Alcohol alters the expression of Soluble *N*-Ethylmaleimide-Sensitive Factor Attachment Protein Receptors (SNAREs) and spontaneous γ-Aminobutyric Acid (GABA) release via activation of the transcription factor Heat Shock Factor 1 (HSF1)

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Abstract

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Many synapses within the central nervous system are highly sensitive and responsive to ethanol. Although the regulation of postsynaptic receptors by alcohol is well studied, the mechanisms underlying the presynaptic effects of alcohol to alter neurotransmitter release remain relatively unexplored. This dissertation addresses whether alcohol-induced changes in transcriptional activity can promote synaptic vesicle fusion and therefore, neurotransmitter release. To identify a transcriptional pathway by which ethanol can regulate neurotransmitter release, we first investigated the effects of acute alcohol on the expression of genes encoding for synaptic vesicle fusion machinery proteins that form the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) complex. The proteins in this complex reside on the vesicle membrane (synaptotagmin 1 and synaptobrevin/vesicle-associated membrane protein, which is also known as VAMP) and the plasma membrane (syntaxin 1 and synaptosomal associated protein of 25 kDa, which is also known as SNAP-25), and their interactions within the SNARE complex trigger vesicle fusion and neurotransmitter release. We found that ethanol treatment of mouse cortical neurons increased the mRNA and protein expression levels of a subset of SNARE

complex proteins, including synaptotagmin 1 (Syt1) and one of the isoforms of synaptobrevin, VAMP2, but not the other isoform, VAMP1. The gene induction of *Syt1* and *Vamp2* by alcohol occurs via activation of the transcription factor heat shock factor 1 (HSF1), while HSF1 transcriptional activity had no effect on *Vamp1* mRNA levels. We then investigated whether ethanol altered neurotransmitter release in cortical neurons, using whole-cell voltage clamp electrophysiology. We found that alcohol increased γ-aminobutyric acid (GABA) release via HSF1, but had no effect on glutamatergic synaptic vesicle fusion. Collectively, these data indicate that alcohol induction of HSF1 transcriptional activity triggers a specific coordinated adaptation in GABAergic presynaptic terminals that ultimately results in increased GABA release. This molecular mechanism could explain some of the transient changes in synaptic function that occur after alcohol exposure, and may underlie some of the enduring effects of chronic alcohol drinking on local circuitry.

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Abbreviations

AC	Adenylyl cyclase
AMPAR	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
AP-1	Activator protein-1
ARE	Alcohol response element
BAC	Blood alcohol concentration
BNST	Bed nucleus of the stria terminalis
CeA	Central nucleus of the amygdala
CRE	cAMP-response element
CREB	cAMP-response element binding protein
CRF	Corticotropin-releasing factor
CNS	Central nervous system
DIV	Days *in vitro*
GABA	γ-aminobutyric acid
$GABA_AR$	Ionotropic γ-aminobutyric acid receptor
GluR1-4	AMPAR subunit 1-4
Hsf1, HSF1	Heat shock factor 1
Hsp, HSP	Heat shock protein
mEPSC	Miniature excitatory postsynaptic current
mIPSC	Miniature inhibitory postsynaptic current
mPSC	Miniature postsynaptic current
NAc	Nucleus accumbens
NMDAR	N-methyl-D-aspartate receptor
NP rats	Alcohol nonpreferring rats
NPY	Neuropeptide Y
NR1-4	NMDAR subunit 1-4
NRSE	Neuron-restrictive silencer element

NRSF	Neuron-restrictive silencer factor
P rats	Alcohol preferring rats
PFC	Prefrontal cortex
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
qPCR	Quantitative polymerase chain reaction
RE-1	Repressor element-1
REST	Repressor element-1 silencing transcription factor
siRNA	Small interfering RNA
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors
Snap25, SNAP-25	Synaptosomal associated protein of 25 kDa
Sp1	Specificity protein 1
Stx1a	Syntaxin 1A
Syp1	Synaptophysin 1
Syt1	Synaptotagmin 1
TTX	Tetrodotoxin
Vamp1, VAMP1	Synaptobrevin/vesicle-associated membrane protein 1
Vamp2, VAMP2	Synaptobrevin/vesicle-associated membrane protein 2
VTA	Ventral tegmental area

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Introduction[1]

Alcohol is an anxiolytic and sedative drug that has a variety of effects on human behavior and physiology. Drinking alcohol can either be beneficial or negative for adult health, with moderate drinking associated with a lower incidence of cardiovascular problems and higher levels of alcohol use linked to liver and kidney disease. In addition, chronic heavy drinking can lead to problems of physical dependence and tolerance that result from physiological adaptation to the drug and are broadly similar to those associated with addiction to other drugs of abuse.

Despite the negative social and health effects of alcoholism, people have consumed the drug for thousands of years (Li et al., 1977). Until recently, however, the mechanisms by which alcohol affects the central nervous system (CNS) were poorly understood and its actions were considered to be "non-specific" in nature. Recent advances in neuroscience have led to significant progress in our understanding of the neural basis of alcohol intoxication and alcoholism (Chandler et al., 1998; Faingold et al., 1998). In particular, the earlier notion that alcohol exerts its effects purely on membrane lipids has been shown to be inconsistent with the experimental evidence accumulated during the last twenty years.

Instead, the recent progress of research in cellular and molecular neuroscience has resulted in some remarkable advances for alcohol research, most notably the identification of a number of candidate target molecules within the CNS that are sensitive to levels of alcohol relevant to acute human intoxication. Advances in our understanding of the mechanisms of neuronal excitability

[1] Part of this introduction was adapted from the published review listed below. I am grateful to Leonardo Pignataro and Neil Harrison for their contributions to the ideas, text and figures of this chapter.
Pignataro L, Varodayan FP, Tannenholz LE, Harrison NL (2009) The regulation of neuronal gene expression by alcohol. *Pharmacology and Therapeutics* **124**(3), 324-335.

and synaptic transmission have shown that ethanol interacts with a variety of neurotransmitter systems, including glutamate (Crowder et al., 2002; Lovinger et al., 1989; Valenzuela and Cardoso, 1999; Woodward, 2000), dopamine (Brodie et al., 1990; Budygin et al., 2001; Gessa et al., 1985; Imperato and Di Chiara, 1986), adenosine (Dar et al., 1983; Diao and Dunwiddie, 1996), and γ-aminobutyric acid (GABA) (Cagetti et al., 2003; Charlton et al., 1997; Follesa et al., 2004a; Follesa et al., 2004b; Sanna et al., 2003; Wu et al., 1995). The actions of ethanol on these systems lead to widespread fluctuations in neurotransmission and may converge to produce the varied behavioral effects of acute alcohol ingestion.

As far as the effects of chronic alcohol intake are concerned, a consensus has formed in the field that the development of alcohol tolerance and dependence result from alterations in brain structure and function over time. Synapses are generally regarded as the most sensitive sites of ethanol action within the CNS, and considerable evidence suggests that over time the transient molecular changes that accompany a single alcohol exposure can persist, as individual neurons respond to each and every alcohol exposure in a systematic and coordinated manner (Koob, 2006; Nestler, 2001). This remodeling of synaptic connections after chronic alcohol exposure is likely to depend upon changes in gene expression, initiated by each alcohol presentation (Koob, 2006; Mulligan et al., 2006; Nestler, 2001; Wilke et al., 1994). In this respect, the adaptations to alcohol resemble to some extent the brain plasticity that is known to occur during long-term exposure to other drugs of abuse, such as cocaine and heroin (Koob, 2004; Nestler, 2005). Brain areas of interest to researchers in the field are therefore those associated with the brain's reward system (ventral tegmental area, nucleus accumbens) as well as the circuits associated with

higher-order cognitive processes, such as decision making and impulse control (prefrontal cortex), levels of arousal (thalamus) and anxiety (amygdala).

The profound adaptations that lead to alcohol abuse and dependence, however, presumably result from a complex chain of events that is initiated in the brain of the drinker long before a state of dependence is reached. Several studies have shown that even a brief exposure to alcohol can modify the expression of a variety of genes in animal models (Bachtell et al., 1999). For example, it has been demonstrated in several strains of mice that a single exposure to low doses of ethanol can induce the expression of immediate-early genes (IEGs) such as *c-fos* (Demarest et al., 1999; Eisenman et al., 2002; Hitzemann and Hitzemann, 1997; Zoeller and Fletcher, 1994). These immediate-early genes encode nuclear proteins with transcriptional activity, which in turn regulate the expression of many other genes. The activation of these transcription factors may be the earliest events in a sequence of changes in gene expression that ultimately result in functional alterations of critical brain circuitry. These functional changes may underlie some aspects of the behavioral tolerance and adaptation that occur in the advanced stages of chronic alcoholism.

Short-term effects of a single alcohol exposure

Alcohol is an anxiolytic and sedative drug that has a variety of dose-dependent effects on human behavior and physiology. Low blood alcohol concentration (BAC) levels of 0.01-0.08 g%, which are equivalent to an ethanol concentration of 2-17 mM, are associated with mild euphoria, increased confidence and assertiveness, anxiety relief and some disinhibition (Koob and Le Moal, 2006; Naranjo and Bremner, 1993). A BAC level of 0.08 g% is set as the limit after which

driving is not allowed across the United States (Freeman, 2007), and a non-dependent 170 pound male would need to consume 4 standard size drinks within 1 hour to reach this limit, while a 140 pound female would need 3 drinks in one hour to reach a BAC of 0.08 g% (NHTSA, 1992). More moderate BAC levels of 0.08-0.15 g% (17-33 mM ethanol) lead to pronounced mood swings and significant disinhibition, as well as impaired judgment, cognition and motor function (Koob and Le Moal, 2006; Naranjo and Bremner, 1993). To reach the upper limits of this range, a non-dependent 170 pound male would need to consume over 6 drinks and a 140 pound female would need to consume 5 drinks in one hour, which are levels commonly defined as binge drinking (Wechsler et al., 2002).

Even heavier bouts of drinking can cause emotional instability, ataxia (including staggering and slurred speech), hypnosis (including impaired sensory response and reaction time), and memory impairments, which are the hallmarks of BAC levels in the range of 0.15-0.30 g% (33-65 mM ethanol) (Koob and Le Moal, 2006; Naranjo and Bremner, 1993). At the higher end of this BAC range, the individual may experience post-intoxication "blackouts", where they are unable to remember events that occurred during the intoxication period. The actions of alcohol as a general anesthetic emerge at the extremely high BAC levels of 0.30-0.40 g% (65-87 mM ethanol), and many of the individuals who drink heavily enough to reach these BAC levels will enter a coma (Koob and Le Moal, 2006; Naranjo and Bremner, 1993). Finally, the lethal dose of alcohol for 50% of the non-dependent population (LD_{50}) is 0.40-0.50 g% (87-109 mM ethanol), though some long-term alcoholics routinely tolerate extremely high blood alcohol concentration levels of over 100 mM ethanol (Urso et al., 1981). Therefore, the behavioral and physiological effects of a single alcohol exposure vary in a dose-dependent manner, ranging from mild levels of

euphoria and anxiolysis to coma and death. In addition to these behavioral and physiological effects of alcohol drinking, a single alcohol exposure can also alter molecular pathways within brain cells and some of these changes may contribute to the development of alcohol abuse and dependence disorders (Koob, 2006; Mulligan et al., 2006; Nestler, 2001; Wilke et al., 1994).

Physiological adaptation to alcohol

In the case of alcohol dependence, it is presumed that alcohol intake induces a series of functional changes that can profoundly alter the activity of the nervous system, and that these changes are manifested both psychologically and physiologically in the alcoholic patient. This process occurs in two stages, as with the development of addiction to other drugs (Koob et al., 1998). The initial behavioral changes are subtle and manifest as alterations in decision making by the individual, leading to increased alcohol intake and frequency of drinking. These initial functional changes may then be consolidated so that the CNS becomes "hard-wired" for a drug-seeking state that is maintained over a long period. As heavier drinking becomes routine, eventually more profound physiological changes occur that lead to a state of enhanced CNS excitability, perhaps in order to counteract the depressant actions of alcohol on the brain. Extreme examples of this process of physiological adaptation to alcohol are observed in the biology of alcoholic individuals. Some long-term alcoholics routinely tolerate extremely high blood alcohol concentrations of over 100 mM, which would cause severe intoxication, profound sedation or death in naïve individuals (Urso et al., 1981). The intense withdrawal symptoms seen in long-term alcoholics also provide strong evidence for the extensive nature of these adaptations; normal CNS activity simply becomes impossible when alcohol is withdrawn from

the chronic drinker, indicating a state known as physical dependence. In addition, the physical manifestations of alcohol withdrawal syndrome (*delirium tremens*, extreme anxiety, hyperalgesia, autonomic activation, physical irritability, and seizures) strongly suggest a shift towards increased neuronal excitability in the alcoholic brain (Veatch and Gonzalez, 1996). The development of extreme behavioral tolerance to alcohol and the intense withdrawal symptoms observed in abstinent alcoholics reveal that the brain undergoes significant functional adaptations in order to cope with the continued presence of the drug. It is likely that some of this systemic adaptation to chronic alcohol intake stems from the accumulation and persistence of individual molecular changes that accompany each and every single alcohol exposure (Koob, 2006; Nestler, 2001).

The key circuitry that governs the development of alcohol dependence

The transition from alcohol use and abuse to dependence is often associated with changes in brain circuitry thought to control motivational state and higher-order cognitive processing (Koob and Le Moal, 2008). Non-dependent individuals tend to exhibit occasional impulse control behaviors, such as increased tension prior to drinking and gratification upon drinking, while chronic alcoholic patients often act compulsively by engaging in repetitive cycles of anxiety and stress prior to drinking and relief upon drinking (APA, 1994). This progressive shift from positive reinforcement to negative reinforcement largely involves the opponent systems of the mesolimbic reward circuitry and the extended amygdala, with overarching "top-down" control on these limbic systems exhibited by the prefrontal cortex (PFC) (Koob and Le Moal, 2005).

In the early stages of alcohol use, a drinker encounters the positive reinforcing effects of alcohol, such as mild euphoria and anxiety relief. These hedonic emotions are thought to result from changes in the brain's natural reward system and are considered strong motivation for future drinking episodes. At the core of the mesolimbic circuitry underlying the reward system is the dopaminergic medial forebrain bundle that projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and PFC (blue straight arrows, Introductory Fig. 1)(Koob, 2005). GABAergic neurons in the VTA also project to the NAc and PFC (red straight arrows, Introductory Fig. 1), and the VTA receives innervation from GABAergic neurons in the NAc (red straight arrows, Introductory Fig. 1) and glutamatergic neurons in the PFC to complete the circuit (green straight arrows, Introductory Fig. 1).

Early studies determined that intoxicating doses of alcohol increased the firing rate of dopaminergic neurons in the VTA, leading to an increase in dopamine release in the NAc (Brodie et al., 1990; Di Chiara, 1998; Gessa et al., 1985). Furthermore, rats genetically selected for alcohol-preferring behaviors (P rats) self-administered ethanol directly into the VTA (Gatto et al., 1994) and infusion of $GABA_A$ receptor antagonists into this region blocked this alcohol intake (Nowak et al., 1998). It is thought that alcohol acts in the VTA to decrease GABAergic interneuron activity and thereby disinhibit dopaminergic neurons, leading to increased dopamine release in the NAc and PFC. Excessive dopamine release in the PFC impairs cognitive function in rodents and monkeys (Arnsten and Goldman-Rakic, 1998; Murphy et al., 1996; Zahrt et al., 1997) and could explain the disinhibition experienced by individuals engaging in a single drinking exposure (BAC levels 0.08-0.15 g%) (Koob and Le Moal, 2006; Naranjo and Bremner, 1993).

As drinkers continue these patterns of high alcohol intake, they will then enter a negative reinforcement state where the motivation to drink stems from a need to ward off the aversive effects of withdrawal. At this stage, the recruitment of brain and hormonal stress systems, termed the antireward system as it limits reward, lead to a shift in the reward set point and the appearance of a negative affective state. The extended amygdala, comprising the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST), is central to this process as it builds associations between previously neutral stimuli and reward or aversion (Holland and Gallagher, 2004). The CeA and BNST contain reciprocal innervations for GABA (red wavy arrows, Introductory Fig. 1) and neuropeptide transmitters, such as corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) (yellow wavy arrows, Introductory Fig. 1) (Gilpin, 2012; Kash, 2012). In addition, the extended amygdala receives significant glutamatergic projections from the PFC and hippocampus (green wavy arrows, Introductory Fig. 1), and dopamingeric and serotonergic inputs from the VTA, lateral hypothalamus and brainstem (blue wavy arrows, Introductory Fig. 1). The extended amygdala also sends inhibitory efferents back to the lateral hypothalamus, VTA and various lower brainstem regions (red wavy arrows, Introductory Fig. 1) (Gilpin, 2012; Heimer and Alheid, 1991; Kash, 2012).

As an individual develops alcohol dependence, the extended amygdala circuitry may become compromised. This leads to a shift in brain reward thresholds (i.e. decreased reward valence), as seen in alcohol dependent rats withdrawn for a few hours to two days (Schulteis et al., 1995). This shift to the negative affective state is accompanied by decreased dopamine and serotonin

neurotransmission in the NAc (Weiss et al., 1996), and decreased GABA and opioid transmission in the CeA of alcohol dependent animals experiencing withdrawal (Koob, 2003). The compensatory depletion of dopamine stores and hypersensitivity of dopamine receptors in the PFC (Fadda et al., 1980) leads to an increased dopaminergic reward response to subsequent alcohol administration in alcoholic patients, as well as feelings of craving and loss of control (Koob and Le Moal, 2006; Modell and Mountz, 1995; Modell et al., 1990; Modell et al., 1993; Naranjo and Bremner, 1993).

The PFC plays a critical role in managing the responses of the mesolimbic circuitry and extended amygdala, and alcohol exposure can lead to frontal cortical dysfunction that reduces "top-down" inhibitory response control on these limbic systems (Abernathy et al., 2010; Jentsch and Taylor, 1999). As the PFC has high functional and structural adaptability, alcohol intake is able to affect immediate goal-directed behavior, as well as mediate future cognitive processing. For example, acute alcohol administration causes immediate PFC-mediated cognitive deficits such as decreased performance in spatial recognition and planning tasks (Weissenborn and Duka, 2003) and poor decision making in a gambling task (George et al., 2005). Furthermore, alcoholic patients suffer from deficits in executive function that depend on the PFC and show reduced cortical matter. In particular, alcohol-dependent patients showed deficits in gambling tasks similar to patients with ventromedial PFC lesions (Bechara et al., 2001) and poor performance in the Wisconsin Card Sorting Task that correlates with reduced glucose metabolism in the medial PFC (Adams et al., 1993). These patients also show reduced gray matter in the dorsolateral PFC (Jernigan et al., 1991) and reduced cortical white matter, especially in the frontal lobe (de la Monte, 1988; Pfefferbaum et al., 1997). Therefore, the characteristics of the PFC that make it

especially suited for higher-order cognitive processing, such as its high structural and functional adaptability, also make the PFC prone to the addictive effects of alcohol as its mediation of goal-directed behavior is influenced by past alcohol experiences (Abernathy et al., 2010; Jentsch and Taylor, 1999).

Actions of alcohol on the synapse

The synapse is the crucial structure within the CNS that allows a neuron to transmit a chemical signal to another neuron, forming a brain circuit. Signal transmission between these neurons can be excitatory, inhibitory or modulatory, depending on the neurotransmitter released from the presynaptic terminal and the receptors expressed on the postsynaptic terminal. The chemical synapse is comprised of three main compartments: synaptic vesicles recruited to the presynaptic active zone, the synaptic cleft, and the postsynaptic specialization within a dendritic spine, each of which play a key role in mediating the type and amount of information transfer (Introductory Fig. 2).

Within the presynaptic terminal, synaptic vesicles are loaded with neurotransmitter, transported to the active zone and fused with the plasma membrane to release their contents into the synaptic cleft (Sudhof, 1995). A variety of proteins embedded in the vesicle membrane mediate these processes, including neurotransmitter transporters and proton pumps that load the vesicle by active transport (step 1, Introductory Fig. 2). The vesicle is trafficked through the interactions of its membrane proteins with the actin cytoskeleton and is tethered near the release site by the vesicle membrane protein synapsin (step 2, Introductory Fig. 2) (Shupliakov et al., 2011). The

synaptic vesicles are docked within the active zone by the interactions among a complement of proteins, including soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) which are located both on the vesicle membrane (v-SNAREs: synaptobrevin/vesicle-associated membrane protein, which is also known as VAMP) (blue, Introductory Fig. 2) and the target plasma membrane (t-SNAREs: syntaxin 1 and synaptosomal associated protein of 25 kDa, which is also known as SNAP-25) (green and red, respectively, step 3, Introductory Fig. 2). During docking, the vesicle membrane protein synaptotagmin 1 (yellow, Introductory Fig. 2) binds to VAMP and plasma membrane phospholipids (Martens et al., 2007). This pulls the two membranes into closer proximity and promotes helical zippering between VAMP, syntaxin 1 and SNAP-25 (step 4, Introductory Fig. 2). When an action potential reaches the presynaptic terminal, the membrane depolarization opens voltage-gated calcium channels and calcium enters the terminal. The calcium ions bind to synaptotagmin 1, causing a conformational change across the SNARE complex, directly triggering vesicle fusion and neurotransmitter release (step 5, Introductory Fig. 2). Membrane proteins, such as synaptophysin, are also involved in the rapid endocytosis of vesicles to replenish the readily releasable pool of vesicles (Kwon and Chapman, 2011).

