver 2002). *Per* and *Cry* encode proteins that accumulate slowly over the course of the day (Figure 1.1a). As PER and CRY proteins accumulate, become phosphorylated, and dimerize, they enter the nucleus and negatively feedback onto CLOCK and Bmal1 to suppress transcription, with protein levels (and transcriptional inhibition) peaking near the middle of the night (Figure 1.1b). Over the remainder of the night, protein and mRNA levels decrease, allowing for another cycle of transcription to begin the next day (Reppert and Weaver 2002). Other genes such as *Rev-erba* and *RORα/β* also coordinate the transcription of clock genes, particularly *Bmal1*. This cycling of transcriptional activation and inhibition maintains the timing of behaviors and physiological processes over 24 hours (h), and provides the mechanism through which the clock can respond to external timing cues.

Photic phase shifts

The phase of the circadian clock adjusts to changes in environmental cues, such as seasonal shifts in day length, or remaining active at irregular times, as well as daylight savings, travelling across time zones, and shift work (Stevens 2009; Kantermann et al

2007). The process through which circadian clocks adjust to timing stimuli is known as phase resetting. In mammals, photic phase shifts occur when light shines on the eyes at night and activates retinal ganglion cells. The signal propagates via the optic nerve to the SCN, causing glutamate to be released from retinal ganglion cells onto SCN neurons. The glutamate then binds to and activates NMDA receptors (Ding et al 1997; Mintz et al 1999; Wang et al 2008), a critical step in the photic phase shifting process (Ebling 1996). Concurrent activation of the receptor tropomyosin receptor kinase B (TrkB) by brain-derived neurotrophic factor (BDNF) is also necessary for phase shifts (Liang et al 1998; Liang et al 2000; Allen et al 2005; Kim et al 2006; Michel et al 2006; Mou et al 2009). This leads to TrkB autophosphorylation, which can activate kinases that phosphorylate NR2B subunits of NMDA receptors. The importance of activating NR2B-containing NMDA receptors for photic phase shifts was shown in experiments that blocked glutamate-induced phase shifts with ifenprodil, an NR2B selective antagonist (Bendova et al 2012). Upon activation of NMDA and TrkB receptors, calcium ions (Ca^{2+}) enter the cell, activating a variety of downstream signaling pathways. Eventually this leads to phosphorylation of the transcription factor cAMP response element binding protein (CREB), resulting in an increase in transcription of multiple genes, including the clock genes, *Per1* and *2* (Kornhauser et al 2002; Yokota et al 2001). Increased *Per* gene transcription will either phase delay or phase advance the clock (or have no effect) depending on the time of *Per* gene transcription (Shigeyoshi et al 1997; Yan et al 1999; Yan and Silver 2002). Therefore, the timing of the light-induced glutamate release in the SCN is paramount for determining whether it will induce a phase delay, a phase advance, or no shift. In vitro experiments investigating the cellular processes associated with photic phase shifts

typically bypass activation of retinal ganglion cells and directly apply glutamate onto SCN tissue (Ding et al 1997; Tischkau et al 2000; Prosser and Glass 2009; Lindsay et al 2014).

Ethanol, ethanol tolerance, and their effects on glutamate and GABA signals

Ethanol consumption paradigms

EtOH research is challenging in part because EtOH has many targets. Acutely, EtOH affects extracellular proteins, receptor proteins, second messengers, transcription factors, and genetic regulators. However, it is well known that acute EtOH primarily inhibits glutamate signaling (Lovinger et al 1989) and enhances GABA signaling (Allan and Harris, 1986; Suzdak et al., 1986; Ticku et al., 1986). This is largely due to acute EtOH's ability to modify glutamate receptor and GABA receptor function, decreasing cation flow through NMDA channels and increasing Cl⁻ ion influx through GABA(A) channels (Roberto et al 2004; Proctor et al 2006; Moykkynen and Korpi 2012; Hughes et al 2013; Allan and Harris, 1986; Suzdak et al., 1986; Ticku et al., 1986; Wei et al 2004; Olsen et al 2007). This change in receptor activity by acute EtOH involves modulation of multiple receptor components including subunit expression, post-translational modification, and localization of receptors. Together, these acute effects of EtOH result in decreased neuronal activity, since glutamate generally increases neuronal activity and GABA inhibits neuronal activity (Crews et al 1996; Diamond and Gordon 1997).

A second complication is that the effects of EtOH can change over time due to the development of tolerance at cellular and physiological levels. While acute EtOH inhibits glutamate signaling, chronic ethanol leads to compensatory increases in glutamate release and/or receptor activation (Hendricson et al 2004; Roberto et al 2004; Clapp et al

2010; Wu et al 2010). Likewise, while acute EtOH enhances GABA(A) receptor signaling, chronic EtOH can decrease GABA signaling (Mihic and Harris, 1997; Kumar et al 2003; Liang et al 2007; Abramian et al 2010; Ron and Messing 2013). Compensatory changes in the brain in response to sustained EtOH exposure occur over varying time periods, leading to a variety of changes in receptor subunit composition, phosphorylation, and localization (Chandler et al 1998). These changes are believed to underlie the development of alcohol dependence and alcohol abuse. However, forcing animals to consume EtOH can generate distinct EtOH effects, as shown in multiple studies (Khanna 1991a; 1993; Grover et al 1994; Ravindran et al 2007; Liang et al 2007; Pietrzykowsky and Triestman 2008; Prosser and Glass 2009; Seggio et al 2009; Gonzalez et al 2012; Lindemeyer et al 2014). Therefore, voluntary EtOH consumption may be preferable to forced EtOH exposure if one is trying to mimic the effects of alcohol consumption in humans. However, in experiments where animals control their level of EtOH consumption, it is important to monitor how much EtOH is consumed, and how much it varies across days/subjects. The EtOH consumption/exposure level is important because proteins have different levels of sensitivity to EtOH. For example GABA(A) receptors containing different subunits exhibit either low EtOH (>100mM) or high EtOH (<50mM) sensitivities (Wei et al 2004; Santhakumar et al 2006; Wallner et al 2006).

Finally, one also should consider the time of day the animals consume EtOH. Mice (and other nocturnal rodents) tend to drink the most during the early night (Brager et al 2010 [mice]; Ruby et al 2009 [hamsters]; Rosenwasser et al 2014 [rats]). However, drinking in the morning vs evening can have distinct effects on EtOH induced behaviors

and cellular mechanisms (Deimling and Schnell 1980; Baird 1998; Damaggio and Gorman 2014).

Based on these and other considerations, many different drinking paradigms have been utilized in the EtOH research field. For instance the chronic intermittent EtOH (CIE) paradigm involves switching between periods of EtOH consumption and periods of withdrawal in order to reliably escalate EtOH intake and overall blood alcohol concentration levels (Becker and Lopez 2004). For our experiments, we have used two paradigms: a single nighttime (12 h) of no-choice EtOH consumption to investigate rapid tolerance, and daily 4 h no-choice EtOH access (starting 1 h prior to lights-off, and continuing 3 h into the night) over different durations of drinking (7-21 days), followed by 0-6 days of EtOH withdrawal. These drinking paradigms allow the mice to control the amount of EtOH they consume, and we have found that the inter- and intra-animal variability is low. Using these protocols, we have investigated how EtOH affects NMDA and GABA receptors in the SCN. To put the experiments in context, background information on NMDA and GABA(A) receptors, how EtOH modifies their function, and their post-translational modifiers is presented here.

NMDA Receptors

Glutamate ionotropic NMDA receptors are heterotetrameric: they all contain two NR1 subunits plus two additional subunits, consisting of any combination of NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B subunits. Upon activation, the ion channel opens, allowing Ca^{2+} , sodium ions (Na^+), and potassium ions (K^+) to flow through the channel (Ca^{2+} and Na^+ generally entering and K^+ ions leaving the cell). This depolarizes the neuron, resulting in enhanced neuronal signaling. Each NMDA receptor subunit has

unique effects on NMDA receptor conductance characteristics. For example, an NR2A containing NMDA receptor has consistent and steady firing, whereas an NR2B containing NMDA receptor has more sporadic, burst firing with slower decay kinetics (Cull-Candy 2004). NR2B-containing NMDA receptors remain open 10 times longer than NR2A-containing NMDA receptors, thus allowing more depolarization and a greater increase in intracellular Ca^{2+} accumulation (Cull-Candy 2004). NMDA receptors containing both NR2A and NR2B subunits exhibit hybrid pharmacological and kinetic characteristics (Paoletti and Neyton 2007, Tovar et al 2013). NMDA receptors containing NR3 subunits are inactive. While the type of subunits defines the overall characteristics of the NMDA receptor, the receptor kinetics can also be altered via post-translational modifications.

One key post-translational modification of NMDA receptors is phosphorylation. Phosphorylation of specific NMDA receptor subunit residues can alter ion movement in and out of the channel or change the localization of the receptor. For example phosphorylation of NR2B tyrosine 1472 (Y1472) increases Ca^{2+} ion influx through the NMDA receptor (Nakazawa et al 2001) and maintains its localization within the synaptic active zone (Goebel et al 2005). This is due to phosphorylated Y1472 enhancing NR2B binding with a scaffolding protein, post-synaptic density-95 (PSD-95; Roche et al 2001; Prybylowski et al 2005; Zhang et al 2008). Upon phosphorylation of Y1472 by Fyn kinase (Fyn; Trepanier et al 2012; Gibb et al 2011), the C-terminus of the NR2B subunit binds to the PSD-95/Discs large/zona occludens-1 (PDZ) binding domain of PSD-95.

On the other hand, phosphorylation of serine 1480 (S1480) breaks the interaction of the NR2B subunit with PSD-95. This leads to internalization of the NMDA receptor followed by the receptor either recycling back to the surface or being targeted for

degradation via ubiquitination (Roche et al 2001; Chung et al 2004). Phosphorylation of S1480 involves calcium/calmodulin dependent protein kinase II (CaMKII) forming a complex with the constitutively active kinase, casein kinase 2 (CK2), to form a tri-molecular complex with NR2B. This localizes CK2 to the S1480 residue, increasing CK2 phosphorylation of S1480 (Sanz-Clemente et al 2013).

EtOH and NMDA Receptors

Many studies have demonstrated that the EtOH-induced modulation of NMDA receptors involve changes in the phosphorylation of Y1472 and S1480 of the NR2B subunit, suggesting that this subunit is highly sensitive to the effects of EtOH (Chandler et al 1998; Wang et al 2010; Wu et al 2010; Clapp et al 2010, Staples et al 2015). However, there are other aspects of NMDA receptors that are affected by EtOH, and these effects vary depending on the duration of EtOH exposure. For example, acute EtOH inhibits NMDA receptor conductance (Roberto et al 2004), while chronic EtOH increases NMDA signaling (Kroener et al 2012) and alters subunit composition of NMDA receptors, with many brain regions exhibiting increases in NR2A, NR2B, and NR1 subunit expression (Clapp et al 2010; Pian et al 2010; Wu et al 2010; Wang et al 2011; Staples et al 2015). Even though the expression of most NMDA receptor subunits is increased during chronic EtOH exposure, some studies suggest EtOH has preferential effects for the NR2B subunit. For example, in the hippocampus, a 15 minute (min) exposure to 100mM EtOH induces H-ras mediated endocytosis of NR2A subunits and increases the ratio of NR2B to NR2A subunit containing NMDA receptors (Suvarna et al 2005). Also, transgenic mice with a conditional knockout of the NR2B subunit do not develop

enhanced neuronal firing in response to chronic EtOH application to the bed nucleus of the stria terminalis (Wills et al 2012).

Ethanol and post-translational modifiers of NMDA receptors

EtOH alters the posttranslational modifications of NMDA receptors largely by altering the activity of kinases and phosphatases that target key residues of NMDA receptors (Barria and Malinow 2005; Prybylowski et al 2005; Honse et al 2003; Suvarna et al 2005). Acute vs. chronic EtOH appears to have differential effects on kinases that phosphorylate NMDA receptors (Xu et al 2006; Kash et al 2009; Chung et al 2004). Unfortunately, direct comparisons are difficult because few studies have compared EtOH modulation of these proteins across multiple EtOH exposure paradigms. With this caveat in mind, in this section we focus mainly on EtOH's modulation of Fyn kinase, CaMKII, and striatal enriched tyrosine receptor phosphatase-61 (STEP-61).

Fyn phosphorylates Y1472 of the NMDA receptor NR2B subunit. Before this can occur, Fyn must be phosphorylated at its tyrosine 420 (Y420) residue in response to mature BDNF activating TrkB (Yamada and Nabeshima 2004; Carreño et al 2011). Neuronal cell adhesion molecule-1 (NCAM-1), a neuronal cell adhesion molecule that regulates synaptogenesis, can also activate Fyn via interactions with receptor-like protein tyrosine phosphatase α (RPTP α , Bodrikov et al 2005). Acute and chronic exposure to EtOH do not alter total Fyn protein expression in the hippocampus and striatum (Wu et al 2010; Darcq et al 2014), but chronic EtOH increases Y420 phosphorylation through inactivation of STEP-61 in the dorsal medial striatum (Darcq et al 2014). EtOH can alter expression of BDNF and TrkB, but the effects are regionally specific, with some regions exhibiting increases in BDNF and TrkB while others have decreases (Davis 2008).

Chronic EtOH also decreases polysialic acid (PSA) containing NCAM-1, which allows for Fyn activation due to PSA blocking NCAM-1 related cell signals (Barker et al 2012).

CaMKII α , another kinase important for NMDA receptor regulation, forms a complex with CK2, allowing for increased phosphorylation of NR2B serine 1480 (S1480). Activation of CaMKII involves increases in calcium influx, causing autophosphorylation of CaMKII α at threonine 286 (T286) (Davies et al 2007). This also causes autophosphorylation of threonine 305 (T305), which reduces activation of CaMKII α (Colbran 1993; Zhang et al 2005). A recent study has demonstrated that phosphorylation of CaMKII α at T286 is associated with acute EtOH-induced increases in c-fos expression, suggesting CaMKII α activation is important for EtOH-related increases in neuronal firing (Schöpf et al 2015). Also chronic ethanol increases T286 phosphorylation, but no changes were noted in CaMKII expression in the amygdala during chronic EtOH or EtOH withdrawal (Christian et al 2012). Because CaMKII α activation via T286 phosphorylation correlates with increases in neuronal activity in the SCN (Kon et al 2014) and many other regions, EtOH-induced changes in CaMKII α T286 phosphorylation could underlie some of its actions on the SCN circadian clock.

STEP-61 decreases phosphorylation of NR2B Y1472. This reduces activation of the NMDA receptor and ultimately leads to receptor internalization. Recent studies have shown that proteosomal degradation of STEP-61 occurs through PSD-95 and BDNF, which target STEP-61 for ubiquitin induced degradation (Won et al 2016; Saavedra et al 2016). Protein kinase A (PKA) also blocks STEP-61 from interacting with its substrates by phosphorylating its serine 221 (S221) residue (Paul et al 2000). Studies have demonstrated that chronic EtOH does not alter total STEP-61 expression, but does

increase phosphorylation at the serine 221 residue, again reducing STEP-61 dephosphorylation of NMDA receptors (Darcq et al 2014). Thus, chronic EtOH could increase NMDA receptor activity in part by decreasing activation of STEP-61.

GABA receptors

As mentioned earlier, many actions of EtOH involve changes in GABA related signals. GABA(A) receptors are pentameric with multiple combinations of receptor subunit types generating up to 19 different GABA(A) receptor isoforms. The most common receptor isoforms include 2 α subunits, 2 β subunits, and either a γ or δ subunit (Olsen and Sieghart 2008). The α and β subunits are important for regulating ion channel conductance properties, while the γ and δ subunits regulate the localization of GABA receptors in and out of the synaptic active zone. Activation of GABA(A) receptors that contain γ subunits, which are typically localized within the synapse, causes phasic inhibitory currents in neurons (Farrant and Nusser 2005), while δ subunit-containing GABA(A) receptors, which generate tonic inhibitory currents, are typically found in extrasynaptic regions. Another important distinction is that δ subunit-containing GABA(A) receptors have a much higher sensitivity to EtOH than γ subunit-containing GABA(A) receptors (Wei et al 2004; Santhakumar et al 2006; Wallner et al 2006). Because activating GABA(A) receptors increases Cl^- conductance, GABA signaling is generally inhibitory, decreasing the signaling of its post-synaptic targets.

Regulation of GABA(A) receptors, like NMDA receptors, occurs through a series of post-translational modifications to key residues. The PKA and protein kinase C (PKC) kinases are well documented regulators of GABA(A) receptors via phosphorylation of specific residues, particularly on the $\alpha 4$ subunit. By phosphorylating serine 408 and serine

409 of the GABA(A) β 3 subunit, PKA decreases synaptic α 4 synaptic expression but increases extrasynaptic α 4 expression (Bohnsack et al 2016). Conversely, PKC γ increases synaptic α 4 expression via phosphorylation of the serine 443 residue (Abramian et al 2010). PKC can also stimulate internalization of α 4 containing GABA(A) receptors by phosphorylating the β 2 subunit (Bright and Smart 2013). Thus regulation of GABA(A) receptor function occurs via modulation of posttranslational modifiers leading to changes in channel flux, localization to the synaptic or extrasynaptic regions, and subunit composition of the receptor.

EtOH and GABA receptors

EtOH alters GABA signaling, and its actions at GABA(A) receptors differ depending on the duration of EtOH exposure in the same manner as seen with NMDA receptors. Acute EtOH's actions on GABA(A) receptors are subunit specific. For example, EtOH enhances influx of Cl⁻ ions through γ subunit-containing GABA(A) receptors (Allan and Harris, 1986; Suzdak et al., 1986; Ticku et al., 1986; Wei et al 2004; Olsen et al 2007) while decreasing tonic currents mediated by δ subunit-containing GABA(A) receptors (Liang et al 2007). The effects of chronic EtOH compared to acute EtOH on GABA(A) receptors are more complex. For instance chronic EtOH reduces the expression of particular GABA(A) subunits, alters their phosphorylation, and leads to internalization of GABA(A) subunits, particularly those receptors containing α 1, β 1, and γ subunits (Kumar et al 2003; Liang et al 2007; Abramian et al 2010; Ron and Messing 2013). In contrast, chronic EtOH lasting at least 40 days of exposure increases α 4 and δ subunit mRNA expression in the hippocampus (Matthews et al 1998; Cagetti et al 2004). In short, acute

EtOH enhances actions of γ containing and inhibits δ containing GABA(A) receptors, while chronic EtOH inhibits γ containing and enhances δ containing GABA(A) receptors.

EtOH and post translational modifiers of GABA(A) receptors

Acute and chronic EtOH can also modify the function of kinases that phosphorylate GABA(A) receptor subunits. Acute EtOH-induced changes in kinase activity alters receptor activation and membrane localization (Brandon et al 2000; Brandon et al 2003; Carlson et al 2013; Carlson et al 2014; Choi et al 2008; Kumar et al 2010). The PKC family of kinases in particular are implicated in EtOH effects on GABA(A) receptors (Wallace et al 2006; Bowers et al 1999). PKC γ is required for ethanol induced increases in the surface localization of $\alpha 4$ subunits in cerebral cortical neurons, but the specific mechanism of interaction through which PKC γ affects GABA(A) $\alpha 4$ subunits is still unknown (Werner et al 2011). Recent research has suggested chronic EtOH influences PKC γ phosphorylation of $\alpha 4$ subunit serine 443, increasing $\alpha 4$ subunit surface localization (Werner et al 2016 referring to Abramian et al 2010). Thus acute and chronic EtOH induced modulation of GABA(A) receptors may involve modulation at the $\alpha 4$ subunit via EtOH's actions on PKC γ .

Other studies have suggested that EtOH alters regulation of GABA(A) $\alpha 4$ subunits by PKA and not PKC. Support for this comes from studies showing that acute EtOH activates both PKA and PKC (Dohrman et al 1996; Messing et al 1991). Further support for this comes from studies confirming acute EtOH induced modulation of $\alpha 4$ trafficking after concurrent activation of PKC and PKA (Kumar et al 2012; Carlson et al 2013). Furthermore, a recent study has shown that direct activation of PKA and not PKC increases the surface localization and tonic current of $\alpha 4/\delta$ GABA(A) receptors, while 4 h

of ethanol exposure reduces $\alpha 4/\delta$ receptor abundance (Carlson et al 2016). Thus EtOH appears to modulate localization of $\alpha 4/\delta$ GABA(A) receptors through multiple mechanisms.

It is possible that the mixed results on PKA and PKC modulation of $\alpha 4/\delta$ GABA(A) receptors are due to different durations of EtOH exposure, with acute EtOH mediating the effects of $\alpha 4/\delta$ via PKA (evident by Carlson et al 2016) and chronic EtOH mediating the effects of $\alpha 4/\delta$ via PKC (evident by Werner et al 2016). As indicated earlier, acute vs chronic EtOH exposure differentially regulates receptor currents and subunit composition, and can also differentially affect kinases and phosphatases. Therefore, investigating changes in PKA and PKC associated with different forms of EtOH tolerance may be worthwhile.

EtOH tolerance

EtOH tolerance is defined as requiring higher concentrations of EtOH to achieve the same effect. Tolerance includes changes at the behavioral level, termed behavioral tolerance, as well as cellular changes, termed physiological tolerance (Chandler et al 1998). Behavioral tolerance to EtOH involves the effects of EtOH across many brain regions, as demonstrated in experiments assessing whether behavioral modifications normally induced by EtOH (e.g. loss of coordination) are changed after continued EtOH exposure (Grieve and Littleton 1979; Pietrzykowski and Triestman 2008). Physiological tolerance typically refers to the effects of EtOH tolerance on peripheral mechanisms (e.g., metabolism) and/or how cellular/molecular components of neurons in specific brain regions are modified during continued exposure. Physiological and behavioral EtOH tolerance develops over multiple time domains in mammals, including minutes (acute

tolerance), hours (rapid tolerance), and days (chronic tolerance). Acute tolerance has been shown to involve changes in NMDA receptor and GABA(A) receptor related currents. For example, while initial exposure of CA1 hippocampal tissue to EtOH reduces isolated field potentials from NMDA receptors, this effect decreases with continued exposure over 15 minutes (Grover et al 1994). Also EtOH potentiation of GABA(A) currents diminishes after 5-60 minutes of EtOH exposure in cell cultures prepared from the cerebellum (Allan and Harris 1987).

Rapid tolerance, the least studied of the three forms, has been proposed to underlie the transition from acute to chronic tolerance (Khanna et al 1991a). Most experiments have focused on the involvement of NMDA and GABA(A) receptors in behavioral rapid tolerance and have not addressed physiological rapid tolerance. Pharmacologically, blocking NMDA receptors with the selective antagonist, MK-801, blocks behavioral rapid tolerance to EtOH (Khanna et al 1991b; 1993). Muscimol, a GABA(A) agonist, also blocks behavioral rapid tolerance to EtOH (Barbosa and Morato 2001). Unfortunately, literature investigating the cellular effects underlying physiological rapid tolerance to EtOH is sparse.

