HSF1 transcriptional activity mediates alcohol induction of Vamp2 expression and GABA release

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INTRODUCTION

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

While the mechanisms underlining the postsynaptic effects of alcohol on a variety of neurotransmitter receptors are well studied (Lovingier, 1997; Harris, 1999), only in the last decade have researchers begun to investigate the effects of acute and chronic ethanol treatment on neurotransmitter release (Criswell and Breeze, 2005; Siggins et al., 2005; Weiner and Valenzuela, 2006). Acute application of ethanol increases γ-aminobutyric acid (GABA) release in the central amygdala (CeA; Melis et al., 2002), cerebellum (Carta et al., 2004) and ventral tegmental area (VTA; Theile et al., 2008), as revealed by increased miniature inhibitory postsynaptic current (mIPSC) frequency and paired-pulse depression. 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., 2004). Despite these findings that alcohol increases GABA release, the effects of alcohol on synaptic vesicle fusion machinery are not well understood.

Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins 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 zipper-up of synaptobrevin and plasma membrane target SNAREs (t-SNAREs: SNAP-25, syntaxin-1), triggering vesicle fusion and

Abbreviations: ANOVA, analysis of variance; CRE, cAMP response element; CRF, corticotrophin-releasing factor; DIV, days in vitro; GABA, γ-aminobutyric acid; Hsf1, HSF1, heat shock factor 1; Hsp, HSP, heat shock protein; mEPSC, miniature excitatory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean; siRNA, small interfering RNA; SNAP-25, Snap-25, synaptosomal-associated protein 25; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Stx1, syntaxin-1; Syt1, synaptotagmin 1; Synt1, synaptophysin 1; TTX, tetrodotoxin; VAMP, Vamp, synaptobrevin/vesicle-associated membrane protein; VTA, ventral tegmental area.
neurotransmitter release. We have found that a subset of genes encoding SNAREs and SNARE-associated proteins are induced by acute alcohol exposure, including synaptotagmin 1 (Syt1), Vamp2, and Snap25 (Varodayan et al., 2011).

In particular, our laboratory showed that alcohol exposure rapidly induced Vamp2 gene expression, but not Vamp1 (Varodayan et al., 2011). These two genes encode distinct isoforms of synaptobrevin, but are not strictly redundant as VAMP2-deficient mice die shortly after birth (Schoch et al., 2001) and mice with a VAMP1 null mutation develop a neuromuscular wasting disease and die within 2 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, including across the entire cortex (Gene Expression Nervous System Atlas [GENSAT; Gong et al., 2007] Project. NINDS Contracts N01NS02331 & N01NS06267). Whereas Vamp1 mRNA levels predominate in the diencephalon, midbrain, brainstem, and spinal cord (Trimble et al., 2007; Charles River Laboratories, Wilmington, MA) as previously described (Gong et al., 2007).

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 (Huetttner and Baughman, 1986) with modifications (Ma et al., 2004; Varodayan et al., 2011). Cortical neurons were cultured for 14–21 days in vitro (DIV) and then exposed to ethanol (final concentrations 10–150 mM; Sigma-Aldrich, St. Louis, MO) or vehicle Dulbecco’s phosphate-buffered saline control (Invitrogen, Carlsbad, CA) for specific time periods (15 min–24 h), by addition directly to the culture medium. All transfection protocols and electrophysiology recordings were performed after 16 DIV.

**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 AND ETHANOL EXPOSURE**

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 (Huetttner and Baughman, 1986) with modifications (Ma et al., 2004; Varodayan et al., 2011).

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**QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qPCR) ANALYSES OF mRNA LEVELS**

qPCR was carried out as previously described (Ma et al., 2004; Pignataro et al., 2007; Varodayan et al., 2011). Briefly, total RNA was isolated from the neurons using TRIzol (Invitrogen) and cDNA was prepared with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The first-strand reverse transcribed cDNA was then used as a template for PCR amplification with the appropriate specific primer pairs listed below. qPCR reactions were carried out with iQ SYBR Green Supermix (Bio-Rad) using a Chromo4 Real-Time PCR machine (Bio-Rad).

In preliminary experiments, the Vamp2 cDNA concentration was normalized against Actb, Gapdh and 18S [gene encoding ribosomal protein 18S] (QuantumRNA Internal Standards, Ambion, Austin, TX) cDNA within the same sample. As the results were not significantly different among the three internal standards, for all subsequent experiments the cDNA concentration for the gene of interest was normalized against the concentration of Actb cDNA within the same sample. The final results were expressed as percentage of increase vs. the control.