Released neurotransmitter traverses across the synaptic cleft by diffusion and binds to neurotransmitter receptors contained within the postsynaptic specialization (step 6, Introductory Fig. 2). The postsynaptic specialization contains a specific complement of neurotransmitter receptors, ion channels, signaling proteins, and scaffolding proteins, which define the postsynaptic neuronal response. This entire postsynaptic specialization can exist within a dendritic spine, a small membranous protrusion from the dendrite that creates a compartment

based on its physical shape (Harris, 2001). The shape of the spine defines a chemical microdomain where large rapid rises in signaling molecules can occur in response to neurotransmitter release, leading to alterations in the sensitivity of the postsynaptic neuronal response (Kasai et al., 2003). Given the importance of the entire synaptic structure to normal CNS function, the effects of alcohol on any particular step of neurotransmission can alter neuronal communication and may lead to compensatory changes within the synapse and across local circuits.

Actions of alcohol on postsynaptic terminal structures

Given the diversity of proteins comprising the synaptic structure, it is not surprising that the effects of alcohol are highly variable, depending on the length of alcohol exposure, cell type and brain region. One consistent finding, however, is that alcohol reduces the number of postsynaptic specializations and spines. *Post mortem* studies of cortical tissue from alcoholic patients showed reductions in the density of dendritic spines on layer V pyramidal neurons (Ferrer et al., 1986) and reduction of dendritic arborization in layer III pyramidal neurons (Harper and Corbett, 1990). Similar morphological changes have been observed in the rat hippocampal CA1 region in withdrawn animals that consumed alcohol for five months (McMullen et al., 1984). Reductions in spine density are generally considered to reflect decreases in synapse number (Moser et al., 1994), and may result from reorganization of the actin cytoskeleton (Bonhoeffer and Yuste, 2002). For example, it has been observed in rat hippocampal cultures that withdrawal after two days of 50 mM ethanol treatment decreased spine number, as indicated by a reduction in colocalized filamentous actin (F-actin) and post-synaptic density protein-95 (PSD95) staining, in

addition to decreasing the size of retained dendritic spines (Carpenter-Hyland and Chandler, 2006). As postsynaptic densities and spines define the functional capabilities of the neuron to receive neurotransmission signals, the effects of alcohol exposure on the postsynaptic specializations alter neuronal communication and thus, local circuitry.

Actions of alcohol on presynaptic terminal structures

Relatively little is known about how ethanol regulates presynaptic terminal structures, but a few studies indicate that alcohol administration may alter presynaptic terminal number. Detailed *post mortem* analysis of the brains of heavy drinkers showed consistent loss of the presynaptic marker, synaptophysin, in layers I and II of the frontal cortical area 10 of Brodman (Brun and Andersson, 2001). In addition one month of chronic intermittent ethanol exposure in rats reduced the number of presynaptic terminals in the CA3 region of the hippocampus, while one month of continuous treatment caused an increase in hippocampal dentate gyrus subgranular presynaptic terminals (Lundqvist et al., 1994). Finally, four days of chronic 25-100 mM ethanol treatment of primary hippocampal rat culture also increased the number of clusters labeled with synapsin and synaptophysin, representing either an increase in the total number or presynaptic terminals or an expansion of cluster, and thus presynaptic terminal, size (Carpenter-Hyland et al., 2004). These effects of alcohol to alter the presynaptic terminal represent a pathway by which alcohol may affect neuronal communication and thus, local circuitry.

Actions of alcohol on postsynaptic terminal functions

In addition to the effects of alcohol on postsynaptic specializations, acute alcohol exposure also alters postsynaptic terminal function by directly affecting the function of individual proteins, such as ligand-gated ion channels. In particular, direct application of alcohol regulates, within seconds to minutes, the function of ligand-gated γ-aminobutyric acid receptors ($GABA_A$Rs) and the family of ionotropic glutamate receptors, including α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs). Chronic alcohol exposure, on the other hand, often leads to changes in ligand-gated ion channel expression and localization profiles, as the postsynaptic terminal compensates for the acute effects of the drug.

The effects of alcohol on $GABA_A$ receptors

Although the behavioral effects of alcohol present a complex picture, many of its actions are shared by other drugs that modulate the actions of GABA; for example, the benzodiazepines produce a similar spectrum of anxiolytic, hypnotic and sedative effects as alcohol (Koob, 2004). For this reason, it has long been hypothesized that ethanol achieves some of its behavioral effects via regulation of the $GABA_A$ receptor. The $GABA_A$R is a member of the cys-loop ligand-gated ion channel superfamily and mediates both fast inhibitory synaptic transmission and a form of tonic extrasynaptic inhibition in the central nervous system (Farrant and Nusser, 2005; Olsen and Sieghart, 2008; Olsen and Sieghart, 2009). The $GABA_A$R is a heteromeric assembly of five subunits (α1-6, β1-3, γ1-3, δ, ε, π and θ) that is encoded by nineteen genes. The most common

$GABA_AR$s contain two α, two β and either a γ2 or δ subunit with the specific subunit composition conferring properties of localization and ligand sensitivity on the receptor. The α1β2γ2 $GABA_AR$s are the most abundant across the CNS, and are highly expressed within the cortex, hippocampus, amygdala, basal ganglia, thalamus, brainstem and cerebellum (Sieghart and Sperk, 2002). These synaptic receptors have a moderate affinity for GABA and mediate phasic inhibitory currents in the presence of high concentrations of GABA. While δ-containing $GABA_AR$s are highly expressed in similar brain regions, like the cerebellum, dentate granule layer of the hippocampus, some cortical areas, thalamus, and the striatum (Sieghart and Sperk, 2002), the subunit partners with the α4 subunit (Korpi et al., 2002; Sur et al., 1999), or in the cerebellum with α6 (Jones et al., 1997; Pirker et al., 2000) to form extrasynaptic receptors (Nusser et al., 1998). As these receptors exhibit a high affinity for GABA, (Saxena and Macdonald, 1994; Wohlfarth et al., 2002), they are activated by the ambient levels of GABA present outside the synaptic cleft and generate a basal tonic inhibitory current (Mody, 2001).

Acute alcohol generally potentiates $GABA_AR$ function in the presence of the agonist GABA, with enhanced receptor activity observed in tissue preparations from the cerebellum (Allan and Harris, 1986), dorsal root ganglion (Nakahiro et al., 1991; Nishio and Narahashi, 1990), cortex (Allan and Harris, 1986; Reynolds and Prasad, 1991; Reynolds et al., 1992; Suzdak et al., 1986), hippocampus (Aguayo, 1990; Reynolds and Prasad, 1991) and spinal cord (Ticku et al., 1986). This ethanol enhancement of channel activity occurs at both synaptic and extrasynaptic $GABA_AR$s and is thought to be mediated by either increased probability of the channel opening (Zhou et al., 1998) or increased affinity of the agonist for the receptor (Tonner and Miller, 1995; Welsh et al., 2009). These effects of alcohol on $GABA_AR$s are linked to changes in protein

phosphorylation by protein kinase C (PKC). Ethanol enhancement of synaptic γ2-containing $GABA_AR$s is specifically mediated by the PKCε subunit (Qi et al., 2007), while alcohol potentiation of tonic currents in the cerebellum (Hanchar et al., 2005), hippocampus (Wei et al., 2004), and thalamus (Jia et al., 2007) is dependent on PKCδ activity (Choi et al., 2008). As enhanced $GABA_AR$ function will increase neuronal inhibition, these potentiating actions of acute alcohol on $GABA_AR$s may underlie some of the anxiolytic, sedative and ataxic properties of the drug.

Many of the effects of acute ethanol treatment on the GABAergic system are tolerated after chronic ethanol exposure, leading to a decrease in the intoxicating effects of the drug. Decreased $GABA_AR$ function after chronic ethanol treatment has been observed in the cortex (Morrow et al., 1990; Sanna et al., 2003), nucleus accumbens (Szmigielski et al., 1992) and spinal cord (Mehta and Ticku, 1988; Ticku et al., 1986). As the total number of $GABA_AR$s does not change during this long-term exposure, $GABA_AR$ functional tolerance may derive from changes in the expression profiles of distinct $GABA_AR$ subunits (Grobin et al., 1998). Investigation of this question has led to contradictory results with two exceptions: one to eight hours of ethanol exposure modestly decreased expression of the gene encoding the α1 subunit (*Gabra1*), while the α4 subunit gene (*Gabra4*) shows a remarkable degree of induction (Cagetti et al., 2003; Devaud et al., 1997; Devaud et al., 1995; Liang et al., 2007; Liang et al., 2008; Liang et al., 2006; Petrie et al., 2001; Sanna et al., 2003). Similarly, alcohol increases the surface expression of the $GABA_AR$ α4 and γ2 subunits and decreases α1 and δ subunits, leading to a situation where newly-formed α4βγ2 $GABA_AR$s may "crowd" α1βγ2 $GABA_AR$s out of the synapse to alter $GABA_AR$ sensitivity to ethanol (Liang et al., 2007). These changes in $GABA_AR$ subunit

composition are also observed in chronic intermittent ethanol treated rats (Liang et al., 2007), suggesting that they play a role in homeostatic adaptation to the continued presence of the drug. As the GABAergic system mediates inhibition across the CNS, both through phasic neurotransmission and by creating a tonic tone, the effects of alcohol this system are likely to play a role in the development of alcohol use disorders and the hyperexcitability experienced by alcoholics experiencing withdrawal.

The effects of alcohol on ionotropic glutamate receptors

The excitatory neurotransmitter L-glutamate acts on NMDA and AMPA receptors to mediate most fast excitatory synaptic transmission. The NMDAR exists in the brain as a heteromeric complex composed of the essential NMDAR1 (NR1) subunit in combination with NMDAR2 (NR2A-D) subunits, although additional NMDAR3 (NR3A-B) subunits have been reported (Cull-Candy et al., 2001). The stoichiometry of the receptor is a tetramer of two NR1 and two NR2 subunits. Selective splicing of NR1 transcripts and the differential expression of NR2 subunits leads to the generation of multiple receptor isoforms with distinct regional distributions and biophysical and pharmacological properties. NMDAR activation requires simultaneous binding of two co-agonists, glutamate and glycine, and the receptor is also "gated" by virtue of a voltage-dependent block by Mg^{2+} (Mayer and Westbrook, 1984; Nowak et al., 1984). Removal of the Mg^{2+} block by membrane depolarization allows influx of Na^{+} and Ca^{2+}, leading to additional depolarization and generating a slow excitatory postsynaptic potential (EPSP) in the neuron (Forsythe and Westbrook, 1988).

Acute ethanol exposure inhibits the excitatory action of glutamate at postsynaptic NMDA receptors (Calton, 1998; Criswell et al., 2003; Hoffman et al., 1989; Loftis and Janowsky, 2003; Lovinger et al., 1989; Martin et al., 1995; Nie et al., 1994; Roberto et al., 2004b; Tabakoff and Hoffman, 1996; Tsai and Coyle, 1998; Woodward, 1999) and there are differences in the ethanol sensitivity of the receptor based upon its subunit composition. The effects of acute alcohol treatment tend to be more potent in NR1/NR2A or NR1/NR2B receptors than in NMDARs containing NR1/NR2C (Chu et al., 1995; Masood et al., 1994). In contrast, chronic ethanol treatment increases NMDAR function (Cebere et al., 1999; Gulya et al., 1991; Läck et al., 2007; Smothers et al., 1997), particularly enhancing the current mediated by NR2B-containing receptors (Floyd et al., 2003; Kash et al., 2009; Roberto et al., 2005; Roberto et al., 2004b). These effects of chronic ethanol treatment are accompanied by increased NR2B mRNA and protein expression (Follesa and Ticku, 1995; Hu et al., 1996; Kash et al., 2009; Roberto et al., 2005; Snell et al., 1996), suggesting that changes in subunit composition of NMDARs may contribute to the increased functional response. Changes in the subcellular localization of NMDARs may also underlie some of the effects of chronic ethanol on receptor function, as ethanol exposure increased the trafficking of preexisting NR2B-containing NMDARs to dendritic spines (Carpenter-Hyland et al., 2004). These changes in NMDAR expression and localization presumably offset the effects of acute ethanol and may be one of the mechanisms that contribute to hyperexcitability during alcohol withdrawal (Hendricson et al., 2007; Nelson et al., 2005).

Much less is understood about the effects of alcohol on AMPA receptors, which contain four potential subunits (GluR1-4) arranged in two sets of identical dimers. Acute alcohol inhibits

AMPAR function (Akinshola et al., 2001; Akinshola et al., 2003; Dildy-Mayfield and Harris, 1992; Nieber et al., 1998), but this inhibition is much less potent compared to the inhibition of NMDAR function (Frye and Fincher, 2000) and does not appear to be dependent on the subunit composition (Lovinger, 1993). The effects of chronic ethanol treatment on AMPARs are also not well characterized, with some groups reporting evidence of increased AMPAR subunit expression (Chandler et al., 1999) and function (Läck et al., 2007; Netzeband et al., 1999), while other studies identified no changes (Smothers et al., 1997). This variation may be attributed to the ethanol exposure model employed and brain region of interest studied. As glutamatergic neurotransmission is central to the process of synaptic plasticity (Kauer and Malenka, 2007), learning and memory (McEntee and Crook, 1993), and higher-order cognition (Watson et al., 2009), the effects of alcohol on this system may play an important role in the development of alcohol use disorders.

Actions of alcohol on presynaptic terminal functions

Only over the past decade have researchers observed that alcohol can stimulate neurotransmitter release. Acute application of ethanol increases GABA release in the CA1 region of the hippocampus (Carta et al., 2003), NAc (Crowder and Weiner, 2002), cerebellum (Carta et al., 2004), CeA (Roberto et al., 2003), VTA (Theile et al., 2008) and substantia nigra pars reticular (Criswell et al., 2008). In these experiments, GABA release increased rapidly with ethanol administration and recovered following drug washout. A parallel study conducted *in vivo* showed a similar increase in GABA release in the VTA of mice injected intraperitoneally with ethanol one day prior to electrophysiology recording (Melis et al., 2002). In addition, Roberto et al.

(2004) reported an increase in GABA release within the CeA of chronically ethanol-treated rats, which was further increased upon acute application of ethanol. This suggests that the chronically ethanol-treated animals did not develop tolerance to the acute effects of ethanol on GABA release, and that separate mechanisms underlie the increases in GABA release that accompany acute and chronic alcohol exposures.

The mechanisms underlying acute ethanol enhancement of GABA release are not yet fully elucidated, but consistent pathways involving G-protein coupled receptors (GPCRs) and protein kinases are emerging. In cerebellar interneurons, increased GABA release after ethanol application is mediated via activation of both the adenylyl cyclase (AC)/protein kinase A (PKA) and phospholipase C (PLC)/PKC pathways and internal calcium store release (Kelm et al., 2007; Kelm et al., 2008; Kelm et al., 2010). In addition, PKCε activity in the CeA leads to CRF binding to the CRF_1 receptors on presynaptic GABA terminals to stimulate neurotransmitter release (Bajo et al., 2008; Nie et al., 2004). These GPCR-mediated pathways provide for a relatively fast GABAergic neuronal response after acute alcohol administration, but the enhanced GABA release that occurs after chronic ethanol exposure is likely to be regulated by longer-lasting changes in gene expression.

There have been relatively few studies investigating the effects of alcohol on glutamate release. One study, however, determined that acute alcohol administration increased glutamate release in the VTA via activation of presynaptic dopamine D1 receptors (Xiao et al., 2008). Other groups have found ethanol inhibition of glutamate release in spinal motor neurons (Ziskind-Conhaim et

al., 2003) and hippocampal neurons (Maldve et al., 2004; Moriguchi et al., 2007). As a whole, these studies have found that various models of alcohol administration can alter neurotransmitter release across multiple cell types, both *in vitro* and *in vivo*, suggesting that these effects of alcohol on the presynaptic terminal may play a critical role in neuronal adaption to the drug.

Molecular mechanisms of synaptic gene regulation by alcohol

Neuronal gene expression is constantly modulated in a precise, coordinated manner throughout development, and in response to external stimuli. In the simplest level of gene regulation, activators and repressors bind to their corresponding elements relatively near the transcription start site and either physically promote or inhibit local transcription. In addition, these regulation factors can bind to more distant *cis*-elements to promote gene expression within specific neuronal populations and silence genes in non-neuronal cells (Quinn, 1996). As the promoter sequences of eukaryotic genes contain multiple transcription factor binding sites, different signaling pathways can converge in a coordinated manner to regulate gene expression. Therefore, the complement and relative positioning of these diverse elements define the promoter architecture, and presumably determine how a given promoter responds to the many transcription factors in the cell.

Despite numerous studies on the effects of alcohol on gene expression by alcohol, the mechanisms of action are known for only a few alcohol-responsive genes. In the rest of this section, we will summarize studies in which detailed analysis of the gene promoter regions have identified *cis*-elements and transcription factors mediating ethanol induction of gene expression.

These signaling pathways are likely to be generalizable to a subset of other alcohol-responsive genes, suggesting that a single alcohol exposure can lead to a coordinated transcriptional response.

Alcohol regulation of gene expression via neuron-restrictive silencer element (NRSE)/repressor element-1 (RE-1)

As discussed earlier, detailed investigation into the effects of ethanol on NMDA subunit expression has revealed that the NR2B subunit is highly regulated by ethanol (refer to earlier section: The effects of alcohol on ionotropic glutamate receptors). Analysis of the regulatory sequence of the mouse NR2B gene (*Grin2b*) has uncovered five highly conserved putative neuron-restrictive silencer element (NRSE) binding sites between –1407 and –2741 bp (Introductory Fig. 3) (Qiang et al., 2005). The NRSE, also known as repressor element-1 (RE-1), has been identified as a key regulator in eukaryotic gene regulation (Ogbourne and Antalis, 1998) that blocks the expression of numerous neuronal-specific genes in non-neuronal cells (Chong et al., 1995; Kraner et al., 1992; Mori et al., 1992; Mori et al., 1990; Paquette et al., 2000; Schoenherr and Anderson, 1995). Other examples of this type of non-neuronal repression include the silencing of genes that encode the voltage-dependent sodium channel (Chong et al., 1995), the synaptic vesicle membrane protein synapsin I (Li et al., 1993), the m4 muscarinic acetylcholine receptor (Wood et al., 1996) and the glutamate receptor subunit GluR2 (Huang et al., 1999). Several studies have shown that this type of transcriptional repression of neuron-specific genes can be mediated by the neuron-restrictive silencer factor (NRSF)/repressor element-1 silencing transcription factor (REST) binding to the NRSE (Bessis et al., 1997; Chong

et al., 1995; Huang et al., 1999; Palm et al., 1998; Palm et al., 1999; Schoenherr and Anderson, 1995; Wood et al., 1996). Specifically, NRSF interacts with the NRSE2 and 3 binding sites of the NR2B gene to repress expression (red box, Introductory Fig. 3) (Qiang et al., 2005), and 100 mM ethanol treatment for five days reduced NRSF expression in neurons and increased NR2B promoter activation (Qiang et al., 2005). As the NRSF/NRSE transcriptional repression system is widely utilized throughout the genome (Schoenherr and Anderson, 1995), it is likely that alcohol exposure disinhibits the expression of several other alcohol-responsive genes as well.

Alcohol regulation of gene expression via cAMP-response element (CRE) and its complementary binding protein (CREB)

The alcohol sensitivity of two other alcohol-responsive genes, tyrosine hydroxylase (*TH*) (Gayer et al., 1991) and dopamine β-hydroxylase (*DBH*) (Hassan et al., 2003) has been found to be governed by cyclic AMP (cAMP)-response element (CRE) binding sites. The enzymes encoded by these two genes are critical for neurotransmitter synthesis (dopamine and norepinephrine, respectively) and are therefore, of obvious importance to the neuropharmacology of alcohol. Specifically, deletion analysis of the 5'-proximal region (–262 to –142 bp) of the human *DBH* gene revealed that alcohol induction of the gene is controlled by a pathway involving the interaction of the complementary binding protein (CREB) with CRE sites. These findings have been extended to a wider set of alcohol-responsive genes that contain CRE sequences within their promoter regions, including *TH* (Hassan et al., 2003) and *Grin2b* (Klein et al., 1998; Rani et al., 2005). Ethanol regulation of *Grin2b* expression is specifically mediated by the CRE site located in the upstream region –410 to –403 bp (blue oval, Introductory Fig. 3) (Klein et al., 1998). Mutation of this site prevents interaction with CREB, and abolishes the stimulatory effect

of chronic ethanol treatment on *Grin2b* expression (Rani et al., 2005). In addition, ethanol treatment is known to increase levels of activated CREB by mediating its Ser133 phosphorylation *via* environmental-regulated kinase (ERK) (Rani et al., 2005). Therefore, alcohol induces a subset of neuronal alcohol-responsive genes via the CRE/CREB transcriptional system, allowing for a coordinated gene response to the presence of the drug.

Alcohol regulation of gene expression via specificity protein 1 (Sp1) and activator protein-1 (AP-1)

Ethanol treatment also regulates the expression of genes encoding several classes of molecular chaperones and proteins that bind to nascent polypeptides to facilitate correct folding (Hsieh et al., 1996; Miles et al., 1991; Miles et al., 1994; Wilke et al., 2000). In particular, ethanol increased heat shock cognate protein 70 (*Hsc70,* also known as *Hsp73*) gene expression in neuroblastoma-glioma hybrid cells (NG108-15 cells) (Miles et al., 1991). *Hsc70* is a member of the heat shock protein (*Hsp*) family, and has 85% identity with human *Hsp70*. The promoter of the *Hsc70* gene has three putative specificity protein-1 (Sp1) transcription factor-binding sites localized at –263, –173 and –67 bp upstream from the transcription initiation site and overlapping with two sets of heat shock regulatory elements (HSE) (Introductory Fig. 4). In particular, the Sp1 site located at –67 bp is necessary for *Hsc70* gene sensitivity to ethanol (red oval, Introductory Fig. 4) and increased expression of Sp1 transcription factor enhanced the transcriptional response induced by alcohol (Wilke et al., 2000). Further studies revealed that the Sp1 site is not sufficient for *Hsc70* gene regulation by alcohol and that the sequence context of the gene's promoter region may contribute to its alcohol sensitivity (Wilke et al., 2000). The

overlap of two HSE sites with the Sp1 site at –67 bp suggests that Sp1 may interact with the transcription factor heat shock factor 1 (HSF1) to confer the observed ethanol sensitivity of the *Hsc70* gene.