Many studies have demonstrated changes to NMDA and GABA(A) currents during chronic tolerance to alcohol. As mentioned, changes in expression and phosphorylation of NMDA receptors and GABA receptors have been reported in chronic tolerance and chronic EtOH (Wu et al 2010; Kroener et al, 2012; Honse et al 2003; Suvana et al 2005; Wang et al 2011; Ravindran et al 2007, Liang et al 2007, Gonzalez et al 2012, Lindemeyer et al 2014). Chronic treatments of EtOH also cause behavioral tolerance with respect to cognitive impairment and to the sedative effects of EtOH (Rafi-Tari et al 1996; Wu et al

2010). Similarly, chronic tolerance has been demonstrated with respect to circadian periodicity and locomotor activity in mice with respect to circadian regulated behaviors (Logan et al 2012). Differences regarding cellular changes in chronic tolerance versus other tolerance types are distinct given the changes that occur to subunit expression and phosphorylation.

Ethanol withdrawal

The sudden withdrawal from EtOH after sustained EtOH exposure induces drastic changes in behavior and physiology that can lead to symptoms like insomnia, psychomotor agitation, hallucination, and delirium tremens (Schuckit 2014). Experimentally in animals, the effects of EtOH withdrawal are typically studied during the first 1-2 days, which corresponds to when the more intense behavioral and physiological effects of EtOH withdrawal occur (Schuckit 2015). This is not to suggest that the effects of withdrawal last only during this timeframe. Some studies have used longer periods of EtOH abstinence that can extend from one week to several weeks. These studies are often performed to determine the duration of EtOH withdrawal induced effects as well as investigate novel effects across longer durations of withdrawal. For example Ding et al 2013 determined that the increased presynaptic glutamate release that develops during EtOH exposure lasts 7 days after withdrawal.

Studies have demonstrated that EtOH withdrawal is associated with increases in extracellular glutamate in various brain regions (Ding et al 2013; Griffin et al 2014). Some have suggested this is due to increased presynaptic release of glutamate that develops during chronic EtOH exposure (Roberto et al 2004; Christian et al 2013). Additionally, physiological and behavioral changes exhibited during EtOH withdrawal are blocked by

glutamate receptor antagonists, suggesting increased activation of glutamate receptors occurs during EtOH withdrawal (Faingold et al 2000; Farook et al 2008). The overt effects of sudden abstinence from EtOH are likely due in part to the inability of glutamate signals to immediately revert to pre-EtOH exposure levels following withdrawal.

The effects of EtOH withdrawal also involve GABA receptors. Changes seen during withdrawal include increases in GABA(A) receptor expression in the nucleus accumbens (Linsenbardt & Boehm 2010). A study investigating the basolateral amygdala demonstrated that surface expression of the $\alpha 4$ and $\gamma 2$ subunits increase after 2 days of withdrawal following chronic intermittent ethanol, while expression of $\alpha 1$, $\alpha 2$, and δ subunits decrease (Lindmeyer et al 2014). Also, the GABA(A) agonist lorazepam reduces handling-induced convulsions that occur after repeated EtOH withdrawals, suggesting that enhancing GABA(A) activity during EtOH withdrawal can diminish withdrawal related pathologies (Veatch and Becker 2005). Thus GABA(A) receptors are a strong candidate for investigating and comparing changes in tolerance and withdrawal to EtOH.

Studying ethanol tolerance and withdrawal-induced sensitization in the SCN circadian clock

Ethanol disrupts circadian clock function

Because circadian rhythms regulate sleep, and alcohol disrupts sleep, we and others hypothesized that alcohol-induced disruptions in sleep are at least partially due to disturbances in circadian regulation (Brower 2001; Prosser et al 2003; Åkerstedt 2003; Chokroverty 2010; Rosenwasser 2010). Because persistent sleep problems after EtOH withdrawal are a strong predictor of relapse drinking (Brower and Perron 2010; Hasler et

al 2010), a better understanding of how EtOH affects sleep could lead to improved treatment for AUD. Over the past 15 years substantial evidence has accumulated showing that EtOH affects circadian rhythms and, conversely, that the circadian clock affects EtOH consumption.

Ethanol disrupts many aspects of circadian regulation. For instance, EtOH alters mRNA levels of the core clock genes *Period1*, *Period2*, and *Period3* (*Per1*, *Per2*, and *Per3*) in adolescent and adult rats (Chen et al 2004; 2006). On the other hand, mutation of core clock genes contributes to AUD, EtOH abuse, and relapse. Mutating the *Per2* gene in mice reduces expression of the glutamate transporter, *Eaat1*, leading to increased extracellular glutamate and increased EtOH consumption (Spanagel et al 2005a). Another study demonstrated that both *Per1* and *Per2* mutant mice have increased drinking and alcohol seeking behaviors (Gamsby et al 2013). In the same study, *Per1* mutants had higher blood alcohol levels than controls 1 h after a single administration of EtOH, suggesting altering clock genes disrupts EtOH metabolism (Gamsby et al 2013). Mice selectively bred for high or low EtOH consumption also have modified core clock gene mRNA expression (Rosenwasser et al 2005; McCulley et al 2013). Thus clock genes have a role in EtOH consumption and EtOH metabolism, while EtOH disrupts clock gene expression.

Human studies have demonstrated more links between circadian disruptions and EtOH. Patients with AUD have a high prevalence of depression. Treating circadian disruption with anti-depressants can reverse circadian abnormalities (Duncan 1996), suggesting AUD and circadian disorders involve disruptions to similar neuronal circuits. Abstinent alcoholics have disruptions of circadian patterning, in that activity patterns tend

to be disorganized with greater effects seen in more severe cases of EtOH withdrawal (Sano et al 1994). Studies assessing mRNA levels of clock genes (*Clock1*, *Bmal1*, *Per1*, *Per2*, *Cry1*, and *Cry2*) from peripheral blood mononuclear cells from AUD patients revealed decreased mRNA expression of all of these genes compared to controls (Huang et al 2010). Mutations in the *CLOCK* and *Per2* genes in humans are also linked to alcoholism (Spanagel et al 2005b; Sjöholm et al 2010). Studies assessing the effects of AUD on free-running rhythms and phase resetting in humans, however, have not been conducted. Thus researchers have turned to animal models to investigate these effects.

Many studies have demonstrated EtOH modulation of free-running clocks and phase resetting in animal models. For instance EtOH consumption disrupts SCN circadian clock free-running period and phase resetting in rodents (Lindsay et al 2014 Brager et al 2010; Ruby et al 2009; Seggio et al 2009; Rosenwasser et al 2005). Research with *Drosophila melanogaster* yields similar results with respect to EtOH induced disruptions in clock periodicity (Seggio et al 2012; Ahmad et al 2013). Other rodent studies have shown that drinking during the night versus during the day can increase EtOH withdrawal induced fatality incidences, alter body temperature, and reduce activity, suggesting that the timing of EtOH consumption has differing effects on the SCN (Damaggio and Gorman 2014; Deimling and Schnell 1980; Baird 1998). With respect to phase resetting, our *in vitro* experiments in mice show direct actions of EtOH within the SCN, including EtOH inhibition of glutamate phase resetting, enhancement of serotonergic phase resetting, and involvement of both TrkB and δ -subunit containing GABA(A) receptors in these effects (Prosser et al 2008; Brager et al 2011; McElroy et al

2009). Although these studies demonstrate acute EtOH actions in the SCN, a critical characteristic of AUD is the development of tolerance.

In the SCN, tolerance is evident as decreased EtOH inhibition of photic/glutamate phase shifts (Prosser and Glass 2009; Lindsay et al 2014; Brager et al 2010). The SCN exhibits all three forms of tolerance: acute, rapid, and chronic, as well as withdrawal-associated glutamate hypersensitivity as indicated in this research (Prosser and Glass 2009; Lindsay et al 2014a; Lindsay et al 2014b; Lindsay et al 2014c). However, the distinct cellular mechanisms underlying each of these conditions are not known. In our studies we have determined how long it takes for rapid vs. chronic tolerance and withdrawal to develop, and have identified distinct changes in NMDA subunit and GABA(A) subunit expression, phosphorylation, and surface expression that occur during EtOH tolerance and withdrawal hypersensitivity. We choose to focus on effects on NMDA and GABA(A) receptors due to the fact that these receptors are key targets of EtOH and they are key regulators of SCN circadian clock functioning. From this, we hope to gain a better understanding of the mechanisms associated with each form of EtOH tolerance so that we may identify key alterations in neuronal function that are necessary for each individual tolerance mechanism.

In our experimental model we assess cellular changes across EtOH tolerance and withdrawal hypersensitization and focus particularly on differences in the developmental timing of EtOH tolerance and withdrawal induced sensitization. Moreover, our assessments are conducted at key time points across the day [Zeitgeber time (ZT) 6, ZT16, and ZT23; Zeitgeber time is based on the light-dark cycle, with ZT0 being lights-on and ZT12 being lights-off in the animal colony.] when baseline neuronal activity differs in

the SCN and when glutamate has distinct effects. At ZT 6, when neuronal activity is highest, glutamate does not modulate clock phase. At ZT 16, when neuronal activity is nearing its trough, glutamate delays clock phase. At ZT 23, when neuronal activity is starting to rise, glutamate phase-advances the SCN clock. Using the SCN, we 1) investigate EtOH tolerance and withdrawal physiologically via assessing glutamate-induced phase resetting; 2) assess changes in receptors and their key regulators across these different states; and 3) compare differences in these EtOH consumption-induced changes across distinct baseline cellular states within a single brain area. We hypothesized that EtOH tolerance and withdrawal induced sensitization are associated with changes in the expression and phosphorylation state of NR2B containing NMDA receptors in the SCN, and that the extent that these changes occur differs across the tolerance and withdrawal paradigms.

Summary of introduction

The expression of EtOH tolerance and withdrawal symptoms are two criteria for the diagnosis of an AUD. The cellular mechanisms associated with EtOH tolerance and withdrawal hypersensitivity are not well understood. Furthermore, little if any research has attempted to compare cellular changes associated with different durations of EtOH use and withdrawal. We use the SCN, the locus of the primary circadian oscillator, to study different types of tolerance using glutamate induced phase shifts as our experimental output. This model was chosen because photic phase shifts depend upon NMDA receptors and are modulated by GABA(A) receptor activation (Mintz et al 1999; Ralph and Menaker 1989; Gillespie et al. 1997; Herzog et al 2017), and EtOH modifies these

receptors as well as the kinases and phosphatases that regulate their function. Thus using glutamatergic phase shifts as an experimental output should constitute an excellent indicator of changes in neuronal physiology due to EtOH tolerance and EtOH withdrawal. We also investigate the changes that occur with respect to NMDA receptor subunit expression, phosphorylation, and localization by comparing tolerance and withdrawal induced sensitization in a single experiment. We planned to perform similar experiments investigating GABA(A) receptors, but were unable due to time constraints. The literature demonstrates that there are changes associated with acute and chronic EtOH. However, there is little known about how these receptors mediate tolerance, and how their regulation is variably altered by different forms of tolerance and withdrawal induced sensitization. Thus, we will investigate different forms of tolerance and withdrawal sensitization to EtOH in the SCN via photic phase shifts, and assess which cellular mechanisms associated with different forms of tolerance and withdrawal induced sensitization are distinct.

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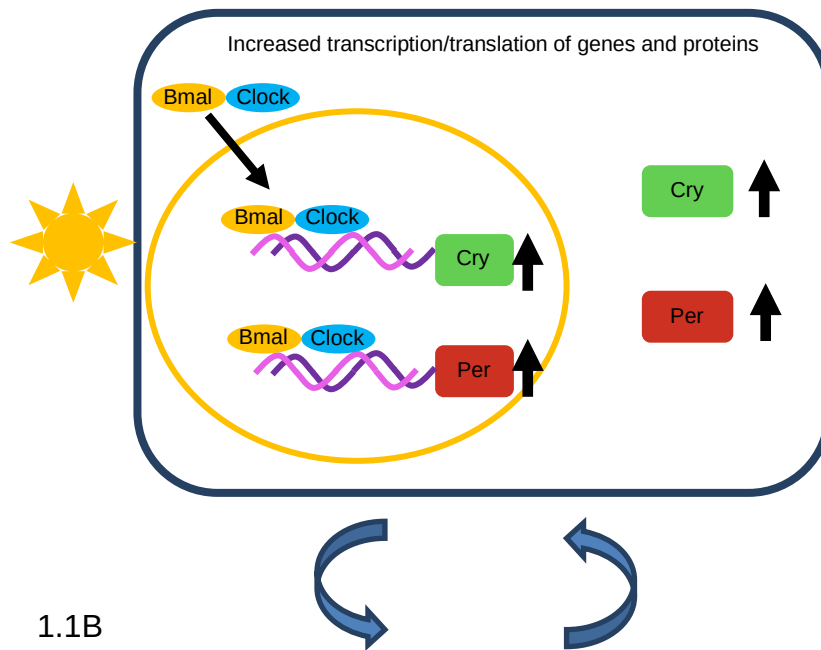
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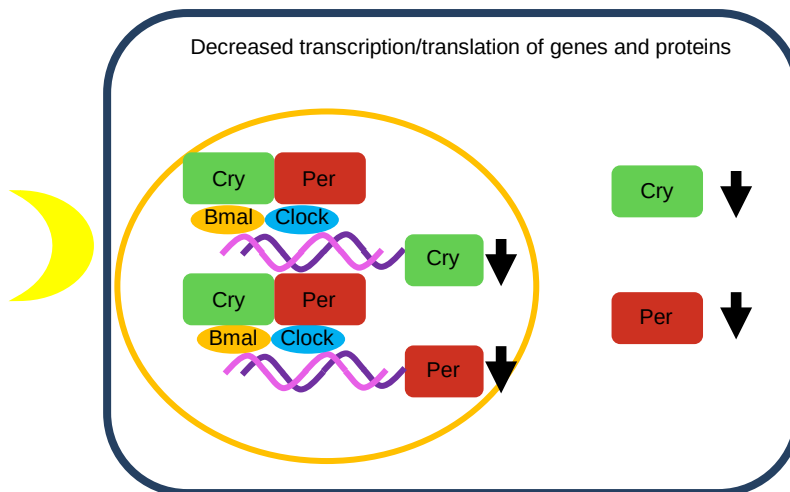
Appendix A

Figure 1.1 The transcription translation feedback loop (TTFL) maintains the timing of circadian rhythms in the majority of mammalian cells. **(A)** At the beginning of the day CLOCK and Bmal1 heterodimerize and translocate into the nucleus of the cell. This complex activates transcription of many genes including period (*Per*) and cryptochrome (*Cry*). As the day progresses, *Per* and *Cry* mRNA become translated into proteins. Once the PER and CRY proteins become phosphorylated, PER and CRY heterodimerize and translocate to the nucleus. The PER:CRY complex then inhibits function of CLOCK and Bmal1. **(B)** As the day ends and night begins, the transcriptional activation by CLOCK:Bmal1 is greatly reduced by the feedback inhibition of PER:CRY. Because of this, protein and mRNA levels decrease over the remainder of the night, which decreases the inhibitory feedback, allowing for another cycle of transcription to begin the next day (blue arrows).

1.1A



1.1B



Chapter 2

The Mammalian Circadian Clock in the Suprachiasmatic Nucleus Exhibits Rapid Tolerance to Ethanol In Vivo and In Vitro

This chapter is adapted for dissertation formatting from the following publication:

Lindsay, J. H., J. D. Glass, M. Amicarelli, and R. A. Prosser (2014). The mammalian circadian clock in the suprachiasmatic nucleus exhibits rapid tolerance to ethanol in vivo and in vitro. *Alcoholism, Clinical and Experimental Research* 38 (3), 760-769.

My primary contributions to this paper were in the following areas (excluding in vivo data that was provided by members of Dr. Glass' research team): designing the experiments, performing experimental procedures, collecting and analyzing data, interpreting data, preparing the figures and writing the manuscript.

Abstract

Background

Ethanol (EtOH) triggers cellular adaptations that induce tolerance in many brain areas, including the suprachiasmatic nucleus (SCN), the site of the master circadian clock. EtOH inhibits light-induced phase shifts in the SCN in vivo and glutamate-induced phase shifts in vitro. The in vitro phase shifts develop acute tolerance to EtOH, occurring within minutes of initial exposure, while the in vivo phase shifts exhibit no evidence of chronic tolerance. An intermediate form, rapid tolerance, is not well studied but may predict subsequent chronic tolerance. Here, we investigated rapid tolerance in the SCN clock.

Methods

Adult C57BL/6 mice were provided 15% EtOH or water for one 12-hour lights-off period. For in vitro experiments, SCN-containing brain slices were prepared in the morning and treated for 10 minutes with glutamate +/- EtOH the following night. Single-

cell neuronal firing rates were recorded extracellularly during the subsequent day to determine SCN clock phase. For in vivo experiments, mice receiving EtOH 24 hours previously were exposed to a 30-minute light pulse immediately preceded by intraperitoneal saline or 2 g/kg EtOH injection. Mice were then placed in constant darkness and their phase-shifting responses measured.

Results

In vitro, the SCN clock from EtOH-exposed mice exhibited rapid tolerance, with a 10-fold increase in EtOH needed to inhibit glutamate-induced phase shifts. Co-application of brain-derived neurotrophic factor prevented EtOH inhibition, consistent with experiments using EtOH-naïve mice. Rapid tolerance lasts 48 to 96 hours, depending on whether assessing in vitro phase advances or phase delays. Similarly, in vivo, prior EtOH consumption prevented EtOH's acute blockade of photic phase delays. Finally, immunoblot experiments showed no changes in SCN glutamate receptor subunit (NR2B) expression or phosphorylation in response to rapid tolerance induction.

Conclusions

The SCN circadian clock develops rapid tolerance to EtOH as assessed both in vivo and in vitro, and the tolerance lasts for several days. These data demonstrate the utility of the circadian system as a model for investigating cellular mechanisms through which EtOH acts in the brain.

Introduction

Alcohol abuse is a debilitating condition linked to depression, sleep disorders, and cognitive defects (Brower 2001; Hesselbrock 1991; Sullivan and Pfefferbaum 2005).

Neurochemically, alcohol affects multiple neurotransmitter systems, including GABA and glutamate signaling (Ariwodola and Weiner 2004; Carta et al 2003; Hancher et al 2005; Wu et al 2010; Zhu and Lovinger 2006). However, ethanol's (EtOH's) effects can exhibit tolerance, or desensitization, as assessed behaviorally (e.g., ataxia and hypothermia; Crabbe et al 1979; Gill and Deitrich 1998) and electrophysiologically (Knott et al 2002). For example, acute tolerance occurs within minutes of exposure to EtOH, while chronic tolerance to EtOH develops over weeks to months (Khanna et al 1991). Not surprisingly, the cellular mechanisms underlying these 2 forms of tolerance appear to be different (Grobin et al 1998; Roberto et al 2006).

Intermediate to these is rapid tolerance, a less-studied phenomenon that develops 8 to 24 hours after initial exposure to EtOH. This form of tolerance is seen with hypnotic, motor, and hypothermic effects of EtOH (Gilliam 1989; Silveri and Spear 1999). While rapid tolerance may be a predictor of subsequent chronic tolerance development (Khanna et al 1991; Radcliffe et al 2006; Wu et al 2001), little is known about its prevalence or cellular basis.

The suprachiasmatic nucleus (SCN) circadian clock is the master 24-hour oscillator that regulates daily behavioral and physiological rhythms in mammals. External cues, particularly light, synchronize the SCN clock to the cyclic environment. Disruptions in clock synchronization are linked to diverse clinical conditions, including sleep disorders, diabetes, and cancer (Blask et al 2003; Crowley 2011; Dauchy et al 1999; Dijk et al 2000; Stevens 2006). As alcohol abuse is associated with chronic sleep problems and other pathologies (Åkerstedt 2003; Anstey et al 2009; Brower 2001; Grewal and Viswanathan 2012; Riley et al 2011), it is important to understand the neurophysiological

mechanisms through which EtOH affects circadian clock phase regulation. In this regard, we and others have shown that EtOH disrupts circadian rhythms through actions within and outside the SCN (Prosser and Glass 2009; Rosenwasser et al 2005; Spanagel et al 2005). For example, acute EtOH administered peripherally or via microdialysis into the SCN inhibits photic phase shifts in vivo (Brager et al 2011), while EtOH applied to the SCN in vitro inhibits glutamate-induced phase resetting (Prosser et al 2008). Likewise, chronic EtOH consumption inhibits photic phase shifts in vivo (Brager et al 2010).

Interestingly, the similar inhibition of SCN photic phase resetting seen with both acute and chronic EtOH in vivo suggests that the circadian system does not develop chronic tolerance to EtOH. This contrasts with our findings regarding acute tolerance in vitro: the SCN develops acute tolerance within 5 to 15 minutes of initial EtOH exposure, as exhibited by a loss of EtOH inhibition of glutamatergic phase resetting (Prosser and Glass 2009). To further explore EtOH tolerance within the SCN and better understand the differences between the in vivo and in vitro conditions, we investigated rapid tolerance to EtOH using both in vivo and in vitro approaches in the mouse.

Materials and methods

Animals

Adult (~8 weeks old) male C57BL/6 mice were obtained from Harlan Laboratories (Frederick, MD) and Jackson Laboratories (Bar Harbor, ME). Animals were singly housed in polycarbonate cages under a 12:12 light/dark (LD) cycle at a light intensity of 270 lux, in a temperature-controlled vivarium (23°C) with food and water provided ad libitum. The experiments followed the National Institutes of Health Guidelines for the Care and Use of

Laboratory Animals and were approved by the University of Tennessee Institutional Animal Care and Use Committee and Kent State University Institutional Animal Care and Use Committee.

EtOH consumption

Mice were trained to drink water from drip-resistant sipping tubes (Medical Associates, Inc., St. Albans, VT) for 2 days prior to introducing EtOH. On the first day of the experiment, the water bottle of each “EtOH-exposed” mouse was replaced with one containing 15% EtOH immediately prior to lights-off. Mice were then allowed to consume alcohol overnight. Control (“EtOH-naïve”) mice had their water bottles replaced with ones containing water. Although we did not monitor the pattern of EtOH/water consumption, our previous studies indicate that mice do the majority of their drinking (EtOH and/or water) in the first half of the night (Brager et al 2010). The following morning at lights-on, the amount of EtOH (or water) consumed overnight was measured, and the animals were weighed.

