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Potential Role of CREB in Ethanol-induced NR2B Gene Transcription in Fetal Mouse Cortical Cells

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Running Title: Chronic ethanol upregulates NR2B gene expression via CREB

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Abbreviations used: NR2B, N-methyl D-aspartate receptor 2B subunit; CRE, cyclic AMP response element; CREB, cAMP response element binding protein; CaM Kinase, Ca2+/calmodulin-dependent protein kinase; ERK, extracellular signal-regulated kinase; ELISA, enzyme-linked immunosorbent assay;

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Abstract

We have previously shown that chronic ethanol treatment causes an upregulation of 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 treated fetal cortical neurons chronically with ethanol and studied its effect on CREB and ERK levels by Western blot and ELISA. We find a significant increase in phosphorylated CREB, without change in total CREB protein, in cells treated with ethanol for 5 days. Chronic ethanol treatment did not increase levels of both total and phospho-ERK in serum-free medium, while 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-bp long double-stranded DNA fragment containing the CRE-like sequence of the NR2B promoter as [^{32}P]-labeled probe, showed an increase in specific CRE binding to nuclear proteins isolated from chronic ethanol-treated cells. A 467-bp long DNA fragment of the NR2B promoter containing the CRE sequence cloned into the luciferase vector exhibited high reporter activity in transient co-transfection 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 likely involved in

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mediating ethanol-induced upregulation of NR2B gene.

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NMDA receptors are increasingly recognized as a major target of ethanol effects on the brain, and have recently been the subject of intense study (see for reviews, 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-D of R2 or R3 (Ishii et al., 1993; Mori and Mishina, 1995; Luo et al., 1997). Chronic ethanol treatment is shown to increase NMDA receptor number and function both *in vivo* and *in vitro* (Snell et al., 1993; Folesa 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 chronic ethanol effects, studies from our laboratory have shown that chronic ethanol treatment causes an upregulation 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 chronic ethanol effects.

Recently, much attention has focused 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/MAPK

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and calcium. CREB is a member of the bZIP superfamily of transcriptional activators that includes ATF and CREM. 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 (CRE), 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 Sassner and Buananno (1996) have described an 800-bp region that includes a 255-bp of the first non-coding 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 earlier studies a stimulatory effect of chronic ethanol on NR2B receptors and NMDA-induced intracellular calcium levels (Folesa and Ticku, 1996; Kumari and Ticku, 2000; Hu

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and Ticku, 1995; Hu et al., 1996), we have studied the effects of chronic 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 chronic ethanol treatment increases CREB activity as shown by an increase in its phosphorylation, ^{32}P-labeled CRE binding to CREB in the gel-shift assay, CRE-regulated activity of luciferase 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.

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Materials and Methods

Cell Culture. Cortical cells were isolated from 14 day-old C57BL/6 mouse fetuses as described previously (Hu and Ticku, 1995). Briefly, cortices were removed from E14 mice and cells isolated by trituration and resuspended in culture medium: minimal essential medium (MEM, Invitrogen, Carlsbad, CA) supplemented with 5% each of fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and heat-inactivated horse serum (GIBCO/Invitrogen), 100 µM Glutamax (Invitrogen), 28 mM D-glucose and 1X antibiotic-antimycotic solution (Sigma Aldrich, St. Louis, MO). Cells (~2.8 x $10^5/cm^2$) were cultured under 5% CO_2 at 37^oC in T-75 or T-25 tissue culture flasks with filter caps that permitted gas exchange or in 24-well tissue culture plates, pre-coated with poly-L-lysine (20 µg/ml). On day 2, cultures were treated with a mixture of 5-fluoro-2'-deoxyuridine and uridine (Sigma, 20 µg/ml each), to inhibit proliferating cells. From day *in vitro* (DIV) 3 onwards cells were switched to a serum-free medium consisting of Neurobasal medium, supplemented with 2% B-27, 100 µM Glutamax (all items obtained from Invitrogen), and 1X antibiotic-antimycotic solution. Chronic ethanol treatment consisted of adding ethanol (75 mM) to the medium from day 3 (3 DIV) for a total of 5 days with a change of media every 2 days. The ethanol treatment was conducted in a CO_2 incubator, which contained an open 30-ml beaker half-filled with ethanol. This system maintained ethanol levels in culture medium for 2-3 days at the original level, as determined using an alcohol assay kit (Sigma). The morphological appearance of cells was monitored

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daily by phase-contrast microscopy and compared to control cells that were untreated in a regular CO_2 incubator.