The NR2B promoter also includes 4 Sp1 sites in the region approximately –504 to –18 bp (Klein et al., 1998) and an activator protein-1 (AP-1) consensus sequence from –1107 to –1084bp (Introductory Fig. 3) (Qiang and Ticku, 2005). Chronic five day treatment of neurons with 75 mM ethanol increased the binding activity of the AP-1 complex to its *cis*-acting element and enhanced the activity of the promoter construct. As a variety of dimerized c-Fos, FosB, c-Jun, JunD and pCREB proteins comprise the AP-1 family (Qiang and Ticku, 2005), the mechanism by which alcohol stimulates AP-1 binding to its promoter element is presently unknown. It is likely that ethanol changes the expression and/or phosphorylation of the proteins involved, thereby affecting the ratios of the different dimers in the AP-1 complex and providing alcohol with several avenues by which to regulate genes containing these Sp1 and AP-1 sites.

Alcohol regulation of gene expression via alcohol response element (ARE) and heat shock factor 1 (HSF1)

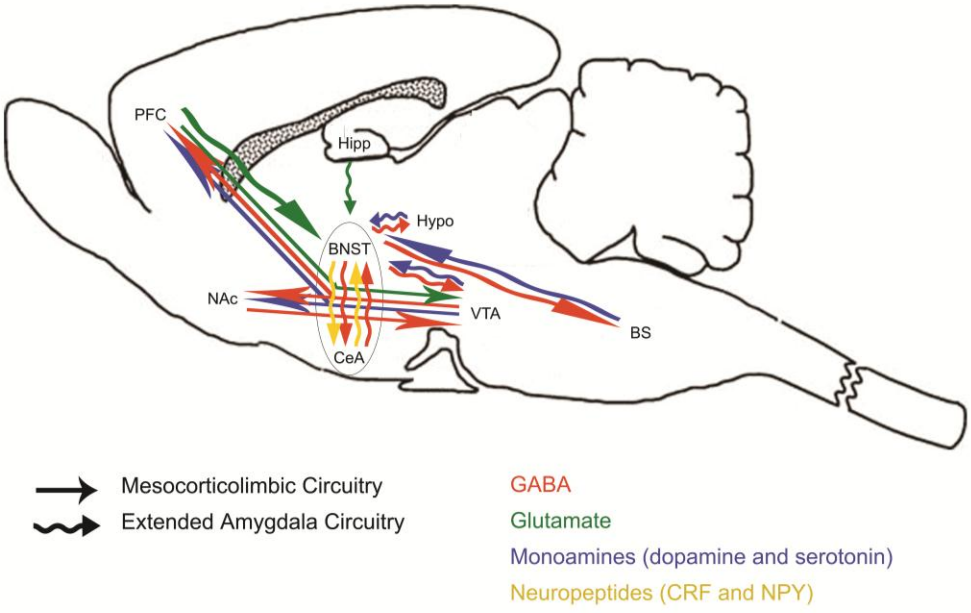
As previously described, α4 mRNA and protein levels are altered by acute and chronic ethanol administration and subsequent withdrawal (refer to earlier section: The effects of alcohol on $GABA_A$ receptors). Our laboratory has identified that alcohol induces *Gabra4* gene expression by activating the transcription factor HSF1 (Pignataro et al., 2007). The *Gabra4* gene has no functional heat shock elements (HSE) and instead we found that HSF1 induces transcription by

binding to a novel *cis*-element on the gene, termed the alcohol response element (ARE) (red box, Introductory Fig. 5). This 11-base pair sequence, located at the end of exon 2, is extremely similar to a consensus sequence found in a subset of *C. elegans* genes that respond to ethanol treatment [tCTGcGTCtCt, where uppercase letters indicate absolute conservation and lower case letters denote a degree of degeneracy at the position] (Kwon et al., 2004). Mutation of the ARE or neuronal transfection with a dominant negative HSF1 mutant gene completely abolished sensitivity of *Gabra4* to ethanol (Pignataro et al., 2007). In addition, acute exposure to alcohol also rapidly increases the transcription of genes known to be HSF1-responsive, including the heat shock protein (*Hsp*) genes *Hsp27*, *Hsp40*, *Hsp70, Hsp90* and *Cryab* (Pignataro et al., 2007). Collectively, these data indicate that a single ethanol exposure can induce HSF1 transcriptional activity to trigger a subset of neuronal alcohol-responsive genes. The activation of HSF1, as well as the other transcription factors discussed in this section, may be an early event in a sequence of changes in gene expression that ultimately result in functional alterations of critical brain circuitry. These functional changes may underlie some aspects of the behavioral tolerance and adaptation that occur in the advanced stages of chronic alcoholism.

In general, researchers within the alcohol field have struggled to link these effects of alcohol on gene transcription with many of the adaptations that present after alcohol administration. We reasoned that a careful study of the effects of alcohol on the gene expression of SNARE complex proteins might reveal a molecular mechanism that can explain the ability of alcohol to alter GABA release. To address this hypothesis and identify a transcriptional pathway by which ethanol can regulate neurotransmitter release, we first investigated the effects of acute alcohol on the expression of genes encoding for synaptic vesicle fusion machinery proteins. We then

addressed whether ethanol alters neurotransmitter release in cortical neurons, using whole-cell voltage clamp electrophysiology. We envision that a mechanism by which alcohol induces the gene expression of SNARE complex proteins to stimulate neurotransmitter release could explain some of the transient changes in synaptic function that occur after alcohol exposure, and may underlie some of the enduring effects of chronic alcohol drinking on local circuitry.

Introductory Figure 1: Diagram of a rodent brain illustrating key brain circuitry underlying the development of alcohol dependence

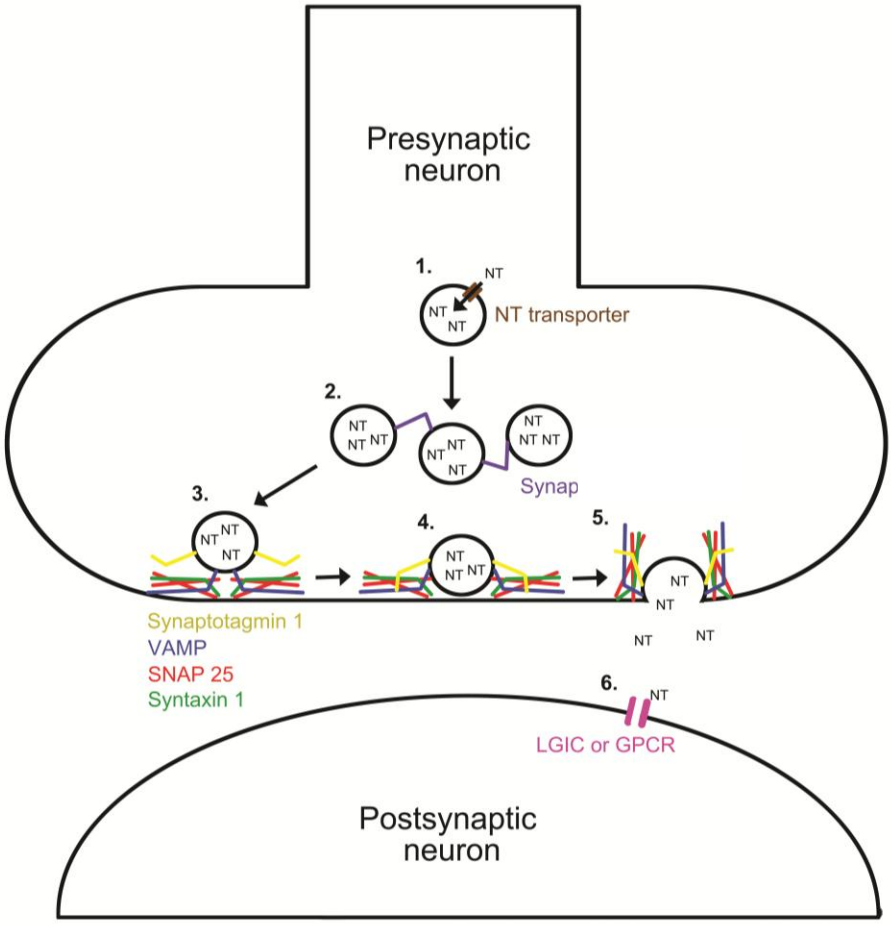


Introductory Figure 1: Diagram of a rodent brain illustrating key brain circuitry underlying the development of alcohol dependence

A sagittal section of a representative rodent brain illustrating the pathways implicated in the reinforcing actions of alcohol. At the core of the mesocorticolimbic circuitry underlying the reward system is the dopaminergic medial forebrain bundle that projects from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and PFC (blue straight arrows). GABAergic neurons in the VTA also project to the NAc and PFC (red straight arrows,), and the VTA receives innervation from GABAergic neurons in the NAc (red straight arrows) and glutamatergic neurons in the PFC to complete the circuit (green straight arrows).

As an individual develops dependence, the antireward system limits reward through its actions in the extended amygdala, comprising the central nucleus of the amygdala (CeA) and the bed nucleus of the stria terminalis (BNST). The CeA and BNST contain reciprocal innervations for GABA (red wavy arrows) and neuropeptide transmitters, such as corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) (yellow wavy arrows). In addition, the extended amygdala receives significant glutamatergic projections from the PFC and hippocampus (green wavy arrows), and dopamingeric and serotonergic inputs from the VTA, lateral hypothalamus and brainstem (blue wavy arrows). The extended amygdala also sends inhibitory efferents back to the lateral hypothalamus, VTA and various lower brainstem regions (red wavy arrows).

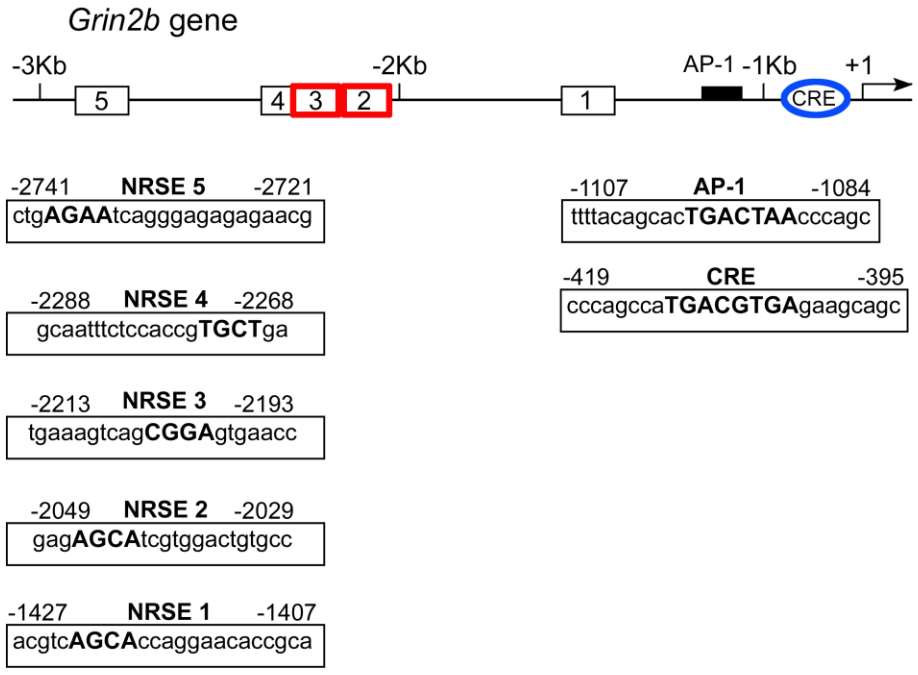
Introductory Figure 2: Neurotransmission at the chemical synapse



Introductory Figure 2: Neurotransmission at the chemical synapse

The chemical synapse is comprised of three main compartments: synaptic vesicles recruited to the presynaptic active zone, the synaptic cleft, and the postsynaptic specialization within a dendritic spine, each of which play a key role in mediating the type and amount of information transfer. Within the presynaptic terminal, synaptic vesicles are loaded with neurotransmitter, transported to the active zone and fused with the plasma membrane to release their contents into the synaptic cleft. A variety of proteins embedded in the vesicle membrane mediate these processes, including neurotransmitter transporters and proton pumps that load the vesicle by active transport (step 1). The vesicle is trafficked through the interactions of its membrane proteins with the actin cytoskeleton and is tethered near the release site by the vesicle membrane protein synapsin (step 2). The synaptic vesicles are docked within the active zone by a complement of SNARE complex proteins which are located both on the vesicle membrane (v-SNAREs: synaptobrevin/vesicle-associated membrane protein, which is also known as VAMP) (blue) and the target plasma membrane (t-SNAREs: syntaxin 1 and synaptosomal associated protein of 25 kDa, which is also known as SNAP-25) (green and red, respectively). During docking, the vesicle membrane protein synaptotagmin 1 (yellow) binds to VAMP and plasma membrane phospholipids. This pulls the two membranes into closer proximity and promotes helical zippering between VAMP, syntaxin 1 and SNAP-25 (step 4). When an action potential reaches the presynaptic terminal, the membrane depolarization opens voltage-gated calcium channels and calcium enters the terminal. The calcium ions bind to synaptotagmin 1, causing a conformational change across the SNARE complex, directly triggering vesicle fusion and neurotransmitter release (step 5). Released neurotransmitter traverses across the synaptic cleft by diffusion and binds to neurotransmitter receptors contained within the postsynaptic specialization (step 6).

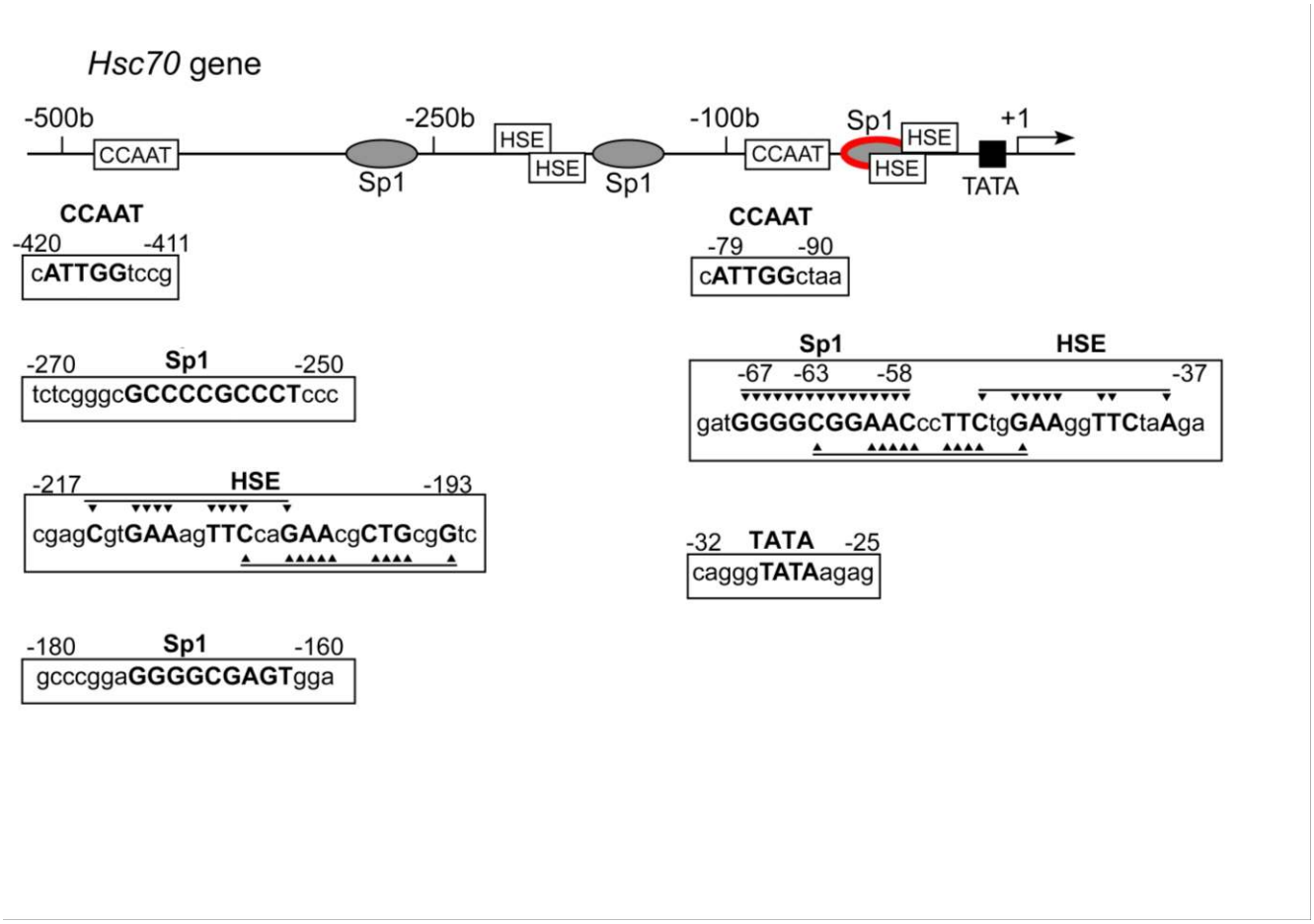
Introductory Figure 3: Location of regulatory elements in the NR2B gene (*Grin2b*)



Introductory Figure 3: Location of regulatory elements in the NR2B gene (*Grin2b*)

Schematic diagram and relative location of the 5 putative NRSE sites present in the mouse *Grin2b* gene. The boxes with the numbers 1-5 represent the location of these elements. Below are the sequence and the specific location of each of these elements with the consensus nucleotides indicated in bold letters. *Grin2b* also poses an AP-1 and a CRE site proximal to the transcription initiation site as indicated in the diagram. The specific location and consensus sequence is indicated in the box below the promoter diagram. Adapted from (Pignataro et al., 2009).

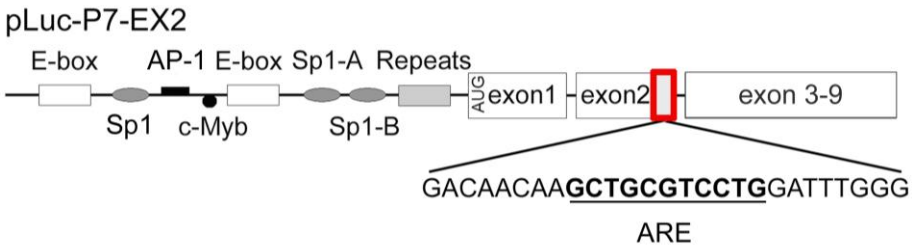
Introductory Figure 4: Organization of the *Hsc70* promoter region



Introductory Figure 4: Organization of the *Hsc70* promoter region

The diagram presents the relative position of regulatory elements in the rat *Hsc70* gene. This gene contains 3 putative Sp1 sites, 4 overlapping HSE sites, two inverted CCAAT boxes and a TATA box. The specific sequences and location of all these sites is indicated in the boxes below the graph with the consensus sites shown bold letters. Adapted from (Pignataro et al., 2009).

Introductory Figure 5: Diagram of the ARE regulatory element essential for the ethanol sensitivity of the *Gabra4* gene



Introductory Figure 5: Diagram of the ARE regulatory element essential for the ethanol sensitivity of the *Gabra4* gene

Schematic of the *Gabra4* gene, containing the *cis*-element ARE binding site for HSF1. The ARE sequence, shown in bold and underlined, is aligned to the consensus sequence found in ethanol sensitive genes in *C. elegans*. Adapted from (Pignataro et al., 2009).

Materials and methods

The Columbia University Institutional Animal Care and Use Committee approved all protocols involving the use of experimental animals in this study.

Cortical neuronal cell culture

Cortical neurons were cultured from mixed gender embryonic day 17-18 C57BL/6 mice (Harlan Laboratories, Indianapolis, IN; Charles River Laboratories, Wilmington, MA) as previously described (Huettner and Baughman, 1986) with modifications (Ma et al., 2004; Varodayan et al., 2011). Lower density cortical cultures for immunohistochemistry experiments were also established and maintained using techniques similar to those used for hippocampal neurons (Banker and Goslin, 1991).

Ethanol and heat stress treatments

Cortical neurons were cultured for 7 - 21 days *in vitro* (DIV) and then exposed to ethanol, heat or vehicle Dulbecco's phosphate-buffered saline control (Invitrogen, Carlsbad, CA) for a specific time (15 minutes - 24 hours). Ethanol (final concentration 10 - 150 mM; Sigma-Aldrich, St Louis, MO) was added directly to the culture medium. Cells were subjected to heat stress by transferring them to an incubator set at 42^{o}C for a period of 1-2 hours.

Quantitative real-time polymerase chain reaction (qPCR) analyses of mRNA levels.

