Cellular/Molecular

Alcohol Regulates Gene Expression in Neurons via Activation of Heat Shock Factor 1

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Drinking alcohol causes widespread alterations in gene expression that can result in long-term physiological changes. Although many alcohol-responsive genes (ARGs) have been identified, the mechanisms by which alcohol alters transcription are not well understood. To elucidate these mechanisms, we investigated *Gabra4*, a neuron-specific gene that is rapidly and robustly activated by alcohol (10–60 mM), both *in vitro* and *in vivo*. Here we show that alcohol can activate elements of the heat shock pathway in mouse cortical neurons to enhance the expression of *Gabra4* and other ARGs. The activation of *Gabra4* by alcohol or high temperature is dependent on the binding of heat shock factor 1 (HSF1) to a short downstream DNA sequence, the alcohol response element (ARE). Alcohol and heat stimulate the translocation of HSF1 from the cytoplasm to the nucleus and the induction of HSF1-dependent genes, *Hsp70* and *Hsp90*, in cultured neurons and in the mouse cerebral cortex *in vivo*. The reduction of HSF1 levels using small interfering RNA prevented the stimulation of *Gabra4* and *Hsp70* by alcohol and heat shock. Microarray analysis showed that many ARGs contain ARE-like sequences and that some of these genes are also activated by heat shock. We suggest that alcohol activates phylogenetically conserved pathways that involve intermediates in the heat shock cascade and that sequence elements similar to the ARE may mediate some of the changes in gene expression triggered by alcohol intake, which could be important in a variety of pathophysiological responses to alcohol.

Key words: **alcohol; heat shock; *Gabra4*; heat shock factor 1 (HSF1); gene expression; ion channels; cortical neurons**

Introduction

Drinking alcohol alters behavior (National Institute on Alcohol Abuse and Alcoholism, 2003), and exposure to the drug in pregnant females and neonates can cause developmental defects in humans and animals (Sulik et al., 1981; Friedman, 1982; Goodlett et al., 2005; Sulik, 2005). The mechanisms by which alcohol achieves its short- and long-term effects on the brain are largely unknown, although ion channels and neurotransmitter receptors have become the targets of intense inquiry (Worst and Vrana, 2005). A number of excellent studies using gene arrays have identified a variety of genes that are upregulated or downregulated by short- or long-term exposure to alcohol in experimental animals and man (Lewohl et al., 2000; Dodd et al., 2006; Mulligan et al., 2006). Despite these investigations, little is known of the molecular mechanisms by which alcohol might alter transcriptional efficiency (Wilke et al., 1994; Hassan et al., 2003). One gene reported to be especially sensitive to alcohol is *Gabra4*, which is expressed at high levels in the CNS, in neurons of the thalamus, striatum, dentate gyrus, and cerebral cortex (Pirker et al., 2000; Roberts et al., 2005, 2006). The product of *Gabra4* is a component of the ligand-gated ion channel that functions as an extrasynaptic receptor for the inhibitory neurotransmitter GABA (Farrant and Nusser, 2005; Jia et al., 2005; Chandra et al., 2006). It has been previously demonstrated that levels of *Gabra4* mRNA and the corresponding protein are upregulated by chronic intermittent administration and subsequent withdrawal from alcohol, in both *in vivo* and *in vitro* studies (Cagetti et al., 2003; Sanna et al., 2003). We reasoned that a careful study of the mechanisms of *Gabra4* regulation at the cellular level might reveal control elements and molecular mechanisms that are responsible for the sensitivity of this gene to alcohol.

Materials and Methods

Cell culture and immunocytochemistry. Cortical neurons were cultured from embryonic day 17–18 C57BL/6 mice as described previously (Huettner and Baughman, 1986) with modifications (Ma et al., 2004). Low-density cortex cultures were established and maintained using techniques similar to those used for hippocampal neurons (Banker and Goslin, 1991). Low-density cultures were used for immunocytochemistry experiments no sooner than 11 d after plating. Immunostaining to detect HSF1 protein was performed with an affinity-purified rabbit anti-HSF1 antibody (0.08 μg/ml; Cell Signaling Technology, Danvers, MA) and a monoclonal anti-α-tubulin antibody (0.2 μg/ml, clone DM1A; Sigma-Aldrich, St. Louis, MO). Cells were mounted with ProLong Gold antifade reagent containing the nuclear stain 4′,6′-diamidino-2-

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phenylindole (DAPI) (Invitrogen, Carlsbad, CA). To assess the contribution of glia to the culture, these were also stained using a mouse monoclonal anti-neuronal nuclei (NeuN) antibody (2 μg/ml; Millipore, Billerica, MA) and a rabbit polyclonal anti-glial fibrillary acidic protein antibody (GFAP, 5.8 μg/ml; Dako, Carpinteria, CA). 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.

Ethanol and heat shock treatment. In most of our experiments (except for the immunocytochemistry, as described above), cortical neurons were cultured for 5–7 d *in vitro* (DIV) and then exposed to ethanol or heat for a specific time (15–240 min). Ethanol (10–150 mM; Shelton Scientific, Peosta, IA) was added directly to the culture medium. Cells were subjected to heat shock by transferring them to an incubator set at 42°C.

Real-time PCR 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°C for 5 min, and RNA was reverse transcribed at 42°C for 50 min, followed by RNase H digestion. Enzymes were subsequently heat-inactivated 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. Quantitative "real-time" reverse transcriptase PCR (Q-PCR) was performed as described previously (Ma et al., 2004). For each sample, the cDNA concentration for the gene of interest was then normalized against the concentration of *Actb* cDNA in 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. A control sample was included in each experiment, in which reverse transcriptase was omitted from the reaction, to monitor for genomic DNA contamination.

Q-PCR primers. The following primers were used for Q-PCR: *Gabra4* forward (5′-CCACCCTAAGCATCAGTGC-3′), reverse (5′-CTGAATGGACCAAGGCATTT-3′); *Actb* forward (5′-TCATGAAGTGTGACGTTGACATCCGT-3′), reverse (5′-CCTAGAAGCATTTGCGGTGCACGATG-3′); *Gabra3* forward (5′-AAGAACCTGGGGACTTTGTGA-3′), reverse (5′-GCCGATCCAAGATTCTAGTGAAG-3′); *Gria1* forward (5′-GTCCGCCCTGAGAAATCCAG-3′), reverse (5′-CTCGCCCTTGTCGTACCAC-3′); *Grin2b* forward (5′-TTCGTGAACAAGATCCGCAG-3′), reverse (5′-ATGTGTAGCCGTAGCCAGTCA-3′); *Hsp27* forward (5′-ATCCCCTGAGGGCACACTTA-3′), reverse (5′-CCAGACTGTTCAGACTTCCCAG-3′); *Hsp40* forward (5′-TTCGACCGCTATGGAGAGGAA-3′), reverse (5′-CACCGAAGAACTCAGCAAACA-3′); *Hsp70* forward (5′-AATTGGCTGTATGAAGATGG-3′), reverse (5′-CATTGGTGCTTTTCTCTACC-3′); *Hsp90* forward (5′-GAACATTGTGAAGAAGTGCC-3′), reverse (5′-CATATACACCACCTCGAAGC-3′); *ryab* forward (5′-GAAGAACGCCAGGACGAACAT-3′), reverse (5′-ACAGGGATGAAGTGATGGTGAG-3′); *Hsf1* forward (5′-AACGTCCCGGCCTTCCTAA-3′), reverse (5′-AGATGAGCGCGTCTGTGTC-3′).

Immunoblotting. The relative abundance of α4 or HSF1 protein was determined by immunoblotting as described previously (Jia et al., 2005). Cellular fractions were isolated with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL). Cellular fractions (15–100 μg of protein) were incubated with antibodies to $GABA_A$ receptor ($GABA_AR$) α4 subunit (10 μg/ml; Novus Biologicals, Littleton, CO) or HSF1 (20 ng/ml; Cell Signaling Technology) together with an antibody against the translation initiation factor eIF4E (0.3 μg/ml; Cell Signaling Technology) or α-tubulin (0.47 μg/ml; Sigma-Aldrich), which were used as internal standards for loading control. Antibodies against heat shock proteins were HSP27 (0.05 μg/ml), HSP70 (0.13 μg/ml), and HSP90 (0.06 μg/ml), all from Cell Signaling Technology. Quantification and normalization were done as described previously (Jia et al., 2005). Briefly, x-ray films were exposed for time periods appropriate for quantification of the proteins within the dynamic range of the signal. Scanned images of the exposed films were quantified using the program Scion Image for Windows β 4.0.2 (Scion, Frederick, MD). Gel lanes were selected and signals transformed into peaks. The area under each peak (gray value) was transformed into an optical density (OD) value using the following function: $OD = \log_{10}(255/(255 - \text{gray value}))$. The OD values of the protein of interest were normalized to the eIF4E or α-tubulin internal standard to compensate for variations in protein loading and transfer.