Brain slice preparation

Coronal brain slices (500 μ) containing the SCN were prepared from EtOH-exposed or EtOH-naïve mice between Zeitgeber Time (ZT) 0 and ZT 4 (where ZT 0 = lights-on and ZT 12 = lights-off in the animal colony) and maintained in a Hatton-style perfusion chamber that received a continuous supply of 95% O₂/5% CO₂. SCN brain slices were perfused continuously with 37°C, oxygenated, glucose/bicarbonate-supplemented Earle's balanced salt solution (EBSS), pH 7.4.

In vitro drug treatments

Drugs, dissolved in EBSS, were bath applied to the brain slices at ZT 16 or ZT 23 (i.e., either 4 or 11 hours after lights-off in the previous LD cycle). Drug treatments were applied by stopping the perfusion and replacing the medium in the chamber with EBSS containing the drug treatment. After 10 minutes, the solution was removed and perfusion restarted. Thus, for EtOH-exposed mice, the *in vitro* drug treatment occurred approximately 24 to 30 hours after the bulk of their EtOH consumption and at a minimum 16 hours after their last exposure to EtOH. Drugs used in the study include glutamate (Sigma-Aldrich, St. Louis, MO), EtOH (Decon Laboratories, Inc., King of Prussia, PA), and brain-derived neurotrophic factor (BDNF) (Alomone Labs, HarHotzuim, Jerusalem, Israel).

Electrophysiology

SCN neuronal activity was recorded extracellularly on day 2 *in vitro* using glass microelectrodes. Electrophysiological recordings and analysis were performed with a DataWave system (Berthold, CO). Individual cells were recorded for 5 minutes, and 4 to 6 neurons were recorded each hour. As in previous studies (Prosser 2003; Prosser and Glass 2009; Prosser et al 2008), the firing rates of individual neurons were combined to generate 2-hour running averages. The time of peak activity was determined by visual estimation to the nearest quarter hour (Prosser 2003; Prosser and Glass 2009; Prosser et al 2008). Phase shifts were calculated as the difference in time of peak of untreated slices from the time of peak of drug-treated slices, because the process of exchanging the medium in itself has no effect on the neuronal activity rhythm (Prosser 2003; Prosser

and Glass 2009; Prosser et al 2008). The overall experimental design is illustrated in Figure 2.1.

Rapid tolerance duration

To determine the duration of rapid tolerance, EtOH-exposed mice received 15% EtOH for 1 night as usual. The next morning, the EtOH was replaced with water. Mice had free access to water for an additional 24, 48, or 72 hours before brain slices were prepared (Figure 2.1). Therefore, in these experiments, the in vitro drug treatments were applied to the brain slices 48, 72, or 96 hours after the bulk of their EtOH consumption, respectively. Brain slice preparation, in vitro drug treatment, and electrophysiological recordings were performed as described previously.

Immunoblots

SCN brain slices were prepared from either EtOH-naïve or EtOH-exposed mice, and drug treatments were performed at ZT 16. Immediately following the drug treatment, slices were collected and frozen on dry ice, then stored at -80°C until protein assays were performed. Protein was extracted from SCN tissue in ice-cold lysis buffer containing 20% radioimmunoprecipitation assay (RIPA) buffer. Protein concentrations were determined using a Bradford assay. Whole cell lysate was added in 40 μg quantities into acrylamide gels for separation via SDS PAGE. Separated proteins were transferred to polyvinylidene difluoride blots and blocked with 5% nonfat dried milk in Tris-buffered saline containing 20% Tween (TBST) for 1 hour at room temperature. Blots were exposed to NR2B (1:1,000; EMD Millipore, Billerica, MA), actin (1:1,000; Santa Cruz Biotechnology, Dallas, TX), NR2B p1472 (1:1,000; EMD Millipore), or NR2B p1303 (1:1,000; EMD Millipore) anti-rabbit polyclonal primary antibodies for 2 hours at room temperature or overnight at 4°C .

Blots were washed 3 times with TBST and then exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies. After 90 minutes, blots were rinsed, luminol solution was applied, and blots were developed using X-ray film exposed to chemiluminescent reaction. Blots were analyzed by determining band intensity relative to background using ImageJ (NIH, Bethesda, MD). All treatments were normalized to actin. Significance was determined using 1-way analysis of variance (ANOVA).

In vivo assessment of rapid tolerance to EtOH

Mice housed under 12:12 LD conditions were individually caged and their general locomotor activity rhythms were measured over a 2-week pre-experimentation period using overhead passive infrared motion detectors interfaced with a data acquisition system (Clocklab; Coulbourn Instruments, Whitehall, PA). As in previous studies (Brager et al 2010), activity data were collected in 1-minute bins; activity onset associated with lights-off (ZT 12) was defined by the initial 6-minute period that (i) coincided with an intensity of activity that exceeded 10% of the maximum rate for the day; (ii) was preceded by at least 4 hours of activity quiescence; and (iii) was followed by at least 60 minutes of sustained activity. Phase shifts were calculated as the difference in projected times of activity onset of baseline entrainment and days following EtOH-photic treatment as determined by (i) back extrapolation of the least-squares line through activity onsets on days 3 to 7 after EtOH-photic treatment and (ii) extrapolation of the least-squares line calculated from activity onset data collected the last 5 days of baseline entrainment (Brager et al 2010; Ruby et al 2009). Mice had access to EtOH during a single night as described earlier. The subsequent night, animals received an intraperitoneal (i.p.) injection of EtOH (2.0 g/kg, v/v in physiological saline) or saline alone immediately

preceding a 30-minute phase-delaying light pulse (25 lux) delivered from ZT 16 to 16.5. Immediately following the light pulse, the animals were released into constant darkness (DD) for 2 weeks to assess the extent of phase-delaying using an Aschoff Type II procedure (Pittendrigh and Daan 1976). Under DD, activity onset is designated as circadian time 12 and is the phase reference point for the onset of the subjective night.

Statistical analysis

Differences in the time of peak neuronal activity were assessed using 1-way ANOVA. In vivo results were analyzed using a standard *t*-test. In all cases, the level of significance was set at $p < 0.05$.

Results

EtOH consumption

Across all the experiments, mice given access to 15% EtOH drank 2.19 ± 0.06 ml of EtOH, which translates to an average consumption of $10.6 \text{ g/kg} \pm 0.32$ EtOH ($n = 112$).

The SCN circadian clock exhibits rapid tolerance to in vitro EtOH: neuronal activity rhythms

We have shown that in vitro glutamate treatment at ZT 16 delays the phase of the SCN neuronal activity rhythm by about 3 hours (Prosser et al 2008). Here, we confirm these results, showing that a 10-minute bath application of glutamate (1 mM) at ZT 16 to brain slices prepared from an EtOH-naïve mouse induced a 3-hour phase delay (Figure 2.2B) relative to the time of peak activity for an untreated slice (Figure 2.2A). Again, as previously demonstrated (Prosser et al 2008) in brain slices from EtOH-naïve mice, treatment with 1 mM glutamate + 20 mM EtOH inhibited the glutamate-induced

phase delay (Figure 2.2C). In slices from EtOH-exposed mice, in vitro glutamate treatment continued to induce a 3-hour phase delay (Figure 2.2E); however, 20 mM EtOH did not block glutamate-induced phase delays in tissue prepared from EtOH-exposed mice. Instead, glutamate continued to induce a 3-hour phase delay (Figure 2.2D). The mean phase shifts induced by these treatments are summarized in Figure 2.2E. Because this loss of EtOH inhibition was seen approximately 24 hours after EtOH consumption, these results are consistent with the induction of rapid tolerance to EtOH.

Next, we investigated rapid tolerance with respect to glutamate-induced phase advances. As shown previously (Prosser et al 2008), bath application of glutamate (1 mM) at ZT 23 to SCN slices induced a 3-hour phase advance. Treating brain slices from EtOH-naïve mice with 20 mM EtOH in conjunction with glutamate inhibited phase advances (mean phase shift = 0.0 ± 0.0 hours, $n = 2$). However, 20 mM EtOH did not inhibit glutamate-induced phase advances in tissue from EtOH-exposed mice (Figure 2.3). Thus, our results with treatments at ZT 23 are consistent with those seen with ZT 16 treatments.

The SCN circadian clock exhibits rapid tolerance to EtOH in vivo: behavioral rhythms

In EtOH-exposed mice, a systemic injection of saline followed by a 30-minute light pulse delivered at ZT 16 induced a 0.62 ± 0.17 hour ($n = 7$) phase delay in the behavioral activity rhythms (Figure 2.4A). Previously, we have shown that systemic administration of 2 g/kg EtOH to EtOH-naïve mice decreases photic-induced phase delays by about 50% (Brager et al 2011). However, this same dose of EtOH administered to EtOH-exposed mice did not significantly inhibit light-induced phase delays in behavioral activity (mean phase delay = 0.86 ± 0.13 hours [$n = 7$], $p = 0.29$ vs. saline injection; Figure 2.4B). Thus,

the rapid tolerance observed in our SCN brain slices is also seen with photic phase shifts of behavioral activity rhythms in intact animals.

Dose-dependency of EtOH inhibition

We have shown that in vitro EtOH inhibits glutamate-induced phase delays in EtOH-naïve mice with an ED₅₀ of about 10 mM (Prosser et al 2008). Here, we investigated whether rapid tolerance to EtOH involves a complete loss of sensitivity to EtOH or just a decrease in sensitivity. Thus, we treated SCN brain slices prepared from EtOH-exposed mice with 1 mM glutamate at ZT 16 together with varying concentrations of EtOH. As seen in Figure 2.5A, the amount of EtOH needed to completely inhibit glutamatergic phase delays increased from 20 to 200 mM, with an ED₅₀ between 150 and 175 mM (Figure 2.5A). A similar shift is seen in the dose–response curve for EtOH treatments at ZT 23. Application of 200 mM EtOH completely blocked glutamatergic phase advances, with the ED₅₀ again between 150 and 175 mM (Figure 2.5B).

BDNF reverses EtOH inhibition of glutamatergic phase shifts

We have shown that co-treatment of BDNF with glutamate and EtOH prevents acute EtOH inhibition of glutamate-induced phase shifts in tissue prepared from EtOH-naïve mice (Prosser et al 2008). Here, we investigated if this occurs in tissue prepared from EtOH-exposed mice. First, we repeated our previous experiments with brain slices from EtOH-naïve mice at ZT 16, showing that co-application of 100 ng/ml BDNF with 1 mM glutamate and 20 mM EtOH resulted in phase delays of 2.3 ± 0.45 hours, $n = 3$ (Figure 2.2E), indicating that BDNF blocked EtOH inhibition. Next, we tested brain slices from EtOH-exposed mice, applying 100 ng/ml BDNF together with 200 mM EtOH and 1 mM glutamate. BDNF rescued glutamate-induced phase delays in brain slices from

EtOH-exposed mice as well (Figure 2.2E). Control experiments indicated that applying EtOH and BDNF together in the absence of glutamate did not induce phase shifts (Figure 2.2E). [Previous work indicates that BDNF and EtOH applied separately do not induce phase shifts (Mou et al 2009; Prosser et al 2008)]. We also investigated these treatments at ZT 23. Similar to our results at ZT 16, adding 100ng/mL BDNF rescued glutamate-induced phase advances blocked by 200 mM EtOH, resulting in a 2.7 ± 0.15 hour phase advance ($n = 3$) (Figure 2.3). Again, control experiments confirmed that EtOH and BDNF did not induce phase shifts when applied concurrently (Figure 2.3). Thus, BDNF reverses EtOH inhibition of in vitro glutamate-induced phase shifts in brain slices from both EtOH-naïve and EtOH-exposed mice.

Duration of rapid tolerance

To determine how long rapid tolerance to EtOH persists in the SCN, we modified the experimental protocol by expanding the time between in vivo EtOH consumption and brain slice preparation. Thus, brain slices were prepared 24, 48, or 72 hours after access to EtOH ended. These brain slices were then treated with EtOH + glutamate the subsequent subjective night at either ZT 16 or ZT 23, as in previous experiments. Thus, for brain slices prepared 24 hours after EtOH access ended, ZT 16 treatments were applied approximately 48 hours after the majority of EtOH consumption, while for slices prepared 48 hours after EtOH access ended, ZT 16 treatments were applied approximately 72 hours after the majority of EtOH consumption. Given this time frame, co-treatment with glutamate and EtOH 48 hours after the majority of EtOH consumption resulted in phase delays of 3.2 ± 0.14 hours, $n = 3$, indicating continued tolerance, while co-treatment with glutamate and EtOH 72 hours after the bulk of EtOH consumption

resulted in no significant phase delay (0.45 ± 0.07 hours, $n = 3$; Figure 2.6A). Thus, rapid tolerance to EtOH inhibition of glutamate-induced phase delays is lost between 48 and 72 hours after the majority of EtOH consumption. With respect to ZT 23 experiments, the tolerance to EtOH appears to last longer. Phase advances were seen after co-administration of EtOH and glutamate 48 and 72 hours after EtOH consumption, (mean phase shifts = 2.875 ± 2.04 hours, $n = 2$ and 3.1 ± 0.29 hours, $n = 3$, respectively) but not when they were co-applied approximately 96 hours after EtOH consumption ended (mean phase shift = 0.0 ± 0.20 hours, $n = 3$) (Figure 2.6B). Thus, for glutamate-induced phase advances, rapid tolerance to EtOH appears to last between 72 and 96 hours after EtOH consumption.

Effects of EtOH on NR2B expression and phosphorylation

Our data suggest that SCN tissue develops tolerance to EtOH at both ZT 16 and ZT 23. To begin investigating potential cellular mechanisms underlying this effect, we focused on N-methyl-D-aspartate (NMDA) receptors in the SCN. Using immunoblot analysis, we first focused on EtOH-naïve SCN tissue. Tissue samples were probed for total NR2B and Y1472-phosphorylated NR2B receptor levels. Blot analysis showed that in EtOH-naïve tissue neither total NR2B expression nor Y1472-phosphorylated NR2B expression were significantly altered by any of the treatments when compared to control ($n = 5$, $p > 0.966$) (Figure 2.7A). Next, we probed for the expression of total NR2B along with NR2B phosphorylated at either Y1472 or S1303 in EtOH-exposed tissue. Y1472 and S1303 are phosphorylated by Src family proteins and CAM kinase II, respectively. Analysis again revealed no significant differences across treatments ($n = 3$, $p > 0.711$) (Figure 2.7B). Thus, we found no differences in the level of total NR2B expression in the

SCN or in the level of NR2B phosphorylation at Y1472 and S1303 in EtOH-naïve and EtOH-exposed tissue.

Discussion

The SCN clock exhibits rapid tolerance to EtOH

Chronic alcohol abuse is characterized by a self-perpetuating spiral of decreasing alcohol sensitivity and increasing consumption (Kalant 1998). A better understanding of the cellular processes underlying alcohol desensitization may help to develop treatments that alleviate or prevent this debilitating cycle of dependence. The propensity to develop rapid tolerance has been linked to the development of chronic tolerance (Khanna et al 1991). Here, we show for the first time that the SCN circadian clock exhibits rapid tolerance to EtOH. Importantly, this tolerance is seen in vivo as well as in vitro. Rapid tolerance in the SCN in vitro is manifest as a 10-fold shift in the dose–response curve for EtOH inhibition of glutamate-induced phase-advance and phase-delay shifts; it is apparent 24 hours after initial exposure to EtOH; and it lasts for several days. Future experiments could determine whether rapid tolerance in vivo exhibits similar characteristics by increasing the concentration of EtOH administered i.p. and by increasing the time interval between EtOH consumption and testing for EtOH inhibition of photic phase shifts.

Time course of rapid tolerance

We have previously shown that EtOH inhibits in vitro glutamate-induced phase delays and phase advances and that the SCN clock develops an acute form of tolerance to EtOH within 5 to 15 minutes (Prosser and Glass 2009). This acute tolerance does not

dissipate with maintained EtOH application for at least 3 hours. That study did not determine the duration of acute tolerance after EtOH removal, but a previous report indicates that acute tolerance is lost 24 hours after EtOH exposure (Allen and Harris, 1987). This time course, in part, helps to differentiate acute tolerance from rapid tolerance.

Rapid tolerance develops more slowly than acute tolerance, being initially seen 8 to 24 hours after exposure (Crabbe et al 1979; Kalant 1998). In this study, our experimental design precludes an accurate estimation of the time course of tolerance development, because we utilized self-administration of EtOH by the mice over the course of a 12-hour lights-off period, and we did not monitor the timing of EtOH consumption. The minimum time between EtOH consumption and when EtOH was applied to the brain slices (or administered to the mice *in vivo*) was 16 hours. However, based on typical nocturnal drinking patterns in mice (Brager et al 2010), the actual time between the 2 EtOH exposures was likely closer to 24 hours. To more narrowly define the time course of tolerance development, the time of EtOH access by the mice would have to be further restricted.

An issue not previously addressed in the literature is the persistence of rapid tolerance. We investigated this issue with both early night phase delays and late night phase advances. For glutamate/EtOH treatments at ZT 16, we found that tolerance lasts at least 48 hours after EtOH exposure but is no longer present after 72 hours. As the experiment requires tolerance assessment at times when glutamate phase shifts the circadian clock, we are limited in our ability to define the time course more precisely. Interestingly, rapid tolerance appears to last between 72 and 96 hours for ZT 23 co-

treatments with glutamate and EtOH. This difference in time course may be related to the distinct cellular mechanisms associated with circadian clock phase delays and phase advances (Gillette and Mitchell 2002). Determining differences in the cellular responses to alcohol through phase delay/advance mechanisms could provide key information on how alcohol affects neuronal cellular dynamics, especially those associated with the development of tolerance.

BDNF reverses EtOH inhibition of glutamatergic phase shifts in EtOH-exposed mice

In these experiments, rapid tolerance in the SCN was characterized by a lack of response to the lower (20 mM) concentration of EtOH, while a 10-fold higher concentration was able to inhibit glutamate-induced phase shifts. This higher EtOH concentration could potentially affect different cellular mechanisms than the lower concentrations: many studies have demonstrated dose-dependent differences in receptor sensitivity to EtOH, including GABA (Wakita et al 2012; Wu et al 2005) and glutamate (Allgaier 2002; Blevins et al 1997; Proctor et al 2006) receptors. Our previous experiments demonstrated that BDNF prevents acute EtOH inhibition of glutamate-induced phase shifts (Prosser et al 2008), suggesting that EtOH acts in part by inhibiting BDNF actions. Therefore, as an initial assessment of potential differences in cellular mechanisms, in these experiments, we investigated whether the inhibition induced by 200 mM EtOH likewise was sensitive to reversal by BDNF. Our results show that BDNF still reverses EtOH inhibition during rapid tolerance, suggesting that similar cell signaling processes underlie EtOH's actions under both conditions.

As application of BDNF can enhance NMDA receptor activity, it is possible that rapid tolerance in the SCN involves changes in either NMDA (glutamate) or TrkB (BDNF)

receptors. Previous studies determined that NMDA receptor antagonists can prevent rapid tolerance (Khanna et al., 1991). Others indicate that NMDA receptors, especially its NR2B subunit, play a major role in the onset of tolerance (Yaka et al., 2003). Both acute and chronic EtOH exposure can alter NMDA receptor biochemistry by decreasing phosphorylation at the Y1472 residue (Wu et al 2010), but whether similar changes occur with rapid tolerance is not known. We hypothesized that rapid tolerance could involve either increased NR2B expression and/or enhanced phosphorylation at NR2B Y1472 residue. However, our data do not support this hypothesis. There are several possible explanations: First, it is possible that the changes underlying rapid tolerance do not include changes in NR2B expression and phosphorylation. If true, this would differentiate rapid tolerance from both acute and chronic tolerance (Läck et al 2005; Miyakawa et al 1997; Nagy 2008; Roberto et al 2006; Trepanier et al 2012). Second, rapid tolerance may involve compensatory responses upstream and/or downstream of NMDA receptors. For example, chronic EtOH induces increases in striatal enriched tyrosine phosphatase-33 (STEP-33) and p38 mitogen activated protein kinase (MAPK), without altering Src, Lyn, and Fyn kinases (Wu et al 2010). It may be worthwhile investigating these molecules in future experiments with rapid tolerance. Third, changes in NMDA receptor membrane localization could play a role in rapid tolerance. Future experiments using biotinylation could begin to address this possibility. Last, rapid tolerance may involve other receptors, such as TrkB or GABA(A) receptors that are known to play critical roles in the SCN. For example, BDNF enhances NMDA currents in the SCN (Kim et al 2006), so rapid tolerance could involve an increase in TrkB signaling. Also, a previous study found that EtOH acts in the SCN at least partially through activating GABA receptors (McElroy et al 2009).

Therefore, it will be important to determine whether rapid tolerance is associated with decreases in GABA signaling in the SCN. Importantly, due to the different durations of rapid tolerance, we observed regarding glutamate-induced phase delays and phase advances, it will be important to assess these cellular mechanisms with respect to phase advances in addition to phase delays.

Summary: the different manifestations of EtOH tolerance

Broadly speaking, to better understand the mechanisms leading to alcohol abuse, it is important to clarify the different forms of tolerance at the behavioral, physiological, and molecular levels. Acute EtOH exposure is associated with transient changes in receptor signaling, whereas chronic EtOH exposure leads to changes in receptor expression and localization, and intracellular signaling processes. Rapid tolerance lies somewhere in between, and its relationship with the other 2 forms of tolerance is uncertain (Lovinger and Roberto 2013). Although we have shown that acute tolerance develops in vitro (Prosser and Glass 2009) determining whether it occurs in vivo is technically difficult. Rapid tolerance, we show here, occurs both in vitro and in vivo with respect to EtOH modulation of the SCN circadian clock. Interestingly, our previous research indicates that chronic tolerance may not develop in vivo (Brager et al 2010). However, results from another study suggest the lack of chronic tolerance seen in our experiments may be due to using no-choice EtOH versus free-choice EtOH drinking (Seggio et al 2009). In light of reports suggesting that developing rapid tolerance correlates with the propensity to develop chronic tolerance (Khanna et al 1991; Radcliffe et al 2006; Wu et al 2001), the possible lack of chronic tolerance in vivo is surprising. There could be compensatory

changes outside the SCN that counteract a decrease in circadian clock sensitivity to chronic EtOH, or the SCN circadian clock could somehow be immune to chronic EtOH tolerance. We should be able to distinguish between these possibilities in our in vitro preparation by investigating whether chronic tolerance is seen in SCN brain slices. It will also be interesting to investigate potential differences in cellular changes depending on the mode of EtOH administration.

Significance

Rapid tolerance to EtOH has been linked to the development of chronic tolerance, a critical step toward alcohol dependency and abuse (Khanna et al 1991; Radcliffe et al 2006; Wu et al 2001). Therefore, determining the physiological and molecular characteristics that differentiate the forms of EtOH tolerance should help elucidate the processes that lead to alcohol abuse, and possibly identify treatments that could prevent that progression. Our demonstration that rapid tolerance is seen with the SCN circadian system at the cellular and behavioral levels through the use of complementary in vivo and in vitro experimental paradigms further proves its usefulness for investigating the direct connections between cellular actions of EtOH and its behavioral manifestations.