Cell Viability Assay. Cell viability was measured using CellTiter 96 Aq_{ueous} 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, MTS 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 medium, the reagent was added at 20 µl /100 µl medium as suggested by the manufacturer and incubation continued for 1 h at 37^{o}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 percent of control A_{490} values.

Cell lysis and Western blotting. Cells were lysed at 4^{o}C in lysis buffer (composition in mM: Tris-HCl 10, pH 7.4, NaCl 100, EDTA 1, EGTA 1, 1% Triton X-100, 10% glycerol, 0.25% SDS, 0.5% deoxycholate, Sigma protease and phosphatase inhibitor cocktails), and centrifuged at 20,000 X g 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 before (Kalluri and Ticku, 2002). The following commercial primary antibodies were used at the recommended

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concentrations: anti-phospho-CREB (p-Ser^{133}) and anti-CREB (Upstate Biotechnology, Lake Placid, NY); anti-ERK1&2 pan antibody and anti-ERK1&2 [pTpY$^{185/187}$} phosphospecific antibody (Biosource International, Camarillo, CA); mouse anti-calmodulin-dependent protein kinase II (CaMKII) monoclonal antibody that detects a 50 kDa band corresponding to the α-isoform (Chemicon International, Temecula, CA); CaM Kinase IV polyclonal antibody (Affinity BioReagents, Golden, CO); anti-actin monoclonal antibody (Oncogene Research products, San Diego, CA), and NMDAR2B monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA). Equal amounts of protein (~25 µg) were separated by SDS-PAGE on 4-12 % NuPAGE Bis-Tris gels (Invitrogen) and transferred to PVDF membranes (Invitrogen). Following incubations with the primary and appropriate secondary antibodies, the chemiluminescence signal developed using Western Lighting Plus reagents (Perkin Elmers Life Sciences, Inc., Boston, MA) was captured on X-ray film (Kodak BioMax-Light/MR film) and the band intensity quantitated using the UVP Biochemi system (UVP Inc., Upland, CA). Data were normalized to the intensity of actin band and represented as percent of control group.

CREB ELISA. We used the ELISA kits specific for phospho-CREB (pS133) and total CREB (BioSource International Inc., Camarillo, CA) 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

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specific for phospho- or total CREB binds to the captured antigen, which then is bound by the HRP-labeled anti-rabbit IgG. The color produced by the activity of HRP 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 µg 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 percent 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 ($pTpY^{185/187}$) 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 represented as the ratio of phospho to total ERK after normalization to cell proteins.

Preparation of nuclear extract and Electrophoretic Mobility Shift Assay (EMSA). Cells collected by scraping into PBS were pelleted at 450 X g for 10 min. All steps were carried out at 4^oC. The cell pellet was washed with 5 volumes of hypotonic buffer (in mM: Hepes 10, pH 7.9, KCl 10, $MgCl_2$ 1.5, DTT 1, 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 10,000 X g for 15 min. The pellet

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consisting of the nuclei was resuspended in a high-salt buffer (in mM: Hepes 20, pH 7.9, $MgCl_2$ 1, NaCl 420, EDTA 0.2, DTT 1, 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 20,000 X g for 30 min and the supernatant, consisting of the nuclear proteins, was aliquoted and stored at –80^{o}C. Protein concentration in the nuclear extract was measured using the Bio-Rad protein Assay reagent.

For preparing the labeled probe, a 25-bp long complementary DNA oligonucleotides consisting of the NMDAR2B CRE sequence (5'-CCAGCTCAT*TGACGTGA*GAAGCAGC-3') were annealed and labeled with [^{32}P]-γ-ATP using T4 polynucleotide kinase. The nuclear extract (10 µg protein) was incubated for 20 min at room temperature with 5 X 10^5 cpm of labeled probe in 20 µl of binding buffer consisting of 10 mM Tris-Hcl, pH 7.6, 50 M NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1 µg/µl bovine serum albumin and 2 µg of ploy-d (I-C). For the supershift assay, 1 µl 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^{o}C prior to addition of the labeled probe. The reaction mix was loaded on 5% nondenaturing polyacrylamide gels and electrophoresis 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^{o}C or to phosphorimager screen (Molecular Dynamics, OR).

