

# Potential Role of cAMP Response Element-Binding Protein in Ethanol-Induced *N*-Methyl-D-aspartate Receptor 2B Subunit Gene Transcription in Fetal Mouse Cortical Cells

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## ABSTRACT

We have shown previously that long-term ethanol treatment causes an up-regulation of *N*-methyl-D-aspartate (NMDA) receptor 2B subunit (NR2B) number and function in cultured fetal mouse cortical neurons. To examine the intracellular signaling pathways involved in this NR2B gene transcription, we have subjected fetal cortical neurons to long-term treatment with ethanol and studied its effect on cAMP response element-binding protein (CREB) and extracellular signal-regulated kinase (ERK) levels by Western blot and enzyme-linked immunosorbent assay. We find a significant increase in phosphorylated CREB, without change in total CREB protein, in cells treated with ethanol for 5 days. Long-term ethanol treatment did not increase levels of both total and phospho-ERK in serum-free medium, whereas it did increase ERK phosphorylation in medium containing serum, without affecting total ERK levels. CREB phosphorylation was increased by ethanol treatment in

both media, irrespective of the presence of serum. Electrophoretic mobility shift assay, using a 25-base pair (bp) double-stranded DNA fragment containing the cyclic AMP response element (CRE)-like sequence of the NR2B promoter as <sup>32</sup>P-labeled probe, showed an increase in specific CRE binding to nuclear proteins isolated from cells undergoing long-term ethanol treatment. A 467-bp DNA fragment of the NR2B promoter containing the CRE sequence cloned into the luciferase vector exhibited high reporter activity in transient cotransfection assay of mouse cortical neurons, and ethanol treatment increased this activity. Introducing site-directed mutation in the CRE sequence significantly reduced the reporter activity relative to the wild-type construct, and it also abolished the stimulatory effect by ethanol. Our results indicate that CREB is probably involved in mediating ethanol-induced up-regulation of NR2B gene.

NMDA receptors are increasingly recognized as a major target of ethanol effects on the brain and have recently been the subject of intense study (for reviews, see Diamond and Gordon, 1997; Woodward, 2000; Kumari and Ticku, 2000; Chandler, 2003). Although it is known that NMDA receptors mediate some of ethanol's damaging effects on the brain, the exact mechanism of that interaction is not known. NMDA receptors have a complex heteromeric composition and are made up of a combination of different subunits, NMDA R1 with the four subunits, A to D of R2 or R3 (Ishii et al., 1993; Mori and Mishina, 1995; Luo et al., 1997). Long-term ethanol treatment is shown to increase NMDA receptor number and function both in vivo and in vitro (Snell et al., 1993; Follasa

and Ticku, 1995, 1996; Hu and Ticku, 1995; Hu et al., 1996; Chen et al., 1997; for reviews, see Fadda and Rossetti, 1998; Carpenter-Hyland et al., 2004). Using cultured fetal mouse cortical neurons as a model to study the effects of long-term treatment with ethanol, studies from our laboratory have shown that such ethanol treatment causes an up-regulation of NR2B receptor at the transcriptional level (Kumari and Ticku, 1998, 2000). Given that multiple intracellular signaling pathways are involved in regulating gene transcription, the study of neuronal signal transduction pathways is of immense importance in understanding the molecular mechanism of long-term treatment with ethanol.

Much attention has been focused recently on cAMP response element-binding protein (CREB), a key transcription factor that lies downstream of major intracellular pathways, such as signaling via cAMP/protein kinase A, ERK/mitogen-activated protein kinase and calcium. CREB is a member

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**ABBREVIATIONS:** NMDA, *N*-methyl D-aspartate; CREB, cAMP response element-binding protein; ERK, extracellular signal-regulated kinase; CRE, cyclic AMP response element; NR2B, *N*-methyl D-aspartate receptor 2B subunit; bp, base pair(s); Luc, luciferase; E14, embryonic day 14; DIV, days in vitro; CaM, Ca<sup>2+</sup>/calmodulin; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; NBM, neurobasal medium containing B-27; PCR, polymerase chain reaction.

of the bZIP superfamily of transcriptional activators that includes activating transcription factor and cAMP response element modulator. It contains a DNA binding domain and a highly conserved leucine zipper dimerization domain (Lonze and Ginty, 2002). CREB activates gene transcription in response to elevation of intracellular cAMP levels brought about by a series of enzyme activations via various receptors, which in turn phosphorylates CREB at Ser133. Activated CREB binds to the cAMP response elements (CREs), represented by the palindromic consensus sequence TGACGTCA found in the 5' flanking region of target genes and initiates a transcription cascade (Bito, 1998; Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001; Weeber and Sweatt, 2002).

For many genes, their tissue and developmental expression is regulated by the presence of positive and negative *cis*-regulatory elements in their 5' flanking region, upstream of the transcriptional start site. For the NR2B gene, Sasner and Buananno (1996) have described an 800-bp region that includes a 255-bp of the first noncoding exon as sufficient to direct neural-specific transcription in the transgenic mouse. Klein et al. (1998) identified a "core promoter" that drives reporter gene expression in both neuronal and non-neuronal cell lines and described several Sp1 sites and one CRE site (−406 to −413) in the 5' flanking region of NR2B. The latter is of specific interest to us here in examining whether NR2B gene regulation is linked to signaling pathways involving CREB in the neuronal cells. Having observed in previous studies a stimulatory effect of long-term treatment with ethanol on NR2B receptors and NMDA-induced intracellular calcium levels (Hu and Ticku, 1995; Follesa and Ticku, 1996; Hu et al., 1996; Kumari and Ticku, 2000), we have studied the effects of long-term treatment with ethanol on CREB and explored the role of the CRE in NR2B gene regulation in the cultured mouse cortical cell model. Our results show that long-term ethanol treatment increases CREB activity as shown by an increase in its phosphorylation, <sup>32</sup>P-labeled CRE binding to CREB in the gel-shift assay, and CRE-regulated activity of luciferase (Luc) reporter construct transfected into cortical cells and that mutation of the CRE motif in the NR2B promoter region decreases its activity and abolishes ethanol-induced increase in the promoter activity.

## Materials and Methods

**Cell Culture.** Cortical cells were isolated from 14-day-old C57BL/6 mouse fetuses as described previously (Hu and Ticku, 1995). In brief, cortices were removed from E14 mice, and cells were isolated by trituration and resuspended in culture medium/minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 5% heat-inactivated horse serum (Invitrogen), 100  $\mu$ M GlutaMAX (Invitrogen), 28 mM D-glucose, and 1 $\times$  antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO). Cells ( $\sim 2.8 \times 10^5/\text{cm}^2$ ) were cultured under 5% CO<sub>2</sub> at 37°C in T-75 or T-25 tissue culture flasks with filter caps that permitted gas exchange or in 24-well tissue culture plates, precoated with 20  $\mu$ g/ml poly-L-lysine. On day 2, cultures were treated with a mixture of 5-fluoro-2'-deoxyuridine and uridine (20  $\mu$ g/ml each; Sigma-Aldrich), to inhibit proliferating cells. From day in vitro (DIV) 3 onward, cells were switched to a serum-free medium consisting of neurobasal medium, supplemented with 2% B-27, 100  $\mu$ M GlutaMAX (all items obtained from Invitrogen), and 1 $\times$  antibiotic-antimycotic solution. Long-term ethanol treatment consisted of adding 75 mM ethanol to the medium from 3 DIV, for a total of 5 days

with a change of media every 2 days. The ethanol treatment was conducted in a CO<sub>2</sub> incubator, which contained an open 30-ml beaker half-filled with ethanol. This system maintained ethanol levels in culture medium for 2 to 3 days at the original level, as determined using an alcohol assay kit (Sigma-Aldrich). The morphological appearance of cells was monitored daily by phase-contrast microscopy and compared with control cells that were untreated in a regular CO<sub>2</sub> incubator.