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Appendix B

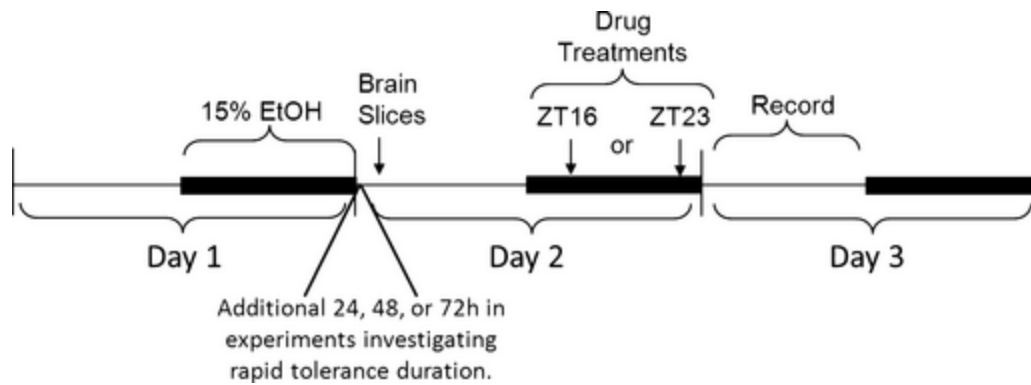
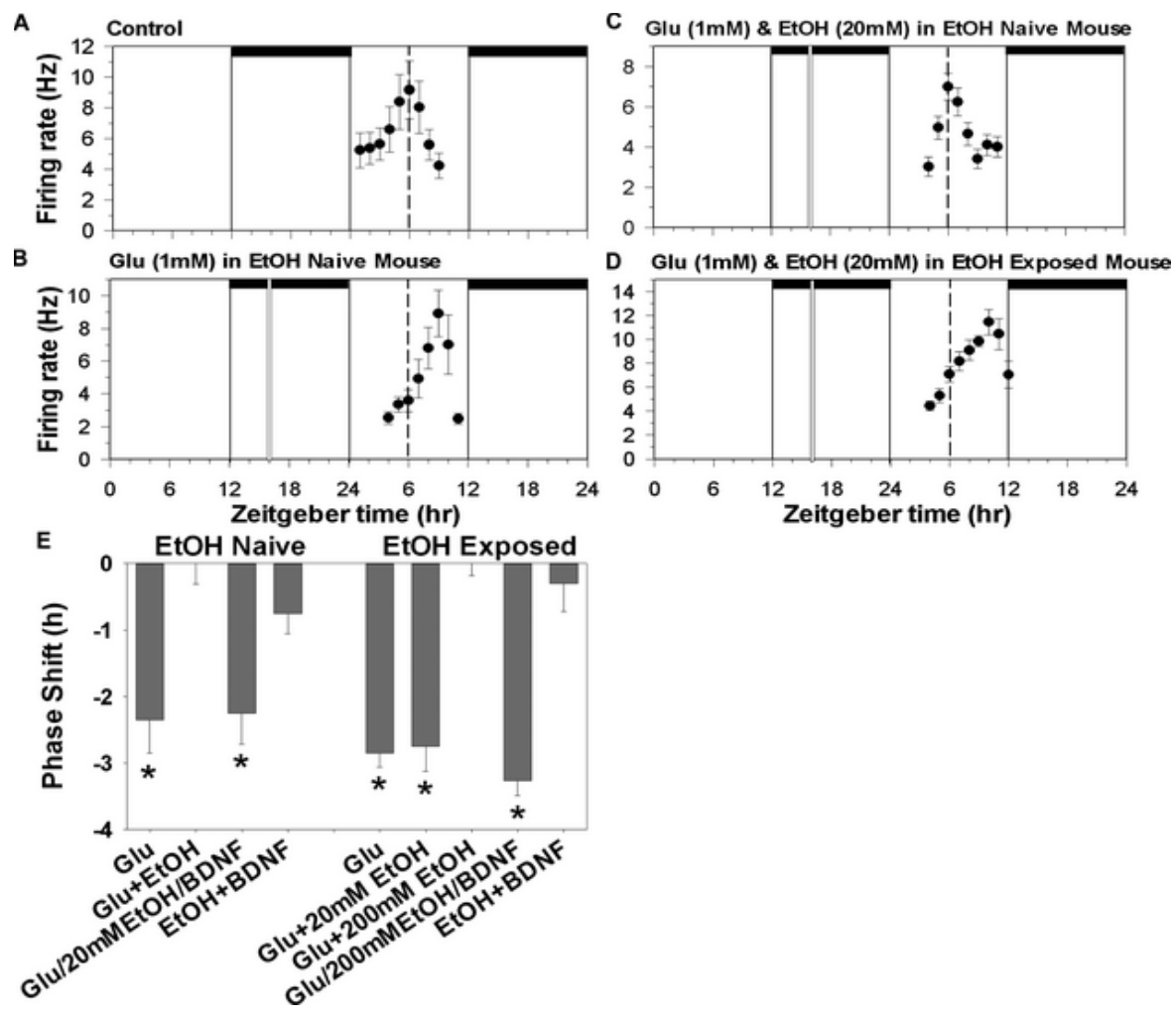


Figure 2.1 In vitro rapid tolerance experimental protocol. On Day 1, mice are allowed overnight access to 15% ethanol (EtOH). At lights-on of Day 2, mice are sacrificed and suprachiasmatic nucleus (SCN) brain slices are prepared. At Zeitgeber Time (ZT) 16 or ZT 23, brain slices receive drug treatment. After 10 minutes, drug treatment is removed, and slices are left undisturbed until the next day. On Day 3, electrophysiological recordings of SCN neuronal activity are conducted. For tolerance duration experiments, an additional 1 to 3 days of water-only access were added to the protocol between the single night of EtOH access and when brain slices were prepared.

Figure 2.2 Rapid tolerance to ethanol (EtOH) inhibition of glutamate-induced phase delays. Shown are the 2-hour mean $\pm$ SEM of suprachiasmatic nucleus neuronal activity obtained in individual experiments. **(A)** Control experiment with no drug treatment showing normal time of peak activity at Zeitgeber Time 6 (ZT 6). **(B)** A 10-min bath application of glutamate (1 mM) at ZT 16 to brain slices prepared from an EtOH-naïve mouse induces a 3-hour phase delay. **(C)** Treating brain slices from an EtOH-naïve mouse with 1 mM glutamate + 20 mM EtOH leads to no shift, indicating that the glutamate-induced phase delay is inhibited. **(D)** The 20 mM EtOH does not block glutamate-induced phase delays in brain slices prepared from an EtOH-exposed mouse. 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. **(E)** Shown are the mean ($\pm$ SEM) phase shifts induced in experiments involving various combinations of glutamate, EtOH, and brain-derived neurotrophic factor (BDNF) bath applied at ZT 16. EtOH inhibits glutamate-induced phase delays in brain slices from EtOH naïve mice, and this effect is reversed by co-application of 100 ng/ml BDNF. In brain slices from EtOH-exposed mice 20 mM EtOH does not block glutamate-induced phase delays, while 200 mM EtOH completely blocks glutamate-induced phase delays. Again, this inhibition by EtOH is reversed by co-application of 100 ng/ml BDNF. Co-treatment with BDNF and EtOH alone had no effect on brain slices from either EtOH naïve or EtOH-exposed mice. One-way ANOVA indicated a significant effect of treatment, $F(29) = 14.4$. *Post hoc (Holm-Sidak) test indicates $p < 0.001$ versus control experiments.



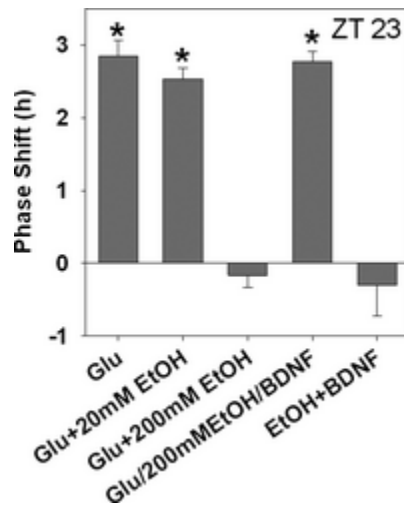


Figure 2.3 Rapid tolerance to ethanol (EtOH) inhibition of glutamate-induced phase advances. Shown is a summary of mean ($\pm$ SEM) phase-shifts induced in experiments involving various combinations of glutamate, EtOH and brain-derived neurotrophic factor (BDNF) bath applied at Zeitgeber Time 23 (ZT 23) in tissue from mice exposed to EtOH 24 hours prior to treatment. EtOH 20 mM does not inhibit glutamate-induced phase advances. However, raising the concentration to 200 mM EtOH inhibits glutamate-induced phase advances. This effect is reversed by co-application of 100 ng/ml BDNF.

* $p < 0.001$ vs. controls, post hoc (Holm-Sidak) test.

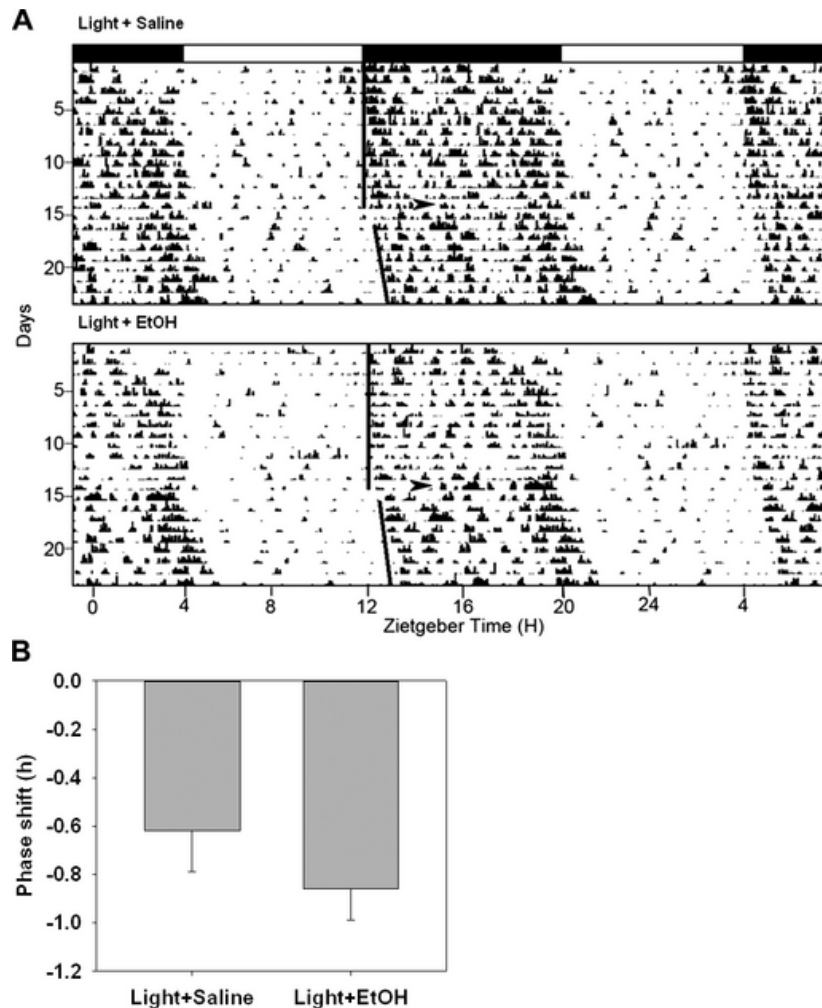


Figure 2.4 Rapid tolerance to inhibition of photic phase shifts in vivo. **(A)** Representative double-plotted actograms of general locomotor activity showing the effects of systemic injection of saline (top) and 2 g/kg ethanol (EtOH) (bottom). Injection was performed immediately prior to a 30 minute light pulse at ZT 16. Black arrows represent time of drug administration and light pulse. **(B)** Shown is the mean phase shift induced by a 30-minute light pulse at ZT 16 with systemic injection of either saline or 2 g/kg EtOH prior to light pulse in mice given access to EtOH the previous night. Bars are the mean $\pm$ SE. Phase delays for saline (0.62 ± 0.17 hours, $n = 7$) and EtOH (0.86 ± 0.13 hours, $n = 7$) are not significantly different ($p = 0.29$).

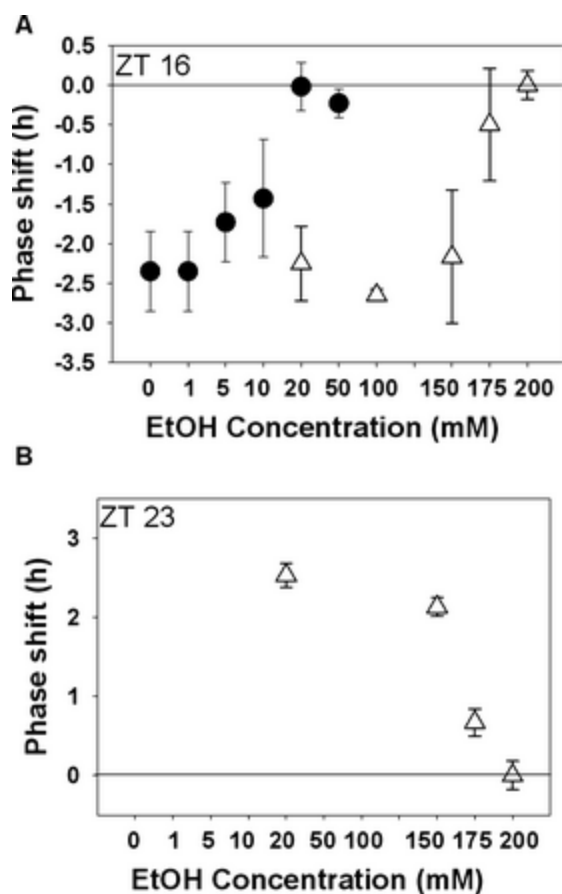


Figure 2.5 (A) Dose–response curves for ethanol (EtOH) inhibition of glutamate-induced phase delays in vitro in brain slices prepared from EtOH–naïve (black dots) and EtOH-exposed (white triangles) mice. (Data from EtOH-naïve mice replotted from Prosser et al. [1] for the ease of comparison.) (B) Dose–response curves for EtOH inhibition of glutamate-induced phase advances in vitro in brain slices prepared from EtOH-exposed mice. In both cases, the glutamate-induced phase shifts in brain slices from EtOH-exposed mice are completely inhibited by 200 mM EtOH, with an ED_{50} between 150 and 175 mM.

Figure 2.6 Rapid tolerance duration for ethanol (EtOH) inhibition of glutamate-induced with phase delays and phase advances. **(A)** Shown are the mean ($\pm$ SEM) phase shifts induced by co-application of 1 mM glutamate and 20 mM EtOH to brain slices prepared from mice that consumed 15% EtOH either 24, 48, or 72 hours prior to drug treatment at Zeitgeber Time 16 (ZT 16). Glutamate-induced phase delays are insensitive to EtOH inhibition at 24 and 48 hours after EtOH consumption but are completely blocked by EtOH 72 hours after EtOH consumption. **(B)** Shown are the mean ($\pm$ SEM) phase shifts induced by co-application of 1 mM glutamate and 20 mM EtOH to brain slices prepared from mice that consumed 15% EtOH either 24, 48, 72, or 96 hours prior to drug treatment at ZT 23. Glutamate-induced phase advances are insensitive to EtOH inhibition at 24, 48, and 72 hours after EtOH consumption but are completely blocked by EtOH 96 hours after EtOH consumption.

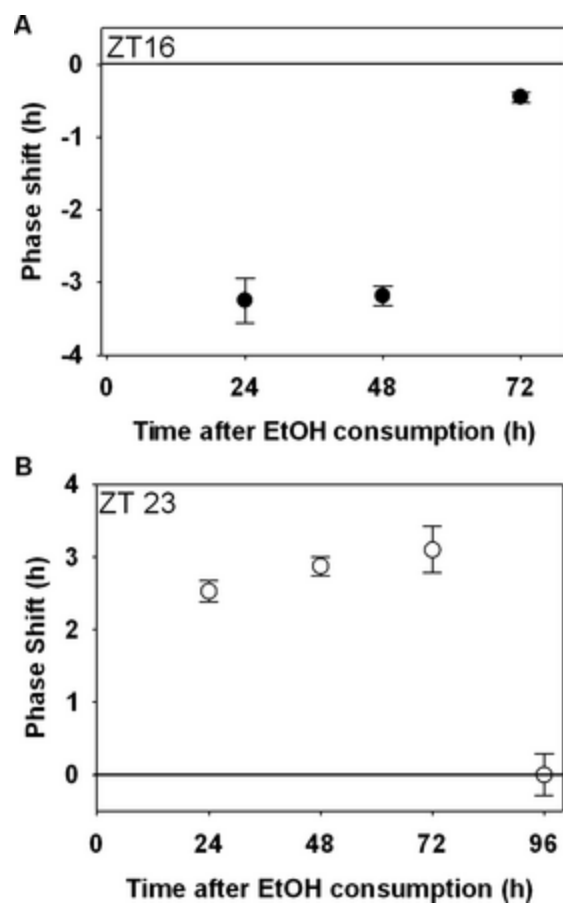
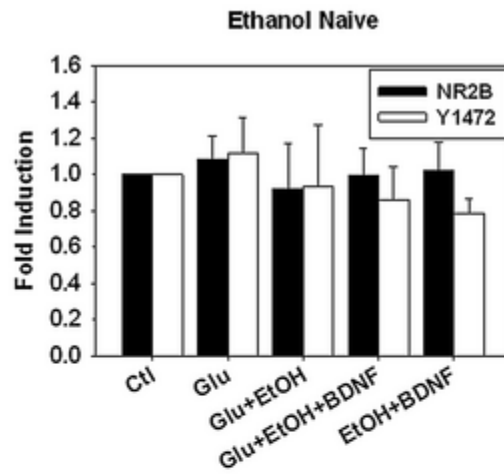
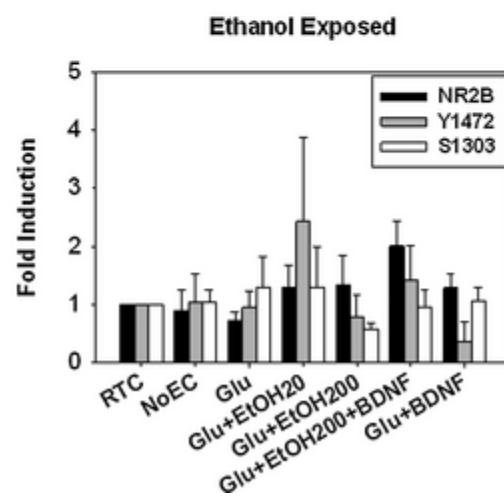


Figure 2.7 The effects of glutamate, ethanol (EtOH), and brain-derived neurotrophic factor (BDNF) application on N-methyl-D-aspartate receptor subunit NR2B and NR2B phosphorylation levels in EtOH-naïve and EtOH-exposed slices. **(A)** NR2B subunit expression and Y1472 residue phosphorylation levels in suprachiasmatic nucleus (SCN) brain slices prepared from EtOH-naïve mice. The treatments are as follows: no-treatment control (Ctl); 1 mM glutamate (Glu); 1 mM glutamate and 20 mM EtOH (Glu+EtOH); 1 mM glutamate, 20 mM EtOH, and 100 ng/mL BDNF (Glu+EtOH+BDNF); 20 mM EtOH and 100 ng/mL BDNF (EtOH+BDNF). Each group represents 4 independent replicates. **(B)** NR2B subunit expression, Y1472 residue phosphorylation, and S1303 residue phosphorylation in SCN brain slices prepared from EtOH-exposed mice. The treatments for EtOH-exposed slices are as follows: rapid tolerance with no treatment (RTC); EtOH-naïve with no treatment (NoEC); 1 mM glutamate (Glu); 1 mM glutamate and 20 mM EtOH (Glu+EtOH20); 1 mM glutamate and 200 mM EtOH (Glu+EtOH200); 1 mM glutamate, 200 mM EtOH, and 100 ng/mL BDNF (Glu+EtOH200+BDNF); 1 mM glutamate and 100 ng/mL BDNF (Glu+BDNF). Each group represents 2 to 3 independent replicates.

A



B



Chapter 3

Changes in NMDA Receptor and NMDA Receptor Signaling-Associated Characteristics During Ethanol Tolerance and Withdrawal in the Suprachiasmatic Nucleus.

Abstract

Alcohol tolerance and withdrawal induced effects are criteria for alcohol use disorders listed by the DSM-V. Although tolerance and withdrawal have been studied extensively over many decades, there is still uncertainty regarding the mechanistic distinctions that characterize the different forms of tolerance. Gaps in the existing literature include a lack of research on the cellular mechanisms underlying each form of EtOH-induced change, inconsistencies in the experimental procedures investigating a specific region across multiple studies, and not having demonstrated multiple tolerance forms in a single brain region. The suprachiasmatic nucleus (SCN) is the primary clock oscillator that regulates the timing of mammalian biological rhythms. Previous work has demonstrated that ethanol (EtOH) blocks photic phase shifts. Interestingly, both acute and rapid tolerance have been shown to develop in the SCN, with respect to EtOH inhibition of glutamate-induced phase shifts. Here, we demonstrate using our in vitro analysis that chronic tolerance and withdrawal induced hypersensitization to glutamate also occur. For our drinking model investigating chronic tolerance, we allowed mice to consume EtOH for 4 h each day, with access to 15% EtOH starting one hour prior to lights out through three hours after lights out. Tolerance developed after 10 days of 4 h EtOH access, as indicated by a decreased sensitivity to EtOH inhibition of glutamate-induced phase shifts. For withdrawal, we used the 4 hour access model for 21 days followed by two days of withdrawal. We observed an increased sensitivity to glutamate-induced phase shifts in SCN tissue following withdrawal, consistent with withdrawal induced hyperexcitability. Additionally, we used Western blotting to compare changes in NMDA receptors and associated proteins across multiple forms of EtOH plasticity, including rapid

tolerance, chronic tolerance, and EtOH withdrawal. We demonstrated an increase in NR2B/NR2A total expression ratios after 21 days of drinking and after EtOH withdrawal, and an increase in phosphorylation of Y1472 during withdrawal. Furthermore, we also detected an increase in mature BDNF and p-TrkB during withdrawal. These results demonstrate that multiple tolerance forms and withdrawal induced hypersensitization occur in the SCN; that chronic drinking and EtOH withdrawal change the subunit composition of NMDA receptors in the SCN; and that increases in mBDNF, TrkB phosphorylation, and Y1472 phosphorylation occur during EtOH withdrawal. Importantly, this study further demonstrates the power of using the SCN as a model system to investigate EtOH induced plasticity.