Promoter-reporter constructs and luciferase reporter assay. The *Gabra4* basal promoter construct (pLuc-P7), the extended promoter construct (pLuc-P7-EX2), and the mutant extended promoter construct (pLuc-P7-EX2M) were generated by PCR methods, as described previously (Ma et al., 2004). For luciferase assay, these constructs were transiently expressed in neurons by transfection, and reporter gene activity was measured as described previously (Ma et al., 2004). Samples were harvested and analyzed 24–48 h after transfection with the luciferase construct. Relative luciferase activity for each construct was calculated as the ratio of firefly luciferase activity (driven by the promoter-reporter construct) to *Renilla* luciferase activity (driven by the CMV promoter), with the pLuc Basic vector (Promega, Madison, WI) used as a negative control (this contains no eukaryotic promoter or enhancer sequence).

EMSA (gel-shift) and supershift. Electrophoretic mobility shift assay (EMSA, "gel shift") experiments were performed with labeled target DNA probes containing the *Gabra4* alcohol response element (ARE) sequence, a scrambled ARE sequence, or a random sequence. Nuclear fractions were isolated from cortical neurons in culture with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) in the presence of a mixture of protease inhibitors [20 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μM EDTA, 1.3 μM bestatin, 0.14 μM E-64, 10 μM leupeptin, and 3 μM aprotinin; Sigma-Aldrich] and a mixture of serine/threonine phosphatase inhibitors (cantharidin, bromotetramisole, and microcystin LR; Sigma-Aldrich). Nuclear fractions were stored at −80°C and used within a week of isolation. EMSA experiments were performed with biotin-labeled DNA probes using the LightShift Chemiluminescent EMSA kit (Pierce Biotechnology). The binding reaction was performed at room temperature in a 20 μl volume containing 10 mM Tris, 50 mM KCl, and 1 mM dithiothreitol, pH 7.5, with the addition of 8.8 μM poly (dA-dT), 2.5% v/v glycerol, 1 mM EDTA, 50 mM NaCl, 8 μg/μl bovine serum albumin, 2–3 μg of nuclear extract protein, and 1 μM biotinylated target DNA probe. Oligos (35 bp) were synthesized (Invitrogen) with a 5′-biotin label and were: *Gabra4* ARE (5′-TTATGACAACAGGCTGCGTCCTGGATTTGGGGGTA-3′), *Gabra4* ARE scrambled (5′-TTATGACAACAGCTCGTGCGCGTGATTTGGGGTA-3′) and random (5′-CCTAGTGGCTTAGTCGATACGTGACTGTACTTAAA-3′). The consensus ARE sequence is underlined. Double-stranded oligos were prepared by annealing complimentary strands in 10 mM Tris, 1 mM EDTA, and 50 mM NaCl, pH 8.0, at 95°C for 5 min and were cooled to room temperature. The free and bound probes were separated by native PAGE in TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 8.3), transferred to a nylon membrane (Bio-Rad) and cross-linked to the membrane with a UV cross-linker (Stratagene). Biotin-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate (Pierce Biotechnology). The specificity of the binding of the probe to the nuclear extract was tested by adding an excess of unlabeled probe to the EMSA binding reactions.

Supershift experiments were performed in similar conditions using the Nushift Kit (Active Motif, Carlsbad, CA). Nuclear extracts were preincubated at room temperature with a selection of antibodies, followed by the addition of the target DNA probe. Antibodies for supershift experiments were selected based on the location of the epitope to ensure that the epitopes were not involved in DNA binding. Antibodies to the following proteins were HSF1 (2 μg; Active Motif), HSF2 (4 μg; Stressgen), ATF2 (6.4 μg; Active Motif), c-Jun (2 μg; Active Motif), and CREB1 (2 μg; Active Motif). The specificity of the supershift was tested by blocking the antibody with a molar excess of control peptide or by substituting the antibody with preimmune serum. To verify these experimental results, we also tested recombinant rat transcription factor proteins (0.75 μg) rHSF1 (Stressgen) and rHSF2 (Abnova, Taipei, Taiwan) for their ability to reproduce the shift of the target DNA probe.

RNA interference experiments. RNA interference experiments were performed with presynthesized small interference RNA, consisting of a

pool of three target-specific 20–25 nt small interfering RNAs (siRNAs) designed to knock down the expression of a particular gene. Cultured cortical neurons were transfected on DIV 5 with *Hsf1* siRNA or control siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA). Transfection was performed with TransFectin (Bio-Rad) as follows: siRNA (2 μg) was added to OPTI-MEM (150 μl; Invitrogen) for 15 min and then combined with a mixture of TransFectin (3 μl) and OPTI-MEM (150 μl) for an additional 15 min. The culture medium was removed and replaced with 300 μl of transfection medium and the neurons were incubated for 2 h at 37°C. Cells were washed once and the transfection medium replaced with conditioned medium; neurons were maintained for another 24 h before ethanol or heat treatment. Control experiments were performed with a scrambled 20–25 nt siRNA (control siRNA), which does not degrade any known mRNA (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

Constitutively active and inactive Hsf1 constructs. We made use of 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 (Zuo et al., 1995), whereas 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, Miami, FL) and cloned into pcDNA3.1$^+$ (Invitrogen). Transfections were performed with 5 μg of DNA and 9 μg of nupherin (Biomol, Plymouth Meeting, PA), and sister cultures were transfected with an empty pcDNA3.1$^+$ as controls.

In vivo *experiments.* Postnatal day 60 CD1 adult mice were injected intraperitoneally with ethanol (20% v/v in saline) at a dose of 3 g/kg body weight. Saline injections of equal volume were used as controls. Animal care was provided according to the guidelines of Weill Cornell Medical College. It has been previously demonstrated that a dose of 3 g/kg in these mice results in a blood ethanol concentration (BEC) of 300 mg/dl (Ikonomidou et al., 2000; Ieraci and Herrera, 2006), representing a BEC of ~65 mM.

Animals were killed at 2, 4, 8, and 24 h after ethanol administration, brains were rapidly removed, and the cerebral cortex was dissected out and frozen on dry ice. RNA was isolated as described above for real-time PCR analysis. cDNA reactions were performed in a final volume of 100 μl containing 5 μg of total RNA. Q-PCR assays were performed as described. Cellular fractions were isolated with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) and were stained with affinity-purified rabbit anti-HSF1 antibody (20 ng/ml; Cell Signaling Technology).

Gene arrays. For gene microarray analysis, 0.5 μg of total RNA was isolated from cultured neurons treated with alcohol or heat, and this RNA was used to make biotin-labeled cRNA using the Illumina cRNA amplification and labeling kit (Ambion, Austin, TX). Preparation of biotin-labeled cRNA consists of a reverse transcription using an oligo(dT) primer bearing a T7 promoter with ArrayScript, a reverse transcriptase that catalyzes the synthesis of virtually full-length cDNA. The cDNA was then used for a second strand synthesis and purified, before *in vitro* transcription with T7 RNA polymerase. This *in vitro* transcription generated biotin-labeled antisense cRNA for each mRNA in a sample, and this was then used for hybridization on the Illumina arrays. cRNA quality was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) before hybridization. Biotin-labeled cRNA was labeled with the fluorescent dye at the Rockefeller University Gene Array Facility and hybridized onto a Sentrix MouseRef-8 24K expression array bead chip (Illumina, San Diego, CA). The arrays were scanned using the Illumina Bead Station laser scanning imaging system; an average of 30 beads per gene transcript was used to generate the expression data, which were converted into text files and normalized before being analyzed using Genespring software (Agilent Technologies).

Database search. For all genes analyzed, mouse genomic DNA was obtained from the National Center for Biotechnology Information (NCBI; National Institute of Health) and Mouse Genomic Informatics (The Jackson Laboratory, Bar Harbor, ME) databases. DNA sequence analyses were performed using the Vector NTI (Invitrogen) and Spidey (NCBI) programs. Candidate genes were designated as those containing the ARE motif, CTGNGTC, anywhere between 2 kb upstream of the ATG and the second exon. DNA sequence analysis for predicting transcription factor binding sites was performed using the AliBaba2 program and TRANSFAC database.

Statistical analysis. Details of the statistical analysis and *p* values of the data are included in the figure legends, as appropriate. In all cases in which immunoblots are shown, the blot is representative of at least three experiments with similar results.

The analysis of HSF1 immunoreactive granules was performed by standard methods (Cotto et al., 1997) using ImageJ 1.36b (NIH, Bethesda, MD). Grayscale 8-bit calibrated-images (0.8–1 μm optical section) were manually adjusted for threshold, and the area and number of HSF1 granules present in the nucleus of each neuron were calculated. Particles smaller than ~0.02 μm^2 were not considered to be HSF1 stress granules and were discarded from the analysis.