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Plasmid Constructs and Transient Transfections. A 467-bp (-451 to +16) DNA fragment of the promoter region of NMDA R2B gene (5144-5610, AF03356, Klein et al, 1998) was generated from the M1 mouse genomic clone using Hind III and Xcm I. This fragment, which contains a CRE-like motif (TGACGTGA$^{-413\ to\ -406}$), was cloned into a promoterless luciferase reporter vector, pGL3-Basic (Promega) upstream of the Luc gene and grown in E.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-$CaPO_4$ complexes using 2 μg/well of pGL3-Basic or NR2B promoter construct DNA. To monitor transfection efficiency, we performed co-transfection using a *Renilla* luciferase vector, phRG-B (40 ng/well). Cells were incubated with the DNA-$CaPO_4$ complex for 1 h in NBM, washed and continued to incubate 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-

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20/20 luminometer. Chronic ethanol treatment was done in two ways: in some experiments, ethanol was added for 3 days prior to transfection and the ethanol treatment continued for 2 more days, for a total of 5 days. In others, transfection was done first on 3 DIV and cells were treated with or without ethanol for 5 days, and luciferase activity assayed. The results calculated from the ratio of firefly to *Renilla* 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 and chronic ethanol-treated cells using Trizol reagent (Invitrogen), and was treated with DNA-Free reagents (Ambion Inc., Austin, TX) to remove any genomic contamination. RNA integrity was examined by electrophoresis on denaturing agarose gel, visualized by the presence of 18S and 28S bands. Two-step RT-PCR was performed: 1 µg of total RNA was first converted to cDNA using random hexamers and TaqMan Reverse Transcriptase reagents (Applied BioSystems Inc. Branchburg, NJ); the cDNA, equivalent to 20 ng 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 Inc., Assay ID: Mm_00433820_m1). 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 Pre-Developed Assay Reagents for eukaryotic 18S rRNA (Hs 99999901_s1, Applied BioSystems). The PCR products were analyzed on 2% agarose gels to confirm the presence of a

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single band of predicted size. From the average difference in cycle threshold (Ct) of the target gene and the 18S control for each sample, the amplification difference between control and chronic ethanol samples was calculated and the relative levels of NR2B gene expression is expressed as fold change compared to control.

Statistical analyses. Data were analyzed as appropriate by Student's *t* test or by One-way ANOVA, with post-test using Student Newman Keuls test, using the Prism program (Graphpad Software, San Diego, CA).

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Results

Effect of Chronic Ethanol 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 since it had no significant effect on cell viability as measured using the CellTiter 96 Aqueous One solution (Promega), while 100 mM ethanol caused >25% loss of cell viability compared to control (Fig. 1B).

Although our laboratory had earlier shown that chronic ethanol treatment increases NR2B receptors, it was important to confirm these results as 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 earlier findings.

Chronic Ethanol Increases CREB, but not ERK Phosphorylation. Immunoblotting using anti-CREB and anti-phospho-CREB (pS^{133}) 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 chronic ethanol treatment. We used specific ELISA kits to measure total and phospho-CREB (pS^{133}) levels in order to enable better quantification of small changes. ELISA

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assays showed that chronic ethanol treatment caused a significant increase (28 ± 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 lysates. As shown in Fig. 4, in both assays there was no significant change in the ratio of phospho- to total ERK due to ethanol treatment. Although phospho-ERK levels determined by ELISA showed a tendency towards decrease in the in ethanol-treated lysates, the differences were not statistically significant. As β-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 an earlier study from our laboratory (Kalluri and Ticku, 2003) had reported that chronic ethanol treatment increased phosphorylation of MAP kinase in fetal cortical neurons cultured in MEM containing 10% each of fetal bovine and horse serum, we compared the effect of ethanol on ERK phosphorylation in cells cultured in neurobasal medium containing B-27 (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 earlier studies, while ethanol had no such stimulatory effect on ERK phosphorylation in serum-free NBM (see also Fig.4). However, the same ethanol treatment increased CREB phosphorylation in both culture media regardless of the presence of serum (data not shown).

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Effect of Chronic Ethanol on CaM Kinases II and IV. We also tested the effect of chronic 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-α in the presence of serum, thus confirming an earlier report by Kalluri and Ticku (2003). However, similar to ERK phosphorylation ethanol treatment in serum-free NBM caused no increase in CaM kinase II levels (Fig. 6 A, 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 (not shown), indicating the presence of specific CaM kinase IV cross-reacting proteins. In cells treated with chronic ethanol in both media with or without serum, there was a clear increase in minor CaM kinase IV band at 80-kDa, while no change was discernible in the major 55-kDa band (Fig. 6 C, D).