**Cell Viability Assay.** Cell viability was measured using CellTiter 96 Aqueous One solution assay (Promega, Madison, WI), which is based on colorimetric method of measuring the formation of a soluble formazan product generated by reduction of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide by dehydrogenase enzymes present only in live cells. For this assay, cells cultured in 24-well plates were treated with or without ethanol at different doses for 5 days. On the day of assay, to cells in 0.5 ml of medium, the reagent was added at 20  $\mu$ l/100  $\mu$ l medium as suggested by the manufacturer and incubation continued for 1 h at 37°C. The absorbance at 490 nm was recorded using a plate reader (Molecular Devices, Sunnyvale, CA). The blank absorbance determined by adding the same volume of reagent to medium without cells was deducted from the cell samples. All assays were done in triplicate, repeated at least three times, and the results expressed as percentage of control A<sub>490</sub> values.

**Cell Lysis and Western Blotting.** Cells were lysed at 4°C in lysis buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.25% SDS, 0.5% deoxycholate, and Sigma protease and phosphatase inhibitor cocktails), and centrifuged at 20,000g to clarify the extract. Protein was determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) and bovine serum albumin as standard. Western blotting was done using extracted proteins as described previously (Kalluri and Ticku, 2002). The following commercial primary antibodies were used at the recommended concentrations: anti-phospho-CREB (p-Ser<sup>133</sup>) and anti-CREB (Upstate Biotechnology, Lake Placid, NY); anti-ERK1/2 pan antibody and anti-ERK1/2 [pTpY<sup>185/187</sup>] phospho-specific antibody (BioSource International, Camarillo, CA); mouse anti-calmodulin-dependent protein kinase II (CaMKII) monoclonal antibody that detects a 50-kDa band corresponding to the  $\alpha$ -isoform (Chemicon International, Temecula, CA); CaM kinase IV polyclonal antibody (Affinity Bioreagents, Golden, CO); anti-actin monoclonal antibody (Calbiochem, San Diego, CA), and NMDA receptor 2B monoclonal antibody (BD Biosciences PharMingen, San Diego, CA). Equal amounts of protein ( $\sim 25$   $\mu$ g) were separated by SDS-PAGE on 4 to 12% Nu-PAGE Bis-Tris gels (Invitrogen) and transferred to PVDF membranes (Invitrogen). After incubations with the primary and appropriate secondary antibodies, the chemiluminescence signal developed using Western Lighting Plus reagents (PerkinElmer Life and Analytical Sciences, Boston, MA) was captured on X-ray film (Kodak BioMax-Light/MR film), and the band intensity was quantitated using the UVP Biochemi system (UVP Inc., Upland, CA). Data were normalized to the intensity of actin band and represented as percentage of control group.

**CREB ELISA.** We used the ELISA kits specific for phospho-CREB (pS133) and total CREB (BioSource International) to quantitate CREB levels in whole cell extracts according to the manufacturer's instructions. In these kits, a monoclonal antibody specific for CREB (regardless of the phosphorylation status) is coated onto 96-well strips and the CREB antigen in samples binds to this capture antibody. In the second step, the polyclonal detection antibody specific for phospho- or total CREB binds to the captured antigen, which then is bound by the horseradish peroxidase-labeled anti-rabbit IgG. The color produced by the activity of horseradish peroxidase enzyme on the chromogenic substrate is then read at 450 nm using a plate reader. The samples are read against a standard curve constructed using known amounts of total or phospho-CREB peptide standards. Results are normalized to micrograms of protein in the cell extract and expressed as the ratio of phospho-CREB to total in the same

sample. The result of ethanol treatment is expressed as percentage of control values. Assays were done in triplicate and repeated with extracts from at least three different experiments.

**ERK ELISA.** ERK ELISAs were performed using the ELISA kits (BioSource International) specific for total ERK and phospho-ERK (pTyr<sup>185/187</sup>) according to the manufacturer's instructions. These assays again were similar to the CREB ELISA, performed using cell lysates in a 96-well plate format. The samples are read against standard curves constructed using known amounts of purified ERK or phospho-ERK peptide standards, and the data are represented as the ratio of phospho- to total ERK after normalization to cell proteins.

**Preparation of Nuclear Extract and EMSA.** Cells collected by scraping into phosphate-buffered saline were pelleted at 450g for 10 min. All steps were carried out at 4°C. The cell pellet was washed with 5 volumes of hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM DTT, and Sigma protease and phosphatase inhibitor cocktails). Cells were disrupted by drawing up and down a 27-gauge needle fitted to a 1-ml syringe in 2 volumes of the hypotonic buffer and centrifuged at 10,000g for 15 min. The pellet consisting of the nuclei was resuspended in a high-salt buffer (20 mM HEPES, pH 7.9, 1 mM MgCl<sub>2</sub>, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 25% glycerol, and Sigma protease and phosphatase inhibitor cocktails), disrupted using a fresh syringe/needle, and proteins were extracted by shaking in cold for 30 min. The crude nuclear extract was centrifuged at 20,000g for 30 min and the supernatant, consisting of the nuclear proteins, was aliquoted and stored at -80°C. Protein concentration in the nuclear extract was measured using the Bio-Rad protein assay reagent.

For preparing the labeled probe, a 25-bp complementary DNA oligonucleotides consisting of the NMDAR2B CRE sequence (5'-CCAGCTCATTGACGTGAGAAGCAGC-3') were annealed and labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase. The nuclear extract (10  $\mu$ g of protein) was incubated for 20 min at room temperature with  $5 \times 10^5$  cpm of labeled probe in 20  $\mu$ l of binding buffer consisting of 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1  $\mu$ g/ $\mu$ l bovine serum albumin, and 2  $\mu$ g of poly-d(I-C). For the supershift assay, 1  $\mu$ l of anti-CREB or anti-phospho CREB was added and the reaction continued for 15 min. For competition assays, the competitor DNA was added for 10 min at 4°C before addition of the labeled probe. The reaction mix was loaded on 5% nondenaturing polyacrylamide gels, and electrophoresis was carried out at 200 V in 25 mM Tris-HCl, pH 8.5, with 190 mM glycerol and 1 mM EDTA. The DNA-protein complexes on the dried gel were visualized by exposing to Kodak BioMax X-ray film with intensifying screen at -80°C or to PhosphorImager screen (Amersham Biosciences Inc., Sunnyvale, CA).

**Plasmid Constructs and Transient Transfections.** A 467-bp (-451 to +16) DNA fragment of the promoter region of NMDA receptor 2B gene (5144-5610, AF03356; Klein et al., 1998) was generated from the M1 mouse genomic clone using HindIII and Xcm I. This fragment, which contains a CRE-like motif (TGACGTGA<sup>-413 to -406</sup>), was cloned into a promoterless luciferase reporter vector, pGL3-Basic (Promega) upstream of the Luc gene and grown in *Escherichia coli* strain GM2163 (New England Biolabs, Beverly, MA). From this wild-type construct, we generated a CRE mutant by replacing G and C with A in the core sequence TGAC to produce TAAA, using the QuikChange II XL site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The plasmid DNA constructs were purified by double banding on CsCl followed by polyethylene glycol precipitation to remove contaminating RNA (Sambrook and Russel, 2001) and verified by sequencing and restriction mapping.