For gene array analysis, a hierarchical clustering algorithm was used to generate the dendrogram, based on the complete-linkage method (Eisen et al., 1998). The distance between two individual samples is calculated by Pearson distance with the normalized expression values. To determine whether any treatments had a significant effect on gene expression behavior across any of the groups under study, we used one-way ANOVA tests, using true biological replicate samples. The list of genes that were differentially expressed at a significant level after the treatments was then subjected to gene ontology analysis using GO/GO SLIMS in Genespring v 7.3 (www.geneontology.org). The gene expression correlations were computed using the Pearson correlation test. The statistical significance of overlap between gene groups was calculated using the standard Fisher's exact test, with the *p* value adjusted with Bonferroni multiple testing correction or hypergeometric probability. This method calculates the probability of overlap corresponding to one or more genes between any given gene list compared against another gene list randomly sampled from a universe of genes. *p* values ≤0.05 were considered statistically

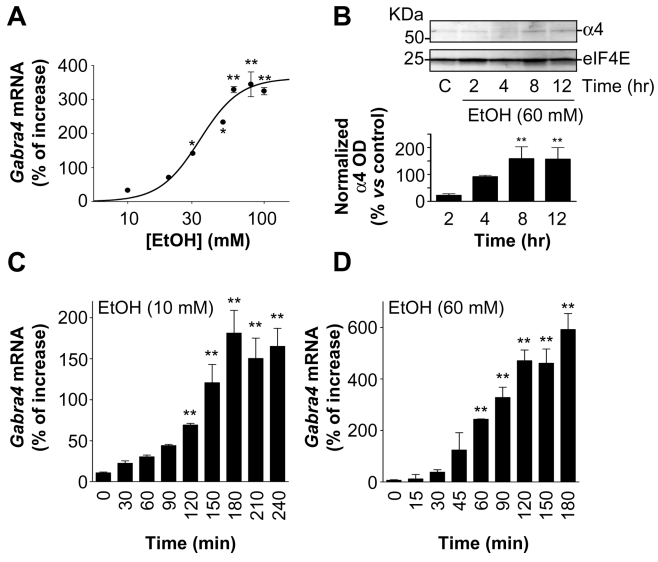

Figure 1. Alcohol (EtOH) rapidly increases *Gabra4* mRNA and α4 protein. ***A***, Concentration–response curve for the effect of EtOH on *Gabra4* expression by Q-PCR in cortical neurons. Data were analyzed by repeated-measures ANOVA and compared with control samples (cells treated with vehicle) by Dunnett's multiple-comparison *post hoc* test, $n \geq 6$). The threshold of EtOH (10 mM, $p < 0.0001$) was defined as the lowest concentration that significantly increased *Gabra4* expression above control value and was obtained by analyzing the tail of the concentration–response curve by one-tailed unpaired *t* test; $n \geq 6$. ***B***, Immunoblot analysis of $GABA_AR$ α4 subunit protein. C, Control. The graph shows the relative abundance of α4 protein in neurons exposed to EtOH for different time periods. The bar graph represents normalized OD (analyzed by 1-way ANOVA vs control with Dunnett's multiple-comparison *post hoc* test, $n \geq 3$). ***C***, ***D***, Increase in *Gabra4* mRNA expression over time for two EtOH concentrations (10 and 60 mM; by 1-way ANOVA vs control, with Dunnett's multiple-comparison *post hoc* test, $n \geq 6$). All data are mean ± SEM (*$p < 0.05$, **$p < 0.01$).

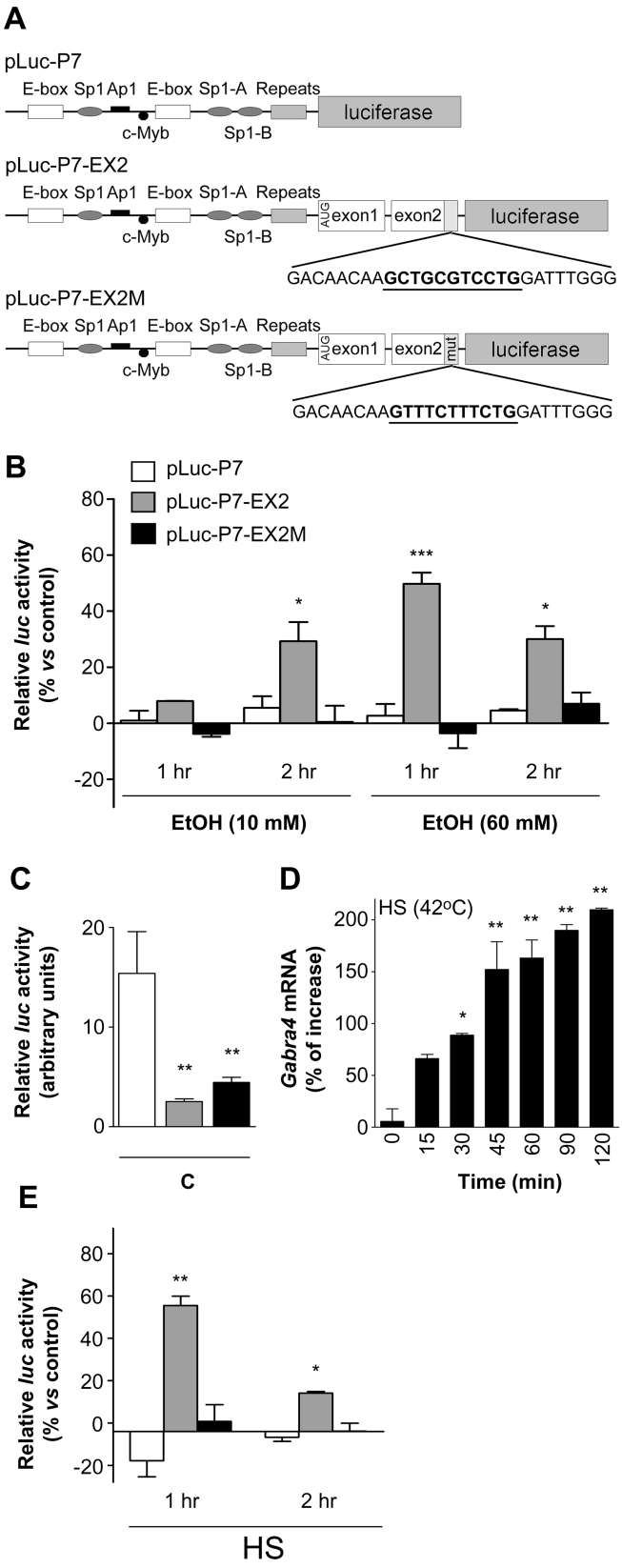

Figure 2. The ARE is essential for EtOH and heat sensitivity of the *Gabra4* promoter in cultured cortical neurons. ***A***, Schematic of the basal *Gabra4* promoter-reporter construct (pLuc-P7) shown together with the extended construct, pLuc-P7-EX2; and a third construct, pLuc-P7-EX2M, containing a mutated ARE sequence. The ARE sequence is shown in bold and underlined. ***B***, The extended *Gabra4* promoter is sensitive to EtOH. EtOH (10 – 60 mM) increased relative *luc* activity in neurons transfected with pLuc-P7-EX2 but not pLuc-P7 or pLuc-EX2M (*significantly different from pLuc-P7/pLuc-P7-EX2M by 1-way ANOVA with Tukey's multiple-comparison *post hoc* test, all pair of columns compared, $n \geq 8$). The values are mean ± SEM expressed as percentage of increase versus control (cells treated with vehicle). ***C***, Relative *luc* activity of pLuc-P7, pLuc-P7-EX2, and pLuc-EX2M constructs transfected in cortical neurons under control conditions (*significantly different from pLuc-P7 by 1-way ANOVA). Values are mean ± SEM ($n \geq 6$), expressed in arbitrary units. ***D***, Activation of *Gabra4* transcription by heat. Cortical neurons incubated at 42°C showed enhanced *Gabra4* mRNA expression as measured by Q-PCR (data analyzed by 1-way ANOVA with Dunnett's multiple-comparison *post hoc* test vs control, cells incubated at 37°C, $n \geq 6$). ***E***, Relative *luc* activity of pLuc-P7, pLuc-P7-EX2, and pLuc-EX2M constructs transfected in cortical neurons exposed to heat shock (HS). The values are mean ± SEM ($n \geq 8$) expressed as percentage of increase versus control (*significantly different from pLuc-P7/pLuc-P7-EX2M by 1-way ANOVA with Tukey's multiple-comparison *post hoc* test, all pair of columns compared, $n \geq 8$). In all cases, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