Electrophoretic Mobility Shift Assay (EMSA) for CREB. In order 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 and chronic ethanol treated cells 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 to the palindromic CRE consensus (TGACGTCA). As shown in Fig. 7 A, the probe bound to specific proteins in the nuclear extract (lane 2, complexes b and c). Binding was markedly reduced when incubated with

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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, since 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 chronic ethanol-treated cells 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 co-transfection 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 a ~15-fold increase in luciferase (Luc) activity, relative to the basal activity ratio of the pGL3-Basic vector. Chronic 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 post-transfection, with Luc activities assayed 5 days after ethanol treatment (data not shown), or when ethanol was added for a total of 5

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days, with transfection conducted during the ethanol treatment period and Luc assayed 48 h post-transfection as in a standard assay protocol (Fig 8). Although, we also observed ~2-fold increase in basal activity ratio of PGL3-Basic construct in ethanol-treated cells, the increase in NR2B promoter activity we observed with chronic ethanol treatment is over and above the effect on basal activity since the results are expressed as fold increase over respective controls.

Mutation of the CRE sequence (Fig. 8B) resulted in significantly lower Luc activity compared to 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 chronic ethanol in upregulation of NR2B gene transcription in mouse cortical neurons. We have provided evidence here for NR2B gene upregulation using a real time PCR assay which showed that chronic 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. Chronic 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

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CREB, we have also utilized ELISA assays to more precisely quantitate the transcription factor levels. This increase in CREB activity could be involved in ethanol-induced NR2B gene transcription, since 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^{133} by a variety of protein kinases, including protein kinase A, protein kinase C, $pp90^{rsk}$, and CaM kinases II/IV (Mayr and Montminy, 2001), and the phosphorylated CREB binds to its coactivator, CREB binding protein (CBP). 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 initiate a cascade of gene transcription (Weeber and Sweatt, 2002).

Ethanol treatment has been shown to affect neuronal CREB phosphorylation, but the results are variable depending on the duration of treatment, brain region and the model system studied. In the NG108-15 neuroblastoma X glioma cell model, acute ethanol induces CREB phosphorylation via cAMP-dependent protein kinase (PKA) and stimulates CRE-mediated gene transcription (Asher et al., 2002; Constantinescu et al., 2002). In adult male rats acute ethanol *in vivo* increased phosphorylated CREB and CRE binding in cerebellar granule cells (Yang et al., 1996), while chronic 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 acute ethanol

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CREB Phosphorylation was attenuated in cerebellar granule cells from chronic ethanol-fed rats, 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 et al., 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, since 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 PKA or PKC (Robertson 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). Chronic ethanol treatment of rat pups on postnatal days 4-7 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 brain (Tsuji et al., 2003). Kalluri and Ticku (2003) recently reported that acute ethanol inhibits, while chronic ethanol increases phosphorylation of MAPK in fetal cortical neurons. Our results in this study

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clearly show that this apparent discrepancy in ethanol effect on ERK phosphorylation is due to presence or absence of serum in the culture media. Serum components seem to have profound effects on the MAP kinase pathway, as also discussed by Morrow et al (2004). Our reason for switching to serum-free medium was due to 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 upregulation 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 in regardless of the presence or absence of serum. Interestingly, similar to ERK phosphorylation CaM kinase II levels were increased by ethanol treatment only in the presence of serum, confirming an earlier 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, while 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 following chronic 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

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phosphorylation. While 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 to 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. An earlier 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 *lac*Z 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 be due to the fact that non-neuronal cell type was used in the earlier study. Interestingly, 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 regulated by cAMP pathway through a

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phosphorylation-dependent activation of CREB (Lau et al., 2004), similar to our data with the NR2B subunit.

Chronic 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, since we observe similar stimulatory effect of ethanol with other NR2B promoter constructs (Qiang et al, 2005).

While 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 CNS 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 (see Kumari and Ticku, 2000 for review). 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/glutaminergic system are of immense importance in understanding the wide-ranging

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neurological effects of alcohol use and abuse, including intoxication, withdrawal seizures, fetal alcohol syndrome, and may lead towards the strategies for suitable pharmacologic intervention.

