Neuroscience 193 (2011) 63–71

ALCOHOL INDUCES SYNAPTOTAGMIN 1 EXPRESSION IN NEURONS VIA ACTIVATION OF HEAT SHOCK FACTOR 1

F. P. VARODAYAN,[a,b] L. PIGNATARO[b] AND N. L. HARRISON[b,c]*

[a]*Department of Neuroscience, Columbia University, 40 Haven Avenue, Room 865, New York, NY 10032, USA*

[b]*Department of Anesthesiology, Columbia University, 622 West 168th Street, PH-511, New York, NY 10032, USA*

[c]*Department of Pharmacology, Columbia University, 630 West 168th Street, PH 7W 318, New York, NY 10032, USA*

Abstract—Many synapses within the central nervous system are sensitive to ethanol. Although alcohol is known to affect the probability of 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 h) 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 up-regulated by ethanol in mouse cortical neurons, and that the distribution of Syt1 protein along neuronal processes is also altered. *Syt1* mRNA up-regulation 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* transcription, while transfection of *Hsf1* small interfering RNA (siRNA) or a constitutively inactive *Hsf1* construct into neurons attenuates the induction of *Syt1* by ethanol. This suggests that the activation of HSF1 can induce *Syt1* 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 (soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor; SNARE) proteins share this property of induction by ethanol, suggesting that alcohol may trigger a specific coordinated adaptation in synaptic function. This molecular mechanism could explain some of the changes in synaptic function that occur following alcohol administration and may be an important step in the process of neuronal adaptation to alcohol.

*Correspondence to: N. L. Harrison, Department of Anesthesiology, Columbia University, 622 West 168th Street, PH-511, New York, NY 10032, USA. Tel: +1-212-304-6459; fax: +1-212-305-3675.
E-mail address: nh2298@columbia.edu (N. L. Harrison).

Abbreviations: ARE, alcohol response element; ARG, alcohol-responsive gene; DIV, days *in vitro*; HSF1, heat shock factor 1; Hsp, heat shock protein; OD, optical density; qPCR, quantitative real-time polymerase chain reaction; siRNA, small interfering RNA; SNAP-25, synaptosomal-associated protein 25; SNARE, soluble NSF attachment protein receptor; Stx1a, syntaxin 1a; Syt1, synaptotagmin 1; Syp1, synaptophysin 1; VAMP, vesicle-associated membrane protein.

Key words: alcohol, synaptotagmin 1 (Syt1), heat shock factor 1 (HSF1), SNARE proteins, gene expression, cortical neurons.

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 post-synaptic effects of alcohol on a variety of neurotransmitter receptors (Lovinger, 1997; Harris, 1999), 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; Roberto et al., 2006; Weiner and Valenzuela, 2006).

Electrophysiological work by several groups indicates that acute application of ethanol increases pre-synaptic 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 motoneurons (Ziskind-Conhaim et al., 2003). In addition, investigators have used confocal microscopy in hippocampal slices pre-loaded 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 toward 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 (ARGs), 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 ARG 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 the forebrain, midbrain, and in most brainstem and spinal cord neurons (Xu et al., 2007). In the presence of calcium, Syt1 binds to both v-soluble NSF attachment protein receptors (v-SNAREs), such as synaptobrevin/VAMP (vesicle-associated membrane protein), and plasma membrane phospholipids (Martens et al., 2007). This brings the two membranes together to promote zippering of VAMP and target

0306-4522/11 $ - see front matter
doi:10.1016/j.neuroscience.2011.07.035

t-SNAREs (synaptosomal-associated protein 25 [SNAP-25], syntaxin-1a) on the plasma membrane, and thus triggers vesicle fusion leading to 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 these mice have attenuated IPSCs in cortical neurons (Xu et al., 2007). The over-expression 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 additional molecular mechanisms by which alcohol can affect neurotransmitter release dynamics and synaptic transmission.