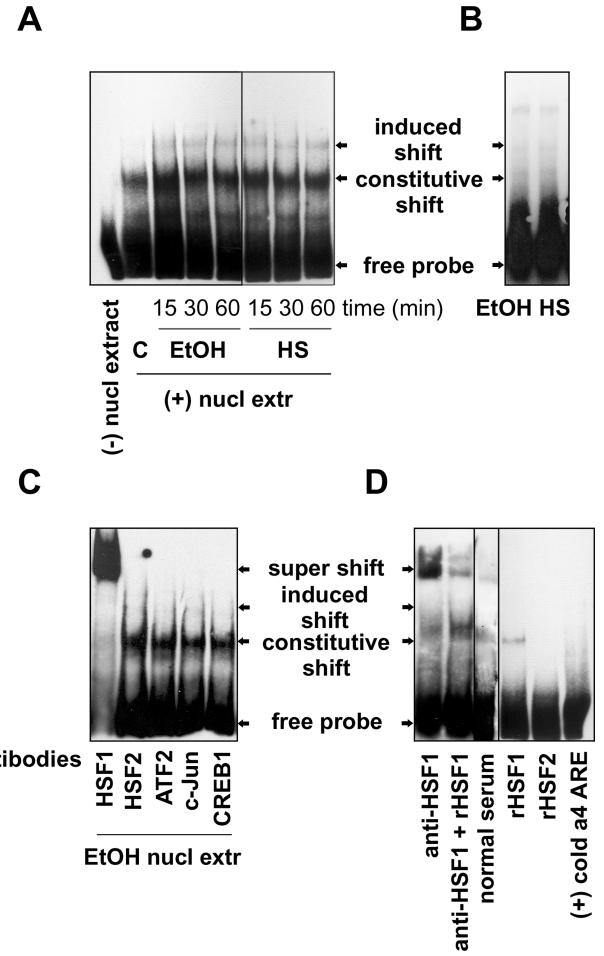

Figure 3. The transcription factor HSF1 binds to the ARE in *Gabra4*. ***A***, EtOH or heat (HS) enhanced the binding of cortical neuron nuclear (nucl) extracts to a DNA probe containing the *Gabra4* ARE sequence (constitutive shift) and induced a second binding activity (induced shift). The shift was dependent on the presence of nuclear extract and on an intact ARE sequence. ***B***, No shift is observed with probes containing either scrambled or random (data not shown) sequences. ***C***, Identification of the nuclear factor(s) that bind(s) to the ARE. A panel of antibodies to transcription factors was screened (see Materials and Methods) but only anti-HSF1 produced a supershift of the protein-DNA complex. ***D***, The transcription factor HSF1 binds to the ARE. The binding of anti-HSF1 antibody to the nuclear protein-DNA complex was reversed by a molar excess of recombinant rat HSF1 protein (rHSF1). Binding to the probe was observed with rHSF1, but not rHSF2, protein and was reversed by unlabeled probe (+ cold α4 ARE). The EMSA is representative of at least three experiments from independent cultures with similar results.

significant. The list of genes in Table 1 was ranked in order of multiple of increase (cutoff ≥1.5), with respect to control untreated cultures.

Supplemental data. Supplemental data are available at www.jneurosci.org as supplemental material.

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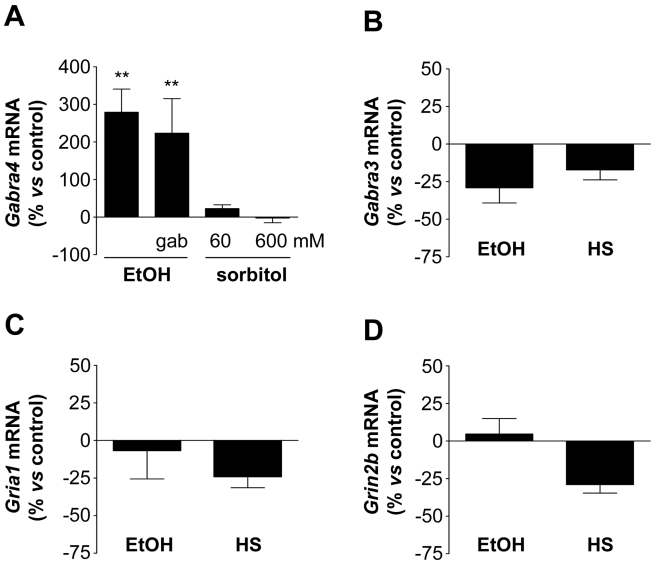

Figure 4. EtOH and heat effects are specific for *Gabra4* and are not attributable to osmotic stress. ***A***, The effect of alcohol or heat (data not shown) on *Gabra4* is not prevented by the $GABA_A$ receptor antagonist gabazine (gab; 20 μM), and cannot be reproduced by the osmotic agent sorbitol (60 – 600 mM; 1-way ANOVA vs control, with Dunnett's multiple-comparison *post hoc* test, $n \geq 6$). ***B***, The expression of *Gabra3* mRNA in neurons is not affected by 60 mM EtOH or heat stress (HS; 1-way ANOVA vs control with Dunnett's multiple-comparison *post hoc* test, $n \geq 9$). ***C***, ***D***, The expression of the glutamate receptor subunit mRNAs, *Gria1* and *Grin2b*, was not affected by alcohol or heat (1-way ANOVA vs control, with Dunnett's multiple-comparison *post hoc* test, $n \geq 6$). All data are mean ± SEM (**$p < 0.01$).

Results

In our initial experiments in mouse cortical neurons *in vitro*, we found that modest concentrations of ethanol (10–20 mM), well within the range of brain concentrations relevant to social use and human intoxication (Urso et al., 1981; National Institute on Alcohol Abuse and Alcoholism, 2003), were effective in rapidly activating the expression of *Gabra4*, as assessed by Q-PCR. This effect of ethanol (EtOH) on *Gabra4* mRNA levels was concentration dependent (Fig. 1*A*), with a sensitivity threshold of 10 mM and half-maximal activation at 43 ± 5 mM. We also observed an increase in the levels of α4 subunit protein, although this was delayed relative to the increase in mRNA (Fig. 1*B*). The activation of *Gabra4* transcription by 10 mM EtOH was rapid, with measurable changes in mRNA levels within 90–120 min (Fig. 1*C*). Higher concentrations of alcohol (60 mM) induced even more rapid activation of *Gabra4* transcription, with a significant increase in mRNA within 60 min or less (Fig. 1*D*). The stimulation by 60 mM EtOH was robust and reproducible (332 ± 23%), and the EtOH effect saturated between 60 and 100 mM (Fig. 1*A*). These EtOH concentrations were not toxic to the neurons; only EtOH concentrations >100 mM caused a modest increase in apoptosis, and hence they were not investigated further (data not shown).

The *Gabra4* promoter has been extensively studied in mouse and rat (Ma et al., 2004; Roberts et al., 2005, 2006), and a basal promoter of ~450 bp containing two functional Sp1 binding sites has been identified in mouse (Ma et al., 2004). When this construct was transfected into cultured cortical neurons, the basal promoter (pLuc-P7) (Ma et al., 2004) was able to drive the expression of the luciferase (*luc*) reporter gene, but *luc* expression was not affected by alcohol (Fig. 2*B*). Similar results were obtained with a second promoter construct containing a longer (~3 kb) fragment upstream of the 5′-flanking region of the *Gabra4* gene (data not shown). In an attempt to locate possible additional regulatory elements in *Gabra4*, we cloned an extended fragment (pLuc-P7-EX2) that included the 450 bp basal promoter together with an additional downstream sequence, including the first and second exons and introns of the gene (Fig. 2*A*). This promoter construct conferred sensitivity of the reporter gene to low and moderate concentrations (10 and 60 mM) of alcohol (Fig. 2*B*). The extended construct also resulted in lower levels of expression of the reporter gene compared with the basal promoter (Fig. 2*C*), suggesting the presence of both positive and negative regulatory elements within the first two exons and introns of *Gabra4*.