In summary, chronic ethanol treatment of fetal cortical cells *in vitro* appears to activate the CREB pathway, which in turn may stimulate NR2B gene transcription. Using this model system to study the neuronal effects of chronic alcohol exposure, our studies have thus far established that chronic ethanol 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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References:

Asher O, Cunningham TD, Yao L., Gordon AS and Diamond I (2002) Ethanol stimulates cAMP-responsive element (CRE)-mediated transcription via CRE-binding protein and cAMP-dependent protein kinase. *J Pharmacol Exp Ther* 301:66-70.

Bito H (1998) The role of calcium in activity-dependent neuronal regulation. *Cell Calcium* 23:143-150.

Carpenter-Hyland EP, Woodward JJ, Chandler LJ (2004) Chronic ethanol induces synaptic but not extrasynaptic targeting of NMDA receptors. *J Neurosci* 24:7859-7868.

Chandler LJ (2003) Ethanol and brain plasticity: receptors and molecular networks of the postsynaptic density as targets of ethanol. *Pharmacol Therap* 99:311-326.

Chen X, Michaelis ML and Michaelis EK (1997) Effects of chronic ethanol treatment on the expression of calcium transport carriers and NMDA/glutamate receptor proteins in brain synaptic membranes. *J Neurochem* 69:1559-1569.

Constantinescu A, Gordon AS and Diamond I (2002) cAMP-dependent protein kinase types I and II differentially regulate cAMP response element-mediated gene expression: implications for neuronal responses to ethanol. *J Biol Chem* 277:18810-18816.

Desai A, Turetsky D, Vasudevan K and Buananno A (2002) Analysis of transcriptional regulatory sequences of the N-methyl-D-aspartate receptor 2A

subunit gene in cultured cortical neurons and transgenic mice. *J Biol Chem* 277:46374-46384.

Diamond I and Gordon AS (1997) Cellular and molecular neuroscience of alcoholism. *Physiol Rev* 77:1-20.

Fadda F and Rossetti ZL (1998) Chronic ethanol consumption: from neuroadaptation to neurodegeneration. *Prog Neurobiol* 56:385-431.

Follesa P and Ticku MK (1995) Chronic ethanol treatment differentially regulates NMDA receptor subunit mRNA expression in the rat brain. *Mol Brain Res* 29:99-106.

Follesa P and Ticku MK (1996) Chronic ethanol-mediated up-regulation of the N-methyl-D-aspartate receptor polypeptide subunits in mouse cortical neuron culture. *J Biol Chem* 271:13297-13299.

Hu XJ and Ticku MK (1995) Chronic ethanol treatment upregulates NMDA receptor function and binding in mammalian cortical neurons. *Mol Brain Res* 30:347-356.

Hu XJ, Follesa P and Ticku MK (1996) Chronic ethanol treatment produces a selective upregulation of NMDA receptor subunit gene expression in mammalian cultured cortical neurons. *Mol Brain Res* 36:211-218.

Ishii T, Moriyoshi K and Sugihara H (1993) Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. *J Biol Chem* 268:2836-2843.

Kalluri H and Ticku MK (2002) Ethanol-mediated inhibition of mitogen-activated protein kinase phosphorylation in the mouse brain. *Eur J Pharmacol.* 439:53-58.

Kalluri H and Ticku MK (2003) Regulation of ERK phosphorylation by ethanol in fetal cortical neurons. *Neurochem Res* 28:765-769.

Klein M, Pieri I, Uhlmann F., Pfizenmaier K and Eisel U (1998) Cloning and characterization of promoter and 5'-UTR of the NMDA receptor subunit ε2: evidence for alternative splicing of 5' non-coding exon. *Gene* 208:259-269.

Kumari M and Ticku MK (1998) Ethanol and regulation of the NMDA receptor subunits in fetal cortical neurons. *J Neurochem* 70:1467-1473.

Kumari M and Ticku MK (2000) Regulation of NMDA receptors by ethanol. *Prog Drug Res* 54:152-189.

Lau GC, Saha S, Faris R and Russek SJ (2004). Up-regulation of NMDAR1 subunit gene expression in cortical neuron via a PKA-dependent pathway. *J Neurochem* 88:564-575.

Lonze BE and Ginty DD (2002) Function and Regulation of CREB Family Transcription Factors in the Nervous System. *Neuron* 35:605-623.

Luo J, Wang RP and Yasuda RP, Dunah AW and Wolfe BB (1997) The majority of N-methyl-D-aspartate receptor complexes in the adult rat cerebral cortex contain at least three different subunits (NR1/NR2A/NR2B). *Mol Pharmacol* 51:79-86.