For transient transfections, mouse cortical neurons were cultured in 24-well plates and transfections were done on DIV 3 or 5 using a calcium phosphate method as described by Desai et al. (2002). We used the Profection calcium phosphate mammalian transfection kit (Promega) to prepare DNA-CaPO<sub>4</sub> complexes using 2  $\mu$ g/well pGL3-Basic or NR2B promoter construct DNA. To monitor transfection

efficiency, we performed cotransfection using a *Renilla reniformis* luciferase vector, phRG-B (40 ng/well). Cells were incubated with the DNA-CaPO<sub>4</sub> complex for 1 h in neurobasal medium containing B-27 (NBM), washed, and incubated for 48 h, and the luciferase activities were assayed using the dual luciferase assay kit according to manufacturer's instructions (Promega) and Turner Designs TD-20/20 luminometer. Long-term ethanol treatment was done in two ways: in some experiments, ethanol was added for 3 days before transfection and the ethanol treatment continued for 2 more days, for a total of 5 days. In other experiments, transfection was done first on 3 DIV, and cells were treated with or without ethanol for 5 days, and luciferase activity was assayed. The results calculated from the ratio of firefly to *R. reniformis* Luc activities are represented as -fold increase over respective pGL3-Basic activity.

**Quantitative Real-Time PCR for NR2B Gene.** Total RNA was isolated from control cells and cells undergoing long-term ethanol treatment using TRIzol reagent (Invitrogen), and was treated with DNA-free reagents (Ambion, Austin, TX) to remove any genomic contamination. RNA integrity was examined by electrophoresis on denaturing agarose gel and visualized by the presence of 18S and 28S bands. Two-step reverse transcription-PCR was performed: 1  $\mu$ g of total RNA was first converted to cDNA using random hexamers and TaqMan reverse transcriptase reagents (Applied Biosystems, Branchburg, NJ); the cDNA, equivalent to 20 ng of RNA, was then used for PCR with TaqMan Universal PCR Master mix and Assays-on-Demand Gene Expression primer and probe set for mouse NR2B (Applied Biosystems). Assays were done in triplicate using the ABI Prism 7900 sequence detection system in a 96-well format. Results were normalized to 18S rRNA, whose amplification was performed in the same way with cDNA samples diluted 1:10, using the TaqMan predeveloped assay reagents for eukaryotic 18S rRNA (Applied Biosystems). The PCR products were analyzed on 2% agarose gels to confirm the presence of a single band of predicted size. From the average difference in cycle threshold of the target gene and the 18S control for each sample, the amplification difference between control samples and those undergoing long-term treatment with ethanol was calculated and the relative levels of NR2B gene expression is expressed as -fold change compared with control.

**Statistical Analyses.** Data were analyzed as appropriate by Student's *t* test or by one-way analysis of variance, with post-test using Student-Newman-Keuls test, using the Prism program (GraphPad Software Inc., San Diego, CA).

## Results

**Effect of Long-Term Ethanol Treatment on Cell Viability, NR2B mRNA, and Protein.** No difference was observed in the appearance of control and cells treated with 75 mM ethanol under the phase contrast microscope (Fig. 1A). This dose of ethanol was chosen because it had no significant effect on cell viability as measured using the CellTiter 96 Aqueous One solution (Promega), whereas 100 mM ethanol caused >25% loss of cell viability compared with control (Fig. 1B).

Although our laboratory had previously shown that long-term ethanol treatment increases NR2B receptors, it was important to confirm these results because we had changed the conditions for primary cortical neuron cultures. We used real-time PCR assay system using specific primers and Taqman probe for NR2B (Assay-on-Demand products; Applied Biosystems) to quantitate mRNA levels. Our results show a significant increase (~20% over control) in NR2B receptor mRNA in cells treated with 75 mM ethanol for 5 days (Fig. 2A). We also found ~40% increase in the 180-kDa band of NR2B protein by Western blot (Fig. 2B), confirming our previous findings.