EXPERIMENTAL PROCEDURES

Cell culture and immunocytochemistry

Cortical neurons were cultured from embryonic day 17–18 C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) as previously described (Huettner and Baughman, 1986) with modifications (Ma et al., 2004). Lower-density cortex cultures were also established and maintained using techniques similar to those used for hippocampal neurons (Banker and Goslin, 1991). The lower-density cultures were used for immunocytochemistry experiments 7–11 days *in vitro* (DIV). Immunostaining was performed with a rabbit polyclonal anti-Syt1 antibody (1:200, Synaptic Systems, Göttingen, Germany) and a mouse monoclonal anti-α-tubulin antibody (1:10,000, clone DM1A, Sigma-Aldrich, St. Louis, MO, USA). Cells were mounted with ProLong Gold anti-fade reagent containing the nuclear stain DAPI (Molecular Probes, Eugene, OR, USA). Images were acquired with an inverted Zeiss Axiovert 200 confocal microscope (LSM 510 META; Carl Zeiss Meditech, Thornwood, NY, USA) equipped with diode (405 nm), argon (458, 477, 488, 514 nm), HeNe1 (543 nm), and HeNe2 (633 nm) lasers.

Ethanol and heat stress treatments

Cortical neurons (7–11 DIV) were exposed to ethanol (10–150 mM; Sigma-Aldrich) for specific time periods (15 min to 24 h), by addition directly to the culture medium. Cells were subjected to heat stress by transferring them to an incubator set at 42 °C for a period of 1–2 h.

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

Total RNA was isolated from cultured neurons using TRIzol (Invitrogen, Carlsbad, CA, USA). cDNA was prepared from total RNA with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). For cDNA preparation, reactions were performed in a final volume of 20 μl; primers were annealed at 25 °C for 5 min, RNA was reverse transcribed at 42 °C for 90 min, followed by heat inactivation at 95 °C for 5 min, and the reaction mixtures were stored at −20 °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 was carried out with iQ SYBR Green Supermix (Bio-Rad) as previously described (Ma et al., 2004). In preliminary experiments, *Syt1* cDNA concentration was normalized against *Actb* and *Gapdh* cDNA, and against *18S* (gene encoding ribosomal RNA 18S) (QuantumRNA Internal Standards, Ambion, Austin, TX, USA) within the same sample. For subsequent work, the cDNA concentration of the gene of interest was normalized against the concentration of *Actb* cDNA within the same sample, and the results were finally expressed as 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: *Syt1* (70 °C) forward (5′-caccgtgggccttaattgc-3′), reverse (5′-tgttaatggcgttcttccctc-3′); *Actb* (82 °C) forward (5′-TCATGAAGTGTGACGTTGACATCCGT-3′), reverse (5′-CCTAGAAGCATTTGCGGTGCACGATG-3′); *Gapdh* (77 °C) forward (5′-aactttggcattgtggaagg-3′), reverse (5′-acacattgggggtaggaaca-3′); *Snap25* (75 °C) forward (5′-CAACTGGAACGCATTGAGGAA-3′), reverse (5′-GGCCACTACTCCATCCTGATTAT-3′); *Stx1a* (77 °C) forward (5′-TCCAAGCTAAAGAGCATTGAGC-3′), reverse (5′-GGCGTTGTACTCGGACATGA-3′); *Syp1* (77 °C) forward (5′-GAGAGAACAACAAAGGGCCAA-3′), reverse (5′-CGGCACATAGGCATCTCCT-3′); *Vamp1* (72 °C) forward (5′-AGCATCACAATTTGAGAGCAGT-3′), reverse (5′-GATGGCACAGATAGCTCCCAG-3′); *Vamp2* (76 °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, USA) and incubated with the following antibodies: rabbit polyclonal anti-Syt1 (1:1500, Synaptic Systems), rabbit polyclonal anti-heat shock factor 1 (HSF1) (1:500, Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-phosphorylated HSF1 (pHSF1, 1:4000, Enzo Life Sciences, Farmingdale, NY, USA), 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, USA), and the VisionWorks LS software (UVP). Digital images were quantified with ImageJ 1.36b (NIH, Bethesda, MD, USA), 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: $OD = \mathrm{Log}_{10}(255/(255 - \text{gray value}))$. The OD values were normalized to the α-tubulin or eIF4E internal standards to compensate for variations in protein loading and transfer.

RNA interference experiments

RNA interference experiments were performed with pre-synthesized small interference RNA (siRNA), consisting of a pool of three target-specific 20- to 25-nucleotide siRNAs designed to knock down the expression of a particular gene. Cultured cortical neurons were transfected on DIV 7 with *Hsf1* siRNA or control siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA, USA). 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 h at 37 °C. Cells were washed once and the transfection medium replaced with conditioned medium;