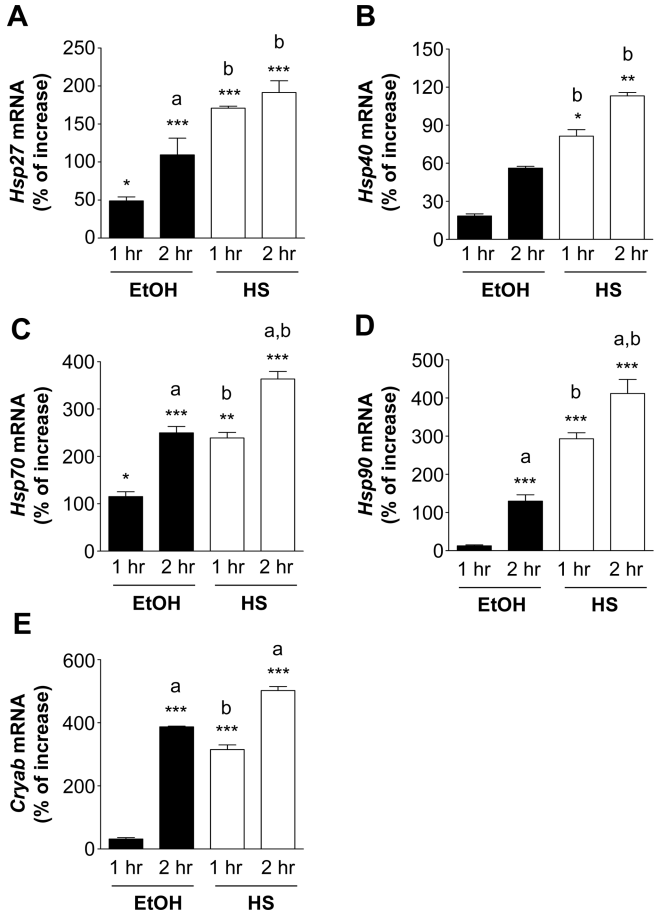

Figure 5. EtOH activates the transcription of several heat shock genes. ***A–E***, EtOH (60 mM) and heat (HS; 42°C) increased the levels of *Hsp27*, *Hsp40*, *Hsp70*, *Hsp90*, and *Cryab* mRNA, as measured by Q-PCR (the global analysis of the data were done by 2-way ANOVA and the data postanalyzed according to variables by 1-way ANOVA with Tukey–Kramer posttest using the error of the 2-way ANOVA; [a]significantly different between exposure times for the same treatment; [b]significantly different between treatments for the same exposure time; $n \geq 9$). Data shown are mean ± SEM (*denotes values significantly increased above control value; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

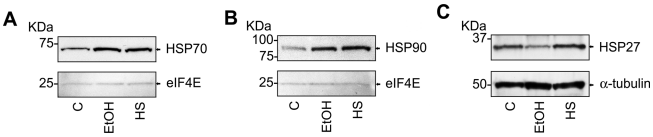

Figure 6. EtOH and heat shock induce the expression of heat shock proteins. ***A–C***, Immunoblot analysis of heat shock protein expression in cortical neurons. EtOH (60 mM) and heat (HS; 42°C) increased the levels of HSP70 and HSP90 but not HSP27. A representative immunoblot for HSP27, HSP70, and HSP90 is shown, with the proteins eIF4E or α-tubulin included as internal standards.

Examination of this additional fragment revealed the presence of an 11 b sequence (which we refer to as the ARE), located at the end of exon 2 in *Gabra4* (Fig. 2*A*) that is strikingly similar to a sequence described previously in *Caenorhabditis elegans* (Kwon et al., 2004). This *cis*-acting element, TCTGCGTCTCT, is conserved in the promoter sequences of many ARGs in *C. elegans*, several of which include genes involved in the heat shock pathway (Kwon et al., 2004). We next analyzed the sequence of the mouse *Gabra4* gene using a program designed to predict transcription factor binding sites. One of the potential candidate factors identified was HSF1, suggesting to us the possibility that the 11 b element might mediate responses to alcohol and heat stress. In fact, we found that the effects of ethanol on *Gabra4* expression could be reproduced by exposing the neurons to a classic "heat shock" stimulus (Fig. 2*D*). The level of *Gabra4* mRNA expression increased within 30 min of incubation of mouse cortical neurons at 42°C (Fig. 2*D*). A similar response to heat was also demonstrated in neurons transfected with the extended promoter-reporter construct (Fig. 2*E*). Mutagenesis of the 11 b ARE (pLuc-P7-EX2M) (Fig. 2*A*) abolished the stimulation of *luc* induced by both alcohol (Fig. 2*B*) and heat (Fig. 2*E*). The effect of both alcohol and heat stress on *Gabra4* expression seems to be mediated by the ARE.

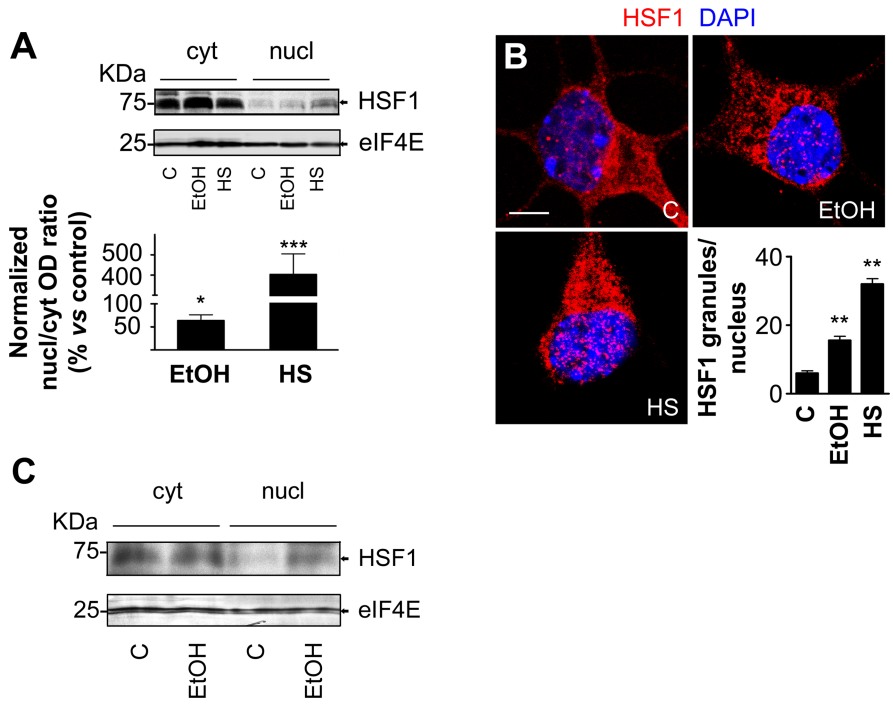

Figure 7. EtOH induces the translocation of HSF1 to the nucleus and the formation of stress granules. ***A***, EtOH stimulates the translocation of HSF1 from the cytoplasm (cyt) to the nucleus (nucl) of cortical neurons. Immunoblot analysis of HSF1 protein in neurons exposed to EtOH or heat shock. The histogram shows the HSF1 OD data (normalized to eIF4E compared by two-tailed *t* test, $n \geq 3$). ***B***, EtOH induces the formation of HSF1 "stress granules" in the nucleus of cortical neurons in culture. Immunocytochemistry of cortical neurons stained with anti-HSF1 antibody (red) and DAPI (blue) reveals the presence of HSF1 aggregates or stress granules in the nucleus. Scale bar, 5 μm. The graph shows the quantification of the number of HSF1 granules per cell nucleus (by 1-way ANOVA with Dunnett's multiple-comparison *post hoc* test vs control cells, $n \geq 20$ cells from two independent cultures). Data are the mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). ***C***, EtOH stimulates the translocation of HSF1 in the mouse cortex *in vivo*. Naive adult mice injected with 3 g/kg EtOH were killed 2 h after injection, cerebral cortex was dissected out, and the protein was isolated. The immunoblot of HSF1 shows a more intense HSF1 immunoreactive band in the nuclear fraction obtained from the EtOH-treated mice compared with the saline-treated control mice. C, control.

We then performed a series of EMSA experiments using nuclear extracts isolated from cultured cortical neurons and a DNA probe containing the *Gabra4* ARE sequence. Under control conditions, we detected a soluble factor that was able to bind to the ARE-containing probe, as revealed by the shift in the mobility of the probe (Fig. 3*A*, constitutive shift). This DNA binding activity was enhanced in nuclear extracts from neurons treated with alcohol or heat. At the same time, an additional shift in probe mobility was observed (Fig. 3*A*, induced shift). This second DNA-protein complex may correspond to the activated form of a transcription factor (Mosser et al., 1990). Mutation of the ARE sequence within the probe abolished both the constitutive and induced shifts (Fig. 3*B*). These results are consistent with the idea that the binding of nuclear factor(s) to the ARE may be a prerequisite for the activation of *Gabra4* transcription.

To identify the transcription factor/s interacting with the ARE, we then performed "supershift" EMSA experiments using a panel of specific antibodies to transcription factors that could potentially bind to the ARE sequence, as predicted by the analysis program. Antibodies against HSF1 produced a supershift of the band corresponding to the DNA-protein complex, whereas antibodies to HSF2, ATF2, c-Jun, and CREB1 failed to alter the mobility of the probe (Fig. 3*C*). This interaction of anti-HSF1 with the DNA-protein complex was specific, because preincubation of anti-HSF1 antibody with its control antigen peptide abolished the supershift of the ARE probe (Fig. 3*D*), as did replacing the HSF1 antibody with preimmune serum (Fig. 3*D*). Finally, we found that addition of exogenous recombinant HSF1 (but not HSF2) protein (Mathew et al., 2001) mimics the shift of the ARE probe produced by the nuclear extracts isolated from cortical neurons (Fig. 3*D*). These experiments suggested that application of alcohol or heat to cultured cortical neurons may activate the transcription of *Gabra4* by promoting an interaction of HSF1, and possibly other proteins, with the ARE sequence.