Introduction

Alcohol use disorder (AUD) is a condition that develops after long periods of ethanol (EtOH) consumption. Many studies have investigated the cellular processes associated with AUD. However, a major complication in AUD research is that the effects of EtOH change as one progresses from short-term to long-term drinking. The acute effects of EtOH include inhibition of glutamate signaling and enhancement of GABA signaling (Roberto et al 2004; Proctor et al 2006; Moykkynen and Korpi 2012; Hughes et al 2013). As EtOH exposure increases, changes in synaptic localization, total expression, and phosphorylation of receptor proteins occur (Hendricson et al 2004; Roberto et al 2004; Clapp et al 2010; Wu et al 2010). These effects are seen throughout the brain, although the specific changes appear to differ regionally. Complicating the picture is that varying EtOH exposure paradigms have been used to investigate changes in different

brain areas, including in 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 the development of tolerance, where increasing amounts of alcohol are consumed to achieve the same effects. Three major forms of EtOH tolerance have been described that differ based on time after initial exposure – acute tolerance (develops 5-15 minutes after drinking), rapid tolerance (develops 8-24 hours after drinking), and chronic tolerance (occurs after days/weeks of ethanol exposure). For example, acute tolerance has been measured 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 circadian activity rhythms and loss of righting reflex (Rosenwasser et al 2014; 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, glutamatergic and γ -aminobutyric acid (GABA) signaling mechanisms adapt to increase and decrease 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 2006; Werner et al 2009). In two separate studies, applying α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptor and NMDA receptor antagonists block rapid tolerance associated with motor coordination and hypothermic effects of ethanol (Rial et al 2009; Khanna et al

1997). Finally, changes in total expression and phosphorylation of key residues of NMDA receptor and GABA receptor subunits have been demonstrated during chronic EtOH tolerance (Wu et al 2010; Kroener et al, 2012; Honse et al 2003; Suvarna et al 2005; Wang et al 2011; Ravindran et al 2007, Liang et al 2007, Gonzalez et al 2012, Lindemeyer et al 2014). Ethanol withdrawal following chronic exposure is typically characterized by insomnia, psychomotor agitation, hallucination, and delirium tremens (Schuckit 2014). At the cellular level, withdrawal induces dramatic changes in glutamate and GABA signaling (Clapp et al 2010; Reynolds et al 2015; Kumar et al 2009; Carlson et al 2016).

In summary, the cellular mechanisms associated with acute, rapid, and chronic EtOH tolerance, and EtOH withdrawal appear to differ (Grobin et al 1998; Radcliffe et al 2006; Roberto et al 2006; Ward et al 2009; Clapp et al 2010; Lindsay et al 2014; Tran et al 2015). However, rarely have the cellular mechanisms underlying each of these forms of EtOH-induced plasticity been assessed in a single study or in a single brain region. Thus our goal was to assess 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 circadian clock in the SCN is the primary endogenous oscillator that regulates 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. Light-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 circadian clock 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; Seggio et al 2007; 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 these experiments. 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.

Materials and methods

Mice

Adult (~8 week old) C57BL/6 adult male mice were obtained from Harlan Laboratories (Frederick, MD). 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 (Medical Associates, Inc., St. Albans, VT).

Brain slice preparation, and drinking schedules

A rapid tolerance drinking schedule 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, 500 μ m 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.

To investigate chronic tolerance, a 4h/day drinking protocol was used. 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 exact experiment. At lights-on after the last day of drinking, coronal brain slices containing the SCN were prepared and maintained as described above.

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 were 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 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 and biotinylation assays did not receive drug treatments but rather were collected at Zeitgeber time (ZT) 6, 16, or 23 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 2003; Prosser et al 2008; Prosser and Glass 2009). Each cell was recorded for 5 minutes, with at least 5 cells recorded per hour over the course of a ten-hour period. Recordings and firing rate analysis were done using DataWave software (Loveland, CO). 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% milk in 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 Kindgdom), 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 goat α -Rabbit (Vector Laboratories, Burlingame, CA), goat α -Mouse (Vector Laboratories), donkey α -Rabbit (LI-COR, Lincoln, NE), or donkey α -Goat (LI-COR) conjugated to horseradish peroxidase or infrared dye (LI-COR). Blots were allowed to incubate for an hour and a half with secondary antibodies. Protein bands were detected on Green x-ray film (Thermo Scientific) in experiments using chemiluminescence or using the Imagestudio program in experiments using fluorescent dye.

Cell surface biotinylation

SCN brain slices removed from the perfusion chamber at ZT 6, 16, or 23 were placed in EBSS containing 1mg/mL NHS-SS-biotin (Thermo Scientific) for 75 min on ice. The reaction was halted by addition of 1mM glycine. Slices were then rinsed with ice cold EBSS and stored at -80°C until protein extraction. The extracted protein samples were run through desalting columns in a centrifuge for 2 min at 4°C. Neutravidin ultralink resin (200mL; Thermo Scientific) was added to samples and incubated overnight at 4°C. The next day the avidin resin was separated from the flow-through via centrifugation and collecting the supernatant (flow-through). Flow-through was kept for control purposes. The avidin resin was then rinsed three times with lysis buffer. Sample and flow-through were boiled for 5 minutes at 100°C. Samples and flow-through were loaded into polyacrylamide gels. Protein samples were then transferred to PVDF after separation of proteins on the gel, and Western blot assays were performed as described above.

Statistical methods

All statistical analysis was performed using SigmaPlot. Individual mice were experimental units for electrophysiological recordings. Tissue from a single mouse (or pooled samples when necessary) were the experimental unit for Western blots and biotinylation experiments. Post-hoc Holm-Sidak analysis using t-test or ANOVA was used between two 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 4 h, we did not measure blood alcohol levels in the mice.

Chronic tolerance to EtOH occurs in the SCN

In brain slices prepared from EtOH naïve mice, firing rates normally peak at ZT6 (Figure 3.1A). 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). Glutamate applied at ZT16 to SCN slices from EtOH naïve mice induces a phase delay of ~3 hours (Figure 3.1B), which is inhibited by co-application of 20 mM EtOH (Figure 3.1C,).

Brain slices prepared from mice that had undergone 4hr EtOH daily consumption for 10 days exhibited no difference in the time of peak neuronal activity compared to brain slices prepared from EtOH naïve mice (difference in time-of-peak vs. tissue from EtOH-naïve mice = -0.77 ± 0.37 n=3, Figure 3.1 E). Likewise, applying 1 mM glutamate alone at ZT16 to brain slices from these mice induced a phase delay of ~3 hours (Figure 3.1E), consistent with the effects seen in brain slices from EtOH naïve mice. However, as seen in Figure 3.1D, co-applying 20mM EtOH and 1mM glutamate to brain slices prepared from these mice did not inhibit the glutamate-induced phase shifts. However, increasing the concentration of EtOH co-applied with 1mM glutamate to 200 mM completely blocked the

glutamate-induced phase shifts in brain slices prepared from the 10-day EtOH drinking mice (-0.42 ± 0.30 , $n=3$ Figure 3.1E). Thus 4 hr daily consumption of EtOH for 10 days decreased EtOH inhibition of in vitro glutamate-induced phase resetting, 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 (150 mM) to inhibit glutamate-induced phase shifts. As seen in Figure 3.1E 150 mM EtOH was also unable to inhibit glutamate-induced phase shifts in brain slices prepared from these mice.

Next we investigated how many days of EtOH drinking are needed to induce chronic tolerance. As shown in Figure 3.1F 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. Thus, at least 8 days of drinking EtOH is needed to generate chronic tolerance as assessed under these conditions.

Sensitization to glutamate develops in the SCN during 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. There was an abrupt shift from no phase-shifting response with 1uM glutamate to a maximum ~ 3 h phase delay with 10 uM glutamate (Figure 3.2A). 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 nearly 3 h (2.42 ± 0.36 hrs, $n=3$) (Figure

3.2A). A similar phase-shift was induced by 10 μ M glutamate applied to SCN tissue under these conditions, indicating that EtOH withdrawal leads to a change in dose responsiveness 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 ethanol consumption without subsequent EtOH withdrawal. As summarized in Figure 3.2A, not only was 1 μ M glutamate unable to induce phase shifts in these brain slices, but a higher (100 μ M) concentration of glut was required to induce a phase delay. Thus, prior to EtOH withdrawal, the SCN clock appears to be slightly less sensitive to glutamate-induced phase shifts while 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 3.2B, withdrawal-induced glutamate hypersensitivity was seen after 17 or 21 days of chronic EtOH, but not after 10 or 14 days. Additionally, we clarified the duration of glutamate hypersensitivity after withdrawal. Withdrawal induced glutamate hypersensitivity still occurs after 4 days but not after 6 days of EtOH withdrawal (Figure 3.2C). Thus the duration of withdrawal associated hypersensitivity lasts between 4-6 days after sudden EtOH abstinence.

EtOH-induced changes in NR2A:NR2B ratio, NR2B phosphorylation, and upstream/downstream NMDA regulators

Previously, we determined that total expression of the NMDA receptor subtype 2 (NR2B) subunit and phosphorylation of the tyrosine 1472 (Y1472) residue of the NR2B subunit do not change 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. A summary of the proteins we assessed and our results is provided in Table 3.1. We performed Western blot assays on SCN tissue from EtOH naïve mice, and from mice receiving 1 night 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 3.3 A and B, no significant changes were observed with respect to total NR2A or NR2B subunit expression in SCN tissue across any of the 5 conditions. However, the ratio of NR2B/NR2A expression was significantly higher after 21 days of drinking with or without 2 days of withdrawal when compared to SCN tissue from naïve mice (21 days: 3.67 ± 0.84 $n=7$, $t_4=3.50$ $p=0.015$; withdrawal 3.45 ± 0.57 $n=7$, $t_4=3.232$, $p=0.027$; Figure 3.3C). We also tested total expression of the NR1 subunit, however no changes were noted (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; $F_4=0.552$, $n=4$ for all conditions; data not shown).

Next we investigated changes in NR2B subunit phosphorylation. Relative to EtOH naïve SCN tissue, we found a significant increase compared to control and overnight conditions in phosphorylation of NR2B Y1472 in SCN tissue from mice that had experienced 21 days of chronic drinking followed by 2 days of withdrawal, but no change in other groups (withdrawal: 4.98 ± 1.53 $n=8$, vs control $t_4=3.23$, $p=0.030$; vs overnight

1.02 $\pm$ 0.33 n=8, $t_4=3.21$ p=0.028 Figure 3.4A). We also tested phosphorylation at NR2B S1480, however no changes were noted (1 $\pm$ 0.00 control, 1.56 $\pm$ 0.39 overnight, 1.07 $\pm$ 0.23 10 day, 1.14 $\pm$ 0.38 21 day, 1.03 $\pm$ 0.33 withdrawal; $F_4=0.570$, n=4 for all conditions data not shown).

Phosphorylation of NMDA receptors has been linked to activation of TrkB receptors. More specifically, TrkB phosphorylates and activates Fyn kinase (Yamada and Nabeshima 2004; Carreño et al 2011), which leads to increased activation of NMDA receptors via Fyn-induced phosphorylation of the NR2B subunits (Nakazawa et al 2001; Goebel et al 2005). Binding of mature brain-derived neurotrophic factor (mBDNF) activates TrkB, leading to phosphorylation of many residues including Y1472 of the NMDA receptor (Mizuno et al 2003). To investigate potential upstream mechanisms leading to changes in phosphorylation of NR2B Y1472 during tolerance and withdrawal, we investigated changes in pro BDNF and mBDNF expression, truncated TrkB and full length TrkB expression, and TrkB phosphorylation in SCN tissue. We found a significant increase in mBDNF total expression during withdrawal compared to EtOH naïve controls (withdrawal 2.07 $\pm$ 0.34, n=6, $t_4=3.104$, p=0.046; Figure 3.4c). Furthermore an increase in pTrkB at Tyr 706 occurred during withdrawal compared to all other conditions (withdrawal 2.55 $\pm$ 0.50, n=4; vs control $t_4=3.401$, p=0.039; vs overnight 1.13 $\pm$ 0.168 $t_4=3.114$, p=0.049; vs 10 day $t_4=3.312$, p=0.042; $t_4=3.231$, p=0.044 vs 21 days; Figure 3.4d). No significant changes were noted for expression of pro BDNF (1 $\pm$ 0.00 control, 1.04 $\pm$ 0.16 overnight, 1.14 $\pm$ 0.16 10 day, 1.10 $\pm$ 0.17 21 day, 1.03 $\pm$ 0.10 withdrawal; $F_4=0.185$, p=0.94, n=6 for all conditions, data not shown), truncated TrkB (1 $\pm$ 0.00 control, 0.95 $\pm$ 0.07 overnight, 0.80 $\pm$ 0.24 10 day, 0.84 $\pm$ 0.24 21 day, 0.95 $\pm$ 0.22

withdrawal; $F_4=0.211$, $p=0.93$, $n=4$ for all conditions data not shown), nor full length TrkB (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; $F_4=0.640$, $p=0.64$, $n=4$ for all conditions, 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; Trepanier et al 2012; Gibb et al 2011). However, we found no significant differences in PSD-95 (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; $F_4=1.398$, $p=0.26$, $n=7$ for all conditions, data not shown) and Fyn (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; $F_4=1.432$, $p=0.25$, $n=6$ for all conditions, data not shown) across all experimental conditions.

Increases in phosphorylation of Y1472 are linked to increases in synaptic localization of NR2B containing NMDA receptor (Nakazawa et al 2001; Goebel et al 2005). To test if increases in surface localization of NR2B subunits occur during tolerance and withdrawal, we used cell surface biotinylation. Although an increase in Y1472 phosphorylation was noted during withdrawal treatments, we observed no changes in surface expression of NR2B subunits across all conditions (1 ± 0.00 control, 1.24 ± 0.29 overnight, 1.28 ± 0.27 10 day, 1.32 ± 0.30 21 day, 1.48 ± 0.30 withdrawal, $F_4=1.069$ $n=6$, $p=0.39$; data not shown).

Discussion

Time course of chronic tolerance and withdrawal

Previously we demonstrated the SCN develops rapid tolerance after mice were 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 circadian periodicity and phase resetting (Prosser et al 2008; Prosser and Glass 2009; Rosenwasser et al 2005; Seggio et al 2007; Seggio et al 2009; Brager et al 2010), 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 to EtOH and withdrawal-induced glutamate hypersensitivity in the SCN. Using the 4-h/day drinking paradigm described here, we find that mice need to consume EtOH for at least 8 days to develop

chronic tolerance. This is consistent with results from other brain regions (Broadwater et al 2011; Matson et al 2014). In contrast, mice need more than 14 days of EtOH consumption to develop withdrawal-induced glutamate hypersensitivity in our experiments. Previous research suggests that the duration of drinking necessary to induce withdrawal hypersensitivity varies across dose and the way EtOH is delivered. For instance withdrawal induced convulsions can develop after as few as four days of EtOH vapor inhalation (Olive and Becker 2008) or after 1 – 2 weeks of drinking in rodents (Roberto et al 2004; Wang et al 2010), with the latter being consistent with the timing of withdrawal induced glutamate sensitization seen here. Importantly, we found that chronic EtOH drinking without withdrawal does not cause glutamate hypersensitization. Together, the differences seen between these drinking paradigms support the hypothesis that distinct cellular adaptations underlie the different forms of EtOH tolerance and withdrawal-induced hypersensitization to glutamate.

To our knowledge, these are the first data carefully delineating these differences in EtOH plasticity. It will be interesting to extend these findings to other brain regions and other EtOH exposure modalities. Doing so will help determine whether the distinctions shown here are unique to the SCN and these drinking paradigms, or if they are more universally applicable. Because both EtOH tolerance and withdrawal can lead to increases in EtOH consumption (Schuckit 1994; Brown et al 1998; Bell et al 2001; Ciccocioppo et al 2003; Das et al 2016), a better understanding of the distinct dynamic cellular processes underlying each of these conditions may improve treatments to alleviate or prevent development of alcohol dependence. Importantly, these data illustrate that investigating multiple EtOH exposure paradigms in a single experiment can provide

a more complete picture of EtOH-induced changes than using a single drinking model. The data from this study also show the power of using the SCN circadian clock to investigate nuanced differences in EtOH effects in the brain.

Prior results indicated that under a no-choice EtOH drinking protocol, the SCN circadian clock in mice does not exhibit chronic tolerance with respect to EtOH inhibition of photic phase shifts *in vivo* in hamsters and mice (Ruby et al 2009; Brager et al 2010). Our data here showing chronic tolerance as assessed *in vitro* raise interesting questions regarding the underlying differences. In Brager et al 2010, mice consumed substantially 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. In addition evidence suggests in an *in vivo* study that 21 days of voluntary EtOH consumption induces no change to phase shift conditions (Seggio et al 2009) as opposed to 21 day forced EtOH which inhibits phase shifts (Seggio et al 2009; also as in Ruby et al 2009 and Brager et al 2010). Seggio et al suggested the phase shifting mechanism is insensitive to chronic voluntary EtOH but is sensitive to chronic forced EtOH. Another study by Rosenwasser et al 2014 demonstrated reduced effects of EtOH on circadian period after sustained high EtOH intake which would be consistent with the development of chronic tolerance, but this effect seems to differ by sex and strain. Thus the specific EtOH consumption paradigm and model used is an important factor to consider for studying chronic EtOH tolerance in the SCN.

Likewise, *in vivo* experiments investigating EtOH withdrawal induced changes in phase shifts have shown mixed results. For instance, enhanced photic phase shifts are seen during withdrawal in hamsters (Ruby et al, 2009), while EtOH 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 in mice (Logan et al 2012). With respect to our results, we showed that withdrawal induced hypersensitization to glutamate occurs in the SCN in vitro. Because sensitivity to photic phase resetting was not assessed using a range of light intensities in the in vivo investigations mentioned above, future experiments assessing withdrawal induced hypersensitization to light will be informative.

Another important difference is that the previous studies investigated photic phase-resetting in vivo vs. the in vitro glutamate-induced phase resetting used in the present study. Because EtOH affects many 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 (Meyer-Bernstein and Morin 1996; Bradbury et al 1997; Meyer-Bernstein and Morin 1999). EtOH has been shown to modify 5-HT levels in rats, increasing 5-HT in the hypothalamus, amygdala, and hippocampus after a 4 week liquid diet (Ara and Bano 2015). In contrast, 5-HT levels decrease in rats after 6 hours of withdrawal following chronic liquid diets in the cerebral cortex and striatum in one study (Uzbay et al 1998), and the hypothalamus, amygdala, and hippocampus in another (Ara and Bano 2015). We have shown that acute EtOH modulates serotonergic signaling in the SCN, as seen by enhanced serotonergic phase shifts in vitro (Prosser et al 2008),

while acute EtOH inhibits serotonergic phase shifts in vivo, likely through extra-SCN actions (Ruby et al., 2009).

Another difference between the data shown here and the previous in vivo study is that hypersensitivity to photic phase resetting was not seen in vivo during withdrawal (Brager et al., 2010). Again this could be due to the methodological differences mentioned above, or to whether the SCN clock is being investigated in vivo vs. in vitro. As with chronic tolerance, it will be important to determine what specific difference(s) account for the differential observance of hypersensitivity. Because the development of chronic tolerance and withdrawal symptoms are thought to be critical steps toward alcohol dependence and abuse (Khanna et al, 1991; Radcliffe et al 2006; Wu et al 2010), it will be important to further explore the factors that determine whether chronic tolerance is seen in the SCN. Importantly, identifying factor(s) that protect the circadian clock from exhibiting chronic tolerance and withdrawal hypersensitivity in vivo could provide insight into treatments for those dependent on alcohol.

Hyperexcitation during withdrawal has been reported across many brain regions (see Burnett et al 2016). This effect of EtOH withdrawal was described by Finn and Crabbe, who suggested that the adaptations that developed in order to offset the effects of EtOH are left unopposed during withdrawal, thus leaving neurons in a hyperactive state (1997). Withdrawal induced hyperexcitability is thought to contribute to synaptic remodeling in the orbital frontal cortex (Badanich et al 2011). Evidence of withdrawal-induced hyperexcitation is also seen in the basal lateral amygdala (BLA), as indicated by shortened paired-pulse ratio intervals and increased medium excitatory post-synaptic current (mEPSC) amplitude and frequency occur during withdrawal (Lack et al 2007).

Furthermore, differences were noted in mEPSC amplitude and frequency in the BLA during chronic drinking versus 24 h of withdrawal (Lack et al 2007), similar to our electrophysiological results supporting physiological disparities in chronic EtOH vs EtOH withdrawal. Thus hypersensitivity to glutamate in the SCN during withdrawal is consistent with hyperexcitable induced states commonly found during EtOH withdrawal in other brain regions.

Unexpectedly, we observed that the SCN clock has a decreased sensitivity to glutamate after 21 days of chronic drinking but prior to EtOH withdrawal. This is somewhat paradoxical, given the glutamate hypersensitivity is seen during withdrawal. Decreased glutamate sensitivity is also not entirely consistent with the increase in NR2B:NR2A ratio and decrease in EtOH sensitivity we see with chronic EtOH (discussed below). We put forth several possible explanations: 1) AMPA receptors that partially mediate the phase shift mechanism become desensitized to glutamate and subsequently internalized during chronic EtOH. Some reports have shown that AMPA receptors are needed for phase shifts in the SCN (Takeuchi et al 1991; Mizoro et al 2010). Unlike NMDA receptors, AMPA receptors become desensitized and internalized after prolonged exposure to glutamate (Traynelis et al 2010; Alsaloum et al 2016). Due to chronic EtOH increasing the levels of extracellular glutamate, AMPA receptors could become internalized, leading to partial desensitization to glutamate during phase shifts as shown in Figure 3.2A. 2) Another explanation could be through cytochrome p450, which metabolizes EtOH to acetaldehyde. It is possible that chronic EtOH induced neurotoxicity via increases in cytochrome p450 2e1 expression (as seen in Zhong et al 2012) causes rapid internalization of NMDA receptors (as seen in Goto et al 2006) in the SCN. In support of

this, one report shows decreased NMDA receptor sensitivity to glutamate in the ventral tegmental area (VTA) after 8 weeks of chronic EtOH (Fitzgerald et al 2012). Yet these results are somewhat counterintuitive to our results showing increases in NR2B to NR2A ratios given that changes in these ratios promote glutamate sensitization. Perhaps this shift in the NR2B:NR2A ratio is indicative of a shift from synaptic NMDA receptors to extrasynaptic receptors, which is somewhat indicative of neurotoxicity (Hardingham and Bading 2010). (Figure 3.2A) and therefore may promote a partial glutamate desensitization in the SCN. 3) Increases in protein expression of glutamate transporters could occur during chronic EtOH in the SCN. This change would be somewhat specific to the SCN as other articles have reported mixed results. For instance CIE in the nucleus accumbens increases glutamate release without altering glutamate transport (Griffin et al 2015). In the nucleus accumbens and VTA of rats, decreases in protein expression of excitatory amino acid transporter-1 but no change in excitatory amino transporter-2 were reported during increased glutamate release due to chronic EtOH (Ding et al 2013). Additionally, chronic EtOH decreases glutamate transporter-1 levels in the PFC (Sari et al 2013). Increases in glutamate transporter expression during chronic EtOH could lead to clearance of lower levels of glutamate, leading to glutamate induced phase shift desensitization in our in vitro experiments.