Mayr B and Montminy M (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nature Rev Mol Cell Biol* 2:599-609.

Misra K. Roy A and Pandey SC (2001) Effects of voluntary ethanol intake on the expression of Ca^{2+}/calmodulin-dependent protein kinase IV and on CREB expression and phosphorylation in the rat nucleus accumbens. *NeuroReport* 12:4133-4137.

Mori H and Mishina M (1995) Structure and function of the NMDA receptor channel. *Neuropharmacol* 34:1219-1237.

Morrow AL, Ferrani-Kile K, Davis MI, Shumilla JA, Kumar S, Maldve R and Pandey SC (2004) Ethanol effects on cell signaling mechanisms. *Alcohol Clin Exp Res* 28:217-227.

Qiang M, Rani CSS and Ticku MK (2005) NRSF regulates the *N*-Methyl-D-aspartate receptor 2B subunit gene in basal and ethanol-induced gene expression in fetal cortical neurons. *Mol Pharmacol* In press **.**

Pandey SC,Zhang D, Mittal N and Nayyar D (1999) Potential role of the gene transcription factor cyclic AMP-responsive element binding protein in ethanol withdrawal-related anxiety. *J Pharmacol Exp Ther* 288:866-878.

Pandey SC, Roy A and Mittal N (2001) Effects of chronic ethanol intake and its withdrawal on the expression and phosphorylation of the CREB gene transcription factor in rat cortex. *J Pharm Exp Ther* 296:857-868.

Reddy AM and Shukla DS (1996) Potentiation of mitogen-activated protein kinase by ethanol in embryonic liver cells. *Biochem Pharmacol* 51:661-668.

Roberson ED, English JD, Adams JP, Selcher JC, Kondratick C and Sweatt JD (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to CREB phosphorylation. *J Neurosci* 19:4337-4348.

Roivainen R, Hundle B and Messing RO (1995) Ethanol enhances growth factor activation of mitogen-activated protein kinases by a protein kinase C-dependent mechanism. *Proc Natl Acad Sci* 92:1891-1895.

Sambrook J and Russel DH (2001) *Molecular cloning*, 3rd Edition, Cold Spring Harbor Laboratory Press, New York.

Sasner M and Buananno A (1996) Distinct N-methyl-D-aspartate receptor 2B subunit gene sequences confer neural and developmental specific expression. *J Biol Chem* 271:21316-21322.

Seiler AEM, Brian NR and Rubin R (2001) Inhibition of insulin-like growth factor I receptor and IRS-2 signaling by ethanol in SH-SY5Y neuroblastoma cells. *J. Neurochem*. 76:573-581.

Shaywitz AJ and Greenberg ME (1999) Creb: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu. Rev Biochem.* 68:821–861.

Snell LD, Hoffman PL and Tabakoff B (1993) Radioligand binding to N-methyl-D-aspartate receptor/ionophore complex: alterations by ethanol *in vitro* and by chronic ethanol ingestion. *Brain Res* 602:91-98.

Sweatt DJ (2001) The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. *J. Neurochem*. 76:1-10.

Tsuji R, Guizzetti M and Costa LG (2003) In Vivo ethanol decreases phosphorylated MAPK and p70S6 kinase in the developing rat brain. Neuroreport 14:1395-1399.

Weeber EJ and Sweatt JD (2002) Molecular Neurobiology of Human Cognition. *Neuron* 33:845-848.

Woodward JJ (2000) Ethanol and NMDA receptor signaling. *Crit Rev Neurobiol* 14:69-89.

Yang X, Diehl AM and Wand GS (1996) Ethanol exposure alters the phosphorylation of cyclic AMP responsive element binding protein and cyclic AMP responsive element binding activity in rat cerebellum. [Journal Article] *J Pharm Exp Ther* 278:338-346.

Yang X, Horn K, Baraban JM and Wand GS (1998) Chronic ethanol administration decreases phosphorylation of cyclic AMP response element-binding protein in granule cells of rat cerebellum. *J Neurochem* 70:224-32.

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Footnote:

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Figure Legends

Fig. 1. A. Phase-contrast micrographs (x200) of control and chronic ethanol-treated (75 mM, 5 days) cultured neurons isolated from E14 mice cortex. B. Effect of chronic ethanol 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 Methods. Results are calculated from the A_{490} values, corrected for blank and expressed as percent of control, mean $\pm$ SEM of at least three experiments each with triplicate wells; * *p<0.01 compared to untreated control.