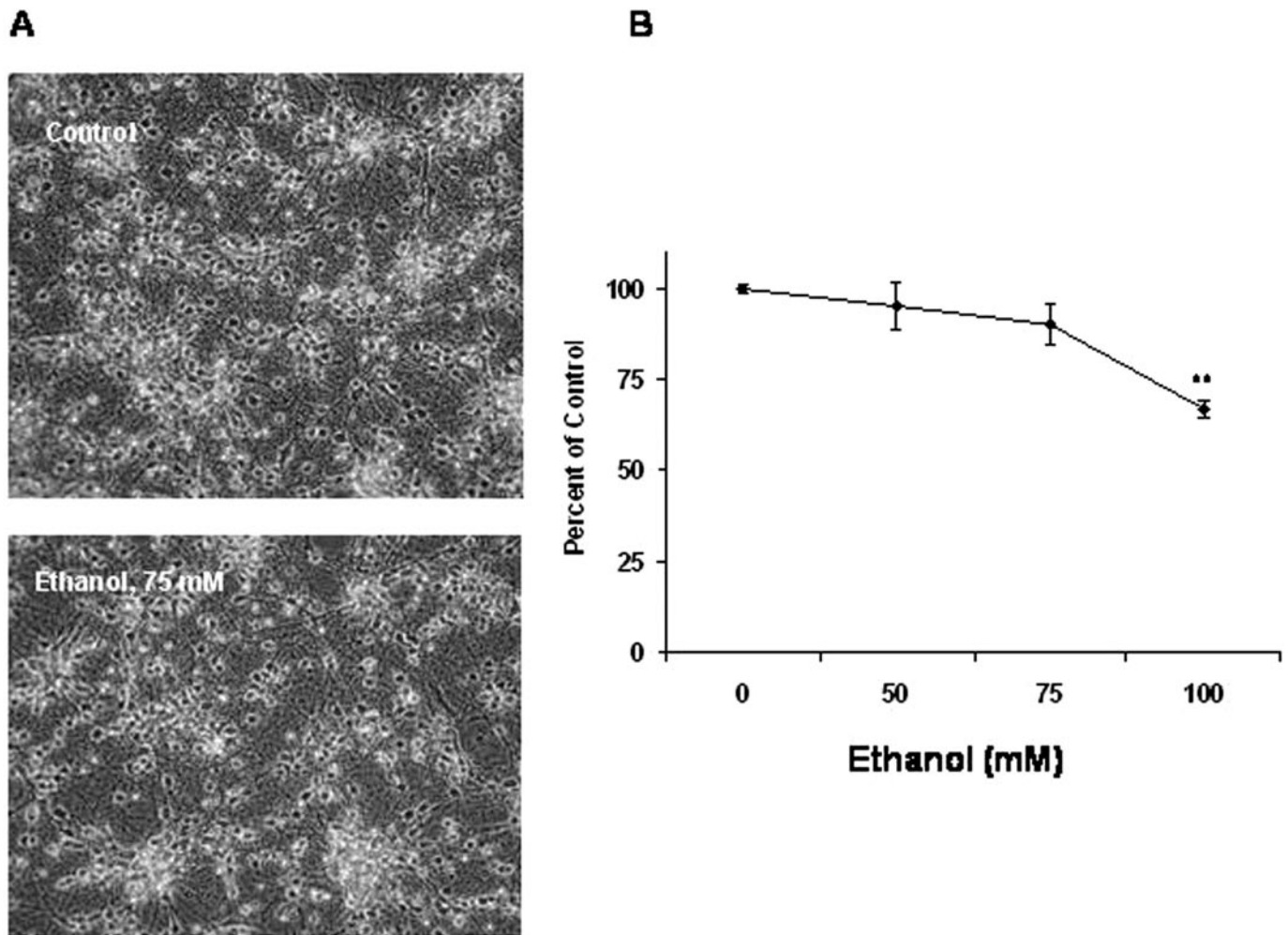


**Long-Term Ethanol Increases Phosphorylation of CREB but Not ERK.** Immunoblotting using anti-CREB and anti-phospho-CREB (pS<sup>133</sup>) antibodies revealed a specific 43-kDa band in cell lysates. Densitometric evaluation of immunoblots indicated a small, but significant increase in phospho-CREB levels, without any effect on total CREB levels (Fig. 3A) in lysates from long-term ethanol treatment. We used specific ELISA kits to measure total and phospho-CREB (pS<sup>133</sup>) levels to enable better quantification of small changes. ELISA assays showed that long-term ethanol treatment caused a significant increase ( $28 \pm 7\%$  over control) in the ratio of phospho-CREB to total CREB (Fig. 3B), in agreement with the immunoblot data.

We also performed Western blots and ELISA for total and phospho-ERK in the above-mentioned lysates. As shown in Fig. 4, in both assays, ethanol treatment caused no significant change in the ratio of phospho- to total ERK. Although phospho-ERK levels determined by ELISA showed a tendency toward decrease in the ethanol-treated lysates, the differences were not statistically significant. Because  $\beta$ -actin levels were not affected by ethanol treatment in these cells (Fig. 4A), they were used as loading control for densitometric quantitation of immunoblots.

Because a previous study from our laboratory (Kalluri and Ticku, 2003) had reported that long-term ethanol treatment increased phosphorylation of mitogen-activated kinase in fetal cortical neurons cultured in minimal essential medium containing 10% each of fetal bovine and horse serum, we compared the effect of ethanol on ERK phosphorylation in cells cultured in NBM with or without the addition of 10% fetal bovine serum. As shown by the ELISA data for total and phospho-ERK (Fig. 5) in cells treated with ethanol in NBM-containing serum, there was an increase in ERK phosphorylation agreeing with our previous studies, whereas ethanol had no such stimulatory effect on ERK phosphorylation in serum-free NBM (also see Fig. 4). However, the same ethanol treatment increased CREB phosphorylation in both culture media regardless of the presence of serum (data not shown).

**Effect of Long-term Ethanol Treatment on CaM Kinases II and IV.** We also tested the effect of long-term ethanol treatment of cells in NBM in the presence or absence of serum on CaM kinase II and IV levels by Western blotting. We found that ethanol treatment increased the 50-kDa band representing CaM kinase II- $\alpha$  in the presence of serum, thus confirming a previous report by Kalluri and Ticku (2003).

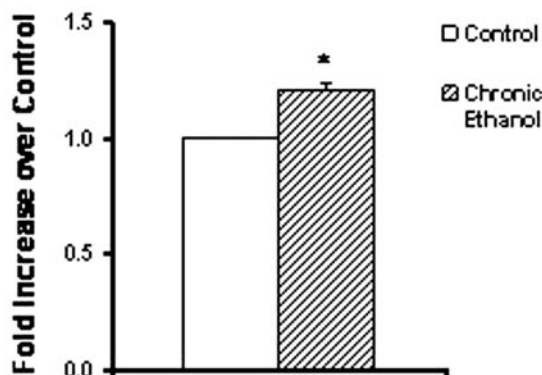


**Fig. 1.** A, phase-contrast micrographs (200 $\times$ ) of control samples and those undergoing long-term ethanol treatment (75 mM, 5 days) cultured neurons isolated from E14 mice cortex. B, effect of long-term ethanol treatment on cell viability. For determining cell viability, cortical cells were cultured in 24-well plates and treated with different doses of ethanol for a total of 5 days as described under *Materials and Methods*. Results are calculated from the  $A_{490}$  values, corrected for blank and expressed as percentage of control, mean  $\pm$  S.E.M. of at least three experiments each with triplicate wells; \*\*,  $p < 0.01$  compared with untreated control.

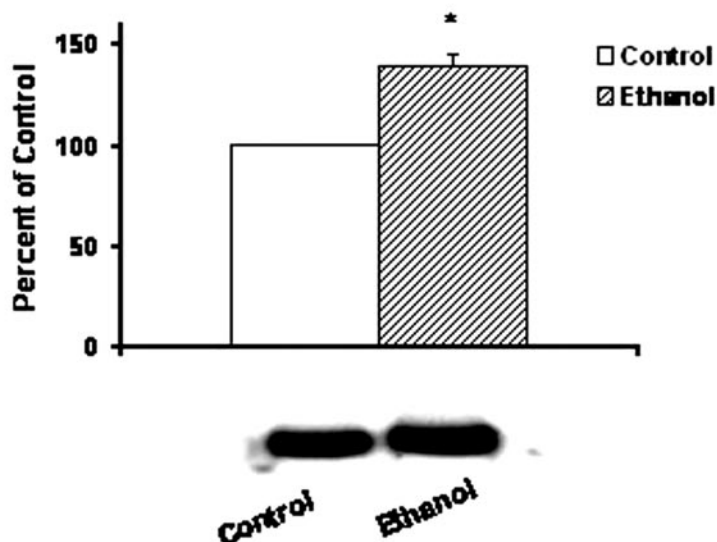
However, similar to ERK phosphorylation ethanol treatment in serum-free NBM caused no increase in CaM kinase II levels (Fig. 6, A and B). Using the same lysates, and a polyclonal antibody to CaM kinase IV, we detected a major

band at 55 kDa and a minor band at 80 kDa, both of which were eliminated when the antibody was first incubated with the immunizing peptide (data not shown), indicating the presence of specific CaM kinase IV cross-reacting proteins. In

### A. NR2B mRNA

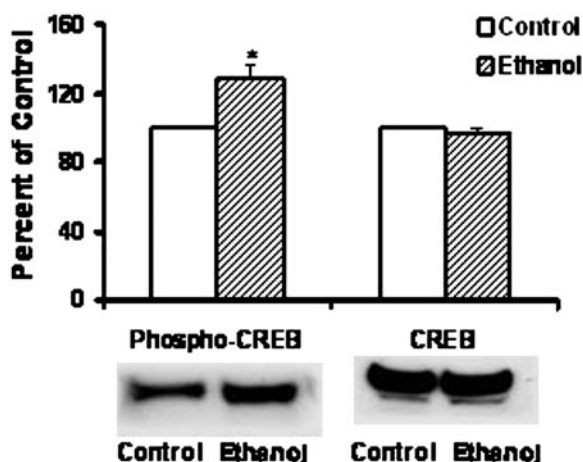


### B. NR2B Protein (180-kDa Band)

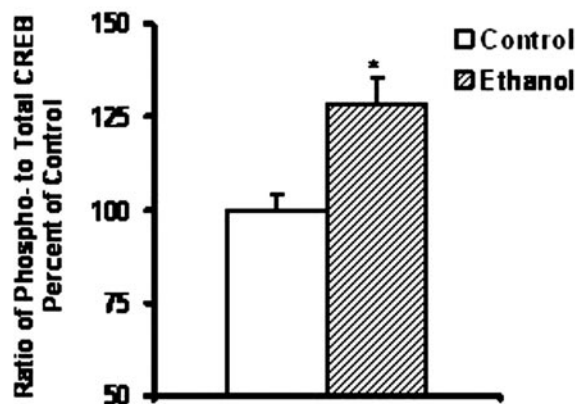


**Fig. 2.** Long-term ethanol treatment increases NR2B mRNA and protein. Cells were treated without (control) or with 75 mM ethanol for 5 days, starting from 3 DIV. A, ethanol effect on NR2B mRNA was examined by isolating total RNA that was reverse transcribed using random hexamers and the cDNA used for real-time PCR with the Assay-on-Demand products (NR2B and 18S rRNA) and TaqMan assay kit, as described under *Materials and Methods*. The relative levels of NR2B expression and the -fold change between control and ethanol-treated cells were calculated from these data. \*,  $p < 0.05$  (Student's  $t$  test). B, cellular proteins were separated by SDS-PAGE on 4 to 12% gels, transferred to PVDF membranes, and probed using a monoclonal antibody to NR2B protein. The 180-kDa band was analyzed by densitometry, and results, expressed as percentage of control, represent mean  $\pm$  S.E.M. of four experiments. \*,  $p < 0.05$  (Student's  $t$  test).