Total RNA was isolated from cultured neurons using TRIzol (Invitrogen). cDNA was prepared from total RNA with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). For cDNA preparation, reactions were performed in a final volume of 20 μl; primers were annealed at 25^{o}C for 5 min, RNA was reverse transcribed at 42^{o}C for 90 min, followed by heat-inactivation at 95^{o}C for 5 minutes and the reaction mixtures were stored at -20^{o}C. The first-strand reverse transcribed cDNA was then used as a template for PCR amplification using the appropriate specific primer pairs listed below. qPCR reactions were carried out with iQ SYBR Green Supermix (Bio-Rad) as previously described (Ma et al., 2004). In preliminary experiments, the *Syt1* and *Vamp2* cDNA concentrations were normalized against *Actb*, *Gapdh* and *18S* [gene encoding ribosomal protein 18S] cDNA (QuantumRNA Internal Standards, Ambion, Austin, TX) within the same sample. For subsequent work, the cDNA concentration for the gene of interest was normalized against the concentration of *Actb* cDNA within the same sample, and the results were finally expressed as a percentage of increase versus the control (untreated neurons or neurons treated with vehicle). In each experiment, the average values of triplicate samples were used for each data point.

qPCR primers

The following primers (and acquisition temperatures) were used for qPCR:

Actb (82^{o}C) forward (5'-TCATGAAGTGTGACGTTGACATCCGT-3'), reverse (5'-CCTAGAAGCATTTGCGGTGCACGATG-3');

Gapdh (77^{o}C) forward (5'-AACTTTGGCATTGTGGAAGG-3'), reverse (5'-ACACATTGGGGGTAGGAACA-3');

Snap25 (75^{o}C) forward (5'-CAACTGGAACGCATTGAGGAA-3'), reverse (5'-GGCCACTACTCCATCCTGATTAT-3');

Stx1a (77^{o}C) forward (5'-TCCAAGCTAAAGAGCATTGAGC-3'), reverse (5'-GGCGTTGTACTCGGACATGA-3');

Syp1 (77^{o}C) forward (5'-GAGAGAACAACAAAGGGCCAA-3'), reverse (5'-CGGCACATAGGCATCTCCT-3');

Syt1 (70^{o}C) forward (5'-CACCGTGGGCCTTAATTGC-3'), reverse (5'-TGTTAATGGCGTTCTTCCCTC-3');

Vamp1 (72^{o}C) forward (5'-AGCATCACAATTTGAGAGCAGT-3'), reverse (5'-GATGGCACAGATAGCTCCCAG-3');

Vamp2 (76^{o}C) forward (5'-GCTGGATGACCGTGCAGAT-3'), reverse (5'-GATGGCGCAGATCACTCCC-3').

Immunoblotting

Relative protein abundance was determined by immunoblotting, as previously described (Jia et al., 2005). Cellular fractions (40-100 mg of protein) were isolated with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL) and incubated with the following antibodies: rabbit polyclonal anti-Syt1 (1:1500, Synaptic Systems, Goettingen,

Germany), rabbit polyclonal anti-HSF1 (1:500, Cell Signaling Technology, Danvers, MA), rabbit polyclonal anti-phosphorylated HSF1 (pHSF1, 1:4000, Enzo Life Sciences, Farmingdale, NY), rabbit polyclonal anti-VAMP1 (1:500, Synaptic Systems), mouse monoclonal anti-VAMP2 (1:2000, Synaptic Systems), mouse monoclonal anti-α-tubulin (1:5000, clone DM1A, Sigma-Aldrich), and rabbit polyclonal anti-eIF4E (1:2500, Cell Signaling Technology). Images were acquired with a refrigerated Chemi 410 CCD camera, the Biospectrum imaging system (UVP, Upland, CA), and the VisionWorks LS software (UVP). Digital images were quantified with ImageJ 1.36b (NIH, Bethesda, MD), with gel lanes selected and their signals transformed into peaks. The area under each peak (gray value) was transformed into an optical density (OD) value using the function: $OD = Log_{10} (255 / (255 – \text{gray value}))$. The OD values were normalized to the α-tubulin or eIF4E internal standards to compensate for variations in protein loading and transfer.

Immunocytochemistry

Lower density cultures were used for immunocytochemistry experiments. Immunostaining was performed with a rabbit polyclonal anti-Syt1 antibody (1:200, Synaptic Systems) and a mouse monoclonal anti-α-tubulin antibody (1:10000, clone DM1A, Sigma-Aldrich). Cells were mounted with ProLong Gold anti-fade reagent containing the nuclear stain DAPI (Molecular Probes, Eugene, OR). Images were acquired with an inverted Zeiss Axiovert 200 confocal microscope (LSM 510 META; Carl Zeiss Meditech, Thornwood, NY) equipped with diode (405 nm), argon (458, 477, 488, 514 nm), HeNe1 (543 nm) and HeNe2 (633 nm) lasers.

RNA interference experiments

RNA interference experiments were performed with presynthesized small interference RNA (siRNA), consisting of a pool of 3 target-specific 20 - 25 nucleotide siRNAs designed to knock down the expression of a particular gene. Cultured cortical neurons were transfected with *Hsf1* siRNA or control siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA). Control experiments were performed with scrambled 20 - 25 nucleotide siRNAs, which do not degrade any known mRNA (Pignataro et al., 2007). Transfection was performed with TransFectin (Bio-Rad) as follows: siRNA (0.33 μg) was added to OPTI-MEM (50 μl; Invitrogen) for 5 min and then combined with a mixture of TransFectin (2.6 μl) and OPTI-MEM (50 μl) for an additional 20 min. The culture medium was removed and replaced with 100 μl of transfection medium and the neurons were incubated for 1 hour at 37^{o}C. Cells were washed once and the transfection medium was replaced with conditioned medium; neurons were maintained for another 24 hours prior to ethanol or heat treatment.

Constitutively active and inactive heat shock factor 1 (Hsf1) constructs

We used a constitutively active form of HSF1 (*Hsf1*-act, BH-S), as well as a dominant-negative mutant form of HSF1 (*Hsf1*-inact, AV-ST). *Hsf1*-act has a long deletion of amino acids 203 - 315 in the regulatory domain of HSF1, while the dominant-negative mutant form of HSF1 has a deletion of amino acids 453 - 523 located in the transcription activation domain (Zuo et al., 1995). Both constructs were generated by Dr. Richard Voellmy (University of Miami) and cloned into pcDNA3.1+ (Invitrogen). Transfections were performed as above with 1 μg of DNA

and 9 μL of nupherin (Enzo Life Sciences), and sister cultures were transfected with the empty pcDNA3.1+ vector as sham controls.

Electrophysiology recordings

Whole-cell voltage clamp patch recordings were used to determine the effects of ethanol on excitatory and inhibitory miniature postsynaptic currents (mPSCs). After ethanol exposure for 4-8 h, cells were washed once with new media to remove ethanol before being incubated in an external solution containing: 124 mM NaCl, 2.5 mM KCl, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose (all Sigma), at 310 mOsm and pH 7.4. mPSCs were recorded in the presence of extracellular tetrodotoxin (TTX; 100 nM; Tocris, Bristol, UK), with excitatory events (mEPSCs) isolated using extracellular SR 95531 hydrobromide (gabazine; 20 μM; Tocris) and inhibitory events (mIPSCs) isolated using extracellular 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX; 10 μM; Tocris) and D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV; 30 μM; Tocris). Patch pipettes were pulled on a Flaming/Browning micropipette puller (Sutter Instrument Company, Novato, CA) from thinwall glass (World Precision Instruments, Saratosa, FL) with a resistance of 3-6 MΩ. The pipettes were filled with an internal solution containing: 140 mM CsCl, 4 mM NaCl, 1 mM $MgCl_2$, 0.05 mM EGTA, 2 mM ATP-Mg^{2+}, 0.3 mM GTP-Na^+, and 10 mM HEPES (all Sigma), at 290 mOsm and pH 7.25. Membrane potentials were clamped at -70 mV and currents were recorded with an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA).

Data were acquired with pClamp 10.3 software (Molecular Devices), filtered at 2 kHz and digitzed at 20 kHz. Each recording was a minimum of 6 minutes long, with the final minute of data analyzed to identify mPSCs. The mPSCs were detected using the Mini Analysis Program 6.0.7 (Synaptosoft, Fort Lee, NJ) with threshold criteria of 5 pA amplitude for the mIPSCs (Cagetti et al., 2003; Liang et al., 2004) and 3 pA for the mEPSCs (Lissin et al., 1999). Frequency of mPSCs was determined from all automatically detected events in the 1 minute recording period. To assess mPSC kinetics, the recording trace was visually inspected and only events with a stable baseline, sharp rising phase and single peak were used.

Statistical, microscopy, genome and electrophysiology analyses

Briefly, the qPCR and immunoblot data were analyzed by one-way ANOVA followed by Dunnett's multiple-comparison *post-hoc* tests. In these experiments, *n* represents the total number of triplicate sample values averaged into each data point, and each data point contains at least three biological replicates. All data are presented as mean ± SEM, and the details of the statistical analysis are also included in the appropriate figure legends. In all cases in which immunoblots are shown, the blot is representative of at least three experiments with similar results.

The analysis of Syt1 immunoreactive puncta was performed by standard methods (Carpenter-Hyland et al., 2004) using ImageJ 1.36b software. Grayscale 8-bit calibrated-images (0.8 to 1 mm optical section) were manually adjusted for threshold, and the area and number of Syt1

clusters present along neurites was calculated. Particles smaller than ~ 0.01 μm^2 were not considered to be Syt1-positive puncta and were discarded from the analysis.

After quantification using ImageJ software, the data were analyzed by one-way ANOVA followed by Dunnett's multiple-comparison *post-hoc* tests. In these experiments, *n* represents the number of cells imaged from at least three biological replicates. All data are presented as mean ± SEM and the details of the statistical analysis are also included in the appropriate figure legends. In all cases in which confocal microscopy images are shown, the image is representative of at least three experiments with similar results.

For all genes analyzed, mouse genomic DNA sequence was obtained from the National Center for Biotechnology Information (NCBI; NIH) database. DNA sequence analyses were performed using Vector NTI (Invitrogen) and putative ARE sites were designated as those containing the ARE motif (tCTGcGTCtCt, uppercase letters indicate absolute conservation) anywhere between 2 kb upstream of the ATG and the 3'-untranslated region.

Electrophysiology numerical data were analyzed using a two-tailed unpaired *t*-test or by one-way ANOVA followed by Dunnett's multiple comparison *post-hoc* tests. In these experiments, *n* represents the number of cells tested from at least three biological replicates. All data are presented as mean ± SEM and the details of the statistical analysis are also included in the appropriate figure legends.

Chapter 1

Alcohol induces synaptotagmin 1 expression in neurons via activation of heat shock factor 1[1]

Abstract

Many synapses within the central nervous system are sensitive to ethanol. Although alcohol is known to affect neurotransmitter release in specific brain regions, the effects of alcohol on the underlying synaptic vesicle fusion machinery have been little studied. To identify a potential pathway by which ethanol can regulate neurotransmitter release, we investigated the effects of acute alcohol exposure (1-24 hours) on the expression of the gene encoding synaptotagmin 1 (Syt1), a synaptic protein that binds calcium to directly trigger vesicle fusion. *Syt1* was identified in a microarray screen as a gene that may be sensitive to alcohol and heat shock. We found that Syt1 mRNA and protein expression are rapidly and robustly stimulated by ethanol in mouse cortical neurons, and that the distribution of Syt1 protein along neuronal processes is also altered. *Syt1* gene induction is dependent on the activation of the transcription factor heat shock factor 1 (HSF1). The transfection of a constitutively active *Hsf1* construct into neurons stimulates *Syt1* gene transcription, while transfection of *Hsf1* siRNA or a constitutively inactive *Hsf1* construct into neurons attenuates the induction of *Syt1* by ethanol. This suggests that the activation of

[1] This chapter is adapted from a manuscript published in 2011. I am grateful to Leonardo Pignataro and Neil Harrison for their contributions to the data and text of this chapter.
Varodayan FP, Pignataro L and Harrison NL (2011) Alcohol induces synaptotagmin 1 expression in neurons via activation of heat shock factor 1. *Neuroscience* **193**: 63-71.

HSF1 can induce *Syt1* gene expression and that this may be a mechanism by which alcohol regulates neurotransmitter release during brief exposures. Further analysis revealed that a subset of the genes encoding the core synaptic vesicle fusion (SNARE) proteins share this property of induction by ethanol, suggesting that alcohol may trigger a specific coordinated adaptation in presynaptic function. This molecular mechanism could explain some of the changes in synaptic function that occur after alcohol administration, and may be an important step in the process of neuronal adaptation to alcohol.

Introduction

Synapses are generally regarded as the most sensitive sites of ethanol action within the central nervous system. While the majority of research has focused on the postsynaptic effects of alcohol on a variety of neurotransmitter receptors (Harris, 1999; Lovinger, 1997), a growing body of evidence suggests that acute and chronic ethanol treatment can also directly modulate neurotransmitter release in a variety of different brain regions (Siggins et al., 2005; Weiner and Valenzuela, 2006).

Electrophysiological work by several groups indicates that acute application of ethanol increases presynaptic γ-aminobutyric acid (GABA) release in the CA1 region of the hippocampus (Carta et al., 2003), nucleus accumbens (Crowder and Weiner, 2002), cerebellum (Carta et al., 2004) and the central amygdala (Roberto et al., 2003), as revealed by increases in the frequency of spontaneous and miniature inhibitory post-synaptic currents (IPSCs). Similar studies suggest that ethanol decreases glutamate release in spinal motor neurons (Ziskind-Conhaim et al., 2003). In addition, investigators have used confocal microscopy in hippocampal slices pretreated with the lipophilic dye FM1-43 to reveal the inhibition of glutamate release by ethanol (Maldve et al., 2004). In light of these and many other studies, it is surprising that there has been little work directed specifically towards investigating how alcohol may regulate the expression of genes that encode the components of the synaptic terminal and proteins that control vesicle fusion.

There are a growing number of alcohol-responsive genes, most of which have been identified using microarray screening and then confirmed using other approaches (Lewohl et al., 2000;

Mulligan et al., 2006). One such candidate alcohol-responsive gene recently identified in a microarray screen is the synaptic vesicle membrane protein synaptotagmin 1 (*Syt1*) (Pignataro et al., 2007). Syt1 acts as a calcium sensor within the space immediately adjacent to the site of synaptic vesicle fusion (Brose et al., 1992), and therefore functions as a critical intermediary in the process of action potential-dependent neurotransmitter release. Syt1 is expressed widely across forebrain, midbrain and most brainstem and spinal cord neurons (Xu et al., 2007). In the presence of calcium, Syt1 binds to both vesicular v-SNAREs (VAMP, also known as synaptobrevin) and plasma membrane phospholipids (Martens et al., 2007). This brings the two membranes together to promote zippering of VAMP and target plasma membrane t-SNAREs (SNAP-25, syntaxin 1A) to trigger vesicle fusion and neurotransmitter release. Synaptophysin 1 regulates this process by associating with VAMP to prevent premature formation of the core SNARE fusion complex (Valtorta et al., 2004).

As Syt1 is intimately involved in the ultimate step of synaptic vesicle fusion, it seems obvious that changes in its expression levels have the potential to alter neurotransmitter release. Syt1-deficient mice show impairment in the fast synchronous component of evoked excitatory postsynaptic currents (EPSCs) in hippocampal neurons (Geppert et al., 1994) and attenuated inhibitory postsynaptic currents (IPSCs) in cortical neurons (Xu et al., 2007). The overexpression of Syt1 in mouse hippocampal cultures increases the probability of evoked vesicle release (Han et al., 2004). Since Syt1 is a key regulator of synaptic vesicle fusion, we reasoned that a careful study of the effects of alcohol on the regulation of Syt1 expression might reveal a molecular mechanism by which alcohol can affect neurotransmitter release.

Results

Alcohol increases Syt1 mRNA and protein expression in cortical neurons

Our initial experiments confirmed earlier findings from microarray work that the *Syt1* gene is an alcohol- and heat stress-responsive gene (Pignataro et al., 2007). We found that exposure of cultured mouse cortical neurons to 60 mM ethanol for 1 hour produced a robust (58 ± 3 %) stimulation of *Syt1* mRNA levels, which was reproduced by a 42^{o}C heat shock treatment for one hour (Fig. 1.1A). The results of ethanol treatment on *Syt1* gene induction were not significantly different when *Syt1* mRNA expression was normalized to three different internal standards, *Actb*, *Gapdh* and *18S* [gene encoding ribosomal RNA 18S] (data not shown). Both alcohol and heat shock treatments also increased Syt1 protein levels (Fig. 1.1B).

The ethanol concentration used in these initial experiments (60 mM) is high, but relevant to human exposure, as chronic alcoholics may routinely tolerate extremely high blood alcohol concentrations of 100 mM or above (Urso et al., 1981). Nevertheless, we wanted to examine the effects of ethanol exposure at concentrations more relevant to social intoxication, so we performed an ethanol concentration-response analysis for *Syt1* mRNA levels. We found that the effect of ethanol (E) on *Syt1* mRNA levels was concentration-dependent (Fig. 1.2A), with a sensitivity threshold of 20 mM and half-maximal activation at 50 ± 1 mM; the ethanol effect saturated at 100 mM. These high ethanol concentrations were not toxic to the neurons, with short-term exposure to ethanol concentrations of over 100 mM resulting in only a modest increase in apoptosis (Pignataro et al., 2007). The time-course of the activation of *Syt1* gene transcription by 60 mM ethanol was surprisingly rapid, increasing mRNA levels significantly

within 30 minutes of exposure (Fig. 1.2B). *Syt1* gene induction further increased during 8 hours of exposure to 60 mM ethanol but then declined, although not to baseline levels, following 24 hours of continuous exposure. Ethanol also increased Syt1 protein, although this effect was delayed relative to the effect on *Syt1* mRNA, with a significant rise in protein occurring after 8-12 hours of exposure (Fig. 1.2C).

Alcohol alters the distribution of Syt1 immunoreactivity in cortical neurons

To visualize changes in Syt1 protein localization after ethanol exposure, we performed immunocytochemistry and confocal microscopy on the cortical cultures. Syt1 protein is embedded in the synaptic vesicle membrane and, as expected, in both control and ethanol-treated neurons we observed punctate clusters of Syt1-positive staining along the neurites (Fig. 1.3A). Ethanol exposure altered the distribution of Syt1 protein, as observed in the representative inset figures. Quantification revealed that ethanol increased the number of identifiable Syt1-positive puncta per 100 µm of neurite length by 2.2-fold, and also increased the average area of the puncta by 1.6-fold (Fig. 1.3B). Both findings are consistent with an increase in the level of Syt1 protein in these neurons.

Alcohol activates HSF1 to induce Syt1 expression in cortical neurons

It has been reported that ethanol and heat induce the *Gabra4* gene via activation of the transcription factor, heat shock factor 1 (HSF1) (Pignataro et al., 2007). HSF1 transcriptional activation is a multi-step process, requiring translocation to the nucleus, followed by

trimerization and inducible hyperphosphorylation (Cotto et al., 1997). It has been demonstrated in primary cortical neuron culture that ethanol and heat shock can induce HSF1 nuclear translocation (Pignataro et al., 2007). To investigate whether HSF1 plays a similar role in *Syt1* gene induction by ethanol, we first assessed the effects of ethanol and heat on HSF1 phosphorylation (pHSF1) and then altered HSF1 protein levels and measured changes in *Syt1* mRNA levels after ethanol exposure. We found that 2 hours of 60 mM ethanol or heat treatment increased the phosphorylation of HSF1 protein, as shown in the inset in Fig 1.4A. The data shows that the proportion of pHSF1 relative to the total expression of HSF1 is increased significantly by ethanol and heat, suggesting activation of this transcription factor (Fig 1.4A). As previous work showed that a 24 hour treatment of cortical neurons with *Hsf1* siRNA produced a > 70% decrease in HSF1 mRNA and protein levels (Pignataro et al., 2007), we used the same HSF1 knock-down protocol here, and observed a reduction in *Syt1* gene induction in response to ethanol exposure (Fig. 1.4B).

To confirm the role of HSF1 in mediating *Syt1* gene induction, we then transfected cortical neurons with a constitutively active *Hsf1* construct (*Hsf1*-act), which is known to directly induce heat shock protein (*Hsp*) gene transcription in the absence of heat stress (Acquaah-Mensah et al., 2001). This construct increased *Syt1* gene expression to a level similar to that seen after 1 hour of ethanol exposure (Fig. 1.4C). Conversely, a dominant-negative *Hsf1* construct (*Hsf1*-inact), which prevents the induction of *Hsp* gene expression (Acquaah-Mensah et al., 2001), abolished the effect of ethanol exposure on *Syt1* mRNA levels (Fig. 1.4C). This confirmed that transcriptionally active HSF1 is both sufficient for stimulation of the *Syt1* gene and necessary for the induction of this gene by ethanol. In the case of *Gabra4* induction by ethanol, it was found

that the activated HSF1 binds to the alcohol response element (ARE) (Pignataro et al., 2007) identified in a subset of alcohol-responsive genes (Kwon et al., 2004; Pignataro et al., 2007). The *Syt1* gene (Gene ID: 20979) contains multiple putative ARE sequences, including multiple repeat ARE-like sequences located within the promoter region and in the first and second introns (Fig. 1.5C).

Alcohol induces a subset of genes encoding synaptic vesicle proteins in cortical neurons

To identify whether *Syt1* induction by ethanol and heat stress is a specific response, we examined the effects of ethanol exposure on genes that code for other proteins intimately involved in synaptic vesicle fusion. We found that the ability of ethanol and heat stress to induce *Syt1* gene expression is replicated among a subset of SNARE complex genes (Fig. 1.5A). We observed that ethanol (60 mM, 1 hour) and heat shock treatment also stimulate SNAP-25 (*Snap25*) and VAMP2 (*Vamp2*) gene expression, while syntaxin 1A (*Stx1a*), synaptophysin 1 (*Syp1*), and VAMP1 (*Vamp1*) were not significantly altered by either treatment. Consistent with these changes in mRNA levels, ethanol and heat shock also increase VAMP2 protein levels, without affecting VAMP1 expression (Fig 1.5B). Sequence analysis of *Vamp1* (Gene ID: 22317) and *Vamp2* (Gene ID: 22318) revealed two putative ARE sequences in each gene, but the sequences in *Vamp1* are located far downstream in the 3'-untranslated region of the gene, while the candidate ARE in *Vamp2* are located in the second intron, as in *Gabra4* (Fig 1.5C). These findings demonstrate that the effects of ethanol are specific to particular synaptic machinery proteins, and not a generalized phenomenon across all proteins expressed at the synaptic terminal.