This effect of alcohol or heat on *Gabra4* expression is not reproduced by very high concentrations of the impermeant osmotic agent sorbitol (60–600 mM) (Fig. 4*A*) but could be reproduced by other membrane-permeant alcohols of longer chain length. Both propanol and butanol (5–20 mM) produced an increase in *Gabra4* expression similar to that observed with EtOH (data not shown). These data suggest that the alcohol effect is not a result of osmotic deformation of the neurons but may result from the activation of specific cellular signaling pathways. The effect of EtOH on *Gabra4* is not prevented by the $GABA_A$ receptor antagonist gabazine (20 μM) (Fig. 4*A*), indicating that the receptor that is the gene product of *Gabra4* is not involved in the activation of this transcriptional response. The effect of EtOH or heat shock is specific among the group of genes encoding the $GABA_AR$ subunits, because Q-PCR analysis showed a robust increase of *Gabra4* mRNA, but almost no effects on the levels of mRNA for *Gabra3* (Fig. 4*B*) or *Gabra6* (data not shown) in neurons treated with 60 mM alcohol or heated to 42°C for 1 h. In addition, the expression of the glutamate receptor subunit mR-

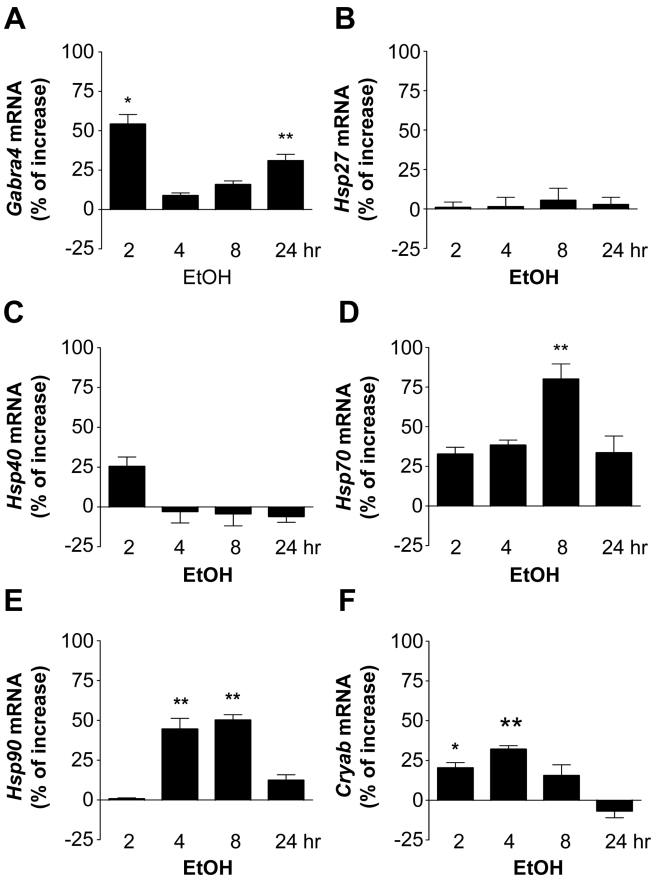

Figure 8. EtOH induces the expression of *Gabra4* and several heat shock proteins in the mouse cerebral cortex *in vivo*. ***A–F***, Adult mice were injected with a single dose of EtOH (3 g/kg, i.p.) and killed at different time points. RNA was isolated from the cerebral cortex, and levels of *Gabra4* and *Hsp* mRNA were measured by Q-PCR as described in Materials and Methods. A single dose of EtOH in naive animals induces the expression of the genes analyzed with the exception of *Hsp27* and *Hsp40*. Data are mean ± SEM (*denotes significantly increased above control value by 1-way ANOVA with Dunnett's multiple-comparison *post hoc* test; $*p < 0.05$, $**p < 0.01$).

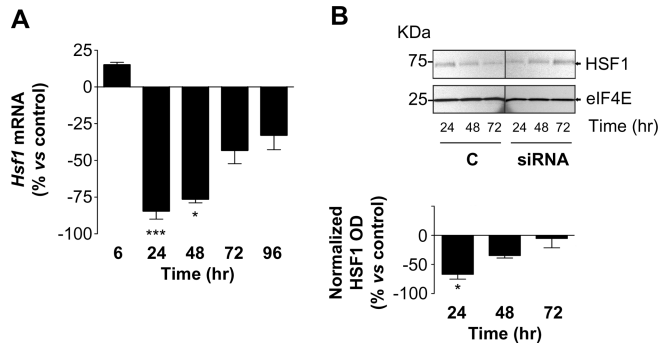

Figure 9. siRNA significantly reduces *Hsf1* mRNA and HSF1 protein expression in neurons. ***A***, *Hsf1* siRNA reduces the levels of *Hsf1* mRNA as measured using Q-PCR. Cortical neurons were transfected with *Hsf1* siRNA or control siRNA, and the levels of *Hsf1* mRNA were then measured and compared with mRNA levels in untransfected cultures. As expected, the control siRNA had no effect on *Hsf1* expression (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). ***B***, *Hsf1* siRNA reduces HSF1 protein in cortical neurons. Immunoblot analysis of HSF1 protein in neurons treated with *Hsf1* siRNA. The histogram shows the HSF1 OD data (normalized to eIF4E). Data are mean ± SEM and were compared with control by two-tailed unpaired *t* test ($n \geq 3$; $*p < 0.05$, $***p < 0.001$). C, Control.

NAs, *Gria1* and *Grin2b*, was not affected by alcohol and heat (Fig. 4*C*,*D*), confirming that this effect of alcohol is specific for *Gabra4*, at least among this group of receptor subunit genes.

These experiments suggest that the effect of alcohol on *Gabra4* may be mediated via activation of the heat shock pathway. To investigate this further, we studied the expression of a variety of heat shock proteins in neurons treated with alcohol or heat. Exposure to 60 mM EtOH or heat for 1 h elicited a rapid increase in the transcription of the heat shock protein genes *Hsp27*, *HSP40*, and *Hsp70* in cultured mouse cortical neurons (Fig. 5*A–C*). *Hsp90* and *Cryab* were also activated by ethanol, although with slower kinetics (Fig. 5*D*,*E*). Immunoblot analysis of heat shock proteins in the cortical neurons confirmed the transcriptional activation induced by EtOH and heat on HSP70 and HSP90 at the protein level, although this is not so clearly evident for HSP27 (Fig. 6). In addition, treatment with alcohol or heat stimulated the translocation of HSF1 from the cytoplasm to the nucleus of neurons, which is a prerequisite for the activation of HSF1-dependent genes (Fig. 7*A*) (Morimoto, 1998), and these stimuli also induced the appearance of HSF1 aggregates (known as "stress granules") (Cotto et al., 1997) in the nucleus (Fig. 7*B*). It is known that astrocytes show a robust response to heat shock, so we investigated the extent of their presence in the cortical cultures. Immunocytochemistry of our cultures at 5 DIV revealed that >95% of cells stained positive for the neuronal marker, NeuN, indicating that these are postmitotic neurons (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

To determine the relevance of these *in vitro* studies to the *in vivo* actions of alcohol, we analyzed the effect of the drug in naive animals. Adult mice were injected intraperitoneally with a single dose of ethanol (3 g/kg), which yields a BEC of ~65 mM, similar to the concentration used in the *in vitro* experiments. Control animals were injected with an equal volume of saline. Alcohol injections produced a rapid and robust activation of *Gabra4* transcription in these animals as assessed by Q-PCR. The data presented in Figure 8 show a biphasic time course, with a very rapid but transient increase in *Gabra4* mRNA within 1–2 h followed by a second phase of increased expression at 24 h. This biphasic pattern of transcriptional activation has also been observed for a group of immediate early genes, for example during the course of brain injury (Herrera and Robertson, 1996; Rickhag et al., 2007). In addition, as we had observed *in vitro*, the administration of EtOH to mice also activated several *Hsp* genes (Fig. 8), with the exception of *Hsp27*. Immunoblot analysis of brain samples obtained from the alcohol-treated mice also showed that EtOH induced the translocation of HSF1 from the cytoplasm to the nucleus, *in vivo* (Fig. 7*C*).