### A. CREB Immunoblot



### B. CREB ELISA



**Fig. 3.** Long-term ethanol treatment increases CREB phosphorylation. A, Western blots for total and phospho-CREB. Lysates were prepared from control and cells treated with 75 mM ethanol for 5 days and equal amount of protein ( $\sim 25 \mu\text{g}$ ) separated on 4 to 12% SDS-PAGE gels, transferred to PVDF membranes and probed with antibodies to phospho-CREB ( $\text{pS}^{133}$ ), total CREB and actin antibodies. The band intensities were determined by densitometry, corrected for loading differences using  $\beta$ -actin and expressed as percentage of control. Data shown are the mean  $\pm$  S.E.M. from three experiments. Representative immunoblots for P-CREB and total CREB are shown on the bottom of corresponding bars. B, ELISA for total and phospho-CREB. Cell extracts from control cells and cells undergoing long-term ethanol treatment were assayed using kits specific for total or phospho-CREB. After normalizing to cell protein, the ratio of phospho-CREB to total CREB was calculated for each group, and the results are expressed as percentage of control. Data shown are the mean  $\pm$  S.E.M for three experiments. \*,  $p < 0.05$  (Student's  $t$  test).

cells subjected to long-term treatment with ethanol in both media with or without serum, there was a clear increase in minor CaM kinase IV band at 80 kDa, whereas no change was discernible in the major 55-kDa band (Fig. 6, C and D).

**EMSA for CREB.** To study whether CREB binds to the CRE-like sequence present in the NR2B promoter region, we carried out gel-shift assay using nuclear extract prepared from control cells and cells undergoing long-term ethanol treatment and a 25-bp oligonucleotide fragment as  $^{32}$ P-labeled probe. This fragment representing -421- to -397-bp region of the NR2B gene (Klein et al., 1998) contains a CRE-like sequence (TGACGTGA), with one base change compared with the palindromic CRE consensus (TGACGTCA). As shown in Fig. 7A, the probe bound to specific proteins in the nuclear extract (lane 2, complexes b and c). Binding was markedly reduced when incubated with either excess unlabeled oligonucleotide (lane 3) or with a consensus CRE oligonucleotide sequence (lane 4), but not with the mutated CRE (TaAaGTGA, lane 5). Specificity of CRE binding was further indicated by the supershifted band (complex a), when incubated with anti-CREB (lane 6) or anti-phospho-CREB (lane 7) antibodies, but not with an unrelated ERK1/2 antibody (lane 8). The complex indicated by the arrow (b) is likely to contain CREB, because this band was greatly reduced by the consensus CRE sequence and completely supershifted by the anti-CREB antibodies. As shown in Fig. 7B, nuclear extracts from cells undergoing long-term ethanol treatment showed a marked increase over control in the binding of labeled probe to CREB, consistent with data from CREB ELISA and Western blotting.

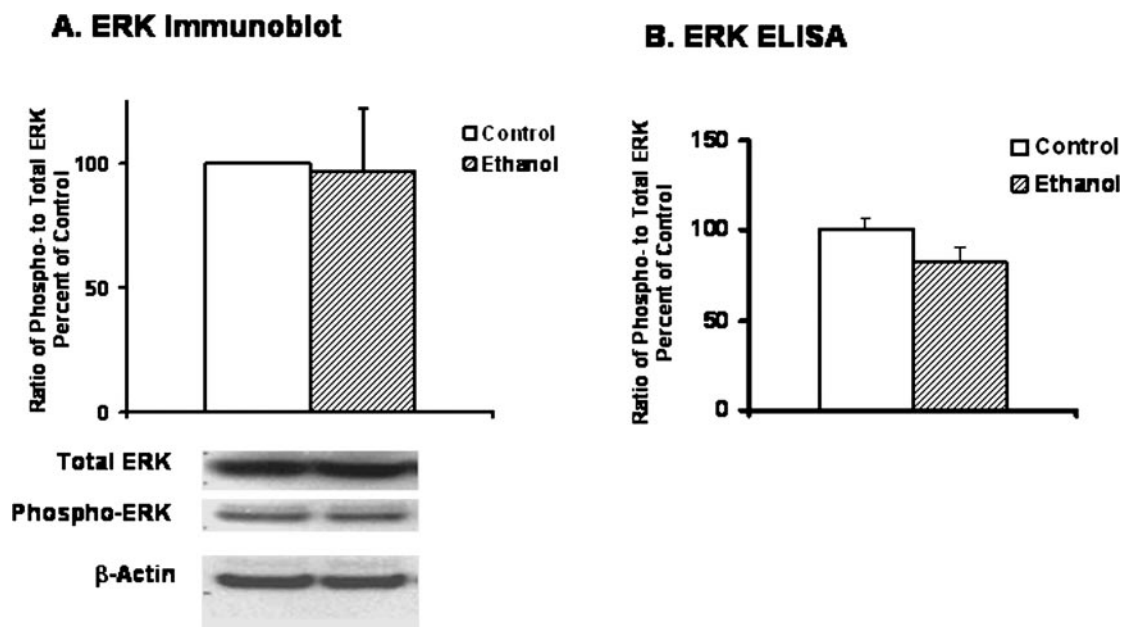
**Mutation in CRE-Like Motif of NR2B Promoter Decreases Transcription.** Using a 467-bp nucleotide fragment of the NR2B gene that includes a CRE sequence (-406 to -413) subcloned into the pGL3-basic vector, we performed

site-directed mutation at the CRE site and examined the luciferase activities in a cotransfection assay of cultured mouse cortical neurons. The promoter construct containing the wild-type CRE motif (Fig. 8A) exhibited high transcriptional activity in the reporter assay as indicated by an ~15-fold increase in Luc activity, relative to the basal activity ratio of the pGL3-Basic vector. Long-term ethanol treatment increased the reporter activity of the wild-type construct by 24% over control cells (Fig. 8C). This effect was observed whether ethanol treatment was initiated after transfection, with Luc activities assayed 5 days after ethanol treatment (data not shown), or when ethanol was added for a total of 5 days, with transfection conducted during the ethanol treatment period and Luc assayed 48 h after transfection as in a standard assay protocol (Fig. 8). Although we also observed an increase of ~2-fold in basal activity ratio of pGL3-Basic construct in ethanol-treated cells, the increase in NR2B promoter activity we observed with long-term ethanol treatment is over and above the effect on basal activity because the results are expressed as -fold increase over respective controls.