As mentioned, many studies have shown increased levels of extracellular glutamate in specific brain regions during chronic EtOH exposure (Gass and Olive, 2008; Brage et al 2012; Ding et al 2012; Ding et al 2013; Griffin et al 2013). This is thought to be due to chronic EtOH inhibition of presynaptic metabotropic glutamate receptors, leading to an increase in glutamate release (Simonyi et al 2004). In contrast, recent

reports demonstrate that astrocytes regulate the amount and timing of extracellular glutamate in the SCN (Brancaccio et al 2017). If chronic EtOH alters the release and/or reuptake of glutamate in the SCN, it would be interesting to investigate whether this involves changes in astrocytic and/or neuronal activity.

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

Having determined the EtOH exposure paradigms needed to induce the various forms of EtOH-induced plasticity, we began to assess the cellular changes occurring with each condition. We focused on NMDA receptors because their activity is necessary for photic phase resetting in the SCN (Ding et al 1997; Mintz et al 1999; Wang et al 2008). We determined that there is a significant increase in the ratio of NR2B to NR2A subunit expression in the SCN after 21 days of drinking that is not seen with either rapid tolerance or 10 days of EtOH consumption. This increase in NR2B:NR2A expression is still seen 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. Changes in NR2B:NR2A ratios are thought to regulate the threshold for synaptic plasticity globally (Philpot et al 1999; Yashiro and Philpot 2008), and have been seen following EtOH exposure. An increase in NR2B:NR2A expression was seen in hippocampal slices 15 minutes after application of 100mM EtOH (Suvarna et al 2005), which would correspond to an acute tolerance paradigm. In contrast, decreased NR2B:NR2A expression is seen in rat hippocampal slices following 7 weeks of EtOH vapor (Staples et al 2015). Others have observed that changes in NMDA receptor subunit

composition in response to chronic EtOH are region specific (Nona et al 2014). With respect to the SCN, an increase in NR2B:NR2A expression is consistent with an enhanced propensity for glutamate-induced phase shifts because 1) NR2B subunits are necessary for glutamate-induced phase shifts (Wang et al 2008) and 2) NR2B-containing NMDA receptors have longer decay kinetics than NR2A-containing receptors (Cull-Candy and Leszkiewicz 2004). Whether this change in NR2B:NR2A expression is necessary for the withdrawal-induced glutamate hypersensitivity 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 be affecting NMDA receptor function. For example, chronic drinking increases membrane localization of NR2B subunits in many regions (Qiang et al 2007). Moreover, EtOH withdrawal has been shown to increase lateral movement of NR2B containing NMDA receptors to extra-synaptic zones (Clapp et al 2010; Wills et al 2012). Our surface biotinylation experiments did not show changes in NR2B surface expression during tolerance nor withdrawal. However we noticed a trend towards increased NR2B surface expression during withdrawal (data not shown), and haven't ruled out changes to surface expression of NR2B subunits during chronic EtOH and withdrawal. Given this, our NR2B results for biotinylation need further validation. In addition our experiments only investigated changes to NR2B subunits. Thus other subunits may show changes in surface expression during chronic EtOH and withdrawal. Also, although assessing surface expression of receptor proteins is a better indicator of potential activity than assessing total expression, it still does not discern synaptic vs extrasynaptic protein levels. Given that increases in

extrasynaptic NR2B are implicated in multiple neurological diseases (Hardingham and Bading 2010; Gladding and Raymond 2011), it would be worthwhile to investigate potential changes in NR2B membrane localization in the SCN in response to different EtOH exposure paradigms using synaptic fractionation.

Our results also demonstrated that the increase in the NR2B:NR2A ratio persists following 2 days of withdrawal from EtOH. Interestingly, withdrawal after chronic EtOH induces hyperexcitability of NMDA receptor related currents in the striatum that lasts up to 9 days after cessation of EtOH (Wang et al 2010). In contrast, no changes in NMDA receptor EPSCs were noted in the central amygdala after 6-8h of withdrawal following chronic EtOH vapor inhalation (Roberto et al 2004), but NMDA receptor-related EPSCs are increased in the BLA 24 hours after abstinence from chronic EtOH (Lack et al 2007). Thus the duration of withdrawal induced hyperexcitability of NMDA receptor currents, and the mechanisms underlying these changes may be region specific. To determine how withdrawal modifies NMDA receptor related signals in the SCN, future investigations assessing synaptic vs extrasynaptic localization of NR2B subunits should be a priority.

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, leading to synaptic recruitment of NMDA receptors (Nakazawa et al 2001; Goebel et al 2005). Consistent with this, we found that during EtOH withdrawal after chronic drinking there is a significant increase in phosphorylation of NR2B Y1472 in the SCN. Interestingly, this change is not seen prior to EtOH withdrawal, so it appears to be induced by the cessation of EtOH consumption.

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 saw no changes in total expression of NR2A and NR2B protein, consistent with our findings (Wang et al 2010, Wu et al 2010). Our results showing increased Y1472 phosphorylation in the SCN is consistent with the glutamate hypersensitivity we observed for the SCN circadian clock. Therefore, pharmacological inhibition of Y1472 phosphorylation during withdrawal may reduce glutamate hypersensitization in the SCN. As with the change in NR2B:NR2A ratio, it will be important to assess whether the change in Y1472 phosphorylation is necessary for the glutamate hypersensitivity we see during EtOH withdrawal.

Phosphorylation of Y1472 has also been shown to enhance membrane localization of NR2B-containing NMDA receptors. Phosphorylation of Y1472 maintains NR2B subunit interactions with the PDZ binding domain of PSD-95 (Roche et al 2001; Prybylowski et al 2005; Zhang et al 2008). This prevents disassociation and subsequent internalization of NMDA receptors via clathrin mediated endocytosis (Roche et al 2001; Chung et al 2004). Although we did not see any changes in PSD-95 expression, others have likewise shown increased Y1472 phosphorylation during withdrawal without a change in PSD-95 expression (Clapp et al 2010, Staples et al 2015). Yet there is evidence PSD-95 can play an important role in EtOH induced plasticity, as PSD-95 KO mice exhibit increased behavioral sensitivity to the sedative effects of EtOH (Camp et al 2011). Also, increased clustering of PSD-95 and colocalization of NR2B containing NMDA receptors occur during chronic EtOH (Chandler et al, 2006; Clapp et al 2010). With respect to our results,

colocalization assays may help confirm increases in PSD-95 interactions with NR2B during EtOH withdrawal.

Increases in mBDNF and TrkB phosphorylation occur during withdrawal

One mechanism through which Y1472 becomes phosphorylated is via TrkB signaling. Binding mBDNF to TrkB induces autophosphorylation of the receptor, allowing activation of downstream signals. The activation of TrkB is neuroprotective, supporting actions that promote neuron survivability (Chao 2003). Our results showed increases in expression of mBDNF and phosphorylation of TrkB during withdrawal. Regarding mBDNF, a previous study demonstrated increases in total expression after 3h of withdrawal to EtOH in the hippocampus and medial prefrontal cortex (Somkuwar et al 2016). Others have reported mixed results regarding EtOH and BDNF/TrkB interactions (e.g., decreased BDNF binding to TrkB in the hippocampus – Kolb et al 2005; increases in expression of BDNF, TrkB, and downstream kinases in the striatum – Logrip et al 2008). Some have suggested these changes are region specific (Kolb et al 2003; Logrip et al 2008; but see Davis 2008), and thus the increases in mBDNF shown here may be specific to the SCN. Regarding phosphorylation of TrkB during withdrawal, a study assessing fetal alcohol syndrome in juvenile mice reported decreases in TrkB phosphorylation (Nona et al 2013). Another study investigating EtOH withdrawal in the rat amygdala saw decreases in TrkB expression, as well as decreased activation of kinases downstream of TrkB signals (Pandey et al 2008). Therefore, increased phosphorylation of TrkB may also be specific to the SCN. These results further elucidate the mechanism associated with increases in Y1472 of NR2B containing NMDA receptors during EtOH withdrawal in the SCN.

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. For example, other receptors may contribute to the effects of EtOH in the SCN. EtOH modifies many glutamate receptors, including α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and kainate receptors (Lack et al 2008; Lack et al 2009; Karpyak et al 2012; Corbit et al 2014; Jin et al 2014). Beckley et al demonstrated AMPA receptor total expression in the nucleus accumbens increases in response to a single 4-hour binge exposure to EtOH (2015). Additionally, EtOH enhances signaling by GABA receptors, particularly the ionotropic GABA(A) receptors, and chronic EtOH exposure alters GABA subunit composition (Ravindran et al 2007; Liang et al 2007; McElroy et al 2009; Gonzalez et al 2012; Lindemeyer et al 2014). We have previously shown that at least some effects of EtOH in the SCN involve activation of δ -subunit containing GABA(A) receptors (McElroy et al 2008). It would also be worthwhile to investigate increases in phosphorylation of Fyn, which is down-stream of mBDNF induced TrkB activation (Yamada and Nabeshima 2004; Carreño et al 2011). We have demonstrated previously that BDNF prevents EtOH inhibition of glutamate-induced phase shifts in EtOH naïve SCN tissue (Lindsay et al 2014) as well as after induction of acute and rapid tolerance (Prosser and Glass 2009; Lindsay et al 2014). It would be worth investigating the mechanisms through which BDNF, TrkB and EtOH interact to modulate glutamate-induced phase shifts. Collectively, investigating AMPA receptors, GABA(A) receptors, and BDNF/TrkB interactions during tolerance and withdrawal in future experiments would likely lead to a greater

understanding of the mechanisms associated with these EtOH induced phenomena in the SCN.

Mechanisms upstream or downstream of NMDA receptors could also contribute to compensatory mechanisms occurring in response to EtOH. For example, chronic EtOH modifies the function of intracellular modulators such as Fyn, glycogen synthase kinase 3 beta (GSK3 β), and STEP-61. Fyn phosphorylates the Y1472 residue and chronic EtOH promotes Fyn activation (Trepanier et al 2012; Gibb et al 2011). Though we saw no changes in total Fyn expression, it is possible that phosphorylation of Fyn, which activates the kinase, may be modulated by EtOH in the SCN. Chronic EtOH increases GSK3 β activity in the cerebellum, dorsal striatum, and neuronal stem cells (Xu et al 2006; Vangipuram and Lyman 2012, Li et al 2013). Chronic EtOH also blocks STEP-61 dephosphorylation of Y1472 of the NR2B subunit in the striatum during chronic EtOH (Darcq et al 2014), but modulation of STEP-61 by EtOH is not seen in all brain regions (Darcq et al 2014). Pertaining to circadian rhythms, Fyn and GSK3 β either regulate the timing of circadian periods or have a role in the photic phase shift mechanism in the SCN (Shima et al 2000; Červená et al 2015). The role of STEP-61 has yet to be investigated in the SCN. Because EtOH modifies the actions of extracellular glutamate and second messengers downstream of NMDA receptors, future investigations will follow up on how extracellular glutamate as well as activation and expression of these proteins change during tolerance and withdrawal in the SCN.

Conclusions

To gain a better understanding of how EtOH addiction develops and how it can be treated, we must understand the dynamic mechanisms that occur as drinking progresses from the short term to long term. Although many studies demonstrate specific changes in response to a single EtOH exposure paradigm, few compare the behavioral and cellular changes across multiple paradigms. Together with our previous studies, we have shown that multiple forms of EtOH-induced plasticity occur in the SCN in response to distinct EtOH exposure paradigms, and that each likely involves unique cellular mechanisms that develop over different timeframes. Here we focused specifically on changes in NMDA receptor subunit total expression, phosphorylation, and surface expression across these states. We see a shift in the ratio of NR2B to NR2A subunits, and an increase in Y1472 phosphorylation that may be critical for glutamate hypersensitivity. Continuing this line of investigation will enhance our understanding of how alcohol abuse leads to disruptions in circadian rhythms. Moreover, the research will fill major knowledge gaps in our understanding of the mechanisms that lead to EtOH addiction, which can lead to novel treatments for alcohol addiction.

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Appendix C

Figure 3.1 Chronic tolerance to ethanol (EtOH) inhibition of glutamate-induced phase delays. (A-D) 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 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 induces a ~2.5 hour phase delay. (C) Co-application of 20mM EtOH with 1mM glutamate blocks glutamate induced phase delays in SCN tissue from an EtOH naïve mouse, indicating that the glu-induced phase delay is inhibited. (D) Co-application of 20mM EtOH and 1mM glutamate does not inhibit glutamate induced phase shifts in SCN slices from a mouse that had undergone the chronic 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. (E) Shown are the mean $\pm$ SEM phase shifts from experiments involving glutamate and EtOH applied to brain slices of mice after 10 days of the chronic drinking protocol. Bath application of 1mM glutamate induces significant phase delays. Co-application of 20mM EtOH does not block phase shifts. Phase shifts still occur with 150mM EtOH, but are inhibited by 200 mM EtOH $n=3-7$. (F) Plotted are the mean peak ($\pm$ SEM) change in phase shifts induced in brain slices of mice drinking 15% EtOH for differing amounts of time in number of days – then treated with 1mMglutamate + 20mM EtOH determines the number of consecutive days of drinking necessary before tolerance occurs. 8 days of drinking is needed for 20mM EtOH to no longer inhibit glu-induced phase shifts. One-way ANOVA indicated a significant effect of treatment, *Post hoc (Holm-Sidak) test indicates $p < 0.05$ versus control experiments. ** $p < 0.01$

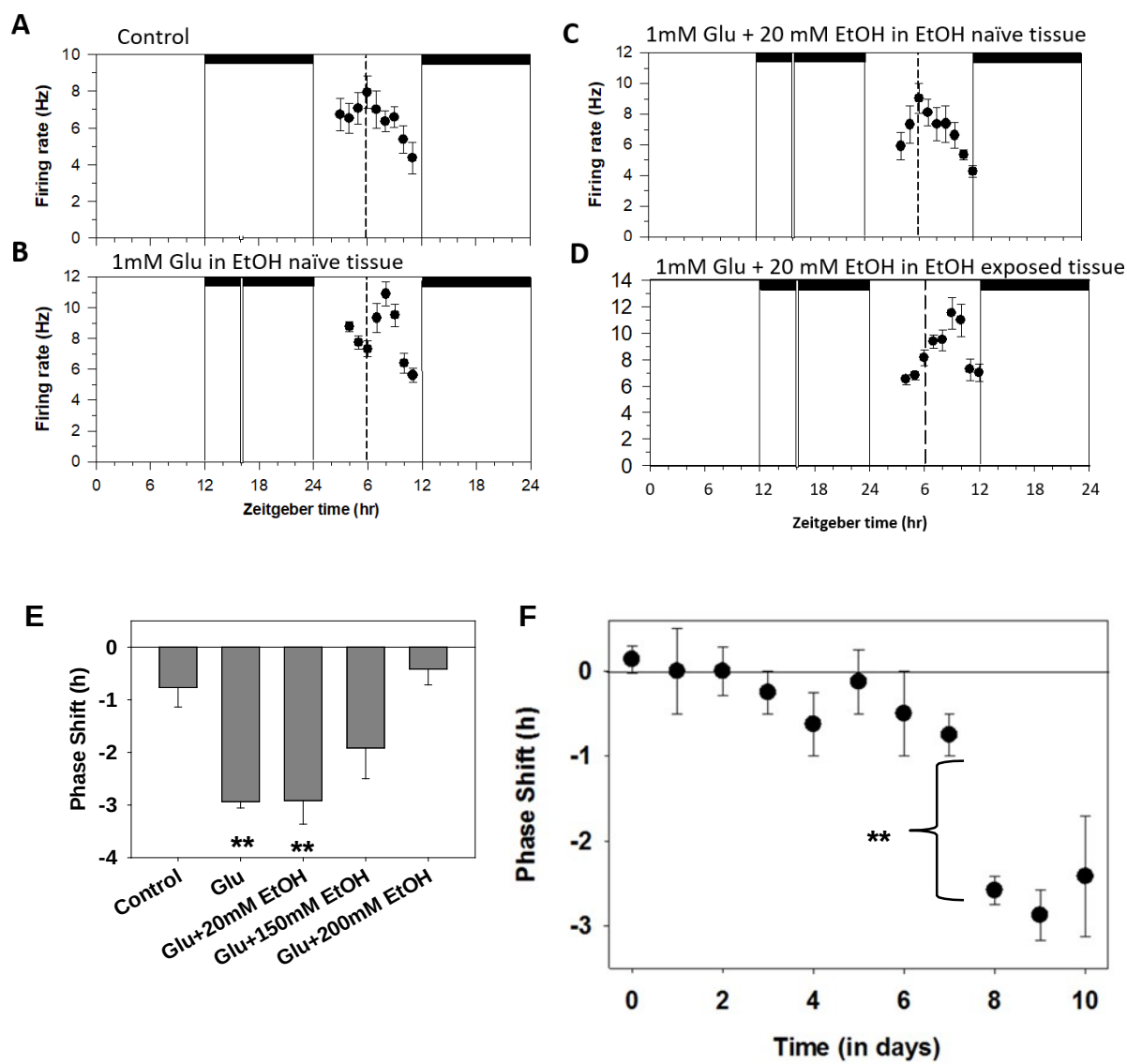


Figure 3.2 Sensitization to glutamate in the SCN after EtOH withdrawal. **(A)** Plotted are the mean peak ($\pm$ SEM) delays after 0uM, 1uM, and 10uM glutamate after no drinking (white bars, n=3), 21 days of drinking (gray bars, n=3), and 21 days of drinking with two days withdrawal (orange bars, n=3). **Post hoc (Holm-Sidak) test indicates $p < 0.01$ versus the 0 uM glutamate EtOH naïve condition. **(B)** Plotted are the mean peak ($\pm$ SEM) firing rates induced by 1uM glutamate treatment in mice that have undergone 2 days of chronic withdrawal from EtOH after varying numbers of days of chronic drinking. **Post hoc (Holm-Sidak) test indicates $p < 0.01$ versus the 10 days of drinking condition. **(C)** Plotted are the mean peak ($\pm$ SEM) firing rates induced by 1uM glutamate treatment in mice that have undergone a varying number of days of chronic withdrawal from EtOH after 21 days of chronic drinking. **Post hoc (Holm-Sidak) test indicates $p < 0.01$ versus the 10 days of drinking condition.

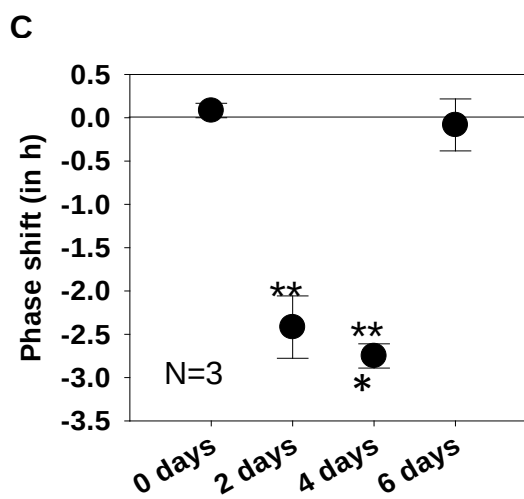
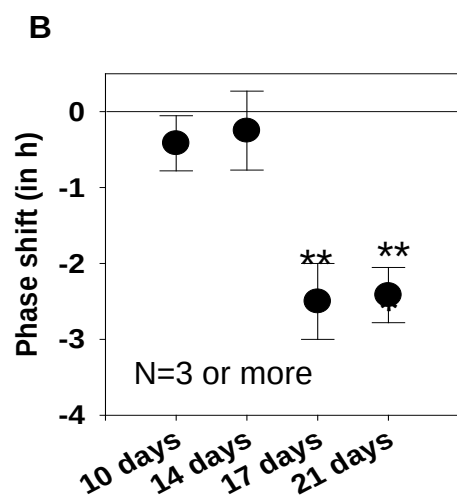
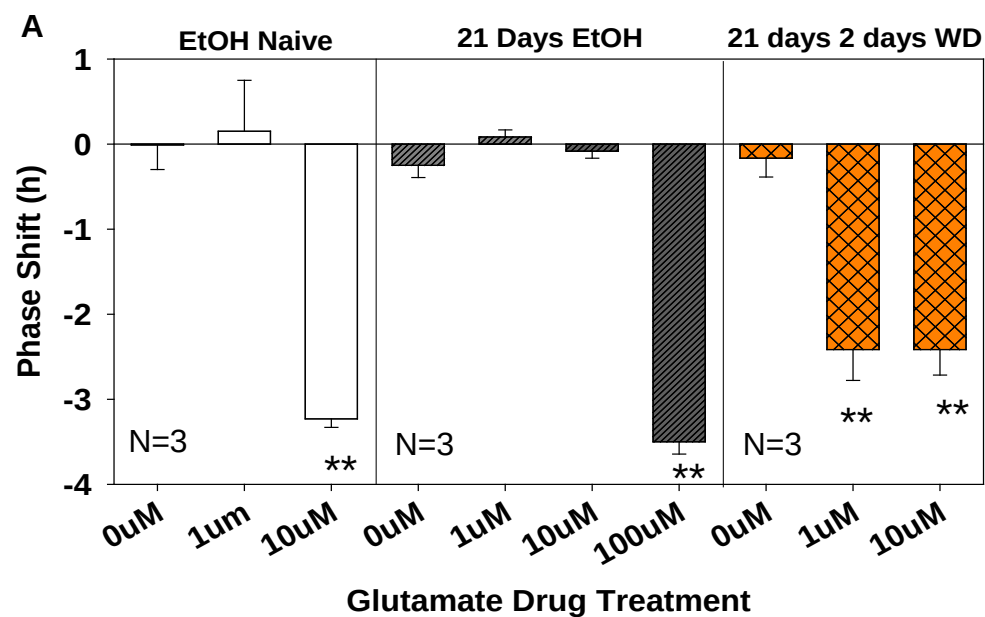


Table 3.1 Summary of Western blot results comparing tissue from EtOH naïve mice with mice exposed to the different EtOH drinking paradigms. Significant changes compared to control conditions were noted for the NR2B/NR2A ratio (2B/2A), phosphorylation of the NR2B subunit at the Y1472 residue (Y1472), total expression of mBDNF (mBDNF), and phosphorylation of TrkB at Y706 residue (pTrkB). All experiments were performed relative to actin and normalized to control. <0.05 represent p-values for results with significant changes compared to control conditions and 'x's represent no significant changes.