The following primers (and acquisition temperatures) were used for qPCR: Actb (82°C) forward (5′-TCATGAAATGTGACGTGTGAC-3′), reverse (5′-CCTAGAAGCCATTTGCGGTCACAGATG-3′); Gapdh (77°C) forward (5′-AACCTTGCGATTGTGAAGAG-3′), reverse (5′-ACACTTGCGGGTGAAGCAACA-3′); Vamp1 (72°C) forward (5′-AGACATCAACATTGAGACACAGTTTGA-3′), reverse (5′-GATGACAGATAGCCTCGCAGG-3′); Vamp2 (76°C) forward (5′-GGTACCGGACGGAGAT-3′), reverse (5′-GATGGCGGATACATCCCTGGG-3′).

**RNA INTERFERENCE EXPERIMENTS**

RNA interference experiments were performed with 20–25 nucleotide small interference RNA (siRNA), as previously described (Pignataro et al., 2007; Varodayan et al., 2011). Briefly, cultured cortical neurons were transfected with Hsf1 or control scrambled siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 37°C. Cells were washed once and the transfection medium was replaced with conditioned medium for another 24 h prior to ethanol or vehicle treatment.

**CONSTITUTIVELY ACTIVE AND INACTIVE HEAT SHOCK FACTOR 1 (Hsf1) CONSTRUCTS**

We made use of a constitutively transcriptionally active form of HSF1 (Hsf1-act, BH-S), as well as a dominant negative mutant form of HSF1 that suppresses HSF1 transcriptional activity (Hsf1-inact, AV-ST), as previously described (Pignataro et al., 2007; Varodayan et al., 2011). Hsf1-act has amino acids 203–315 deleted in the regulatory domain of HSF1 (Zuo et al., 1995), while Hsf1-inact has a deletion in the transcription activation domain of amino acids 453–523 (Zuo et al., 1995). Both constructs were generated by Dr. Richard Voellmy (University of Miami) and cloned into pcDNA3.1+ (Invitrogen). Transfections were performed with 1 μg of DNA and 9 μL of nuphenin (Enzo Life Sciences, Farmingdale, NY), and sister cultures were transfected with the empty pcDNA3.1+ vector as sham controls, as described previously (Pignataro et al., 2007; Varodayan et al., 2011).

**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 5–15 min or 4–8 h, cells were washed once with fresh media to remove ethanol before being incubated in an external solution containing: 124 mM NaCl, 2.5 mM KCl, 2 mM
MgSO₄, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, 26 mM NaHCO₃ and 10 mM glucose (all Sigma), at 310 mOsm, and pH 7.4. mPSCs were recorded in the presence of tetrodotoxin (TTX; 100 nM; Tocris, Bristol, UK), with excitatory events (mEPSCs) isolated using 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, Sarasota, FL) with a resistance of 3–6 MΩ.

The qPCR 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. 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 ± s.e.m and the details of the statistical analyses are included in the appropriate figure legends.

RESULTS

ALCOHOL INCREASES VAMP2 GENE EXPRESSION

Our initial experiments confirmed our previous finding that Vamp2 is an alcohol-responsive gene (Varodayan et al., 2011). We found that ethanol induction of Vamp2 mRNA levels was concentration-dependent (Figure 1A), with the Vamp2 gene responding modestly to ethanol concentrations more relevant to social intoxication (10–30 mM) and strongly to the high ethanol concentrations similar to those measured in blood samples of chronic alcoholics (80–100 mM) (Urso et al., 1981). The ethanol effect on Vamp2 gene expression showed a half-maximal activation at 40 ± 6 mM (33 ± 4% increase compared with ethanol-naïve control) and saturated at 80 mM (57 ± 5% increase). These brief exposures to high ethanol concentrations were not toxic to the neurons, as treatment with 100 mM ethanol caused little, if any, apoptosis, as previously reported (Pignataro et al., 2007). The time course of the activation of Vamp2 transcription by 60 mM ethanol was rapid, with Vamp2 gene expression significantly increased at 30 min of exposure (22 ± 4% increase; Figure 1B). Vamp2 mRNA levels continued to rise during 8 h of 60 mM ethanol exposure (87 ± 10% increase) and were further increased at 24 h of continuous exposure (103 ± 9% increase).