Discussion

Over the last decade, several studies have demonstrated that ethanol can alter neurotransmitter release, especially that of GABA, in the central nervous system (Siggins et al., 2005; Weiner and Valenzuela, 2006). The mechanisms of these effects have received limited attention, but in the cerebellum, the increased release of GABA on to granule cells appears to be secondary to an increase in Golgi cell firing rate (Carta et al., 2004). The effects of ethanol on the synaptic vesicle fusion machinery that governs neurotransmitter release have remained largely unstudied, but a recent microarray screen identified a number of alcohol-responsive genes that encode synaptic proteins. In this way, *Syt1* was first suggested to be an alcohol-responsive gene that is also sensitive to heat shock (Pignataro et al., 2007), but microarray studies can sometimes produce either false negative or false positive results. Our detailed analysis using qPCR confirmed that acute exposure to ethanol increases *Syt1* gene expression in cortical neurons by activating the transcription factor HSF1. In addition, the effects of acute ethanol exposure on *Syt1* transcription are time-dependent, with mRNA levels peaking around 6-8 hours, and then fading between 8 and 24 hours after the onset of exposure. The increase in Syt1 protein during acute alcohol exposure might be expected to result in changes in transmitter release from these neurons, and this possibility is currently being investigated.

Syt1 is an alcohol-responsive gene

There are few, if any, comparable studies available on the effects of acute alcohol *in vitro* or short-term drinking *in vivo* on synaptic gene expression. A *post-mortem* human microarray study found that *Syt1* gene expression was decreased in the nucleus accumbens, but not the frontal

cortex, of alcoholic brains compared to control cases (Flatscher-Bader et al., 2005). We have not yet examined the effects of longer-term exposure to alcohol on Syt1 expression in the cortical neurons, so it is very difficult to compare our data with those collected from human brain tissue.

Little information is currently available on Syt1 expression from *in vivo* alcohol drinking studies. Syt1 protein levels were reduced in the cerebellum of rat pups exposed to binge-like ethanol by oral-gastric intubation, but remained unchanged in the medial septum/diagonal band, cerebral cortex, hippocampus and brain stem (Hsiao et al., 2002). A study of rats genetically selected for either alcohol self-administration preference (AA) or alcohol avoidance (ANA), showed a decrease in *Syt1* gene expression in the frontal cortex of the AA rats (Worst et al., 2005). The discrepancies among these sets of data and our own work are likely to stem from variations in model systems, brain regions of interest, and alcohol exposure paradigms. Future efforts in our laboratory will therefore be aimed at studying synaptic gene expression after longer term exposure to alcohol in the cortical neurons, and in animals engaged in short-term drinking.

Increases in Syt1 protein levels after ethanol exposure may alter synaptic structure

Alcohol exposure increased Syt1 immunoreactivity in cortical neurons, with the Syt1 immunoreactivity seen as distinct puncta on long neurites and around the cell somata, which is a characteristic of synaptic regions (Gitler et al., 2004; Matthew et al., 1981). Of note, a similar study using 4 days of chronic ethanol treatment of primary hippocampal cultures also revealed an increase in the size and density of synapsin- and synaptophysin-positive clusters (Carpenter-Hyland et al., 2004). Our findings that ethanol increases Syt1-labeled puncta number and size are

completely novel, and we cannot as yet determine whether these changes in immunoreactivity represent an increase in the number of Syt1 protein molecules per synaptic vesicle, larger numbers or aggregations of synaptic vesicles, or increases in synaptic vesicle size. A similar increase in the number of SNARE-positive clusters after the induction of long-term potentiation (LTP) in hippocampal synapses leads to increased glutamate release, possibly through the acquisition of new presynaptic terminals (Antonova et al., 2001; Bozdagi et al., 2000).

A molecular mechanism underlying alcohol induction of the Syt1 gene

The *Syt1* gene shares its alcohol and heat stress-responsive properties with *Gabra4*, which is induced by ethanol when activated HSF1 binds to a specific sequence within the second intron, a sequence we have termed the ARE (Pignataro et al., 2007). The ARE is an 11 base pair *cis*-regulatory element (tCTGcGTCtCt, uppercase letters indicate absolute conservation) that was first identified in a subset of alcohol-responsive genes in *C. elegans* (Kwon et al., 2004; Pignataro et al., 2007). The element is a consensus binding site for HSF1, though its sequence is distinct from the classical heat shock element (HSE) (Pignataro et al., 2007). The *Syt1* gene contains multiple candidate ARE sequences, and neuronal transfection of a constitutively active *Hsf1* construct (*Hsf1*-act) induces the *Syt1* gene in a similar manner to ethanol exposure. *Hsf1*-act is known to induce heat shock protein genes (*Hsps*) in the absence of heat stress (Acquaah-Mensah et al., 2001). Conversely, a dominant-negative *Hsf1* construct (*Hsf1*-inact), which cannot induce the gene expression of heat shock proteins (Acquaah-Mensah et al., 2001), abolished the effect of ethanol exposure on the *Syt1* gene. As transcription of the *Gabra4* and *Hsps* genes are dependent on HSF1, the results of these experiments strongly suggest that *Syt1* induction by

alcohol is mediated *via* the heat shock pathway, and that alcohol acts upstream of HSF1 activation. Since the ARE is a highly conserved sequence, it is very likely that HSF1 binds many of these sites throughout the *Syt1* gene. This raises the interesting question of whether only some of the HSF1-bound ARE sites are able to promote *Syt1* gene transcription, and future work in the laboratory will address these potential position dependent effects of the ARE.

The mechanism ethanol employs to activate HSF1 remains unknown. It is well established that heat stress triggers the formation and aggregation of misfolded proteins that are attended to by heat shock protein chaperones (Morimoto et al., 1998). In the process, cytoplasmically-sequestered HSF1 is freed and translocates to the nucleus where it can act as a transcription factor. Other changes in the cellular environment, such as Ca^{2+} influx and acidification, can also induce an increase in HSF1 transcriptional activity (Mosser et al., 1990), and it is possible that ethanol acts indirectly through one of these mechanisms to activate HSF1.

Ethanol alteration of gene expression via HSF1 has profound implications for neuronal physiology, as HSF1 transcriptional activity is inherently linked to improved cell survival in conditions of stress (Morimoto et al., 1998). More recent data, however, identifies a role for HSF1 in non-stressed conditions as HSF1-deficient mice have impaired adult neurogenesis and spinogenesis in the dentate gyrus, and aberrant affective behavior, due to the loss of HSF1 induction of polysialyltransferase gene transcription in the hippocampus (Uchida et al., 2011). HSF1 also has been identified as a circadian transcription factor that induces *Hsp* transcription at the onset of nocturnal behavior and influences the period length of the mammalian clock (Reinke

et al., 2008). Therefore, HSF1 induction of *Syt1* may be connected to the heat stress pathway or may represent a novel role for HSF1 modulating synaptic function.

Alcohol induces a complement of SNARE genes

Syt1 does not act alone in promoting neurotransmitter release, participating with several other key synaptic proteins to mediate vesicle fusion. Given the large variation in the effects of alcohol on neurotransmitter release that is observed across the brain, it is possible that alcohol differentially regulates some of these other synaptic terminal proteins. We were therefore interested in specifically assessing whether genes that encode other key vesicle fusion machinery proteins are regulated by ethanol and heat shock. We found that ethanol and heat shock also induced the *Snap25* and *Vamp2* genes, while mRNA for synaptophysin 1 (*Syp1*), syntaxin 1A (*Stx1a*) and *Vamp1* remained unchanged under both treatment conditions. In the microarray experiment conducted by Pignataro et al. (2007), similar synaptic vesicle fusion machinery genes were also shown to be sensitive to alcohol and heat shock, such as *Vamp2* and *Vamp8* (but not *Vamp1*).

When considered in this light, ethanol and heat stress induction of *Vamp2*, but not *Vamp1*, is particularly interesting. These two genes encode distinct VAMP isoforms and are differentially expressed in the CNS (Nystuen et al., 2007). *Vamp2* is expressed throughout the mouse brain, particularly in the cortex, whereas *Vamp1* predominates in regions of the diencephalon and midbrain. Closer analysis of synaptobrevin expression in the cerebral cortex, however, found that VAMP1 and VAMP2 are co-expressed at axon terminals (Bragina et al., 2010). Though both

genes contain potential ARE sequences, the candidate ARE in *Vamp2* are located in the second intron in a similar position as in *Gabra4*, while in *Vamp1* the sequences are all located far downstream in the 3'-untranslated region of the gene. The selective induction of *Vamp2*, but not *Vamp1*, by ethanol, is therefore also likely to be dependent on the location of the ARE sequence.

Molecular heterogeneity among synaptic terminals can be achieved through diverse SNARE expression and abundance, and VAMP1 and VAMP2 co-expression occurs at different rates in GABAergic and glutamatergic axon terminals of cortical neurons (Bragina et al., 2010). Furthermore, a single cortical neuron can contain a few synapses expressing only one VAMP and others containing both VAMP1 and VAMP2, indicating that an individual neuron can segregate SNAREs to specific terminals (Morgenthaler et al., 2003). The functional significance of these results is that a single neuron, depending on its specific cell-type and postsynaptic targets, can customize a particular synapse by altering key release characteristics, such as the number of release sites (active zones) per synapse, the number and size of synaptic vesicles docked ready for release, and release probability (Atwood and Karunanithi, 2002). Therefore, a mechanism for differential regulation of various SNARE proteins, including the VAMP isoforms, by alcohol has the potential to provide the neuron with exquisite control over neurotransmitter release.

A potential mechanism to explain some of the effects of alcohol on neurotransmitter release

The biological implications of these findings in terms of the functional consequences of *Syt1* regulation by ethanol are unclear. Flatscher-Bader et al. (2005), noted a decrease in *Syt1* gene

expression in the nucleus accumbens of alcoholics, consistent with studies showing a dampening of the mesolimbic dopamine system after chronic alcohol consumption. Our present work shows that acute ethanol induces *Syt1* gene transcription, and it is well established that Syt1 protein over-expression in mouse hippocampal neurons increases the probability of neurotransmitter release (Han et al., 2004). This suggests a potential molecular mechanism by which the rate of vesicle fusion is increased, leading to an increase of neurotransmitter release during prolonged alcohol exposure. The specificity of this effect among variable synapses and cell types (GABA *vs*. glutamate; interneuron *vs.* principal cells) is obviously a key issue and remains to be investigated. Future experiments will be aimed at determining, using molecular and electrophysiological approaches, whether alcohol is able to regulate Syt1 and other key synaptic proteins universally or whether this effect is restricted to specific neurotransmitter profiles, neuronal cell types or brain regions.

Figure 1.1: Ethanol and heat shock alter Syt1 expression in cortical neurons

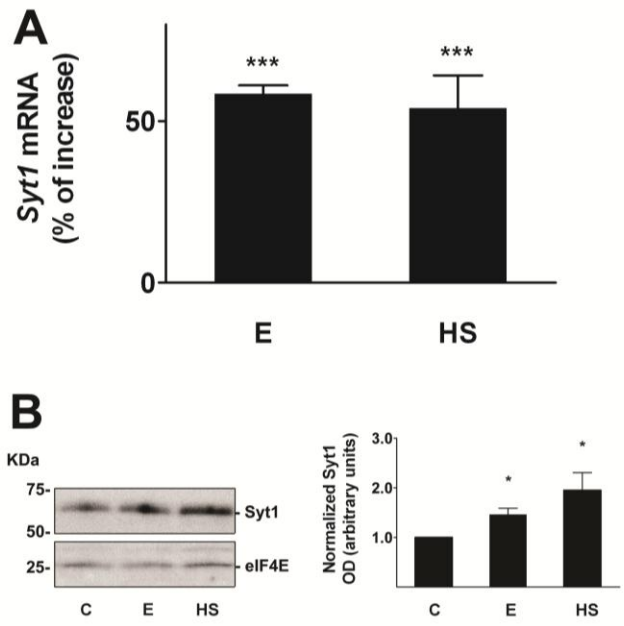


Figure 1.1: Ethanol and heat shock alter Syt1 expression in cortical neurons

A, Increase in *Syt1* mRNA expression after treatment for 1 hour with 60 mM ethanol (E) or 42^{o}C heat shock (HS), as measured by qPCR. The data were normalized to *Actb* cDNA, and compared with control samples treated with vehicle using one-way ANOVA and Dunnett's multiple-comparison *post-hoc* test ($n \geq 6$; $F(2, 29) = 63.82$; $p < 0.0001$). All data are mean ± SEM (*** significantly different at the level of $P < 0.001$).

B, Increase in Syt1 protein after treatment with ethanol or heat shock. The graph shows the relative abundance of Syt1 protein in neurons exposed for 2 hours to 60 mM ethanol (E), a 42^{o}C heat shock (HS) or vehicle control (C). The bar graph represents normalized optical density (OD) relative to the control sample. Ethanol and heat shock data were compared with control by one-way ANOVA, with Dunnett's multiple-comparison *post-hoc* test ($n \geq 3$; $F(2, 12) = 5.43$; $p < 0.05$). All data are mean ± SEM (* significantly different at the level of $P < 0.05$).

Figure 1.2: Ethanol increases Syt1 mRNA and protein expression

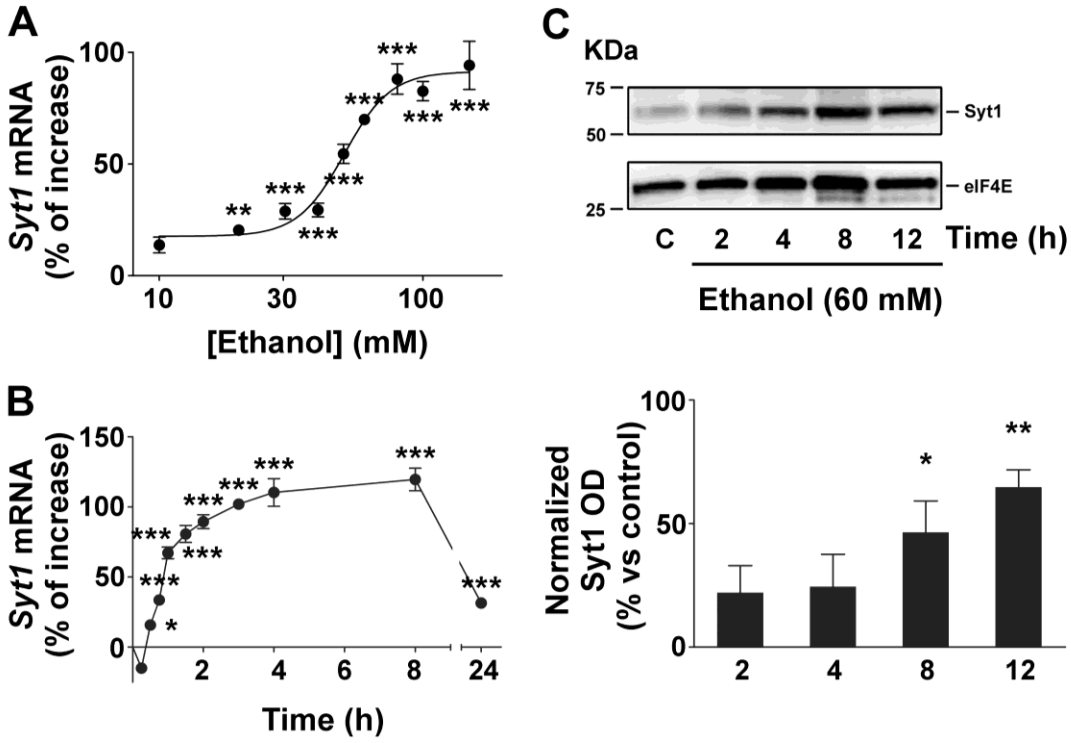


Figure 1.2: Ethanol increases Syt1 mRNA and protein expression

A, Increase in *Syt1* mRNA expression after 1 hour treatment with different concentrations of ethanol, as measured by qPCR. The data were normalized to *Actb* cDNA, and the ethanol-exposed samples were compared with control samples using one-way ANOVA and Dunnett's multiple-comparison *post-hoc* test ($n \geq 6$; $F(9, 70) = 49.90$; $p < 0.0001$). The half-maximal activation of *Syt1* was calculated as 50 ± 1 mM, and the ethanol sensitivity threshold was found to be 20 mM ($p < 0.001$). This threshold is the lowest ethanol concentration that significantly increased *Syt1* expression above the control value and was obtained by analyzing the tail of the concentration-response curve with one-tailed unpaired *t* test. All data are mean ± SEM (** significantly different at the level of $P < 0.01$, *** $P < 0.001$).

B, *Syt1* mRNA expression increases according to the increasing time periods of 60 mM ethanol exposure, as measured by qPCR. The data were normalized to *Actb* cDNA, and the ethanol-exposed samples were compared with control samples using one-way ANOVA and Dunnett's multiple-comparison *post-hoc* test ($n \geq 6$; $F(10, 98) = 103.01$; $p < 0.0001$). All data are mean ± SEM (* significantly different at the level of $P < 0.05$, *** $P < 0.001$).

C, Increase in Syt1 protein after treatment with ethanol over extended periods of time. The representative western blot shows the relative abundance of Syt1 protein in neurons treated with 60 mM ethanol for different time periods of exposure, or vehicle control (C). The bar graph represents normalized Syt1 optical density (OD) for each ethanol-exposed sample relative to the control sample. The data were compared with control values by one-way ANOVA with

Dunnett's multiple-comparison *post-hoc* test ($n \geq 3$; $F(4, 15) = 6.14$; $p < 0.01$). All data are mean ± SEM (* significantly different at the level of $P < 0.05$, ** $P < 0.01$).

Figure 1.3: Ethanol treatment increases Syt1-positive clusters of immunoreactivity in cortical neurons

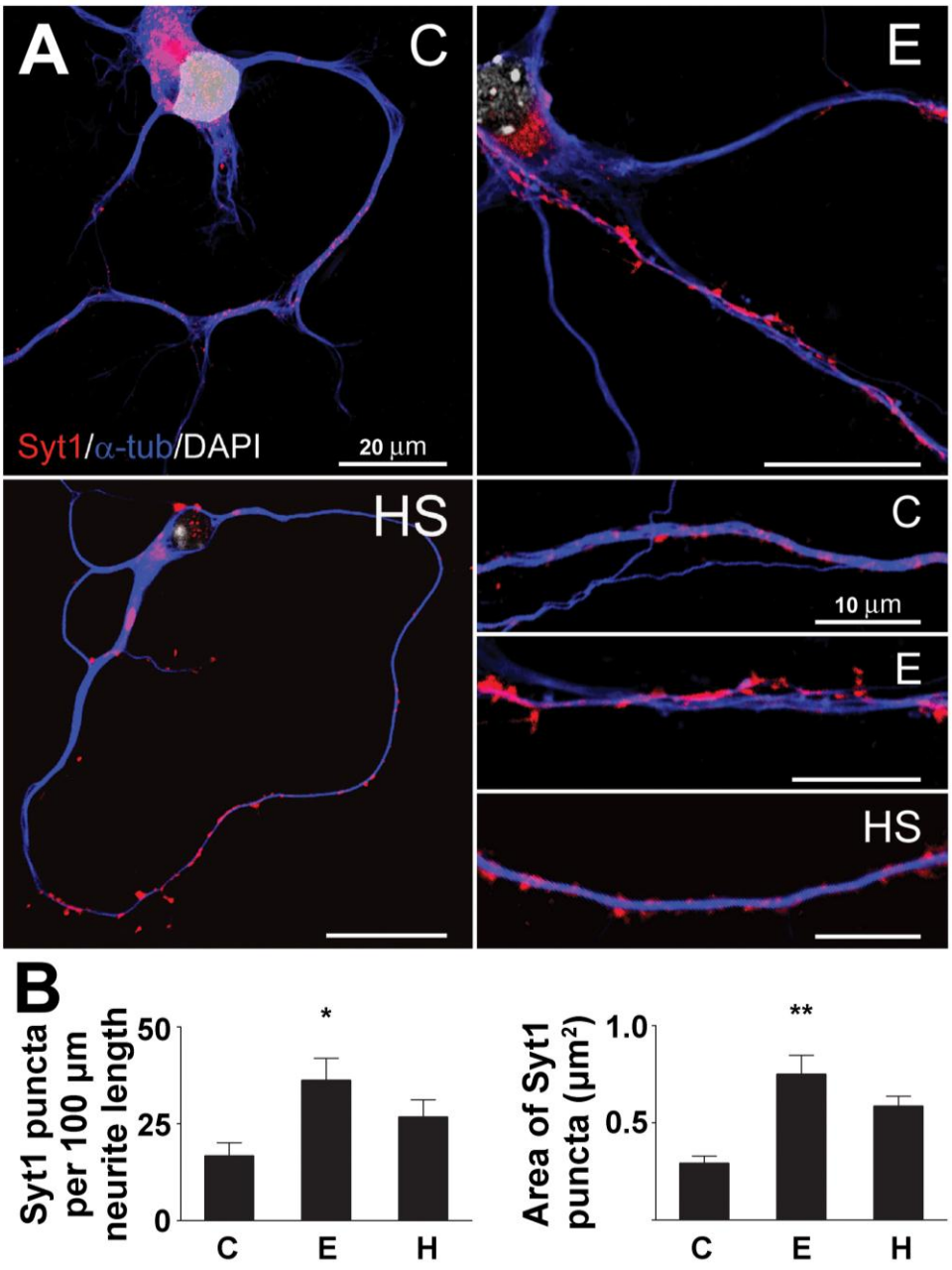


Figure 1.3: Ethanol treatment increases Syt1-positive clusters of immunoreactivity in cortical neurons

A, Visualization of cortical neurons after treatment with 60 mM ethanol (E), a 42^{o}C heat shock (HS) or vehicle control (C). Immunocytochemistry was performed using anti-Syt1 and anti-a-tubulin antibodies, and DAPI nuclear staining

B, Increase in the number and size of Syt1-positive clusters after neurons were treated with ethanol. The graphs show the number of Syt1-positive clusters per 100 mm neurite length and the average cluster size after neurons were exposed to 60 mM ethanol (E), a 42^{o}C heat shock treatment (HS) or vehicle control (C). The quantification was performed with ImageJ software. The data were compared with control values by one-way ANOVA with Dunnett's multiple-comparison *post-hoc* test ($n \geq 6$; length: $F(2, 20) = 3.75$; $p < 0.05$; area: $F(2, 567) = 4.98$; $p < 0.01$). All data are mean ± SEM (* significantly different at the level of $P < 0.05$, ** $P < 0.01$).