	NR2A	NR2B	2B/2A	Y1472	pBDNF	mBDNF	pTrkB
Overnight	x	x	x	x	x	x	x
10 day	x	x	x	x	x	x	x
21 day	x	x	p<0.05	x	x	x	x
Withdrawal	x	x	p<0.05	p<0.05	x	p<0.05	p<0.05

	NR1	S1480	Fyn	PSD95	FL TrkB	Trnc TrkB
Overnight	x	x	x	x	x	x
10 day	x	x	x	x	x	x
21 day	x	x	x	x	x	x
Withdrawal	x	x	x	x	x	x

Figure 3.3 Total expression of NR2B, NR2A, and NR2B:NR2A ratio after rapid, chronic 10 days, chronic 21 days, and withdrawal procedures. **(A)** Above, plotted are the mean band densities ($\pm$ SEM) for total expression of NR2A normalized to control tissue and relative to actin (n=7 for all conditions). Below are representative images for NR2A band densities across all treatments, respectively. **(B)** Above, plotted are the mean band densities ($\pm$ SEM) for total expression of NR2B normalized to control tissue relative to actin (21D n=7; other variables n=11). Below are representative images for NR2B band densities across all treatments, respectively. **(C)** Plotted are the mean ratios of band densities for NR2B to NR2A total expression normalized to control tissue and relative to actin (n=7 for all conditions). Tissue from control untreated mice (ctl), mice drinking overnight (RT), mice chronic drinking for 10 days (CT), mice chronic drinking for 21 days and mice chronic drinking for 21 days with 2 days withdrawal (WD) treatments to SCN slices. *Post hoc (Holm-Sidak) test indicates $p < 0.05$ versus control experiments.

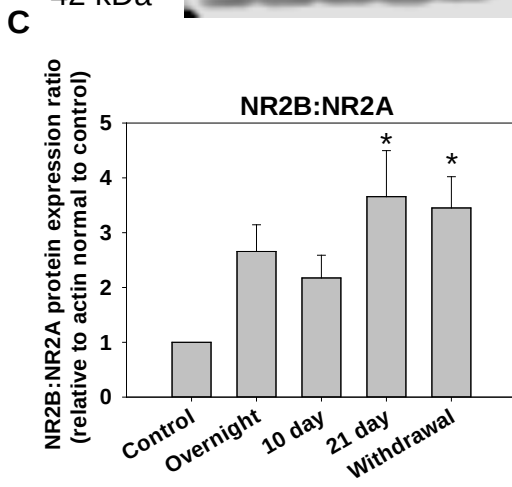
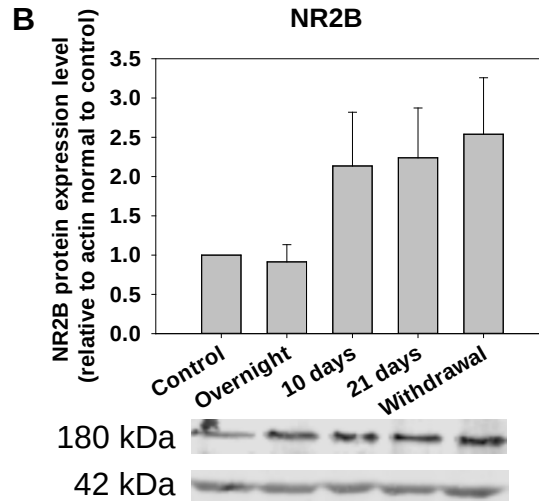
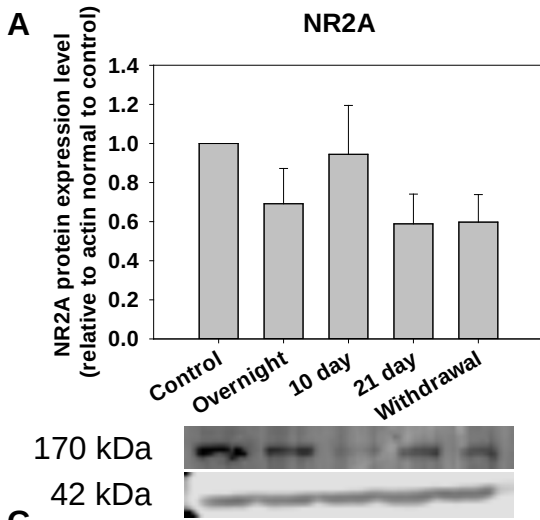
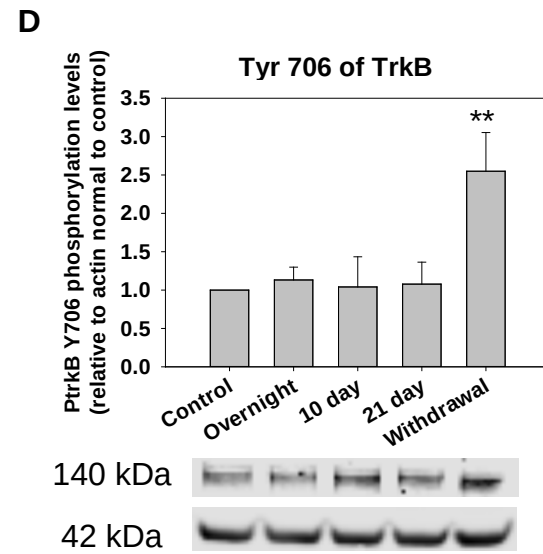
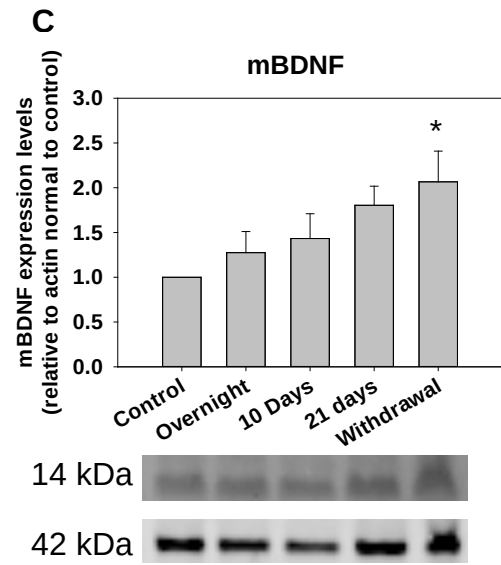
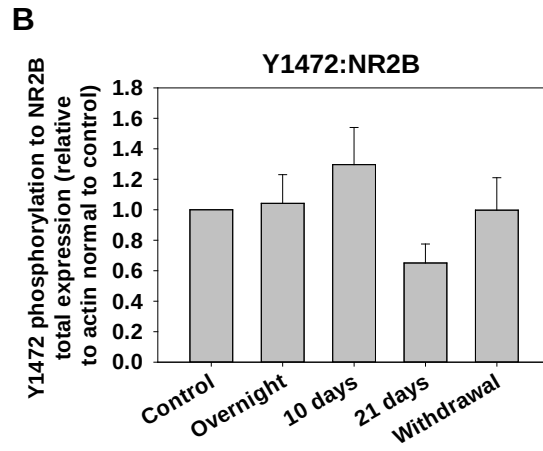
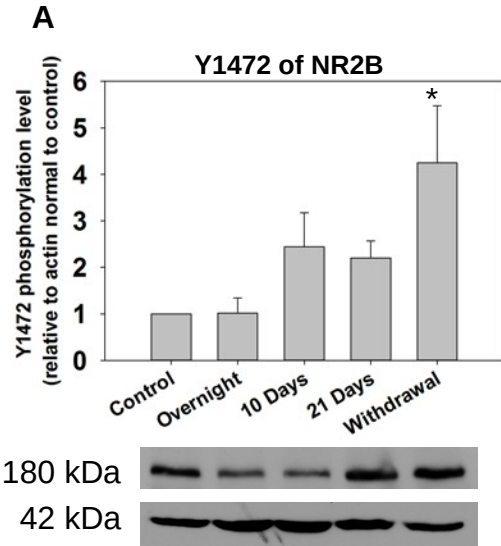


Figure 3.4 Total expression of NR2A and PSD-95 after rapid, chronic 10 days, chronic 21 days, and withdrawal drinking procedures. **(A)** Plotted are mean band densities ($\pm$ SEM) for Y1472 phosphorylation of the NR2B subunit of the NMDA receptor normalized to control tissue and relative to actin across different conditions (n=3 for 21D, n=8 for other conditions). Below are representative images for Y1472 band densities 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 phosphorylation to NR2B total 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 total protein expression normalized to control tissue and relative to actin across different conditions (n=6 for all conditions). Below are representative images for mBDNF band densities 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 tyr 706 phosphorylation of the TrkB receptor normalized to control tissue and relative to actin across different conditions (n=3 for 21D, n=8 for other conditions). Below are representative images for tyr 706 band densities across all treatments, respectively. **Post hoc (Holm-Sidak) test indicates $p < 0.05$ versus all conditions.



Chapter 4

General Conclusions

Summary of conclusions

The goals of this research project were to investigate EtOH induced rapid tolerance, chronic tolerance, and withdrawal induced sensitization in the SCN, and compare the cellular changes induced under each condition. Many research groups investigating EtOH-induced responses in the brain utilize only one model for EtOH exposure. Although these previous studies have shown that EtOH alters cellular processes like glutamate signaling, they provide little insight into the progression of changes that develop across different durations of EtOH exposure. Consistent with these studies, previous results in our lab demonstrate that EtOH inhibits glutamate signaling in the SCN, as assessed by its ability to inhibit glutamate-induced phase-shifts of the SCN circadian clock in vitro, as well as inhibiting photic phase resetting in vivo (Prosser 2003). We also showed the circadian clock develops acute tolerance to EtOH inhibition of glutamate phase shifts in vitro (Prosser and Glass 2009). Based on these results, we hypothesized that rapid tolerance, chronic tolerance, and withdrawal induced sensitization also occur in the SCN. Furthermore, we believed that the circadian clock would be an excellent model system for investigating the cellular changes associated with rapid tolerance, chronic tolerance and withdrawal induced sensitization.

Given that we observed acute EtOH tolerance in the SCN in vitro (Prosser and Glass 2009), it seemed surprising that chronic applications of EtOH continue to inhibit photic phase shifts in vivo (Rosenwasser et al 2005; Seggio et al 2007; Seggio et al 2009; Ruby et al 2009; Brager et al 2010; Brager et al 2011). From this, we wondered whether these differences were due to procedural differences in our in vitro vs in vivo assessments, or if they were due to the presence or lack of extra-SCN influences under

the two conditions. With this in mind, we set out to determine whether rapid tolerance, chronic tolerance, and withdrawal induced sensitization occur in the SCN, and if they do, to determine whether they involve changes in glutamate receptors. We hypothesized that EtOH-induced plasticity in the SCN is associated with changes in NR2B containing NMDA receptors in the SCN, and that the extent and manner of these changes differ across multiple forms of EtOH plasticity.

In the experiments presented in chapter two, we demonstrated that rapid tolerance to EtOH develops within 24 hours of initial EtOH access. This effect is seen with respect to both phase delays (ZT16) and phase advances (ZT23), and is exhibited both *in vivo* and *in vitro*. The dose of EtOH necessary to inhibit glutamate induced phase shifts during rapid tolerance is ten-fold the normal amount. Rapid tolerance lasts between 48 and 72 hours for the phase delays, and between 72 and 96 hours for the phase advances. In chapter three, we demonstrated that chronic tolerance can be seen using our *in vitro* phase-resetting paradigm, and that it develops after a minimum of 8 days of drinking. The dose of EtOH necessary to inhibit glutamate induced phase shifts during chronic tolerance is 10-fold the normal amount, consistent with what we observed with rapid tolerance. We also demonstrated that an increased sensitivity to glutamate-induced phase shifts is seen in the SCN *in vitro* when assessed 2 days after EtOH withdrawal following at least 14 days of EtOH consumption. This withdrawal induced glutamate hypersensitivity is still apparent at 4 days but not at 6 days after abstinence.

Having clearly defined the timing and dosing parameters associated with these forms of EtOH plasticity, we proceeded to investigate the underlying cellular mechanisms. We determined that distinct changes in NMDA receptor subunits accompany the different

forms of plasticity in the SCN: an increase in the ratio of NR2B:NR2A subunits is seen after 21 days of drinking and during EtOH withdrawal, but not after a single night of forced EtOH consumption or even after ten days of drinking using the drinking in the dark paradigm. Increased phosphorylation at the NR2B tyrosine1472 (Y1472) residue is seen after EtOH withdrawal, but not after 21 days of drinking. Upstream of Y1472 phosphorylation, we determined increases in mBDNF total protein expression and TrkB phosphorylation occur during withdrawal, but not in any other conditions, matching results showing Y1472 phosphorylation. Surprisingly, in none of the conditions did we see changes in NR1 total protein expression, NR2B S1480 phosphorylation, nor did we see changes in PSD-95 and total protein expression of Fyn. Taken together, these results demonstrate that distinct physiological and cellular responses accompany each form of EtOH tolerance and EtOH withdrawal, and suggest that EtOH-induced plasticity in the SCN may differ from that seen in other brain regions.

Rapid tolerance to EtOH develops in the SCN

Rapid tolerance occurs after acute tolerance and before chronic tolerance. Some studies have suggested that the development of rapid tolerance may be a good predictor of subsequent chronic tolerance development (Khanna et al 1991a; Wu et al 2001; Radcliffe et al 2006). Like the other forms of EtOH tolerance, a decrease in EtOH-induced hypnotic, motor, and hypothermic effects is seen during rapid tolerance (Gilliam 1989; Silveri and Spear 1999). What distinguishes rapid tolerance from other forms of EtOH tolerance besides its timing could be the underlying cellular substrates. Pharmacologically, blocking NMDA receptors with the selective antagonist, MK-801,

blocks behavioral manifestations of rapid tolerance (Khanna et al 1991b; 1993). A separate study demonstrated that NR2A knock-outs but not NR2B knock-outs reduce but do not eliminate the decreased sleep/sedative effects of EtOH seen during rapid tolerance, supporting the notion that NR2A subunits play a role in rapid tolerance to EtOH induced sedation (Palachick et al 2008). However, there is no information on changes in protein expression and/or phosphorylation of NMDA receptor subunits during rapid tolerance. Thus we attempted to identify physiological characteristics and changes in NMDA receptor subunits and other proteins that could regulate NMDA receptor function during rapid tolerance.

The findings in chapter two demonstrate that rapid tolerance is exhibited by the SCN both in vivo and in vitro 24 hours after access to EtOH. These results are consistent with other studies investigating the timing of rapid tolerance development with respect to EtOH-induced hypothermia, ataxia, and sleep promotion in various rodent models (Khanna et al 1991a; Radcliffe et al 2006; Sharma et al 2014). In our study, the duration of rapid tolerance differed between glutamate-induced phase delays and phase advances, lasting 48-72 hours and 72-96 hours, respectively. To our knowledge, this is the first time the duration of rapid tolerance has been determined. Though we did not address why the duration of tolerance differs between phase delays and phase advances, these phase-shifting processes have distinct cellular mechanisms that could be differentially modified by EtOH tolerance (Gillette and Mitchell 2002).

As mentioned, little is known about modulation of NMDA receptors and NMDA receptor related signals during rapid tolerance. We hypothesized that proteins that regulate NMDA receptor function may be modulated during rapid tolerance. A major

component of NMDA receptor regulation is BDNF-induced TrkB activation (Chao 2003; Mizuno et al 2003). TrkB phosphorylates and activates Fyn kinase (Yamada and Nabeshima 2004; Carreño et al 2011), which leads to increased activation of NMDA receptors via Fyn-induced phosphorylation of the NR2B subunits (Nakazawa et al 2001; Goebel et al 2005). In the SCN, BDNF signaling is needed for glutamate/photic phase shifting (Liang et al 2000; Kim et al 2006; Mou et al 2009; Cooper et al 2017). We have demonstrated that application of exogenous BDNF prevents acute EtOH inhibition of phase shifts (Prosser et al 2008). We subsequently demonstrated that exogenous BDNF prevents EtOH inhibition of glutamatergic phase shifts during rapid tolerance. Despite these data pointing to the importance of BDNF/TrkB/NMDA signaling in glutamate-induced phase shifts, we found no rapid tolerance-associated changes with respect to NMDA receptor NR1, NR2A, or NR2B subunit expression, NR2B phosphorylation at Y1472, S1303, or S1480, or TrkB, BDNF, PSD-95, and Fyn kinase total expression.

These results raise a number of possibilities: 1) Rapid tolerance may not involve changes in NMDA receptors. This would mean that the cellular changes underlying rapid tolerance are different from those associated with both acute and chronic tolerance (Läck et al 2005; Miyakawa et al 1997; Nagy 2008; Roberto et al 2006; Trepanier et al 2012). Given the distinct time courses of these different forms of tolerance, this would not be especially surprising. 2) Rapid tolerance may involve NMDA receptor-associated compensatory responses not investigated here. For example, chronic EtOH induces increases in STEP-33 and p38 MAPK, without altering Src, Lyn, and Fyn kinases (Wu et al 2010). Though we did not see changes in phosphorylation of NR2B Y1472, it is possible STEP-33 and p38 MAPK alter phosphorylation of other residues on NR2B or

different subunits of the NMDA receptor during rapid tolerance. On another note, acute EtOH typically alters phosphorylation of NR1 subunit residues (Xu et al 2010; Roh et al 2011). These changes may still be present during rapid tolerance. 3) Compensatory responses during rapid tolerance may involve other receptors like GABA(A) δ receptors. In support of this, we previously demonstrated the acute actions of EtOH in the SCN involve GABA(A) δ receptors (McElroy et al 2009). Future investigations of rapid tolerance in the SCN could focus on any of these cellular mechanisms.

Chronic tolerance to EtOH develops in the SCN

Chronic tolerance takes longer to develop than acute and rapid tolerance. Reports have shown chronic tolerance in rodents with respect to EtOH metabolism and EtOH-induced ataxia after 10 days to 2 weeks of EtOH exposure (Broadwater et al 2011; Matson et al 2014). Manifestations of chronic tolerance are also seen after longer durations of continued use, including tolerance to the hypnotic effects of EtOH (e.g., 20 days in Matthews et al 2008; 60-90 days in Darbra et al 2002; 120+ days in Cagetti et al 2003). At the cellular level, long-term EtOH exposure has been shown to induce changes in both NMDA and GABA(A) receptor activity. Studies demonstrate that NMDA receptor-associated Ca^{2+} currents increase while GABA receptor-associated Cl^- currents decrease during chronic tolerance, which in both cases can lead to a hyperexcitable state (Iorio et al 1992; Buck et al 1991). Although changes in NMDA and GABA receptor signaling are seen during both acute and rapid tolerance (Allan and Harris 1987; Buck et al 1991; Grover et al 1994; Silveri and Spear 1999; Silveri and Spear 2004), the changes in NMDA- and GABA-receptor ion currents appear to occur exclusively during chronic tolerance or

at least have yet to be identified in acute and rapid tolerance. These data support the conclusion that the mechanisms underlying acute and rapid EtOH tolerance vs. chronic EtOH tolerance are distinct.

It is possible that the shorter duration of EtOH exposure associated with acute and rapid tolerance only allows for changes in the activity of second messengers, while chronic EtOH exposure allows for transcriptional/translational changes (Kalant 1996; Pietrzykowski and Treistman 2008). Thus, total expression of receptor subunits and phosphorylation of key residues of NMDA receptor and GABA receptor subunits are seen during chronic EtOH tolerance while they have not been reported during acute and rapid tolerance. Specifically, increases in NR2B expression and phosphorylation (Suvarna et al 2005; Wang et al 2011), increases in surface localization of GABA $\alpha 4$ subunits (Liang et al 2007), and increases in GABA $\beta 2$ and $\gamma 2$ subunit phosphorylation have been reported during chronic tolerance (Ravindran & Ticku 2006). Importantly however, none of these prior studies compared multiple forms of EtOH-induced plasticity within a single study, which makes drawing clear distinctions between their cellular substrates more difficult.

Our electrophysiological results show that the SCN develops chronic tolerance to EtOH as seen by a decrease in EtOH inhibition of glutamate-induced phase shifts in vitro. This is similar to our in vitro results regarding acute and rapid tolerance in the SCN (Prosser and Glass 2009). However, this differs from in vivo studies showing that chronic EtOH continues to inhibit photic phase shifts (Ruby et al 2009; Brager et al 2010). In comparing these in vivo studies with our in vitro experiments there are a few methodological differences. In the previous studies, the mice had higher daily EtOH

consumptions (20g/kg/day vs. 2.84g/kg/day), they were drinking EtOH for a longer overall period (14 days vs. our 10 days), drinking was not restricted to 4 h each day, and tolerance was not assessed using different intensities of the phase-shifting stimulus (light). A separate study demonstrated that chronic forced EtOH consumption, where more EtOH is consumed, inhibits photic phase delays *in vivo*, while chronic voluntary consumption, where less EtOH is consumed, does not inhibit phase shifts (Seggio et al 2009). With this in mind, a study in our lab suggests that EtOH also enhances serotonergic phase shifts, and that this occurs at least in part through inhibiting glutamatergic signaling (Prosser et al 2008). Interestingly, serotonin receptors have been shown to regulate glutamate release into the SCN (Pickard et al 1996; Smith et al 2001; Sprouse et al 2004). Therefore, the effects of EtOH in the SCN may include increasing serotonergic signaling and decreasing glutamate release. This is supported by reports showing increased serotonin 1a receptor sensitivity during chronic EtOH (Esteban et al 2001). Thus, *in vivo* experiments would show results consistent with glutamatergic phase shift inhibition during chronic EtOH at higher EtOH concentrations, but not at lower EtOH concentrations as assessed in Seggio et al 2009. It will be important to further explore the critical variables determining whether chronic tolerance is seen in the SCN *in vivo*. Importantly, identifying factor(s) such as serotonin that potentially protect the circadian clock from exhibiting chronic tolerance *in vivo* could provide insight into treatments for those dependent on alcohol.

As with rapid tolerance, we characterized the change in sensitivity and timing parameters of both chronic tolerance and withdrawal induced glutamate hypersensitivity. We determined that chronic tolerance is characterized by a 10 fold right-ward shift in the

dose response curve for EtOH inhibition of glutamate induced phase shifts, similar to the changes in sensitivity seen with rapid tolerance. We also determined that, using our assessment protocol, it takes 8 days of drinking to develop chronic tolerance. To our knowledge, this is the first experiment to carefully assess how many days of consecutive drinking are necessary for chronic tolerance to develop. Previous studies have sometimes used 7-14 days of EtOH consumption to investigate chronic tolerance (Broadwater et al 2011; Matson et al 2014). Although this duration of EtOH exposure is generally consistent with our results, it might be worthwhile for researchers to adopt a paradigm that includes at least 8 days of EtOH consumption when investigating chronic tolerance.