HSF1 TRANSCRIPTIONAL ACTIVATION MEDIATES ALCOHOL INDUCTION OF VAMP2 GENE EXPRESSION

A subset of alcohol-responsive genes are known to be up-regulated via activation of the transcription factor, heat shock factor 1 (HSF1; Pignataro et al., 2007, 2013; Varodayan et al., 2011). To investigate whether HSF1 mediates Vamp2 gene induction by ethanol, we altered HSF1 protein expression and assessed changes in Vamp2 mRNA levels after ethanol treatment. We found that knock-down of HSF1 protein, using neuronal transfection with Hsf1 siRNA, decreased Vamp2 gene induction after ethanol exposure (from 61 ± 10% increase to 20 ± 7%; Figure 2A). Transfection with control siRNA had no effect on basal Vamp2 mRNA levels (Figure 2A).

Previous work from our laboratory demonstrated that the Vamp1 gene was not induced when primary cortical culture was exposed to 60 mM ethanol for 1 h (Varodayan et al., 2011). Here we found that the knock-down of HSF1 protein, using neuronal transfection of Hsf1 siRNA, had no effect on Vamp1 mRNA levels.

To confirm the role of HSF1 in mediating Vamp2 gene induction, we used a constitutively active Hsf1 construct (Hsf1-act).

FIGURE 1 | Ethanol increases Vamp2 gene expression. (A) Vamp2 mRNA levels increase after 1 h treatment with different concentrations of ethanol, as measured by qPCR. The half-maximal activation of Vamp2 was calculated as 40 ± 6 mM [n ≥ 6; F₁₀, ₇₇ = 20.45; p < 0.0001]. (B) Vamp2 mRNA levels increase after 60 mM ethanol exposure over time [n ≥ 6; F₁₀, ₁₉₅ = 39.58; p < 0.0001; *p < 0.05; ***p < 0.001].
This construct encodes a transcriptionally active HSF1 protein that can directly induce heat shock protein (Hsp) gene transcription in the absence of heat stress (Zuo et al., 1995; Xia et al., 1999). Neuronal transfection of this construct increased Vamp2 gene expression to a level similar to that seen after 1 h of 60 mM ethanol exposure (42 ± 6% increase; Figure 2B). Conversely, a dominant-negative Hsf1 construct (Hsf1-inact), which encodes a transcriptionally inactive HSF1 protein that suppresses stress-induced Hsp gene expression (Zuo et al., 1995; Xia et al., 1999), abolished the effect of ethanol exposure on Vamp2 mRNA levels (from a 62 ± 7% increase to 11 ± 4%; Figure 2B). Hsf1-inact transfection alone had no effect on basal Vamp2 gene expression (Figure 2B). These experiments reveal that HSF1 transcriptional activity 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.

ALCOHOL INCREASES mIPSC FREQUENCY

As Vamp2 is one of several alcohol-responsive genes that encode proteins intimately involved in synaptic vesicle fusion (Varodayan et al., 2011), we explored whether ethanol alters neurotransmitter release. To investigate this potential mechanism, we used whole-cell voltage clamp electrophysiology to record mPSCs in ethanol exposed cultured cortical neurons treated with 100 nM TTX to block action potential-dependent neurotransmitter release. In these experiments, increased mPSC frequency indicates alterations in the presynaptic terminal leading to an increased probability of 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 h 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 compared to control neurons, as seen in the representative traces and bar graph (fC = 0.42 ± 0.08 Hz, fE = 1.11 ± 0.23 Hz; Figure 3A upper panel, B). Ethanol had no effect on mIPSC amplitude (AC = 10.68 ± 0.93 pA, AE = 10.98 ± 0.74 pA; Figure 3A lower panel, C) or the rise time constant (tRc = 3.21 ± 0.22 ms, tRe = 3.24 ± 0.16 ms), but shortened the decay time constant (tDc = 12.59 ± 2.05 ms, tDe = 8.19 ± 0.78 ms; Table 1). The mIPSCs were totally blocked by...
the perfusion of 20 μM gabazine and partially recovered upon washout in all 5 cells tested, indicating that these events are GABAAergic. Similar experiments conducted after 5–15 min of 60 mM ethanol exposure revealed no change in mIPSC frequency (fC = 0.47 ± 0.08 Hz, fE = 0.55 ± 0.13 Hz; nC = 13, nE = 17) or amplitude (AC = 9.40 ± 0.95 pA, AE = 8.03 ± 0.78 pA; nC = 13, nE = 17), suggesting that this mechanism of ethanol-induced GABA release may require the prolonged processes of transcription and translation.