Figure 1.4: The induction of the *Syt1* gene by ethanol requires transcriptionally activated HSF1

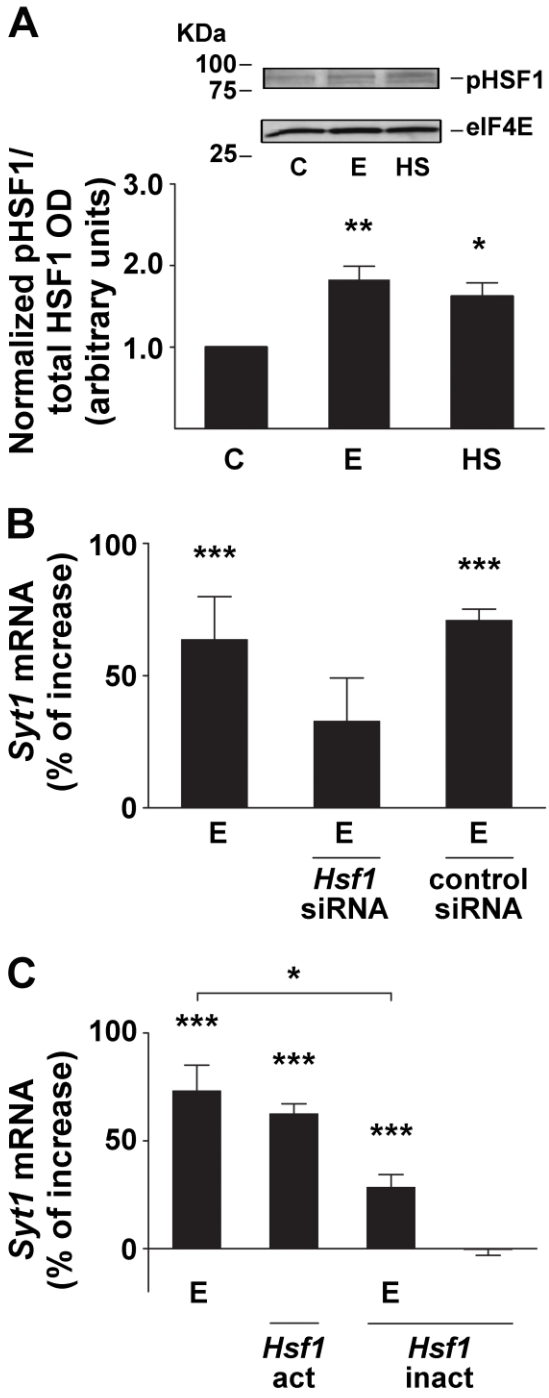


Figure 1.4: The induction of the *Syt1* gene by ethanol requires transcriptionally activated HSF1

A, Increase in phosphorylated HSF1 (pHSF1) protein after ethanol and heat exposure. The inset shows a representative Western blot of the relative abundance of pHSF1 protein in neurons treated with 60 mM ethanol (E) or 42^{o}C heat shock (HS) for 2 hours of exposure, or vehicle control (C). The bar graph represents normalized optical density (OD) of pHSF1 relative to total HSF1 OD. Ethanol and heat shock data were compared with control by one-way ANOVA, with Dunnett's multiple-comparison *post-hoc* test ($n \geq 3$; $F(2, 6) = 10.15$; $p < 0.05$). All data are mean ± SEM (* significantly different at the level of $P < 0.05$, ** $P < 0.01$).

B, Knock-down of HSF1 protein inhibits *Syt1* gene induction by ethanol. Pretreatment of neurons with *Hsf1* siRNA reduced the effects of 60 mM ethanol exposure (E) on *Syt1* gene transcription, while pretreatment with control siRNA had no effect on *Syt1* gene induction by ethanol. The data were normalized to *Actb* cDNA, and the ethanol-exposed samples were compared with control samples using one-way ANOVA and Dunnett's multiple-comparison *post-hoc* test ($n \geq 6$; $F(3, 10) = 17.50$; $p < 0.001$). All data are mean ± SEM (*** significantly different at the level of $P < 0.001$).

C, Stimulation of *Syt1* gene expression by ethanol is mediated by transcriptionally activated HSF1. Cortical neurons transfected with a constituitively active *Hsf1* construct (*Hsf1*-act) showed an increase in *Syt1* mRNA expression, similar to the gene's induction by 60 mM ethanol (E). Transfection of a constituitively inactivated form of *Hsf1* (*Hsf1*-inact) reduced the effects of

ethanol on *Syt1* gene induction. *Hsf1*-inact transfection alone had no effect on *Syt1* expression. The data were normalized to *Actb* cDNA, and the treated samples were compared with control sham-treated samples using 1-way ANOVA and Dunnett's multiple-comparison *post-hoc* test ($n \geq 6$; $F(4, 155) = 36.56$; $p < 0.0001$). All data are mean ± SEM (* significantly different at the level of $P < 0.05$, *** $P < 0.001$).

Figure 1.5: Ethanol induction of SNARE gene and protein expression levels correlate with ARE position

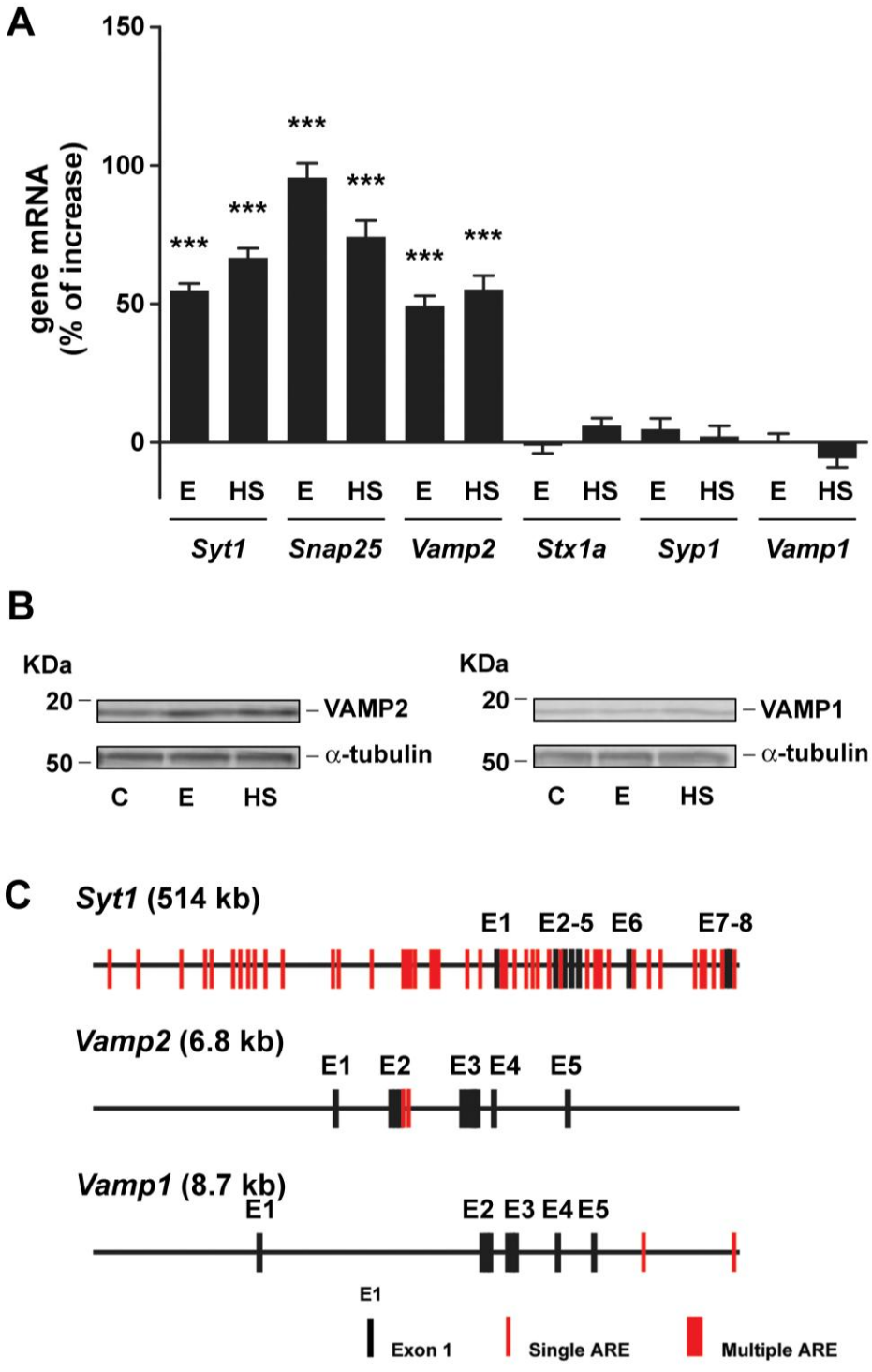


Figure 1.5: Ethanol induction of SNARE gene and protein expression levels correlate with ARE position

A, Ethanol and heat shock induce a subset of genes encoding synaptic vesicle fusion proteins. *Snap25* and *Vamp2* mRNA expression levels increased after treatment for 1 hour with 60 mM ethanol (E) or 42^{o}C heat shock (HS), as measured by qPCR. *Stx1a*, *Syp1*, and *Vamp1* mRNA levels were not significantly altered by either treatment. The data were normalized to *Actb* cDNA, and the treated samples were compared with control samples using one-way ANOVA and Dunnett's multiple-comparison *post-hoc* test, ($n \geq 6$; *Syt1*: $F(2, 125) = 187.4$; $p < 0.0001$; *Vamp2*: $F(2, 160) = 85.72$; $p < 0.0001$; *Snap25*: $F(2, 112) = 165.3$; $p < 0.0001$; *Syp1*: $F(2, 125) = 1.03$; $p = 0.36$; *Vamp1*: $F(2, 152) = 1.50$; $p = 0.23$; *Stx1a*: $F(2, 128) = 1.61$; $p = 0.20$). All data are mean ± SEM (*** significantly different at the level of $P < 0.001$).

B, Ethanol and heat shock increase VAMP2 protein levels, but have no effect on VAMP1 expression. The graphs shows the relative abundance of VAMP1 and VAMP2 protein in neurons treated with 60 mM ethanol (E) or 42^{o}C heat shock (HS) for 2 hours of exposure, or vehicle control (C).

C, Schematic representation of the *Syt1*, *Vamp2* and *Vamp1* genes reveals that the two ethanol sensitive genes contain alcohol response elements (ARE) in their upstream promoter regions. These putative regulation sites may bind heat shock factor 1 (HSF1) to mediate gene induction by ethanol. In *Vamp1*, an ethanol-insensitive gene, the ARE sequences are all located far downstream in the 3'-untranslated region of the gene, while the ARE in *Vamp2* are located in the

second intron. The relative positions of the introns, exons and ARE are conserved in this illustration.

Chapter 2

Alcohol alters the expression of SNAREs and spontaneous GABA release via activation of the transcription factor HSF1

Abstract

The synapse is known to be highly sensitive and responsive to alcohol. While alcohol regulation of postsynaptic receptors is well studied, the mechanisms underlying its effects on neurotransmitter release are relatively unexplored. To identify a pathway by which ethanol can regulate neurotransmitter release, we investigated the mechanism underlying the rapid gene induction by acute alcohol of *Vamp2*, but not *Vamp1*, in primary mouse cortical culture. These two genes encode for isoforms of synaptobrevin, a vesicular soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein that is required for synaptic vesicle fusion. We found that alcohol induction of the *Vamp2* gene is mediated via the transcription factor heat shock factor 1 (HSF1). Neuronal transfection of a transcriptionally active *Hsf1* construct stimulates *Vamp2* gene expression, while transfection of a dominant-negative *Hsf1* construct abolishes the induction of *Vamp2* mRNA levels by ethanol. These alterations in HSF1 activity had no effect on *Vamp1* gene expression. As the *Vamp2* gene encodes a major SNARE protein, we investigated whether acute ethanol altered neurotransmitter release in cortical neurons using whole-cell voltage clamp electrophysiology in the presence of tetrodotoxin (TTX) to record miniature postsynaptic currents (mPSCs). We found that alcohol increased γ-aminobutyric acid

(GABA) release via HSF1, but had no effect on glutamatergic synaptic vesicle fusion. As a whole, these data indicate that alcohol induction of HSF1 transcriptional activity triggers a specific coordinated adaptation in GABAergic presynaptic terminals. This mechanism could explain some of the transient changes in synaptic function that occur after alcohol exposure, and may underlie some of the enduring effects of chronic alcohol drinking on local circuitry.

Introduction

Alcohol abuse and dependence is a major global health problem, but there is little understood about the neuroadaptations that underlie the development of this disease. Considerable evidence suggests that over time the transient molecular changes that accompany a single alcohol exposure can persist, as individual neurons respond to each and every alcohol exposure in a systematic and coordinated manner (Koob, 2006; Nestler, 2001). In particular, the synapse is highly responsive to alcohol, and alterations in synaptic function may lead to changes in local circuitry.

While the mechanisms underlying the postsynaptic effects of alcohol on a variety of neurotransmitter receptors are well studied (Harris, 1999; Lovinger, 1997), only in the last decade have researchers begun to investigate the effects of acute and chronic ethanol treatment on neurotransmitter release (Criswell and Breese, 2005; Siggins et al., 2005; Weiner and Valenzuela, 2006). Acute application of ethanol increases presynaptic γ-aminobutyric acid (GABA) release in the hippocampus (Carta et al., 2003), nucleus accumbens (Crowder and Weiner, 2002), cerebellum (Carta et al., 2004), central amygdala (CeA) (Roberto et al., 2003) and ventral tegmental area (VTA) (Theile et al., 2008) as revealed by increases in the number of spontaneous miniature inhibitory postsynaptic currents (mIPSCs). In addition, mIPSC frequency is increased in the VTA of mice administered a single ethanol dose one day prior to recording (Melis et al., 2002) and in the CeA of chronically ethanol-treated rats (Roberto et al., 2004a). Despite these findings that alcohol alters neurotransmitter release, the effects of alcohol on the

genes that encode for synaptic vesicle fusion machinery have not been thoroughly studied and are not well understood.

Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) play a critical role in neurotransmitter release. During synaptic vesicle fusion, synaptotagmin 1 binds to the vesicular SNARE (v-SNARE) synaptobrevin/vesicle-associated membrane protein (VAMP) and plasma membrane phospholipids (Martens et al., 2007). This pulls the two membranes into closer proximity and promotes zippering of synaptobrevin and target plasma membrane SNAREs (t-SNARES: SNAP-25, syntaxin 1), triggering vesicle fusion and neurotransmitter release. We have found that a subset of genes encoding for SNAREs are induced by acute alcohol exposure, including the vesicle membrane protein synaptotagmin 1 (*Syt1*), as well as *Vamp2* and *Snap25* (Varodayan et al., 2011).

In particular, our laboratory showed that acute alcohol exposure rapidly induced *Vamp2* gene expression, but not *Vamp1* (Varodayan et al., 2011). These two genes encode distinct isoforms of synaptobrevin, a key SNARE protein due to its essential roles in synaptic vesicle fusion (Liu et al., 2011; Schoch et al., 2001) and vesicle endocytosis to replenish the readily releasable vesicle pool (Deak et al., 2004). The two proteins, however, are not strictly redundant as VAMP2-deficient mice die shortly after birth (Schoch et al., 2001) and mutant mice with a VAMP1 null mutation develop a neuromuscular wasting disease and die within two weeks (Nystuen et al., 2007). It is possible that these outcomes are linked to differential patterns of *Vamp* gene expression throughout the body and in particular, the central nervous system. *Vamp2* gene

expression is high throughout the rodent forebrain, particularly in the cortex, whereas *Vamp1* mRNA levels predominate in regions of the diencephalon, midbrain, brainstem and spinal cord (Nystuen et al., 2007; Trimble et al., 1990). Closer analysis of synaptobrevin expression in the cerebral cortex, however, found that VAMP1 and VAMP2 are co-expressed at different rates in both GABAergic and glutamatergic axon terminals, suggesting that there are underlying cell type specific differences in their patterns of expression (Bragina et al., 2010; Morgenthaler et al., 2003).

As synaptobrevin is intimately involved in synaptic vesicle fusion, changes in its expression levels may alter neurotransmitter release. The effects of increased synaptobrevin levels are not well characterized, but overexpression of VAMP2 increased the fusion of large dense core secretory vesicles to allow for neurite-like sprouting in neuronally differentiating PC12 cells (Shirasu et al., 2000). We reasoned that a careful study of the effects of alcohol on *Vamp2* might reveal a molecular mechanism by which alcohol can alter neurotransmitter release.

Results

Alcohol increases Vamp2 mRNA in cortical neurons

Our initial experiments confirmed our previous findings that the *Vamp2* gene is an alcohol-responsive gene (Varodayan et al., 2011). We found that the effect of ethanol on *Vamp2* mRNA levels was concentration-dependent (Fig. 2.1A), with the *Vamp2* gene responding mildly to ethanol concentrations more relevant to social intoxication and strongly to the high ethanol concentrations similar to those measured in blood samples of chronic alcoholics (Urso et al., 1981). The ethanol effect on *Vamp2* gene expression had a half-maximal activation at 40 ± 6 mM and saturated at 80 mM. These high ethanol concentrations were not toxic to the neurons, as treatment with 100 mM ethanol caused only modest increases in apoptosis (Pignataro et al., 2007). The time course of the activation of *Vamp2* gene transcription by 60 mM ethanol was rapid, with *Vamp2* gene expression significantly increased at 30 minutes of exposure (Fig. 2.1B). *Vamp2* mRNA levels continued to rise during 8 hours of 60 mM ethanol exposure and was further increased following 24 hours of continuous exposure. As the results of 60 mM ethanol treatment for 1 hour were not significantly different when *Vamp2* gene expression was normalized to three different internal standards, *Actb*, *Gapdh* and *18S* [gene encoding ribosomal RNA 18S] (data not shown), all qPCR experiments presented in this manuscript used *Actb* cDNA as the internal standard.

Alcohol activates HSF1 to induce Vamp2 gene expression in cortical neurons

Ethanol is known to induce a subset of alcohol-responsive genes via activation of the transcription factor, heat shock factor 1 (HSF1) (Pignataro et al., 2007; Varodayan et al., 2011).

To investigate whether HSF1 plays a similar role in *Vamp2* gene induction by ethanol, we altered HSF1 protein levels and assessed changes in *Vamp2* mRNA levels after ethanol treatment. We found that knock-down of HSF1 protein levels, using neuronal transfection with *Hsf1* siRNA, decreased *Vamp2* gene induction after ethanol exposure (Fig. 2.2A).

Previous work from our laboratory demonstrated that *Vamp1*, a gene that encodes for an isoform of synaptobrevin, was not induced when primary cortical culture was exposed to 60 mM ethanol for 1 hour (Varodayan et al., 2011). As such, the knock-down of HSF1 protein using neuronal transfection of *Hsf1* siRNA had no effect on *Vamp1* mRNA levels (Fig. 2.2C).

To confirm the role of HSF1 in mediating *Vamp2* gene induction, we used a constitutively active *Hsf1* construct (*Hsf1*-act), which encodes a transcriptionally active HSF1 protein that can directly induce heat shock protein (*Hsp*) gene transcription in the absence of heat stress (Acquaah-Mensah et al., 2001). Neuronal transfection of this construct increased *Vamp2* gene expression to a level similar to that seen after 1 hour of 60 mM ethanol exposure (Fig. 2.2B). Conversely, a dominant-negative *Hsf1* construct (*Hsf1*-inact), which encodes a transcriptionally inactive HSF1 protein, abolished the effect of ethanol exposure on *Vamp2* mRNA levels (Fig. 2.2B). These experiments reveal that HSF1 transcriptional activity directly stimulates *Vamp2* mRNA levels and mediates ethanol induction of the *Vamp2* gene. In the case of the *Vamp1* gene, altering HSF1 transcriptional activity by neuronal transfection with either *Hsf1*-act or *Hsf1*-inact and ethanol treatment had no effect on mRNA levels (Fig. 2.2D).