The contribution of the transcription factor HSF1 to the activation of *Gabra4* transcription was investigated by performing a knock-down of HSF1 using siRNA. We found that 24 h treatment of cortical neurons with *Hsf1* siRNA was sufficient to produce a >70% decrease in *Hsf1* mRNA and HSF1 protein (Fig. 9*A*,*B*). The knock-down of HSF1 produced a concomitant reduction of the activation of *Gabra4*, *Hsp70* (Fig. 10*A*,*B*), and *Hsp27* (data not shown) transcription in response to both alcohol and heat. To confirm the apparent requirement of HSF1 for the stimulation of *Gabra4* expression, we transfected cortical neurons with a constitutively active *Hsf1* construct (*Hsf1*-act), which has previously been shown to induce *Hsp* genes in the absence of stress (Acquaah-Mensah et al., 2001). This construct was able to mimic the transcriptional activation of *Gabra4* by EtOH and heat (Fig. 10*C*). Conversely, transfection with a dominant-negative *Hsf1* construct (*Hsf1*-inact), which cannot be activated, abolished the

effect of EtOH and heat shock on *Gabra4* induction (Fig. 10*C*). Because the activation of *Hsp* gene transcription is known to be dependent on HSF1, the results of these experiments strongly suggest that the stimulation of *Gabra4* expression by alcohol is mediated by the activation of the heat shock pathway, which must, presumably, occur at some point upstream of HSF1 activation.

These provocative results on the interaction of EtOH and heat shock pathway prompted us to investigate whether this stimulation was unique to *Gabra4* or whether there were other genes that can be activated by both EtOH and heat. We therefore performed parallel gene microarray experiments on mouse cortical neurons exposed to alcohol or heat. The microarray data, not unexpectedly, revealed a large number of genes that were acutely upregulated by alcohol (supplemental Table 1, available at www.jneurosci.org as supplemental material) and an even larger number that were activated by heat shock (supplemental Table 2, available at www.jneurosci.org as supplemental material). Nine genes showed a dramatic response (>50% stimulation) to both treatments (Table 1). Expression of all nine genes is highly specific to neurons, and all of them contain one or more ARE-like sequence, located either in the 5′-flanking domain (as in *Hsp70*) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) or downstream in an intro/exon region (as in *Gabra4*). This group of nine genes is especially interesting because it includes several genes of which the products are involved in synaptic transmission: *Syt1*, encoding synaptotagmin I, a calcium-binding protein involved in neurotransmitter release, and *Spnb2*, encoding spectrin β2, a calcium sensor involved in vesicle docking to the plasma membrane. The other genes encode proteins that are important in synapse formation and plasticity, such as neurogranin (*Nrgn*), cadherin 13 (*Cdh13*), and glycoprotein m6a (*Gpm6a*); in microtubule assembly (microtubule-associated protein 1B; *Mtap1b*) or in protein trafficking (SEC23A; *Sec23a*).

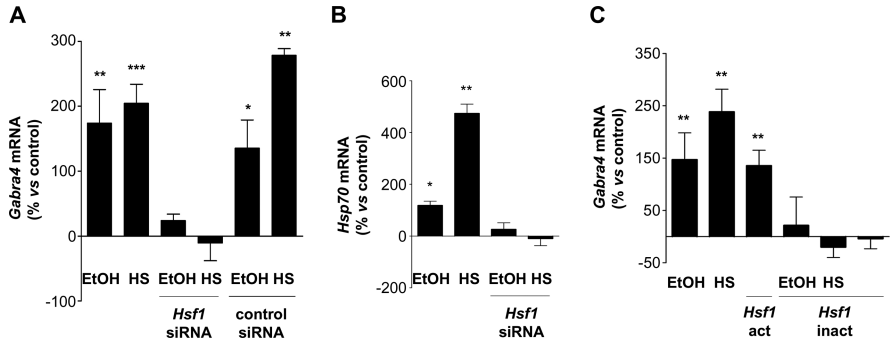

Figure 10. HSF1 is required for the induction of *Gabra4* and *Hsp70* by EtOH or heat. ***A***, ***B***, Knock-down of HSF1 inhibits the activation of *Gabra4* and *Hsp70* transcription. Treatment of neurons with *Hsf1* siRNA dramatically reduced the activation of *Gabra4* and *Hsp70* transcription by EtOH and HS, whereas treatment with control siRNA was without effect (by 1-way ANOVA vs control cells, with Dunnett's multiple-comparison *post hoc* test, $n \geq 3$). ***C***, Cortical neurons transfected with the constitutively active *Hsf1* (*Hsf1* act) construct show an increase in *Gabra4* mRNA expression similar to the induction seen with EtOH or heat shock (HS). The dominant-negative form of *Hsf1* (*Hsf1* inact) completely abolishes the induction of *Gabra4*. *Hsf1*-inact alone had no effect on *Gabra4* expression (by 1-way ANOVA vs control cells, with Dunnett's multiple-comparison *post hoc* test, $n \geq 6$). Data are mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table 1. Genes activated by alcohol and heat stress in cultured cortical neurons, listed in order of induction by EtOH

Gene	Description	GenBank accession number	Ranking	
			EtOH	HS
Gpm6a	Glycoprotein m6a	NM_153581	5	141
Mtap1b	Microtubule-associated protein 1B	NM_008634	6	72
Nrgn	Neurogranin	NM_022029	26	8
4831417L10	ELMO domain-containing 1	NM_177769	27	23
Spnb2	Spectrin β2, transcript variant 1	NM_175836	36	60
Gpc5	Glypican 5	NM_175500	39	6
Sec23a	SEC23A (*Saccharomyces cerevisiae*)	NM_009147	44	369
Syt1	Synaptotagmin I	NM_009306	46	20
Cdh13	Cadherin 13	NM_019707	47	21

Table of the nine genes most responsive to activation by both alcohol and heat as assessed by microarray analysis, listed in order of EtOH induction.

Discussion

It is widely accepted that acute or chronic exposure to alcohol produces alterations in the normal homeostasis of gene expression in the brain (Lewohl et al., 2000; Worst and Vrana, 2005). Many neurotransmitter systems are affected by ethanol, including the GABAergic system, although an abundance of studies on the effect of alcohol on $GABA_A$ receptor subunit gene expression have resulted often in contradictory results, with the exception of the *Gabra4* gene. Most studies have reported an increase in *Gabra4* expression after acute or chronic ethanol administration, both *in vitro* and *in vivo* (Worst and Vrana, 2005), suggesting an important potential role for this gene in the effects of alcohol, or in the homeostatic adaptation to the presence of the drug.

Our detailed analysis of the mechanisms of *Gabra4* regulation by alcohol in cortical neurons has revealed that alcohol can activate critical elements of the heat shock pathway, a suggestion that is consistent with a variety of other experimental observations. For example, in a number of microarray studies, alcohol treatment appears to result in the activation of some of the *Hsp* genes (Lewohl et al., 2000; Gutala et al., 2004; Worst and Vrana, 2005). A very recent pathway-focused microarray analysis revealed that chronic application of alcohol to mouse cortical neurons for 5 d increases the expression of several heat shock proteins (*Hsp70*, *Hspa8*, and *Hsp84*) together with some of the components of the platelet-derived growth factor pathway (Wang et al., 2007). The results presented here indicate that *Gabra4* transcriptional activation by acute ethanol or heat shock is dependent on the activation of the transcription factor HFS1 and subsequent binding to the ARE.

The activation of HSF1 occurs as a sequential process involving trimerization, acquisition of DNA binding activity, and inducible phosphorylation. The mechanism of HSF1 activation has not been completely elucidated, but there is consensus that the heat shock proteins HSP40, HSP70, and especially HSP90 are involved in binding HSF1 within the cytoplasm of unstressed cells and thereby repressing its activity as a transcription factor (Morimoto, 1998; Tonkiss and Calderwood, 2005). After activation by heat stress or other stimuli, the conformational changes of the heat shock proteins

release free HSF1, which allows its translocation to the nucleus (Morimoto, 1998). Among other effects, the activated HSF1 then triggers the transcription of the *Hsp* genes. In the present work, we have demonstrated that ethanol promotes the translocation of HSF1 to the nucleus of the cortical neurons and concomitantly activates a series of genes, including the *Hsp* genes. This finding suggests that many of the genes upregulated by alcohol (ARGs) may be transcriptionally activated through this mechanism, although it is likely that a variety of alternative pathways are also involved. For example, another important ARG, the *Grin2b* gene (encoding the glutamate receptor NR2B subunit), is regulated by alcohol over a slower time scale than reported here, and this occurs via a classical neuron-restricted silencing mechanism (Qiang et al., 2005), whereas other ARGs may be activated by another well described pathway involving the cAMP-response element binding protein, CREB (Hassan et al., 2003).

It is of considerable interest that a significant number of genes appear to be coregulated by alcohol and heat shock treatment *in vitro*, and it is especially noteworthy that some of these are involved in the dynamics of neuronal architecture and synaptic structure. In agreement with our results, other *in vivo* microarray studies have also reported changes in genes related to neuronal structure and function, as well as in the heat shock pathway (Lewohl et al., 2000; Gutala et al., 2004).