To study the effects of ethanol on excitatory currents (mEPSCs), we used 20 μM gabazine to block GABAergic receptor-mediated events. We found no change in mEPSC frequency (fC = 0.44 ± 0.13 Hz, fE = 0.40 ± 0.07 Hz; nC = 22, nE = 22) or amplitude (AC = 6.10 ± 0.13 pA, AE = 6.79 ± 0.38 pA; nC = 22, nE = 22) after 60 mM ethanol exposure for 4–8 h. Details of mEPSC kinetics are displayed in Table 1.

**HSF1 TRANSCRIPTIONAL ACTIVITY MEDIATES ALCOHOL INDUCTION OF mIPSC FREQUENCY**

To investigate whether HSF1 transcriptional activity mediates the increased mIPSC frequency observed after ethanol exposure, we altered HSF1 protein expression and assessed mIPSC kinetics. Neuronal transfection of Hsf1-act increased mIPSC frequency similar to the frequency observed after ethanol exposure (fC = 0.18 ± 0.01 Hz, fE = 0.61 ± 0.19 Hz, fHsf1act = 0.63 ± 0.11 Hz; Figure 4A). Conversely, the dominant-negative Hsf1-inact construct abolished the effect of ethanol exposure on mIPSC frequency (fC = 0.34 ± 0.05 Hz, fE = 0.88 ± 0.25 Hz, fHsf1inact = 0.37 ± 0.04 Hz, fHsf1inact+E = 0.51 ± 0.19 Hz), while Hsf1-inact transfection alone had no effect on mIPSC frequency (Figure 4C). No changes were observed in amplitudes (Figures 4B,D), rise times or decay times after transfection with either the Hsf1-act or Hsf1-inact constructs. These experiments reveal that HSF1 transcriptional activity increases GABA release and mediates ethanol induction of mIPSC frequency. In summary, in this study we have shown that ethanol acts via HSF1 to increase the gene expression of a specific subset of proteins involved in synaptic vesicle fusion and stimulate 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 SNARE complex proteins is induced by alcohol exposure. In particular, we found that alcohol differentially regulates two genes encoding synaptobrevin isoforms, rapidly inducing the Vamp2 gene, but not Vamp1, and 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 acts via HSF1 to alter neurotransmitter release. We found that HSF1 transcriptional activity mediates ethanol-induced GABA release, but has no effect on glutamatergic synaptic vesicle fusion.

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**Table 1** A single ethanol exposure alters mPSC kinetics.

| Treatment | Frequency (Hz) | Amplitude (pA) | Rise time (ms) | Decay time (ms) |
|-----------|----------------|----------------|----------------|-----------------|
| mIPSC C (n = 22) | 0.42 ± 0.08 | 10.68 ± 0.93 | 3.21 ± 0.22 | 12.59 ± 2.05 |
| E (n = 19) | 1.11 ± 0.23* | 10.98 ± 0.74 | 3.24 ± 0.16 | 8.19 ± 0.78* |
| mEPSC C (n = 22) | 0.44 ± 0.13 | 6.10 ± 0.13 | 1.94 ± 0.16 | 0.79 ± 0.15 |
| E (n = 22) | 0.40 ± 0.07 | 6.79 ± 0.38 | 2.67 ± 0.22* | 1.02 ± 0.15 |

*P < 0.05.

Data are obtained from neurons exposed to 60 mM ethanol for 4–8 h and control neurons.

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**FIGURE 4** Increased GABA release after ethanol exposure requires HSF1 transcriptional activity. (A) HSF1 transcriptional activity increases the probability of GABA release. Hsf1-act transfection increased mIPSC frequency, similar to the level seen with 60 mM ethanol exposure for 4–8 h (E). Control cultures were sham transfected with an empty pcDNA3.1 construct [C; nC = 15, nE = 19; F2, 48 = 3.46; P < 0.05]. (B) HSF1 activity does not alter the mean mIPSC amplitude in neurons transfected with an Hsf1-act construct, exposed to ethanol (E) or control sham transfected [C; nC = 15, nE = 17; F2, 48 = 0.32; P = 0.73]. (C) Ethanol stimulation of mIPSC frequency is mediated by activated HSF1. Hsf1-inact transfection reduced the effects of ethanol (E) on mIPSC frequency. Hsf1-inact transfection alone had no effect on mIPSC frequency compared to control cultures sham transfected with empty pcDNA3.1 construct [C; nC = 16, nE = 10; F2, 48 = 12.3, F2, 48 = 2.56; P = 0.07]. (D) HSF1 activity does not alter the mean amplitude of mIPSCs in neurons transfected with an Hsf1-inact construct, exposed to ethanol (E) or vehicle control [C; nC = 16, nE = 10, F2, 48 = 12; F2, 48 = 0.0639; P = 0.60; **P < 0.05, ***P < 0.001, or n.s. denotes no significance].
A SINGLE ALCOHOL EXPOSURE INDUCES SNARE GENE EXPRESSION