Mutation of the CRE sequence (Fig. 8B) resulted in significantly lower Luc activity compared with the respective activities of the wild-type construct in both control and ethanol-treated cells (Fig. 8C). However, the Luc activities of the CRE mutant construct were similar in control and ethanol-treated cells, indicating an absence of ethanol-induced increase in promoter activity when CRE was mutated (Fig. 8C).

## Discussion

Our results indicate that CREB may have a role as an intracellular mediator of the effect of long-term ethanol treatment in up-regulation of NR2B gene transcription in



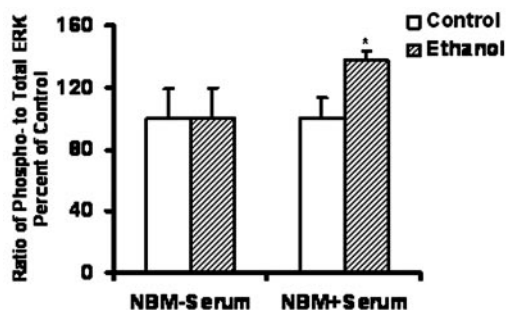
**Fig. 4.** Lack of effect of ethanol on ERK phosphorylation. A, immunoblots for  $\beta$ -actin, total, and phospho-ERK1/2 were performed using lysates from control cells and cells undergoing long-term ethanol treatment. Representative immunoblots are shown, along with densitometric quantitation performed as described in Fig. 3 legend for CREB. Data shown are the mean  $\pm$  S.E.M. from three experiments. B, ELISA assays for total and phospho-ERK were done on whole cell extracts prepared from control cells and cells undergoing long-term ethanol treatment. Ratio of phospho-ERK to total ERK expressed as percentage of control represent mean  $\pm$  S.E.M. of triplicates from three different experiments.



mouse cortical neurons. We have provided evidence here for NR2B gene up-regulation using a real-time PCR assay that showed that long-term ethanol treatment increases levels of NR2B mRNA. We have also confirmed previous findings from

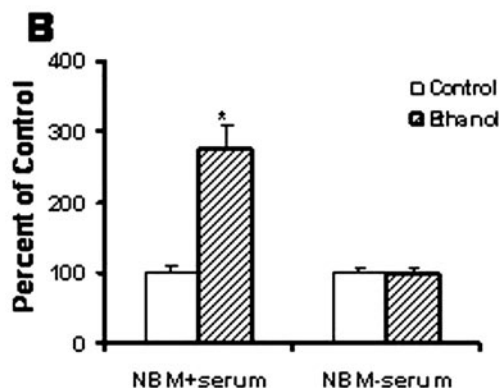
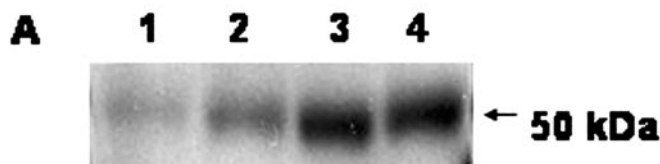
our laboratory by showing an increase in the NR2B protein levels by Western blotting. Long-term ethanol treatment also increased the phosphorylation status of CREB, without affecting the total CREB protein level. In addition to Western blotting to assess levels of the total and phosphorylated CREB, we have also used ELISA assays to more precisely quantitate the transcription factor levels. This increase in CREB activity could be involved in ethanol-induced NR2B gene transcription, because mutation of the CRE sequence found in the promoter of NR2B reduced the CREB-mediated gene expression, determined using a CRE-regulated luciferase reporter construct. CREB is phosphorylated on Ser<sup>133</sup> by a variety of protein kinases, including protein kinase A, protein kinase C, pp90<sup>rsk</sup>, and CaM kinases II/IV (Mayr and Montminy, 2001), and the phosphorylated CREB binds to its coactivator, CREB binding protein. This in turn causes an increased binding to specific DNA sequence known as cyclic AMP response element (CRE motif, TGACGTCA), commonly found in the promoter region of genes under the control of cAMP, and initiates a cascade of gene transcription (Weeber and Sweatt, 2002).

Ethanol treatment has been shown to affect neuronal CREB

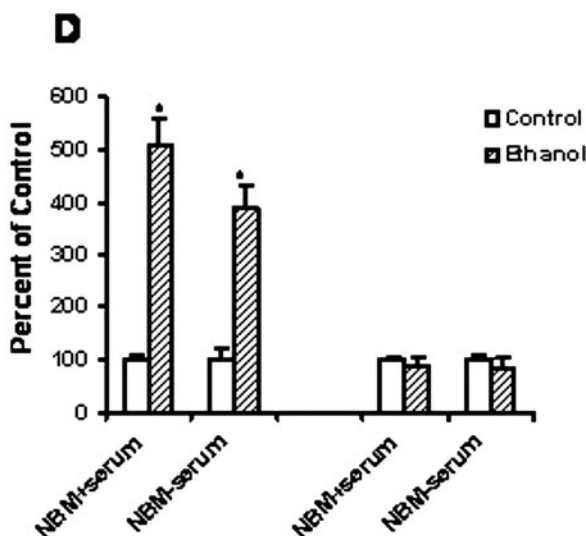
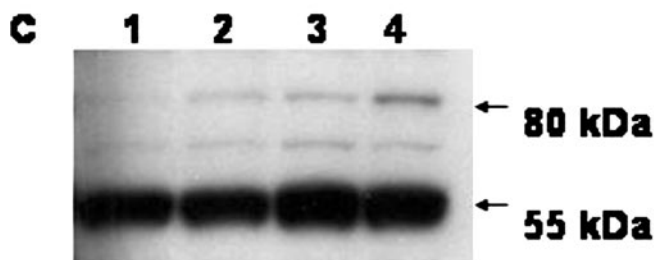


**Fig. 5.** Ethanol stimulates ERK phosphorylation in the presence of serum. Cells from E14 cortex cultured in NBM in the presence or absence of 10% fetal bovine serum were treated with or without 75 mM ethanol for 5 days from DIV 3 to 7. Total and phospho-ERK levels were assayed by ELISA as described under *Materials and Methods*. Ratio of phospho-ERK to total ERK was calculated and results expressed as percentage of control represent mean  $\pm$  S.E.M. of triplicates from three different experiments. \*,  $p < 0.05$  (Student's  $t$  test).