Alcohol increases the frequency of mIPSC events in cortical neurons

As *Vamp2* is one of several alcohol-responsive genes that encode for proteins intimately involved in synaptic vesicle fusion (Varodayan et al., 2011), we wanted to identify whether ethanol exposure altered presynaptic neurotransmitter release. To investigate these potential changes, we recorded spontaneous miniature post-synaptic currents (mPSCs) in cultured cortical neurons treated with ethanol, using whole-cell voltage clamp electrophysiology in the presence of 100 nM TTX to block action potential-dependent neurotransmitter release. In these experiments, increased mPSC frequency indicates alterations at the presynapse leading to increased synaptic vesicle fusion and neurotransmitter release, while increased mPSC amplitude reflects an increase in postsynaptic receptor sensitivity to the released neurotransmitter, possibly due to changes in receptor subunit composition or the number of receptors present (De Koninck and Mody, 1994; Otis et al., 1994).

We first evaluated the effects of 60 mM ethanol exposure for 4-8 hours on inhibitory currents (mIPSCs) by recording in the presence of 30 µM D-APV and 10 µM NBQX to block glutamatergic events. Notably, we found that ethanol increased the frequency of mIPSCs in neurons compared to control cells, as seen in the representative traces and bar graph (n_C = 20, n_E = 25; Fig. 2.3A upper panel, B). Ethanol had no effect on mIPSC amplitude ((n_C = 120, n_E = 240; Fig. 2.3A lower panel, C) or the rise time constant, but shortened the decay time constant. Details of the mIPSC kinetics are displayed in Table 1. These mIPSCs were blocked completely by the perfusion of 20 uM gabazine, with recovery on washout (Fig. 2.3D).

Ethanol exposure had no effect on frequency of excitatory currents (mEPSCs) recorded in the presence of 20 μM gabazine to block GABAergic events (n_C = 26, n_E = 24), but slightly increased mEPSC amplitude (n_C = 183, n_E = 240). Details of mEPSC kinetics are displayed in Table 1.

Alcohol activates HSF1 to increase mIPSC frequency in cortical neurons

To investigate whether HSF1 transcriptional activity mediates the increased mIPSC frequency after ethanol exposure, we altered HSF1 protein levels and assessed mIPSC kinetics. Neuronal transfection of *Hsf1*-act to increase HSF1 activity led to an increase in mIPSC frequency to a level similar to that seen after ethanol exposure (n_C = 16, n_E = 24, $n_{Hsf1act}$ = 25; Fig. 2.4A). Conversely, the dominant-negative *Hsf1*-inact construct abolished the effect of ethanol exposure on mIPSC frequency (n_C = 16, n_E = 10, $n_{Hsf1inact}$ = 12, $n_{Hsf1inact+E}$ = 16; Fig. 2.4B). These experiments reveal that HSF1 transcriptional activity increases GABA release and mediates ethanol induction of mIPSC frequency. In summary, we have shown that ethanol activates HSF1 to increase the gene expression of a specific subset of proteins involved in synaptic vesicle fusion, and GABA release.

Discussion

Ethanol alters GABA release throughout the central nervous system (Criswell and Breese, 2005; Siggins et al., 2005; Weiner and Valenzuela, 2006), but the underlying mechanisms are largely unknown. We recently showed that a subset of genes encoding for SNARE complex proteins is induced by acute alcohol exposure. In particular, we found that alcohol differentially regulates two genes encoding for synaptobrevin isoforms, rapidly inducing *Vamp2*, but not *Vamp1*, and we were therefore interested in the mechanism underlying this difference (Varodayan et al., 2011). Here we show that HSF1 transcriptional activity mediates ethanol induction of *Vamp2* gene expression in cortical neurons. Since *Vamp2* is intimately involved in synaptic vesicle fusion, we then investigated whether alcohol exposure can alter neurotransmitter release via HSF1. We found that ethanol activates HSF1 to increase GABA release, but has no effect on glutamate release.

A single alcohol exposure induces SNARE gene expression

We have previously shown that acute alcohol exposure induces gene expression of some SNARE proteins, including *Vamp2*, *Syt1* and *Snap25*, but not *Vamp1*, *Stx1a* and *Syp* (Varodayan et al., 2011). In this study, we investigated the mechanism underlying *Vamp2* induction by alcohol due to its essential role in fast calcium-triggered synaptic vesicle fusion (Schoch et al., 2001) and its differential induction by alcohol compared to *Vamp1*.

There are few, if any, comparable studies available on the effects of alcohol on *Vamp2* gene expression. Worst et al. (2005) reported that *Vamp2* mRNA levels were lower in cerebellar

cultures treated with ethanol. The same group compared 2 sets of rats genetically selected for either alcohol preference (AA and P) or alcohol avoidance (ANA and NP) and found increased *Vamp2* gene expression in the frontal cortex of the AA and P rats (Worst et al., 2005). Interestingly, a recent transcriptome profiling study used tissue from alcoholic human brains cortices to identify *Vamp2* as a hub gene that is likely to have high functional significance in biological processes associated with alcohol dependence (Ponomarev et al., 2012).

A molecular mechanism underlying the effects of a single alcohol exposure on SNARE gene expression

We found that *Vamp2* gene induction by ethanol is mediated by the transcription factor HSF1 in cortical neurons. Specifically, increasing HSF1 activity induced *Vamp2*, similar to ethanol exposure, while a dominant-negative HSF1 abolished this effect. These changes in HSF1 transcriptional activity had no effect on *Vamp1* mRNA levels. Several laboratories have reported an association between alcohol exposure and HSF1-dependent gene induction, including microarray studies where alcohol treatment increased *Hsp* gene expression (Gutala et al., 2004; Lewohl et al., 2000; Worst et al., 2005) and our work which showed that ethanol increased *Hsp* mRNA and protein levels in neuronal cultures (Pignataro et al., 2007). In addition, we have previously reported that *Syt1* and the gene encoding for the α4 subunit of the $GABA_A$ receptor (*Gabra4*), are induced by alcohol via HSF1. As a whole, these experiments strongly suggest that HSF1 transcriptional activity mediates the effects of alcohol on a subset of alcohol-responsive genes, including some SNARE proteins. As the SNARE proteins are intimately involved in

synaptic vesicle fusion, this raises the interesting question of whether the neuronal response to alcohol includes alterations in neurotransmitter release.

A single alcohol exposure causes a wave of transient presynaptic adaptations leading to changes in GABA release

Changes in GABA release after ethanol exposure have been reported in the last decade (Criswell and Breese, 2005; Siggins et al., 2005; Weiner and Valenzuela, 2006). We found that mIPSC frequency increased in cortical neurons exposed to 60 mM ethanol for 4-8 hours, indicating that these durations of ethanol treatment increased GABA release. The Morrow laboratory observed that mIPSC frequency was unchanged after cultured cortical rat neurons were exposed to 50 mM ethanol for 4 hours or 1-7 days (Fleming et al., 2009; Werner et al., 2011). Since our currents were recorded between these time points, the results as a whole suggest that the increase in mIPSC frequency after a single dose of ethanol is a transient neuronal adaptation that arises within hours and recovers within days. Additional studies have found that bath application of 100 mM ethanol to cultured cortical rat neurons lowered the frequency of mIPSCs (Moriguchi et al., 2007), and that the frequency of mIPSCs in cultured hippocampal rat neurons decreased with 4 hours of withdrawal from a short ethanol exposure (60 mM, 30 minutes), but recovered after withdrawal for 12 hours (Shen et al., 2011). Studies conducted *in vivo* also showed changes in mIPSC frequency across the rodent brain. Melis et al. (2002) observed an increase in mIPSC frequency in the VTA of mice injected intraperitoneally with ethanol one day prior to recording. Chronic ethanol-treated rats showed a similar increase in mIPSC frequency in the CeA and the

frequency was further increased by the application of ethanol, indicating that the acute and chronic effects of ethanol on GABA release are differentially mediated (Roberto et al., 2004a).

Overall, these data define a model of presynaptic adaptations to a single dose of alcohol, where GABA release is dampened within minutes, but recovers by the hour. An intermediate phase of adaptation arises within hours and more GABA is released into the synapse, but these changes return to baseline levels within days. A final wave of changes in neuronal activity occurs during withdrawal from alcohol as GABA release is once again dampened within hours, but recovers within the day. These transient changes in neurotransmitter release could lead to more permanent synaptic modifications, especially as the cycle is repeated with multiple exposures to alcohol.

A molecular mechanism underlying some of the effects of a single alcohol exposure on GABA release

The mechanisms underlying the effects of intermediate durations of ethanol exposure on presynaptic GABA release remain largely unstudied. Our detailed analysis revealed that ethanol increases the number of mIPSC events via HSF1. Specifically, transcriptional activation of HSF1 in cortical neurons increased mIPSC frequency, similar to 4-8 hours of ethanol exposure. Conversely, a dominant-negative HSF1 protein abolished the effect of ethanol on mIPSC frequency. These findings indicate that intermediate ethanol treatment of cultured cortical neurons increases presynaptic vesicular GABA release via HSF1, although it is likely that a variety of alternate mechanisms underlie the similar changes observed after different ethanol

exposure models and across brain regions. For example, acute ethanol application in the cerebellum increases the number of mIPSC events in granule cells due to an initial increase in Golgi cell firing rate (Carta et al., 2004) and in interneurons via activation of both AC/PKA and PLC/PKC pathways and internal calcium store release (Kelm et al., 2007; Kelm et al., 2008; Kelm et al., 2010). PKCε activity is also required in the CeA for corticotrophin releasing factor (CRF) to activate CRF_1 receptors on presynaptic GABA terminals and stimulate release (Bajo et al., 2008; Nie et al., 2004). The effects of acute alcohol administration on these pathways provide for a relatively fast GABAergic neuronal response, but the enhanced GABA release that occurs after chronic ethanol exposure is likely to be regulated by longer-lasting changes in gene expression.

A single alcohol exposure causes a wave of transient postsynaptic adaptations leading to changes in GABA receptor sensitivity

The synapse is a highly responsive structure and perturbations in presynaptic activity are typically met with an adaptive postsynaptic response, and vice versa. We found that treatment of cortical neurons with ethanol for 4-8 hours shortened mIPSC decay time, an indication of changes in postsynaptic $GABA_A$ receptor subunit composition. A decrease in mIPSC decay time was also reported after rat cortical neurons were exposed to ethanol for 4 hours and 1 day, but these changes recovered after 2-7 days (Fleming et al., 2009; Werner et al., 2011). Using a similar *in vivo* paradigm, the Spigelman laboratory administered rats with a single ethanol dose and studied the effects of withdrawal in the hippocampus. The mIPSC decay time decreased within 12 hours of withdrawal and this effect persisted for 7 days, but recovered by day 14

(Liang et al., 2007). This group also found that these changes in mIPSC kinetics coincided with changes in the surface expression of $GABA_A$ receptor subunits. In particular, an increase in α4 expression could cause α4βγ2 $GABA_A$ receptors to "crowd" α1βγ2 $GABA_A$ receptors out of the synapse, leading to changes in $GABA_A$ receptor sensitivity to ethanol (Liang et al., 2007). We have previously found that ethanol treatment of cultured cortical neurons increased α4 expression (Pignataro et al., 2007), indicating that similar changes in $GABA_A$ receptor subunit composition and sensitivity may be occurring in our current study. Overall these data define a model of postsynaptic adaptation to a single dose of ethanol in which there is increased surface expression of a more sensitive α4βγ2 $GABA_A$ receptor within hours, and recovery within days. These transient changes in subunit composition could lead to more permanent synaptic modifications, especially as the cycle is repeated with multiple exposures to alcohol.

Multiple ethanol exposures could lead to persistent adaptation at the GABA presynapse

Overall, these data show that a single ethanol exposure can increase HSF1-mediated transcription of a subset of alcohol-responsive genes, including SNAREs, in a coordinated manner and lead to an increase in GABA release. Given the work of others, it appears that these functional neuronal adaptations are transient, arising over several hours and recovering within days. One may imagine, however, that multiple ethanol exposures could lead to persistent adaptation at the GABA presynapse, resulting in enduring changes in local circuitry. Although we have found that ethanol acts via HSF1 to alter neurotransmitter release specifically in GABAergic, and not glutamatergic, neurons, the specificity of this effect among variable synapses and brain regions is still a key issue that needs to be addressed.

Acknowledgements

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Figure 2.1: Ethanol increases *Vamp2* mRNA expression in cortical neurons

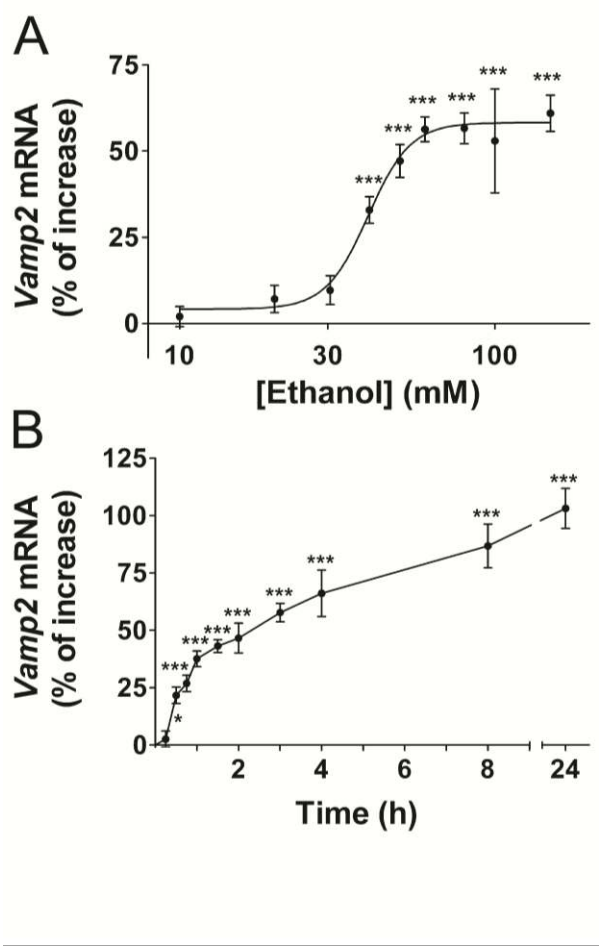

Figure 2.1: Ethanol increases *Vamp2* mRNA expression in cortical neurons

A, Increase in *Vamp2* mRNA expression after 1 hour treatment with different concentrations of ethanol, as measured by qPCR. The half-maximal activation of *Vamp2* was calculated as 40 ± 6 mM. The data were normalized to *Actb* cDNA, and all pairs of columns were compared using one-way ANOVA and Dunnet's multiple-comparison *post-hoc* test ($n \geq 6$; $F(9, 72) = 20.45$; $p < 0.0001$).

B, Increase in *Vamp2* mRNA expression after 60 mM ethanol exposure over time, as measured by qPCR. The data were normalized to *Actb* cDNA, and all pairs of columns were compared using one-way ANOVA and Dunett's multiple-comparison *post-hoc* test ($n \geq 6$; $F(10, 195) = 39.58$; $p < 0.0001$). All data are mean ± SEM (*significantly different at the level of $P < 0.05$, *** $P < 0.001$).

Figure 2.2: Ethanol induction of *Vamp2* gene requires transcriptionally activated HSF1

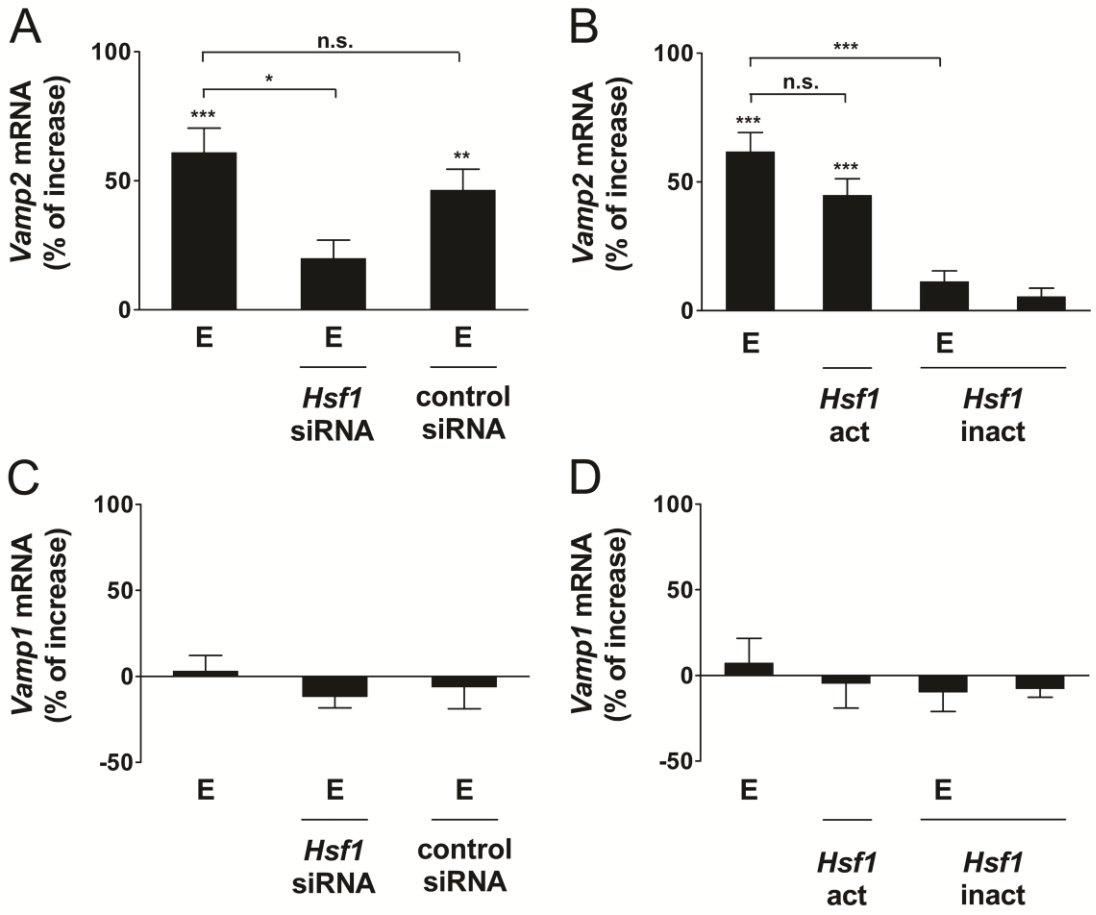

Figure 2.2: Ethanol induction of *Vamp2* gene requires transcriptionally activated HSF1

A, Knock-down of HSF1 inhibits *Vamp2* gene induction by ethanol. Overnight pretreatment of neurons with *Hsf1* siRNA reduced the effects of 60 mM ethanol exposure for 1 hour (E) on *Vamp2* mRNA levels, while pretreatment with control siRNA had no effect on *Vamp2* gene induction by ethanol. The data were normalized to *Actb* cDNA, and all pairs of columns were compared using one-way ANOVA and Dunnett's multiple-comparison *post-hoc* test ($n \geq 6$; $F(3, 44) = 13.55$; $p < 0.001$).

B, Stimulation of *Vamp2* gene expression by ethanol is mediated by transcriptionally activated HSF1. Cortical neurons transfected with a constituitively transcriptionally active *Hsf1* construct (*Hsf1*-act) showed an increase in *Vamp2* mRNA expression, similar to the gene's induction by 60 mM ethanol for 1 hour (E). Transfection of a constituitively transcriptionally inactivated form of *Hsf1* (*Hsf1*-inact) reduced the effects of ethanol on *Vamp2* gene induction. *Hsf1*-inact transfection alone had no effect on *Vamp2* expression. Control cultures were sham transfected with an empty pcDNA3.1+ construct. The data were normalized to *Actb* cDNA, and all pairs of columns were compared using one-way ANOVA and Dunnett's multiple-comparison *post-hoc* test ($n \geq 6$; $F(4, 73) = 27.53$; $p < 0.001$).

C, Knock-down of HSF1 protein has no effect on *Vamp1* gene induction by ethanol. *Vamp1* mRNA levels remain unchanged after neuronal transfection with *Hsf1* or control siRNA and 1 hour of 60 mM ethanol exposure (E). The data were normalized to *Actb* cDNA, and all pairs of

columns were compared using one-way ANOVA and Dunnett's multiple-comparison *post-hoc* test ($n \geq 6$; $F(3, 47) = 0.29$; $p = 0.84$).

D, Alterations in HSF1 transcriptional activity do not alter *Vamp1* gene expression. Transfection of cortical neurons with *Hsf1*-act or *Hsf1*-inact and treatment with 60 mM ethanol for 1 hour (E) had no effect on *Vamp1* mRNA levels. The data were normalized to *Actb* cDNA, and all pairs of columns were compared using one-way ANOVA and Dunnett's multiple-comparison *post-hoc* test ($n \geq 6$; $F(4, 58) = 0.35$; $p = 0.85$). All data are mean ± SEM (*indicates significantly different at the level of $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, or n.s. denotes no significance).