We have previously shown that acute alcohol exposure rapidly induces transcription of some SNARE complex proteins, including the Vamp2, Syt1 and Snap25 genes, but not the Vamp1, Stx1a, and Syp genes (Varodayan et al., 2011). In this study we investigated the mechanism underlying Vamp2 gene induction by alcohol. There are few, if any, comparable studies on the effects of alcohol on Vamp2 gene expression. Interestingly, a recent transcriptome profiling study used tissue from alcoholic human brain 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 ethanol induction of the Vamp2 gene is mediated by HSF1 activity. Transcriptional activation of HSF1 is a multistep process that involves: HSF1 translocation from the cytoplasm, where it is sequestered by chaperone proteins, to the nucleus; HSF1 trimerization and inducible hyperphosphorylation; and HSF1 binding to a DNA element to stimulate transcription (Cotto et al., 1997). We have previously shown that 60 mM ethanol exposure of primary cortical culture induces HSF1 translocation into the nucleus (Pignataro et al., 2007), phosphorylates HSF1 (Varodayan et al., 2011) and stimulates Hsp gene expression (Pignataro et al., 2007), indicating that ethanol promotes HSF1 transcriptional activity. Several other laboratories have also reported an association between alcohol exposure and HSF1-dependent gene induction, including microarray studies where alcohol treatment increased Hsp gene expression (Lewohl et al., 2000; Gutala et al., 2004; Worst et al., 2005). In addition, we have previously reported that ethanol acts via HSF1 to induce the Syt1 gene and the gene encoding the α4 subunit of the GABA_A receptor (Pignataro et al., 2007; Varodayan et al., 2011). As a whole, our current studies 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 h, but not 5–15 min, suggesting that this mechanism of ethanol-induced GABA release may require the prolonged processes of transcription and translation. Similar experiments by the Morrow laboratory found an unchanged mIPSC frequency in cultured cortical rat neurons exposed to 50 mM ethanol for either 4 h or 1–7 days (Fleming et al., 2009; Werner et al., 2011). As a whole, these results suggest that the increase in mIPSC frequency after a single ethanol exposure may be a transient neuronal adaptation. Studies conducted in vivo also showed changes in mIPSC frequency across the rodent brain, with Melis et al. (2002) observing 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 this frequency was further increased by the bath application of ethanol, indicating that the acute, and chronic effects of ethanol on GABA release are differentially mediated (Roberto et al., 2004). Overall, these data define a model of transient presynaptic adaptation, where ethanol promotes HSF1 transcriptional activity to induce a temporary increase in GABA release. This transient change in neurotransmitter release may 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 ethanol exposure on GABA release have been largely unstudied. Our detailed analysis revealed that ethanol treatment of cultured cortical neurons increases GABA release via HSF1 transcriptional activity, although it is likely that a variety of alternate and overlapping mechanisms underlie the similar changes observed after different ethanol exposure models and across brain regions. For example, ethanol application in the cerebellum rapidly increases the number of mIPSC events in interneurons via activation of both AC/PKA and PLC/PKC pathways and internal calcium store release (Kelm et al., 2007, 2008, 2010). The effects of alcohol administration on these kinase pathways provide for a relatively fast GABAergic neuronal response, while the enhanced GABA release that occurs after chronic ethanol exposure is likely to be regulated by longer-lasting changes in gene expression that are triggered by HSF1 and other transcription factors.
similar changes in GABA_\alpha 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 may be a temporary increase in the expression of α4-containing GABA_\alpha receptors. This transient change 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 SYNAPSE**

The data presented here show that a single ethanol exposure induces Vamp2 gene expression and stimulates GABA release via HSFI transcriptional activity. Repeated ethanol exposure could result in a persistent adaptation at the GABAergic synapse and lead to enduring changes in the local circuitry that may play a role in the development of alcohol abuse and dependence. It is interesting to note that ethanol’s effects on HSFI appear to alter neurotransmitter release in GABAergic, and not glutamatergic, neurons, and the apparent specificity of this effect among a variety of synapses merits further study.

**AUTHOR CONTRIBUTIONS**

Participated in research design: Florence P. Varodayan and Neil L. Harrison. Conducted experiments: Florence P. Varodayan. Performed data analysis: Florence P. Varodayan. Wrote or contributed to the writing of the manuscript: Florence P. Varodayan and Neil L. Harrison.

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