### CaM Kinase II



### CaM Kinase IV

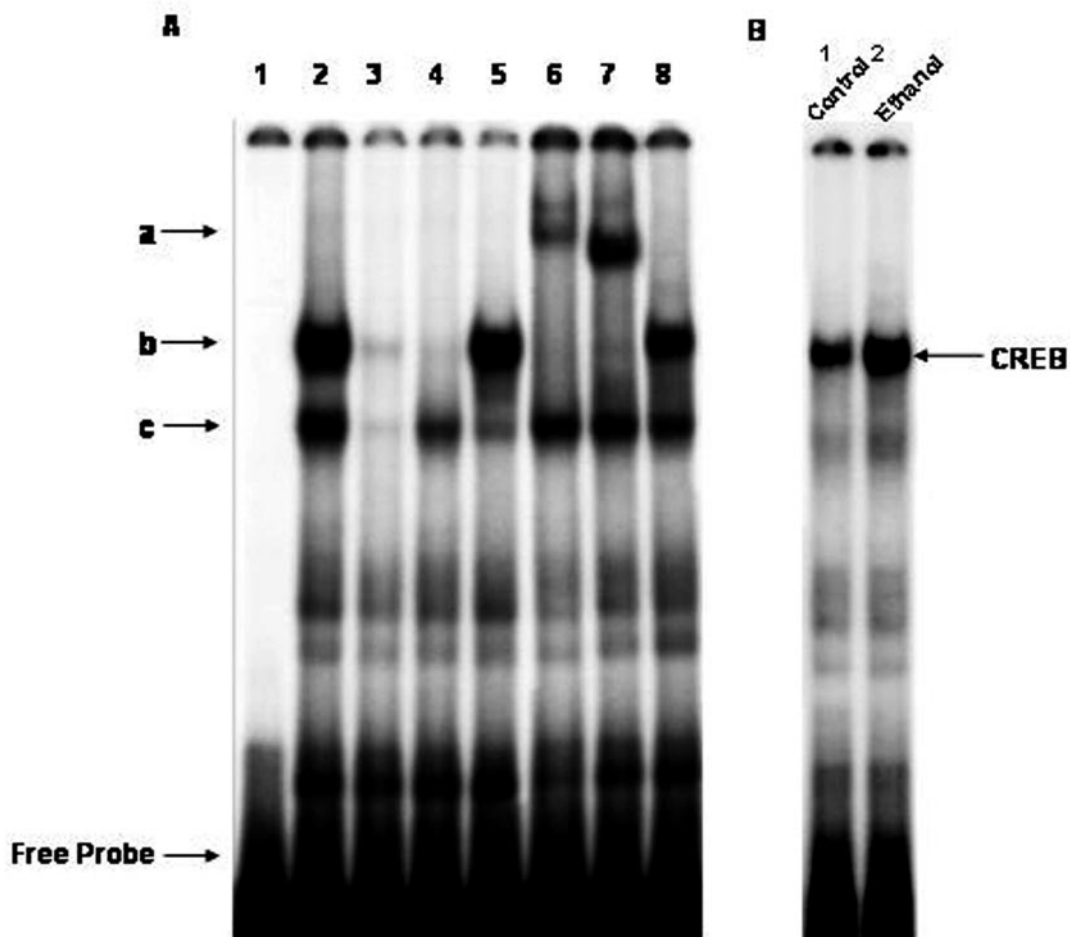


**Fig. 6.** Effect of long-term ethanol treatment on CaM kinases II (A and B) and IV (C and D). The lysates generated under culture conditions as described in Fig. 5 were used for immunoblotting for CaM kinases II and IV. Representative immunoblots for CaMKII (A) and CaMKIV (C) are shown. Lanes 1 to 4 refer in sequence to control, NBM + serum, ethanol, NBM + serum, control, NBM - serum, ethanol, and NBM - serum. The band intensities were determined by densitometry, corrected for loading differences using  $\beta$ -actin and expressed as percentage of control. Data shown are the mean  $\pm$  S.E.M. from three experiments. \*,  $p < 0.01$  (Student's  $t$  test).

phosphorylation, but the results are variable depending on the duration of treatment, brain region, and the model system studied. In the NG108-15 neuroblastoma  $\times$  glioma cell model, short-term treatment with ethanol induces CREB phosphorylation via cAMP-dependent protein kinase and stimulates CRE-mediated gene transcription (Asher et al., 2002; Constantinescu et al., 2002). In adult male rats, short-term treatment with ethanol *in vivo* increased phosphorylated CREB and CRE binding in cerebellar granule cells (Yang et al., 1996), whereas long-term ethanol feeding for 5-week period decreased basal CREB phosphorylation, with no apparent change in levels of kinases or phosphatases, enzymes that alter the phosphorylation status of CREB. However, when challenged with short-term treatment with ethanol, CREB phosphorylation was attenuated in cerebellar granule cells from rats undergoing long-term treatment with ethanol, with a concomitant reduction in levels of protein kinase A and calcium/calmodulin-dependent protein kinase IV (Yang et al., 1998). Interestingly in adult rat cortex, Pandey et al. (1999, 2001) have reported that ethanol withdrawal, not ethanol treatment, produced significant decrease in phosphorylated CREB. They observed changes in

CREB that were brain region-specific (Misra and Pandey, 2001) and suggest a role for CREB in anxiety-like behavior and reward mechanism of alcohol drinking (Morrow et al., 2004).

ERK is another intracellular signaling component extensively studied as a target of ethanol effects on the brain. ERK is also implicated in CREB phosphorylation; however, its effect is deemed indirect, because Ser133 of CREB is not a substrate for ERK (Sweatt, 2001). It is reported that ERK effect on CREB phosphorylation is more likely to be mediated by RSK2, a member of the pp90rsk family of S6 kinases or by cAMP-dependent protein kinase or protein kinase C (Roberson et al., 1999). Again with ERK, a variety of effects of ethanol have been reported, depending on the model system used. Ethanol has been shown to either inhibit (Seiler et al., 2001; Kalluri and Ticku, 2002) or potentiate ERK activity (Roivainen et al., 1995; Kalluri and Ticku, 2003). Long-term ethanol treatment of rat pups on postnatal days 4 to 7 resulted in decreased phosphorylation of ERK and p70S6 kinase in the cerebral cortex, and ERK was suggested to play a role in alcohol-induced neurotoxicity on the developing



**Fig. 7.** EMSA showing binding of CRE motif in NR2B promoter to nuclear proteins from neuron cultures. A, 25-bp double-stranded oligonucleotide sequence (5'-CCCAGCTCATGACGTGAGAAGCAGC-3') in the promoter region of NR2B (5176–5199 bp; GenBank accession no. AF03356) that includes a CRE motif (–406 to –413, shown in bold) was labeled with [ $\gamma$ - $^{32}$ P]ATP and used as probe in the gel-shift assay with nuclear extract from neuron cultures. A representative autoradiogram of dried gel is shown. Lane 1 shows probe only without any nuclear extract. The nuclear extract (10  $\mu$ g of protein) was incubated with  $5 \times 10^5$  cpm of the probe (lanes 2–8) without any competitor (lane 2) or with competitors and/or antibodies, as follows: lane 3, 100 $\times$  unlabeled wild-type oligonucleotide; lane 4, consensus CRE oligonucleotide (5'-AGGGATTGCCTGACGTCAGAGAGCTAG-3'; lane 5, mutated CRE oligo (5'-CCCAGCTCATaAaGTGAGAAGCAGC-3', the mutated bases shown in lowercase); lane 6, anti-CREB antibody; lane 7, anti-phospho CREB; and lane 8, anti-ERK antibody. The DNA complex containing the specific CREB protein is indicated by arrow (b) and supershifted complex by arrow (a). B, binding of labeled probe to nuclear extract from control cells (lane 1) and ethanol-treated cells (lane 2); arrow indicates increased CREB binding by long-term ethanol treatment.



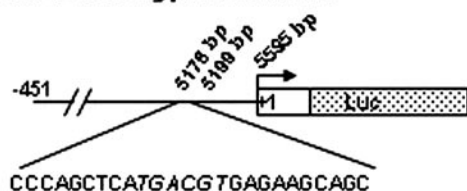
brain (Tsuji et al., 2003). Kalluri and Ticku (2003) recently reported that short-term treatment with ethanol inhibits phosphorylation, whereas long-term ethanol treatment increases phosphorylation of mitogen-activated protein kinase in fetal cortical neurons. Our results in this study clearly show that this apparent discrepancy in ethanol effect on ERK phosphorylation is caused by the presence or absence of serum in the culture media. Serum components seem to have profound effects on the mitogen-activated protein kinase pathway, as also discussed by Morrow et al. (2004). Our reason for switching to serum-free medium was better cell survival in long-term cultures and that ethanol (25–75 mM) had no adverse effect on cell viability (Fig. 1), and we did observe an up-regulation of NR2B gene (Fig. 2).