Figure 2.3: Ethanol exposure increases mIPSCs in cortical neurons

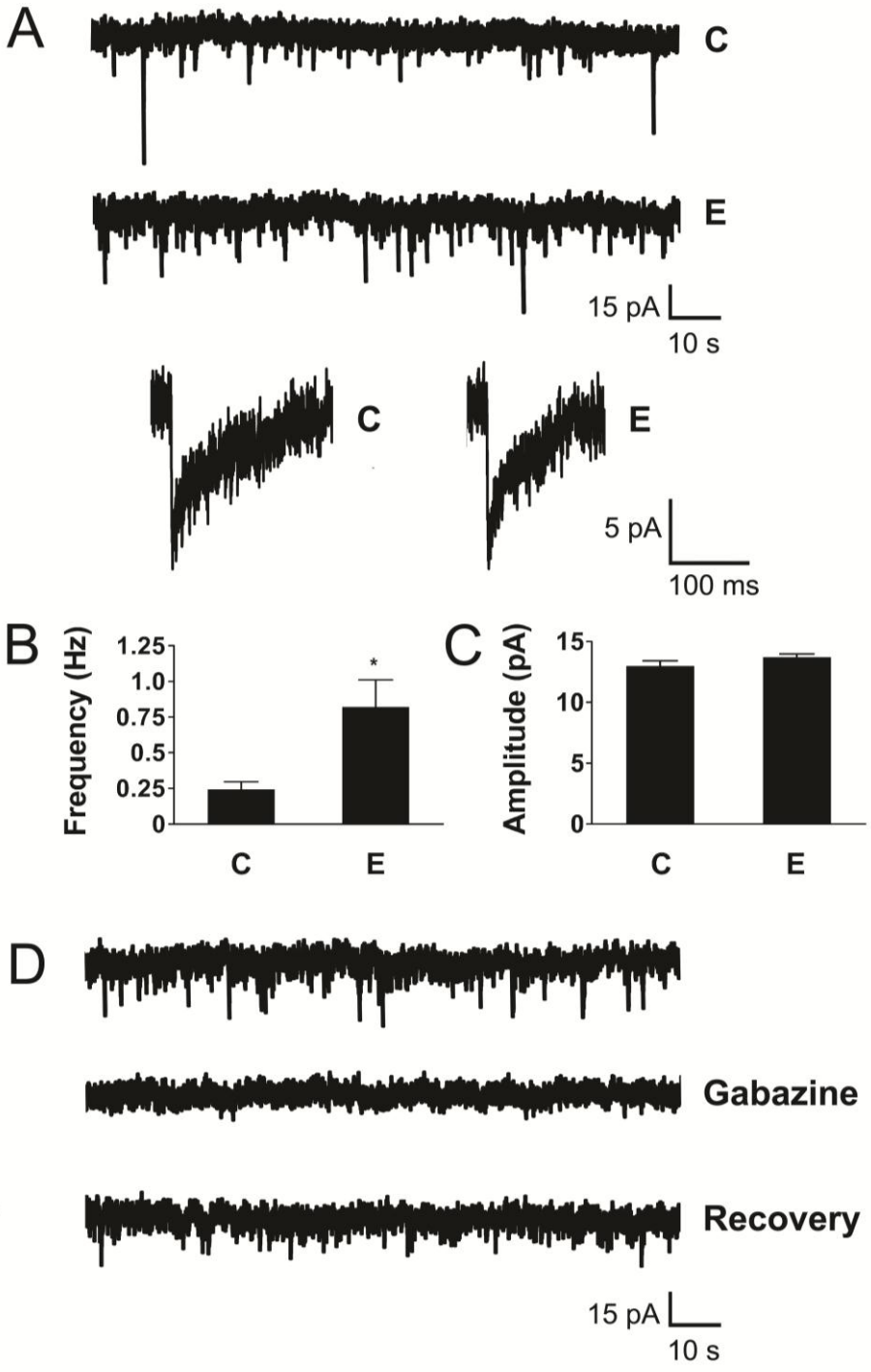

Figure 2.3: Ethanol exposure increases mIPSCs in cortical neurons

A, Whole-cell voltage clamp electrophysiology recordings from representative neurons show that ethanol increases the number of mIPSCs. The current traces in the upper panel were recorded in a neuron exposed to 60 mM ethanol for 4-8 hours (E) and a vehicle control neuron (C). Magnified representative mIPSC events from control and ethanol-treated neurons are shown in the lower panel.

B, Ethanol increases the frequency of mIPSCs averaged across several neurons. The graph shows the mean frequency of spontaneous mIPSCs in neurons treated with ethanol (E) or vehicle control (C). The treated samples were compared with control samples using a two-tailed unpaired *t*-test ($n_C = 20$, $n_E = 25$; $t\ (43) = 2.67$).

C, Ethanol does not alter the amplitude of mIPSCs averaged across several neurons. The graph shows the mean amplitude of spontaneous mIPSCs in neurons exposed to ethanol (E) or vehicle control (C). Details of mIPSC kinetics are displayed in Table 1. The treated samples were compared with control samples using a two-tailed unpaired *t*-test ($n_C = 236$, $n_E = 670$; $t\ (904) = 1.37$; $p = 0.17$). All data are mean ± SEM (*significantly different at the level of $P < 0.05$).

D, Whole-cell voltage clamp electrophysiology recordings from representative neurons demonstrate that the mIPSCs are generated by GABA neurotransmission. The current traces

were recorded in a single neuron exposed to 60 mM ethanol for 4-8 hours. The mIPSCs are blocked in the presence of 20 uM gabazine and recover after drug washout.

Figure 2.4: The increase in mIPSC frequency after ethanol exposure requires transcriptionally activated HSF1

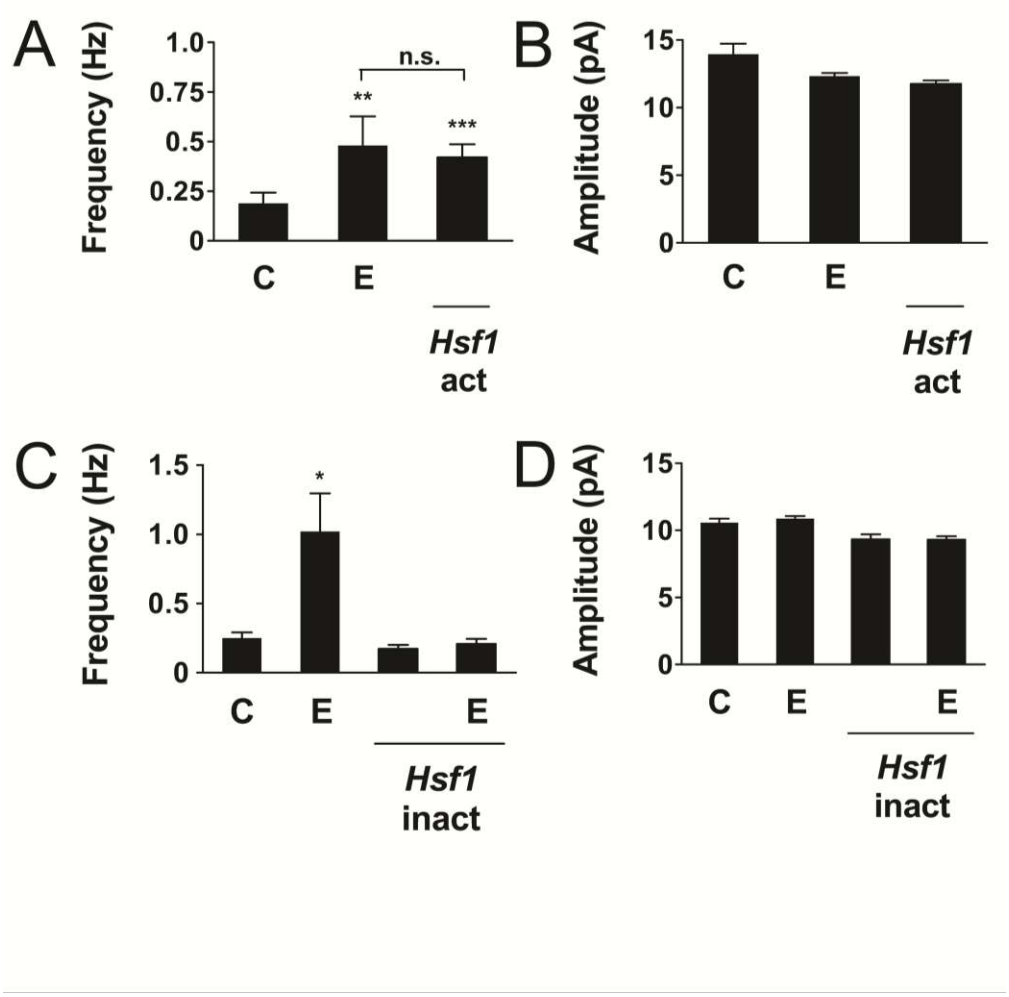

Figure 2.4: The increase in mIPSC frequency after ethanol exposure requires transcriptionally activated HSF1

A, HSF1 transcriptional activity increases the frequency of mIPSCs averaged across several neurons. Cortical neurons transfected with a constituitively transcriptionally active *Hsf1* construct (*Hsf1*-act) showed an increase in mIPSC frequency, similar to the level seen with 60 mM ethanol exposure for 4-8 hours (E). Control cultures were sham transfected with an empty pcDNA3.1+ construct (C). All pairs of data columns were compared using one-way ANOVA and Dunnett's multiple comparison *post-hoc* test ($n_C = 16$, $n_E = 24$, $n_{Hsf1act} = 25$; $F(2, 62) = 6.48$; $p < 0.01$).

B, HSF1 activity does not alter the amplitude of mIPSCs averaged across several neurons. The graph shows the mean amplitude of spontaneous mIPSCs in neurons transfected with an *Hsf1*-act construct, exposed to ethanol (E) or control sham transfected (C). All pairs of data columns were compared using one-way ANOVA and Dunnett's multiple comparison *post-hoc* test ($n_C = 120$, $n_E = 240$, $n_{Hsf1act} = 154$; $F(2, 511) = 1.78$; $p = 0.18$).

C, The increase in mIPSC frequency after ethanol exposure is mediated by activated HSF1. Transfection of a constituitively inactivated form of *Hsf1* (*Hsf1*-inact) reduced the effects of ethanol (E) on the frequency of mIPSC events. *Hsf1*-inact transfection alone had no effect on mIPSC frequency, as compared to control cultures sham transfected with empty pcDNA3.1+ construct (C). All pairs of data columns were compared using one-way ANOVA and Dunnett's

multiple comparison *post-hoc* test (n_C = 16, n_E = 10, $n_{Hsf1inact}$ = 12, $n_{Hsf1inact+E}$ = 16; F (3, 49) = 11.25; $p < 0.0001$).

D, HSF1 activity does not alter the amplitude of mIPSCs averaged across several neurons. The graph shows the mean amplitude of spontaneous mIPSCs in neurons transfected with an *Hsf1*-inact construct, or exposed to ethanol (E) or vehicle control (C). All pairs of data columns were compared using one-way ANOVA and Dunnett's multiple comparison *post-hoc* test (n_C = 135, n_E = 160, $n_{Hsf1inact}$ = 71, $n_{Hsf1inact+E}$ = 60; F (3, 422) = 5.950; $p = 0.06$). All data are mean ± SEM (*indicates significantly different at the level of $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, or n.s. denotes no significance).

Table 2.1: A single ethanol exposure alters mPSC kinetics

Data are obtained from neurons exposed to 60 mM ethanol for 4-8 h and control neurons (n = 20-26).

	Treatment	Frequency (Hz)	Amplitude (pA)	Rise time (ms)	Decay time (ms)
mIPSC	C	0.24 ± 0.05	13.0 ± 0.4	2.82 ± 0.08	16.2 ± 0.8
	E	0.83 ± 0.19*	13.7 ± 0.3	3.02 ± 0.06	12.2 ± 0.4***
mEPSC	C	0.17 ± 0.04	5.6 ± 0.1	0.97 ± 0.05	0.93 ± 0.07
	E	0.20 ± 0.05	6.3 ± 0.2***	1.17 ± 0.05**	1.08 ± 0.07

*P < 0.05, ** P < 0.01, *** P < 0.001 vs. control neurons (two-tailed unpaired t-test).

Conclusion

Summary of findings

This dissertation provides evidence that HSF1 transcriptional activity is involved in ethanol-induced changes in SNARE gene expression and neurotransmitter release. The data presented in Chapter 1 focus on the transcriptional mechanism underlying the effects of ethanol on SNARE genes. We found that synaptotagmin 1 (Syt1) mRNA and protein expression are rapidly and robustly increased after ethanol treatment of mouse cortical neurons. The induction of the *Syt1* gene is dependent on the activation of the transcription factor heat shock factor 1 (HSF1), with neuronal transfection of a transcriptionally active *Hsf1* construct increasing *Syt1* mRNA levels and transfection of a dominant-negative *Hsf1* construct abolishing *Syt* gene induction by ethanol. Further analysis revealed that a subset of the genes encoding the core SNARE proteins share the property of induction by ethanol, including one of the isoforms of synaptobrevin (*Vamp2*), but not the other (*Vamp1*).

In Chapter 2 we demonstrated that ethanol activation of HSF1 also induced *Vamp2* gene expression and increased GABA release. Specifically, we found that ethanol activates HSF1 transcriptional activity to rapidly induce the *Vamp2* gene, but does not change *Vamp1* mRNA levels. As *Syt1* and *Vamp2* encode key SNARE proteins, we investigated whether ethanol activation of HSF1 affected neurotransmitter release using whole-cell voltage clamp electrophysiology. We found that alcohol increased γ-aminobutyric acid (GABA) release via HSF1, but had no effect on glutamatergic synaptic vesicle fusion. Collectively, these data indicate that a single ethanol exposure can induce HSF1 transcriptional activity to trigger a

specific coordinated adaptation in GABAergic presynaptic terminals. This mechanism could explain some of the transient changes in synaptic function that occur after alcohol exposure, and may underlie some of the enduring effects of chronic alcohol drinking on local circuitry.

A model for how a single alcohol exposure causes synaptic adaptations leading to changes in GABA release

The experimental findings presented in this dissertation are discussed in detail within the final section of each chapter. In this section, we will describe an integrated model based on our interpretation of the data and relevant literature.

A single alcohol exposure produces a profound transcriptional response with the induction of a variety of gene classes encoding for signaling molecules, molecular chaperones, neurotransmitter receptors, transcription factors and cytokines (Miles et al., 1994). Some of these changes in gene expression may be attributed to the actions of ethanol on the transcription factor HSF1. Ethanol promotes the translocation of cytoplasmically-sequestered HSF1 to the nucleus, where it acquires transcriptional activity in a sequential process involving trimerization, acquisition of DNA binding activity and inducible phosphorylation (Cotto et al., 1997). The active HSF1 then triggers the transcription of several alcohol-responsive genes, including SNARE proteins (*Syt1* and *Vamp2*), the α4 subunit of the $GABA_A$ receptor (*Gabra4*) (Pignataro et al., 2007), molecular chaperone heat shock proteins (*Hsps*) (Pignataro et al., 2007) and presumably several other genes that regulate neuronal processes. This coordinated transcriptional response may lead to changes

in specific neuronal functions, depending upon the classes of genes induced. In particular, increased Syt1 and VAMP2 protein expression may directly increase GABA release from the presynaptic terminal by increasing the probability of synaptic vesicle fusion.

Therefore, HSF1 activation is an early event in a sequence of changes, including increased gene expression of a subset of SNARE complex proteins and increased GABA release, which ultimately result in altered neuronal communication across local circuitry. Over time, as an individual experiences multiple alcohol exposures, some of these synaptic adaptations may endure, resulting in functional alterations of critical brain circuitry. These functional changes may underlie some aspects of the behavioral tolerance and adaptation that occur in the advanced stages of chronic alcoholism.

Alternative interpretations of the effects of alcohol on GABA transmission

The evidence presented in this dissertation to support the model that alcohol activates HSF1 to increase GABA release was collected by recording mIPSCs under whole-cell voltage clamp in the presence of TTX to block action potential-dependent neurotransmitter release. In these experiments, each mIPSC represents a single or small group of presynaptic vesicles spontaneously fusing with the plasma membrane to release neurotransmitter and generate a postsynaptic current. Thus, increases in mIPSC frequency are generally interpreted as stemming from alterations at the presynapse that increase the probability of synaptic vesicle fusion (De Koninck and Mody, 1994; Otis et al., 1994), perhaps due to increased SNARE expression. In support of this model, the Sudhof laboratory has shown that Syt1 over-expression in mouse

hippocampal neurons increased the probability of neurotransmitter release (Han et al., 2004). A different method was used to measure the probability of neurotransmitter release in this study and the synaptic responses evoked by action potentials were compared to those elicited with application of hypertonic sucrose to measure the size of the readily releasable pool.

Alternatively, mIPSC frequency could be increased by the extension of existing synaptic contacts or the formation of new functional synapses. Several imaging studies on mature neuronal cultures have observed the clustering of synaptic vesicles into nascent active zones, which exhibit evoked vesicle recycling (Ahmari et al., 2000; Friedman et al., 2000) and the growth of new dendritic spines within hours (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999). All of these studies, however, only identified these new synaptic structures using morphological criteria and failed to demonstrate a capacity for synaptic transmission. This relatively fast growth of synaptic structures is also dependent on the variety and abundance of free synaptic terminal proteins available for assembly, which can determine the amount of synaptic growth, as well as its ability to function appropriately. As a result, it is unclear whether several hours of alcohol treatment could increase the size or number of functional synapses to generate a three-fold increase in mIPSC frequency.

A third potential mechanism for increasing mIPSC frequency depends completely on the adaptation of preexisting non-functional synapses to confer neurotransmission. These “silent” synapses have not been identified within the inhibitory neurotransmission system, but are common among glutamatergic synapses. A silent synapse contains all the essential pre- and

postsynaptic proteins for neurotransmission, but remains functionally silent (Gasparini et al., 2000; Gomperts et al., 1998; Isaac et al., 1995; Kerchner and Nicoll, 2008; Voronin and Cherubini, 2004). Some glutamatergic synapses are silent due to a lack of AMPARs in their postsynaptic specializations. The existing NMDARs cannot conduct current, even in the presence of glutamate, due to the channels being blocked by extracellular Mg^{+} ions. AMPAR recruitment to the postsynaptic specialization can "unsilence" the synapse, as the AMPARs will bind glutamate and generate a large enough depolarizing current to unblock the NMDARs. Other mechanisms of silencing glutamateric synapses are due to a low probability of glutamate release or insufficient concentrations of glutamate available for release (Gasparini et al., 2000; Voronin and Cherubini, 2004). While inhibitory silent synapses have not been identified, it is possible that similar silencing mechanisms are unmasked by alcohol treatment, resulting in increased mIPSC frequency.

Potential explanations for the specificity of the effects of alcohol on GABA neurotransmission

There are several potential explanations for the difference in the actions of ethanol to stimulate GABA release, but not affect glutamate release. As we found that ethanol also differentially regulates the two isoforms of synaptobrevin, rapidly inducing the *Vamp2* gene, but not *Vamp1*, it is exciting to consider that these two phenomena may be related. The *Vamp1* and *Vamp2* genes are known to be co-expressed in both GABAergic and glutamatergic axon terminals of cortical neurons (Bragina et al., 2010), excluding the possibility that each synaptobrevin isoform is exclusively expressed in one particular cell type. It is quite possible, however, that GABAergic

and glutamatergic cortical neurons express different amounts of *Vamp1* and *Vamp2* mRNA under basal conditions, and that ethanol stimulation of the *Vamp2* gene only leads to functional changes in the synaptobrevin protein at the GABA presynaptic terminal. In order to better understand the specificity of the effects of ethanol postulated by this model, it is necessary to measure the expression of each synaptobrevin isoform in pure GABAergic and glutamatergic neuronal populations under basal conditions and after ethanol exposure.

An entirely different explanation for the specificity of the effects of ethanol is that the drug acts, either directly or indirectly, on GABA-related pathways to increase GABA release. For example, alcohol may specifically induce HSF1 transcriptional activity in GABAergic neurons via unknown actions on a cell-type specific receptor or second messenger pathway, or alcohol may increase vesicular GABA transporter (vGAT) activity to alter quantal size (Edwards, 2007; Reimer et al., 1998).

Future directions

Many interesting questions related to alcohol activation of HSF1 transcription to increase SNARE gene expression and GABA release remain unanswered. Understanding the mechanism ethanol employs to activate HSF1 is of immediate interest. HSF1 is bound to the chaperone proteins, HSP40, HSP70 and HSP90, in the cytoplasm of unstressed cells (Morimoto et al., 1998; Tonkiss and Calderwood, 2005). After heat exposure there is a general increase of misfolded proteins in the cytoplasm, triggering the release of HSF1 from the HSPs and its translocation into

the nucleus (Morimoto et al., 1998). Other changes in the biochemical environment can also trigger HSF1 activation, including, most notably, increased free calcium (Mosser et al., 1990). As Kelm et al. (2007, 2008, 2010) have determined that ethanol can release internally stored calcium by activating inositol triphosphate (IP_3) and ryanodine receptors downstream of the AC/PKA and PLC/PKC pathways, experiments in the near future should examine whether ethanol activation of HSF1 is dependent on internal calcium release via these pathways.

Overall, the data presented in this dissertation show that a single ethanol treatment of cortical neurons *in vitro* increased HSF1-mediated transcription of SNARE proteins and GABA release. It would be interesting to extend these findings by assessing HSF1 activity, SNARE expression and GABA release in mice administered with a single dose of ethanol. While it is not known whether ethanol exposure increases GABA release in the cortex *in vivo*, it has been demonstrated that GABA release increases in the VTA of mice administered a single ethanol dose one day prior to recording (Melis et al., 2002). Manipulations of HSF1 activity to demonstrate the mechanism underlying the actions of alcohol, could be achieved by using drugs that either activate or inhibit HSF1, or viral vectors that lead to the overexpression of constituitively active or inactive HSF1.

As the functional neuronal adaptations presented in this dissertation appear to be transient, it would also be interesting to determine whether multiple ethanol exposures could lead to more enduring changes in GABA release, both in primary cortical culture and in self-administering mice. A similar *in vivo* experiment in the Siggins laboratory found increased GABA release in

the central amygdala of chronically ethanol treated rats (Roberto et al., 2004a). Once again, manipulations in HSF1 activity would provide mechanistic information about the adaptations at the GABA presynaptic terminal that may result in enduring changes in local circuitry.

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