There are still many unanswered questions regarding the cellular changes associated with chronic tolerance. As with rapid tolerance, it is possible that chronic tolerance does not require changes in NMDA receptor expression. Although other studies have reported changes in NMDA receptors during chronic tolerance (Nagy 2008; Roberto et al 2006; Wu et al 2010), most involved much longer periods of EtOH exposure. So it is not clear whether the changes induced in the SCN by chronic EtOH exposure differ from those of other regions or whether the difference is due to different durations of EtOH exposure. A second possibility is that chronic tolerance in the SCN involves changes in other receptors. Many reports have demonstrated modulation of GABA(A) receptors and AMPA receptors during chronic EtOH tolerance in the amygdala and cortex (Liang et al 2007; Ravindran et al 2007; Liang et al 2009; Lindemeyer et al 2014; Nimitvilai et al 2016; see below). Chronic tolerance may also involve processes upstream and/or downstream of NMDA receptors that were not investigated here. We saw no changes in total expression of Fyn, however other reports have demonstrated that phosphorylation of Fyn

is increased during chronic EtOH (Gibb et al 2011). Seeing that our results demonstrated increases in mBDNF and TrkB phosphorylation during withdrawal, increased phosphorylation of Fyn via TrkB signals during withdrawal is likely. Finally, we did not assess glutamate levels in the SCN, but others have reported increases in extracellular glutamate associated with chronic EtOH exposure (Rossetti and Carboni, 1995; Ding et al 2012; Ding et al 2013; Griffin et al 2013), which could affect signaling in the SCN through homeostatic synaptic changes. Investigating these possibilities should help distinguish the cellular changes associated with chronic tolerance from those of other forms of tolerance.

Withdrawal from EtOH increases circadian clock sensitivity to glutamate-induced phase resetting.

The behavioral effects of withdrawal from EtOH are distinct from those associated with EtOH tolerance. For example, withdrawal can lead to insomnia, psychomotor agitation, hallucination, and seizures (Schuckit 2014), while tolerance is primarily characterized by a loss or reduction in EtOH-induced behaviors, such as decreased EtOH induced sleep, ataxia, and hypothermia (Khanna et al 1991a; Radcliffe et al 2006; Sharma et al 2014). Nevertheless, the cellular changes seen with EtOH withdrawal and EtOH tolerance overlap. Some studies have demonstrated that withdrawal is associated with increases in extracellular glutamate (Ding et al 2013; Griffin et al 2013), a response also seen during chronic EtOH (Roberto et al 2004; Christian et al 2013). Conversely, studies investigating the hippocampus and bed nucleus of the stria terminalis (BNST) have shown that synaptic localization of NR2B subunits decreases during EtOH withdrawal (Clapp et

al 2010; Wills et al 2012), which contrasts with increased synaptic localization of NR2B subunits during chronic EtOH (Clapp et al 2010). With this in mind, we assessed the same glutamate signaling-associated proteins during EtOH withdrawal as we assessed after rapid and chronic tolerance.

We determined that glutamate hypersensitivity, characterized by a 10 fold left-ward shift in the dose response curve for glutamate induced phase delays, is seen after at least 14 days of EtOH consumption followed by 2-6 days of EtOH withdrawal. Others have shown that withdrawal induced convulsions can develop after as few as four days of EtOH vapor inhalation in mice (Olive and Becker 2008), although this paradigm involves a more intensive exposure to EtOH and thus potentially much higher blood ethanol concentration levels than the drinking in the dark paradigm we used. Others have shown EtOH withdrawal associated seizures after EtOH drinking for 2 weeks (Roberto et al 2004) or 1-2 weeks (Wang et al 2010) in rodent models, which is more consistent with our results. Some studies have reported EtOH withdrawal induced convulsions lasting as little as 4-10 hours after EtOH withdrawal (Farook et al 2008; Olive and Becker 2008) while other effects, such as locomotor sensitization, last upwards of 11 days after EtOH withdrawal (Abrahao et al 2013). Importantly, our data showing that chronic drinking prior to withdrawal does not lead to glutamate hypersensitization is consistent with other studies. This suggests that withdrawal induced plasticity requires a period of abstinence to develop.

The increase in NR2B:NR2A expression we observed after 21 days of drinking is still seen during EtOH withdrawal. This increase could be one component of the mechanism underlying withdrawal induced glutamate hypersensitivity. Only after

withdrawal did we also see an increase in phosphorylation of the NR2B Y1472 residue. Increases in the phosphorylation of NR2B Y1472 potentiate NMDA receptor currents and lead to synaptic membrane recruitment of these receptors (Nakazawa et al 2001; Goebel et al 2005). Once the NMDA receptors are localized to the membrane, phosphorylation of Y1472 also maintains their localization within the synapse (Goebel et al 2005) via interactions with the scaffolding protein, PSD-95 (Roche et al 2001; Prybylowski et al 2005; Zhang et al 2008). These changes are associated with hyperexcitable states in neurons and have been demonstrated in other brain regions during EtOH withdrawal. For example, increases in NR2B Y1472 in the hippocampus and the dorsomedial striatum have been reported during EtOH withdrawal (Pawlak et al 2005; Wang et al 2010). Thus, the glutamate hypersensitivity seen during withdrawal could be due to a combination of increased NR2B:NR2A expression and increased NR2B Y1472 phosphorylation. These changes would be expected to increase NMDA receptor excitatory postsynaptic potentials as seen in the amygdala, dorsomedial striatum and hippocampus (Roberto et al 2004; Wang et al 2010; Wu et al 2010). An important test of this scenario would be to investigate synaptic localization of NR2B containing NMDA receptors during EtOH withdrawal. Although we did follow up using cell surface biotinylation, we did not see a significant increase with respect to NR2B subunit surface expression (n=6, data not shown). However, these results did show trending increases in NR2B surface expression during withdrawal. Therefore, we cannot exclude the possibility that chronic EtOH and withdrawal modulate surface expression of NMDA receptor subunits.

One mechanism through which Y1472 phosphorylation occurs is via TrkB receptor activation. proBDNF becomes cleaved to generate its mature form by many proteins,

including plasmin after its activation by tissue plasminogen activator (tPA) (Pang et al 2004). Mature BDNF is then capable of binding TrkB, inducing TrkB dimerization, and allowing autophosphorylation. We showed an increase in mBDNF total protein expression during withdrawal, which is consistent with tPA dependent increases in Y1472 during EtOH withdrawal (Pawlak et al 2005). Furthermore, an increase in phosphorylated TrkB at Tyr 706 occurred during withdrawal. Other studies have demonstrated similar results. For instance, increases in BDNF protein expression have been reported in the rat hippocampus and medial prefrontal cortex after a 3h withdrawal from chronic intermittent ethanol ([CIE], Somkuwar et al 2016). Another investigation in the mouse dorsal lateral striatum showed that p75, a neurotrophin factor receptor that pro BDNF binds and opposes TrkB signals, increases after cessation of 21 days of 2 bottle access CIE for 24 h, suggesting EtOH withdrawal induces neuroadaptive responses (Darcq et al 2016). Yet we showed increases in TrkB phosphorylation, suggesting withdrawal induced sensitization to glutamate in the SCN is acting through TrkB signals. Others have shown that acute ethanol blocks TrkB phosphorylation mediated NR2B containing NMDA receptor activation (Kolb et al 2005). Thus the nature of this response may be compensatory, as the removal of EtOH due to withdrawal may leave previous inhibition of TrkB phosphorylation by EtOH unchecked (see Figure 4.1).

We have summarized our data demonstrating changes in the glutamatergic phase shift mechanism across control conditions, overnight/10 day chronic drinking, 21 day chronic drinking, and 21 days of drinking followed by withdrawal (Figure 4.1). The changes across each condition are represented in a timeline to gain perspective on when each EtOH induced plasticity develops.

Future directions

Ethanol-induced plasticity

We demonstrated that rapid tolerance, chronic tolerance, and withdrawal hypersensitivity all occur in the SCN. However, we were unsuccessful in fully delineating the cellular mechanisms contributing to these responses. We believe it is likely that additional receptors participate in these phenomena. Some likely candidates are discussed below.

GABA(A) receptors

GABA(A) receptors reduce neuronal activation throughout the brain, and generally act to counteract glutamate-induced excitation. In the SCN pharmacological inhibition of GABA(A) receptors can enhance photic/glutamate phase shifts, while GABA(A) receptor activation can inhibit these phase shifts, confirming GABA(A) receptor regulation of photic phase shifts (Ralph and Menaker 1989; Gillespie et al. 1997; Herzog et al 2017). GABA(A) receptors contain 5 total subunits, with the most common isoforms including 2 α subunits, 2 β subunits, and either a γ or δ subunit (Olsen and Sieghart 2009). The α and β subunits are important for regulating ion channel conductance properties, while the γ and δ subunits regulate the localization of GABA receptors in and out of the synaptic active zone, respectively. The characteristics of γ and δ containing-GABA(A) receptors also differ in that they generate phasic and tonic currents, respectively (Farrant and Nusser 2005).

Post-translational modifications such as phosphorylation contribute to the regulation of GABA(A) receptors. Phosphorylation of key residues of different subunits of GABA(A) receptors modify localization to the membrane and receptor kinetics (Abramian

et al 2010; Bright and Smart 2013; Bohnsack et al 2016). Therefore, post-translational modifications of GABA(A) receptors can lead to substantial changes in the regulation of neuronal activity.

As with glutamate signaling, EtOH modifies GABA signaling in a manner that differs based on the duration of EtOH exposure. Acute EtOH enhances GABA receptor ion conductance (Wei et al 2004; Olsen et al 2007), while chronic EtOH reduces GABA receptor activation and expression, and alters phosphorylation of GABA(A) subunits (Kumar et al 2003; Liang et al 2007; Bright and Smart 2013; Abramian et al 2010; Ron and Messing 2013). EtOH can also modify the function of kinases and phosphatases that regulate GABA(A) receptor mobilization to and from the membrane (Brandon et al 2000; Brandon et al 2003; Carlson et al 2013; Carlson et al 2014; Choi et al 2008; Kumar et al 2010). Changes in GABA(A) receptors have been shown during acute, rapid, and chronic tolerance, including changes in GABA(A) receptor currents, subunit expression, and phosphorylation (Allan and Harris 1987; Buck et al 1991; Barbosa and Morato 2001; Ravindran et al 2007, Liang et al 2007, Gonzalez et al 2012, Lindemeyer et al 2014). One target of EtOH induced effects we are especially interested in exploring is δ subunit containing GABA(A) receptors. Previously we showed that application of R015-4513, a GABA(A) δ subunit selective antagonist, prevents EtOH inhibition of glutamate induced phase delays, suggesting that EtOH acts in part on GABA(A) receptors to inhibit photic phase shifts (McElroy et al 2009). Following up on these experiments by investigating potential changes in the expression levels and localization of GABA(A) δ subunits during EtOH tolerance and withdrawal may reveal more on these tolerance mechanisms.

AMPA receptors

Glutamatergic AMPA receptors contribute substantially to regulating neuronal excitation. Each AMPA receptor is composed of 4 subunits, forming a heterotetramer that assembles in a 'dimer of dimers' configuration (Mayer 2005). The different subunit types include GluR1-R4 and always assemble in pairs. Phosphorylation of these receptors modifies membrane localization, ion channel conductance, and the probability the channel is open (Derkach et al 1999; Banke et al 2000; Hayashi et al 2000; Boehm et al 2006). Some studies have demonstrated that in vivo and in vitro applications of AMPA phase shift the clock, suggesting an important role for AMPA receptors in photic phase shift mechanisms (Takeuchi et al 1991; Mizoro et al 2010).

Many reports have shown that EtOH modulates AMPA receptors. Though not as sensitive to acute EtOH as NMDA receptors (Möykkynen and Korpi 2012), acute EtOH inhibits AMPA receptor currents in adolescent rodents (Zhu et al 2007; Puglia and Valenzuela 2010). In contrast, chronic EtOH enhances the amplitude of AMPA receptor small excitatory postsynaptic currents in the basolateral amygdala of rats (Läck et al 2007). Chronic EtOH and EtOH withdrawal also alter AMPA subunit expression, subunit trafficking, and phosphorylation in the rodent amygdala and cortex (Christian et al 2012; Agoglia et al 2015; Nimitvilai et al 2016; Salling et al 2016). Regarding EtOH tolerance, AMPA receptor antagonists block formation of rapid tolerance associated with motor coordination behaviors in mice (Rial et al 2009). Also, chronic intermittent EtOH suppresses acute EtOH inhibition of AMPA receptor induced potentials in the orbitofrontal cortex of mice, suggesting AMPA receptors contribute to tolerance (Nimitvilai et al 2016). Because chronic EtOH and EtOH withdrawal modulate phosphorylation, trafficking, and

conductance of the AMPA receptor, AMPA receptors would be worth investigating using our multiple model approach.

Serotonin Receptors

Serotonin (5-HT) receptors mediate a variety of neurological processes including learning and memory, mood, and locomotion. In the SCN, 5-HT receptors mediate non-photic phase shifts (Medanic and Gillette 1992; Prosser 2003; Sprouse et al 2004). 5-HT receptors can also modify SCN responses to light, particularly the 5-HT1A, 5-HT1B, 5-HT3, 5-HT5A, and 5-HT7 receptor subtypes. These receptors modulate SCN neuron activity via inputs from other regions and within the SCN. For example, applying the selective 5-HT1B receptor agonist TFMPP to the SCN inhibits light induced phase shifts by reducing presynaptic glutamate release (Pickard et al 1997), while 5-HT1B KO mice have reduced responses to photic phase shifts (Sollars et al 2006). Activation of 5HT3 receptors on retinohypothalamic tract terminals induces photic phase shifts by increasing the release of glutamate on to SCN neurons (Graff et al 2006). In contrast, activation of 5-HT7 receptors in SCN neurons reduces amplitude of excitatory post-synaptic currents, and thus inhibits postsynaptic glutamate responses in the SCN (Smith et al 2001; Sprouse et al 2004).

EtOH also affects serotonin receptor function, particularly 5-HT1A, 5-HT1B, and 5-HT3 receptor subtypes, and the effects of EtOH differ depending on exposure duration. Behaviorally, mice with 5HT1A and 5HT1B receptor knockouts have increases in EtOH consumption and EtOH intoxication (Lovinger 1997). Acute EtOH also enhances 5-HT3 receptor activation (Vengeliene et al 2008). Conversely, chronic EtOH increases expression of the 5-HT1A (Burnett et al 2014) and 5-HT1B receptors in the macaque

hippocampus (Pandey et al 1996). Unfortunately, little is known about the effects of chronic EtOH on 5-HT₃ receptors, although recent evidence shows that alcoholic patients have increased 5-HT₃ receptor A subunit mRNA expression compared to non-alcoholics (Xu et al 2016). It appears that no research has been done on the effects of chronic EtOH and EtOH withdrawal on 5-HT₃ receptors. Because acute and chronic EtOH alter serotonin receptor subtypes critical for regulating phase resetting in the SCN, it would be worthwhile to investigate changes in these receptors during EtOH tolerance and withdrawal.

The effects of EtOH metabolites in the SCN

Acetaldehyde, the first metabolite of EtOH metabolized from cytochrome p450 in the brain, causes many problems associated with EtOH abuse. For example, acetaldehyde accumulation is neurotoxic (Takeuchi et al 2003), can promote spreading of tumors by inhibiting DNA repair (Seitz and Meuller 2015), and induces addictive like behavior in animal models by activating dopamine neuron firing in the mesolimbic system (Foddai et al 2004; Deehan et al 2013; Brancato et al 2014). The effects of acetaldehyde on dopamine neurons in the ventral tegmental area are thought to occur by modulation of A-type K⁺ currents and shortening the interval between hyperpolarization-activated inward rectifying currents (Melis et al 2007). A-type K⁺ channels are also found in the SCN, where they regulate SCN neuron excitability (Itri et al 2010; Granados-Fuentes et al 2012). Thus, it is possible that at least some of our effects are caused by acetaldehyde rather than EtOH. To test this, we could co-apply catalase inhibitors with glutamate and EtOH to the SCN in vitro during the early or late night. If the glutamate-induced phase shifts are not blocked, this would suggest glutamate phase shift inhibition involves

acetaldehyde. Follow-up experiments could also investigate whether tolerance to inhibition of phase shifts and withdrawal induced sensitization to glutamate is induced by drinking an acetaldehyde/water solution at various concentrations, depending on the type of tolerance or sensitization under question.

Astroglia and glutamate release

A recent paper by Brancaccio et al., (2017) showed that the timing of extracellular glutamate release is pivotal for maintaining circadian timekeeping. During the night, the level of extracellular glutamate peaks in the SCN, a process controlled by astrocytes. Other players in this mechanism include excitatory amino acid transporters (EAAT) responsible for glutamate reuptake, and NR2C containing NMDA receptors that may be located on GABAergic presynaptic terminals, which would allow the extracellular glutamate to regulate GABA release (Brancaccio et al 2017). As in other brain regions (Gass and Olive, 2008; Brager et al 2012; Ding et al 2012; Ding et al 2013; Griffin et al 2013) chronic EtOH could modulate extracellular glutamate levels in the SCN. Experiments assessing potential changes in total expression of EAAT and NR2C subunits during EtOH tolerance and withdrawal, as well as extracellular glutamate levels, could provide evidence for this possibility.

Conclusions

We have demonstrated the utility of the SCN as a model system for investigating EtOH. Not only does EtOH have clearly identified acute effects both in vivo and in vitro, but multiple forms of EtOH-induced plasticity develop in the SCN in response to different drinking paradigms. While many labs have investigated the cellular changes that occur

during tolerance, none have demonstrated changes across multiple forms of tolerance as well as withdrawal induced sensitization within a cohesive set of experiments.

Many studies, including this one, have demonstrated that the effects of EtOH are extremely varied, across time and across brain region. In light of this, future investigations evaluating novel targets for AUD should consider using multiple drinking models that compare different drinking durations. Assessing the effects using a single paradigm may reveal only some of the critical changes induced by EtOH consumption, and may limit the translational potential of the research.

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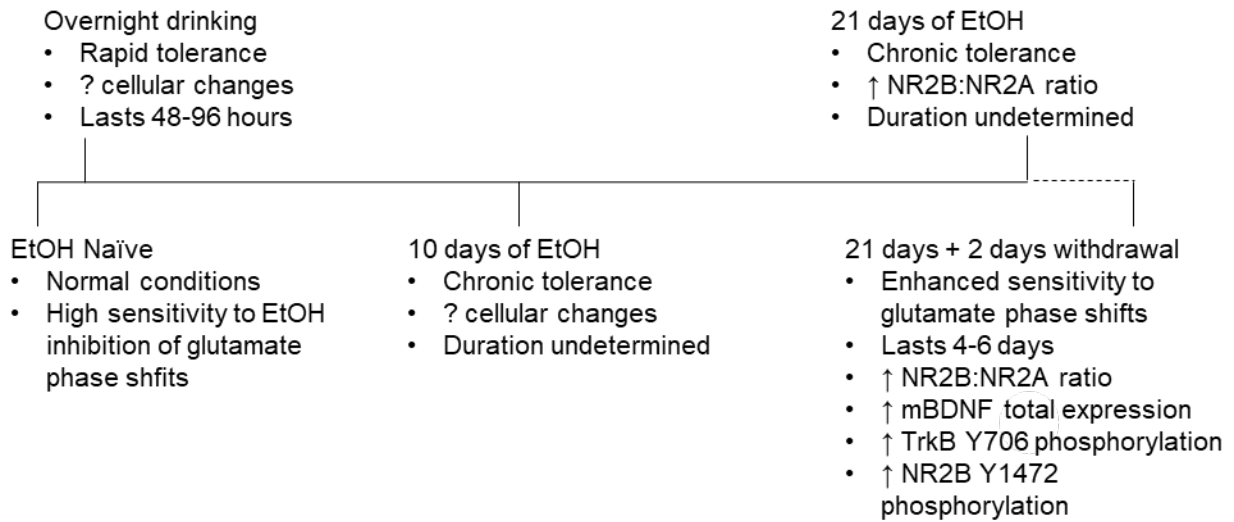
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Appendix D

Figure 4.1 The timeline of overnight/10 days of EtOH, 21 days of EtOH, and EtOH withdrawal in the SCN. Under normal conditions, the glutamate light in vivo or glutamate in vitro phase shifts the clock during the early night (phase delays) or late night (phase advances). Further down the timeline represents the development of rapid tolerance and chronic tolerance after an overnight binge or 4h access for ten days. SCN tissue becomes tolerant to inhibitory effects of EtOH during these conditions. The cellular actions that promote this response are still under consideration and thus no figures represent these two EtOH induced plasticities. Next, After 21 days of 4h EtOH access, EtOH induced plasticity leads to an increase in the ratio of total protein expression of NR2B to NR2A. Though this change is not required for tolerance as an increase in the NR2B to NR2A expression ratio does not occur within the timeframe for tolerance development (overnight for rapid or 10 days of 4h access for chronic), it may be a prerequisite of EtOH withdrawal induced plasticity. During withdrawal (dotted line on the timeline), less glutamate is needed to induce phase shifts, which is evident of EtOH withdrawal induced hypersensitization to glutamate. At the cellular level, increases in the NR2B:NR2A ratio persist in addition to increases in phosphorylation of the Y1472 residue of the NR2B subunit. Upstream, increases in total protein expression of mBDNF and phosphorylation of Tyr 706 of TrkB receptors likely promote the increase in Y1472 phosphorylation. These changes may be in part due to prolonged and sudden abstinence from EtOH, allowing for compensation at the level of TrkB and NMDA receptor signals, and thus leading to SCN neuron hyperexcitation. These changes should promote increases in synaptic NR2B-containing NMDA receptors as well as other receptors and second messengers that were not assessed in this project.



Vita

Jonathan Lindsay was born in Knoxville where he graduated from Farragut High School in May of 2005. He then went to college at the University of Tennessee at Knoxville where he majored in Biochemistry, Cellular, and Molecular Biology. Here Jonathan met his lovely wife, Hannah. Jonathan received his BS in biology with a concentration in BCMB in December of 2010. In the fall of 2011, Jonathan began graduate school, remaining at the University of Tennessee at Knoxville. Jonathan rotated in the Roberts, Prosser, and Alexandre labs but ultimately chose Dr. Prosser's lab due to his interest in neuroscience. Jonathan continued his rotation project on the effects of rapid tolerance to alcohol in the suprachiasmatic nucleus where he later published the results of this project in *Alcoholism: Clinical and Experimental Research*. Jonathan's project expanded to studying the cellular differences across rapid tolerance, chronic tolerance, and withdrawal to alcohol. In August of 2015, Jonathan and Hannah were blessed with a daughter, who they affectionately named Rosemary, after Hannah's grandmother. Jonathan finally completed his project on ethanol's effects on the suprachiasmatic nucleus, 7 years after beginning graduate school.