Unlike the diverse effects on ERK phosphorylation related to serum factors, ethanol had a stimulatory effect on CREB phosphorylation and NR2B gene expression regardless of the presence or absence of serum. It is interesting that, as with ERK phosphorylation, CaM kinase II levels were increased by ethanol treatment only in the presence of serum, confirming a previous study from our laboratory (Kalluri and Ticku, 2003). In serum-free medium, ethanol treatment had no effect on both ERK phosphorylation and CaM kinase II levels, whereas it increased CREB phosphorylation. Thus, with regard to ethanol effects, we see dissociation between ERK and CREB phosphorylation and a positive correlation between CaM kinase II and ERK phosphorylation, but not with CREB phosphorylation. CaM kinase IV is another important kinase in neuronal tissues activated by calcium influx, and it has been implicated in CREB phosphorylation (Mayr and Montminy, 2001). Although we did not find a concomitant increase in the major (55-kDa) CaM kinase IV protein after long-term

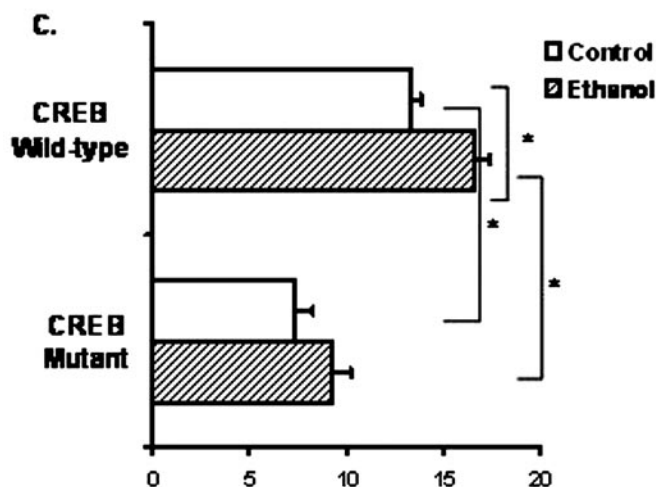
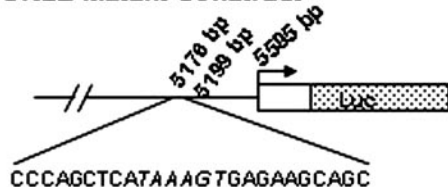
ethanol treatment, there was an increase in another specific protein at 80-kDa that cross-reacted with the CaM kinase IV antibody, and this seems to correlate with CREB phosphorylation. Although our studies hint at a possible involvement of CaM kinase IV in ethanol-induced CREB phosphorylation, further studies using specific inhibitors are warranted for a more definitive proof.

Our most compelling evidence for implicating CREB in NR2B gene transcription comes from the promoter studies. As shown by the gel-shift assay the radiolabeled CRE sequence found in the NR2B promoter (with one base difference compared with the consensus CRE) bound to specific proteins in the nuclear extracts; the specificity of this binding was verified by competitor oligonucleotides and by using antibodies in supershift studies. The luciferase reporter plasmid construct that contained the wild-type CRE sequence from the 5' flanking region of the NR2B gene was highly active in the transient transfection assay, and more importantly, mutating the CRE sequence caused a clear reduction in its reporter activity. A previous study describing the cloning and characterization of the 5'-untranslated region of the NR2B gene (Klein et al., 1998) has reported on promoter activity of constructs that includes the CRE region, in driving *lacZ* reporter gene expression in NIH3T3 mouse fibroblast cell line. However, the mutation of CRE did not result in any alteration in the reporter activity in transfected NIH3T3 cells. This disparity in results could exist because the non-neuronal cell type was used in the previous study. It is interesting that, using rat embryonic cortical neuron cultures, a recent study describes the effect of CRE mutations in the promoter region of the NR1 subunit of the NMDA receptor, and they report that the transcription of NR1 is regu-

### A. CREB Wild-type Construct



### B. CREB Mutant Construct



**Fig. 8.** Mutation in the CRE sequence of the NR2B promoter decreases luciferase reporter activity in transient transfection assay in cultured neurons. **A**, CREB wild-type construct was prepared by cloning a 467-bp restriction fragment of the NR2B gene (–451 to +16 bp, relative to +1, the transcription start site, indicated by a curved arrow at 5595 bp; GenBank accession no. AF033356) upstream of the Luc gene (indicated by shaded bar) of the promoterless pGL3-Basic vector. This construct contained a CRE-like sequence at –406 to –413 bp, indicated by bold letters. **B**, the above-mentioned construct was used as template to introduce site-directed mutations, resulting in base change from TGAC to TAAA in the core CRE motif (changes shown by the underlined bases). The DNA constructs purified by double CsCl banding were sequence verified and used for transient transfection of primary cultured neurons. **C**, Luc activity of control cells and cells undergoing long-term ethanol treatment. Cells were treated with 75 mM ethanol for a total of 5 days, with cotransfection assays performed on 3 DIV, and the dual luciferase activities assayed 48 h after transfection. From the ratio of firefly to *R. reniformis* Luc activities, the -fold increase over basal pGL3-Basic was calculated for each group. The results are the mean  $\pm$  S.E.M. of three to four replicates in at least four experiments. \*,  $p < 0.05$ , by analysis of variance and Student-Newman-Keuls post test for comparisons as indicated in the figure.

lated by cAMP pathway through a phosphorylation-dependent activation of CREB (Lau et al., 2004), similar to our data with the NR2B subunit.

Long-term ethanol treatment increased the activity of the NR2B promoter construct in the transfected cortical cells. This stimulatory effect of ethanol was not observed when CRE motif was mutated, indicating that an intact CRE site is necessary for an ethanol-induced increase in promoter activity. However, other transcription factors in addition to CREB may also be involved in regulating NR2B gene transcription, because we observe similar stimulatory effect of ethanol with other NR2B promoter constructs (Qiang et al., 2005).

Although there is differential expression of both NR1 and NR2 subunits during development, NR2B subunit expression displays the most prominent developmental change, and both these subunits that are part of the NMDA receptor channel play an important role in central nervous system development and synaptic plasticity. Although the mRNA for NR2A subunit is present, NR2A polypeptide is not expressed in our cultures isolated from E14 mice (Follesa and Ticku, 1996). Furthermore, studies have demonstrated that NMDA receptors composed of NR1/NR2B subunits display a high sensitivity to ethanol in cultured cortical neurons and cerebellar granule cells (for review, see Kumari and Ticku, 2000). Thus, exposure to ethanol during neuronal development could have profound effects on CREB-mediated NR2B receptor function, including glutamate-induced excitotoxicity. Given the vital role for CREB in memory formation, studies on CREB-mediated signaling mechanism in ethanol effects on NMDA/glutamatergic system are of immense importance in understanding the wide-ranging neurological effects of alcohol use and abuse, including intoxication, withdrawal seizures, and fetal alcohol syndrome, and they may lead toward the strategies for suitable pharmacological intervention.

In summary, long-term ethanol treatment of fetal cortical cells in vitro seems to activate the CREB pathway, which in turn may stimulate NR2B gene transcription. Using this model system to study the neuronal effects of long-term alcohol exposure, our studies have thus far established that long-term ethanol treatment leads to an increase in CREB phosphorylation, NR2B receptors, and NMDA-induced intracellular levels of  $[Ca^{2+}]$ , in fetal cortical cells.

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