The biological implications of these findings are not yet completely clear. The well known "fetal alcohol syndrome" (FAS) is the result of the exposure of fetuses or neonates to alcohol during a critical period of brain development (Sulik et al., 1981; Goodlett et al., 2005) and is associated with brain damage, cognitive deficits, and craniofacial abnormalities, in both mouse and man (Chen et al., 2005; Sulik, 2005). It is less widely appreciated that similar developmental abnormalities, including neural tube defects, can also be triggered by excessive heat during gestation (Edwards, 2006). One intriguing possibility that should be investigated is that inappropriate activation of the heat shock pathway by alcohol might play a role in the abnormalities in brain development associated with FAS. Another possibility is that the activation of the heat shock pathway cascade by moderate levels of alcohol can actually promote neuronal survival, as reported for *Hsp* gene activation previously (Tonkiss and Calderwood, 2005; Dodge et al., 2006).

References

Acquaah-Mensah GK, Leslie SW, Kehrer JP (2001) Acute exposure of cerebellar granule neurons to ethanol suppresses stress-activated protein kinase-1 and concomitantly induces AP-1. Toxicol Appl Pharmacol 175:10–18.

Banker G, Goslin K (1991) Culturing nerve cells. Cambridge, MA: MIT.

Cagetti E, Liang J, Spigelman I, Olsen RW (2003) Withdrawal from chronic intermittent ethanol treatment changes subunit composition, reduces synaptic function, and decreases behavioral responses to positive allosteric modulators of $GABA_A$ receptors. Mol Pharmacol 63:53–64.

Chandra D, Jia F, Liang J, Peng Z, Suryanarayanan A, Werner DF, Spigelman I, Houser CR, Olsen RW, Harrison NL, Homanics GE (2006) GABA-A receptor alpha 4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. Proc Natl Acad Sci USA 103:15230–15235.

Chen SY, Charness ME, Wilkemeyer MF, Sulik KK (2005) Peptide-mediated protection from ethanol-induced neural tube defects. Dev Neurosci 27:13–19.

Cotto JJ, Fox SG, Morimoto RI (1997) HSF1 granules: a novel stress-induced compartment of human cells. J Cell Sci 110:2925–2934.

Dodd PR, Buckley ST, Eckert AL, Foley PF, Innes DJ (2006) Genes and gene expression in the brains of human alcoholics. Ann NY Acad Sci 1074:104–115.

Dodge ME, Wang J, Guy C, Rankin S, Rahimtula M, Mearow KM (2006) Stress-induced heat shock protein 27 expression and its role in dorsal root ganglion neuron survival. Brain Res 1068:38–48.

Edwards MJ (2006) Hyperthermia and fever during pregnancy. Birth Defects Res A Clin Mol Teratol 76:507–516.

Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95:14863–14868.

Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat Rev Neurosci 6:215–229.

Friedman JM (1982) Can maternal alcohol ingestion cause neural tube defects? J Pediatr 101:232–234.

Goodlett CR, Horn KH, Zhou FC (2005) Alcohol teratogenesis: mechanisms of damage and strategies for intervention. Exp Biol Med (Maywood) 230:394–406.

Gutala R, Wang J, Kadapakkam S, Hwang Y, Ticku M, Li MD (2004) Microarray analysis of ethanol-treated cortical neurons revealed disruption of genes related to the ubiquitin-proteasome pathway and protein synthesis. Alcohol Clin Exp Res 28:1779–1788.

Hassan S, Duong B, Kim KS, Miles MF (2003) Pharmacogenomic analysis of mechanisms mediating ethanol regulation of dopamine beta-hydroxylase. J Biol Chem 278:38860–38869.

Herrera DG, Robertson HA (1996) Activation of c-fos in the brain. Prog Neurobiol 50:83–107.

Huettner JE, Baughman RW (1986) Primary culture of identified neurons from the visual cortex of postnatal rats. J Neurosci 6:3044–3060.

Ieraci A, Herrera DG (2006) Nicotinamide protects against ethanol-induced apoptotic neurodegeneration in the developing mouse brain. PLoS Med 3:e101.

Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Hörster F, Tenkova T, Dikranian K, Olnel JW (2000) Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287:1056–1060.

Kwon JY, Hong M, Choi MS, Kang S, Duke K, Kim S, Lee S, Lee J (2004) Ethanol-response genes and their regulation analyzed by a microarray and comparative genomic approach in the nematode *Caenorhabditis elegans*. Genomics 83:600–614.

Lewohl JM, Wang L, Miles MF, Zhang L, Dodd PR, Harris RA (2000) Gene expression in human alcoholism: microarray analysis of frontal cortex. Alcohol Clin Exp Res 24:1873–1882.

Ma L, Song L, Radoi GE, Harrison NL (2004) Transcriptional regulation of the mouse gene encoding the alpha-4 subunit of the GABAA receptor. J Biol Chem 279:40451–40461.

Mathew A, Mathur SK, Jolly C, Fox SG, Kim S, Morimoto RI (2001) Stress-specific activation and repression of heat shock factors 1 and 2. Mol Cell Biol 21:7163–7171.

Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 12:3788–3796.

Mosser DD, Kotzbauer PT, Sarge KD, Morimoto RI (1990) In vitro activation of heat shock transcription factor DNA-binding by calcium and biochemical conditions that affect protein conformation. Proc Natl Acad Sci USA 87:3748–3752.

Mulligan MK, Ponomarev I, Hitzemann RJ, Belknap JK, Tabakoff B, Harris RA, Crabbe JC, Blednov YA, Grahame NJ, Phillips TJ, Finn DA, Hoffman PL, Iyer VR, Koob GF, Bergeson SE (2006) Toward understanding the genetics of alcohol drinking through transcriptome meta-analysis. Proc Natl Acad Sci USA 103:6368–6373.

National Institute on Alcohol Abuse and Alcoholism (2003) State of the science report on the effects of moderate drinking. Bethesda, MD: National Institutes of Health.

Jia F, Pignataro L, Schofield CM, Yue M, Harrison NL, Goldstein PA (2005) An extrasynaptic GABA-A receptor mediates tonic inhibition in thalamic VB neurons. J Neurophysiol 94:4491–4501.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101:815–850.

Qiang M, Rani CS, Ticku MK (2005) Neuron-restrictive silencer factor regulates the *N*-methyl-D-aspartate receptor 2B subunit gene in basal and ethanol-induced gene expression in fetal cortical neurons. Mol Pharmacol 67:2115–2125.

Rickhag M, Teilum M, Wieloch T (2007) Rapid and long-term induction of

effector immediate early genes (BDNF, Neuritin and Arc) in peri-infarct cortex and dentate gyrus after ischemic injury in rat brain. Brain Res 1151:203–210.

Roberts DS, Raol YH, Bandyopadhyay S, Lund IV, Budreck EC, Passini MA, Wolfe JH, Brooks-Kayal AR, Russek SJ (2005) Egr3 stimulation of GABRA4 promoter activity as a mechanism for seizure-induced up-regulation of GABA(A) receptor alpha4 subunit expression. Proc Natl Acad Sci USA 102:11894–11899.

Roberts DS, Hu Y, Lund IV, Brooks-Kayal AR, Russek SJ (2006) Brain-derived neurotrophic factor (BDNF)-induced synthesis of early growth response factor 3 (Egr3) controls the levels of type A GABA receptor alpha 4 subunits in hippocampal neurons. J Biol Chem 281:29431–29435.

Sanna E, Mostallino MC, Busonero F, Talani G, Tranquilli S, Mameli M, Spiga S, Follesa P, Biggio G (2003) Changes in $GABA_A$ receptor gene expression associated with selective alterations in receptor function and pharmacology after ethanol withdrawal. J Neurosci 23:11711–11724.

Sulik KK (2005) Genesis of alcohol-induced craniofacial dysmorphism. Exp Biol Med (Maywood) 230:366–375.

Sulik KK, Johnston MC, Webb MA (1981) Fetal alcohol syndrome: embryogenesis in a mouse model. Science 214:936–938.

Tonkiss J, Calderwood SK (2005) Regulation of heat shock transcription in neuronal cells. Int J Hyperthermia 51:433–444.

Urso T, Gavaler JS, Van Thiel DH (1981) Blood ethanol levels in sober alcohol users seen in an emergency room. Life Sci 28:1053–1056.

Wang J, Gutala R, Sun D, Ma JZ, Sheela RCS, Ticku MK, Li MD (2007) Regulation of platelet-derived growth factor signaling pathway by ethanol, nicotine, or both in mouse cortical neurons. Alcohol Clin Exp Res 31:357–375.

Wilke N, Sganga M, Barhite S, Miles MF (1994) Effects of alcohol on gene expression in neural cells. EXS 71:49–59.

Worst TJ, Vrana KE (2005) Alcohol and gene expression in the central nervous system. Alcohol 40:63–75.

Zuo J, Rungger D, Voellmy R (1995) Multiple layers of regulation of human heat shock transcription factor 1. Mol Cell Biol 15:4319–4330.