Fig. 2. Chronic ethanol 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 which was reverse transcribed using random hexamers and the cDNA used for real time PCR with the Assay-on-Demand products (NR2B: Mm00433820_m1 and 18S rRNA: Hs99999901_s1) and TaqMan assay kit, as described under 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 4-12% 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 percent of control, represent mean $\pm$ SEM of 4 experiments. *p<0.05 (Student's *t* test).

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Fig. 3. Chronic 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 (~25 µg) separated on 4-12% SDS-PAGE gels, transferred to PVDF membranes and probed with antibodies to phospho-CREB (pS^{133}), total CREB and actin antibodies. The band intensities were determined by densitometry, corrected for loading differences using β-actin and expressed as percent of control. Data shown are the mean ± SEM 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 and chronic ethanol treated cells 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 percent of control. Data shown are the mean ± S.E.M for three experiments. *$p < 0.05$ (Student's *t* test).

Fig. 4. Lack of effect of ethanol on ERK phosphorylation. A. Immunoblots for β-actin, total and phospho-ERK1/2 were performed using lysates from control and chronic ethanol treated cells. Representative immunoblots are shown, along with densitometric quantitation performed as described above for CREB. Data shown are the mean ± SEM from 3 experiments. B. ELISA assays for total and phospho-ERK were done on whole cell extracts prepared from control and chronic ethanol-treated cells. Ratio of phospho-ERK to total ERK expressed as

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percent of control represent mean ± SEM of triplicates from three different experiments.

Fig. 5. Ethanol stimulates ERK phosphorylation in the presence of serum. Cells from E14 cortex cultured in neurobasal+B27 medium (NBM) in the presence or absence of 10% fetal bovine serum were treated with or without 75 mM ethanol for 5 days from DIV 3-7. Total and phospho-ERK levels were assayed by ELISA as described under Methods. Ratio of phospho-ERK to total ERK was calculated and results expressed as percent of control represent mean ± SEM of triplicates from three different experiments. *$p < 0.05$ (Student's *t* test).

Fig. 6. Effect of chronic ethanol treatment on CaM kinases II and IV. 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 1-4 refer in sequence to: control, NBM + serum, ethanol, NBM + serum, control, NBM - serum, ethanol, NBM - serum. The band intensities were determined by densitometry, corrected for loading differences using β-actin and expressed as percent of control. Data shown are the mean ± SEM from three experiments. *$p < 0.01$ (Student's *t* test).

Fig. 7. EMSA showing binding of CRE motif in NR2B promoter to nuclear proteins from neuron cultures. A. The 25-bp double stranded oligonucleotide sequence, (5'-CCCAGCTCA**TGACGTGA**GAAGCAGC-3') in the promoter region

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of NR2B (5176-5199 bp, Genbank Accession number AF03356) that includes a CRE motif (-406 to -413, shown in bold) was labeled with [^{32}P]-γ-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 µg protein) was incubated with 5 X 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 X unlabeled wild-type oligonucleotide; lane 4: consensus CRE oligonucleotide (5'-AGGGATTGCC**TGACGTCA**GAGAGCTAG-3'; lane 5: mutated CRE oligo (5'-CCCAGCTCA**TaAaGTGA**GAAGCAGC-3', the mutated bases shown in lower case); 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 chronic ethanol treatment.

Fig. 8. Mutation in the CRE sequence of the NR2B promoter decreases luciferase reporter activity in transient transfection assay in cultured neurons. A. The 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 number AF033356) upstream of the Luc gene (indicated by shaded bar) of the

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promoterless pGL3-Basic vector. This construct contained a CRE-like sequence at -406 to -413 bp, indicated by bold letters. B. The above 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. Luciferase activity (Luc) of control and chronic ethanol treated cells. Cells were treated with 75 mM ethanol for a total of 5 days, with co-transfection assays performed on 3 DIV, and the dual luciferase activities assayed 48 h post-transfection. From the ratio of firefly to *Renilla* Luc activities, the fold increase over basal pGL3-Basic was calculated for each group. The results are the mean $\pm$ SEM of 3-4 replicates in at least 4 experiments. *$p<0.05$, by ANOVA and Student Newman Keuls post-test for comparisons as indicated in the figure.

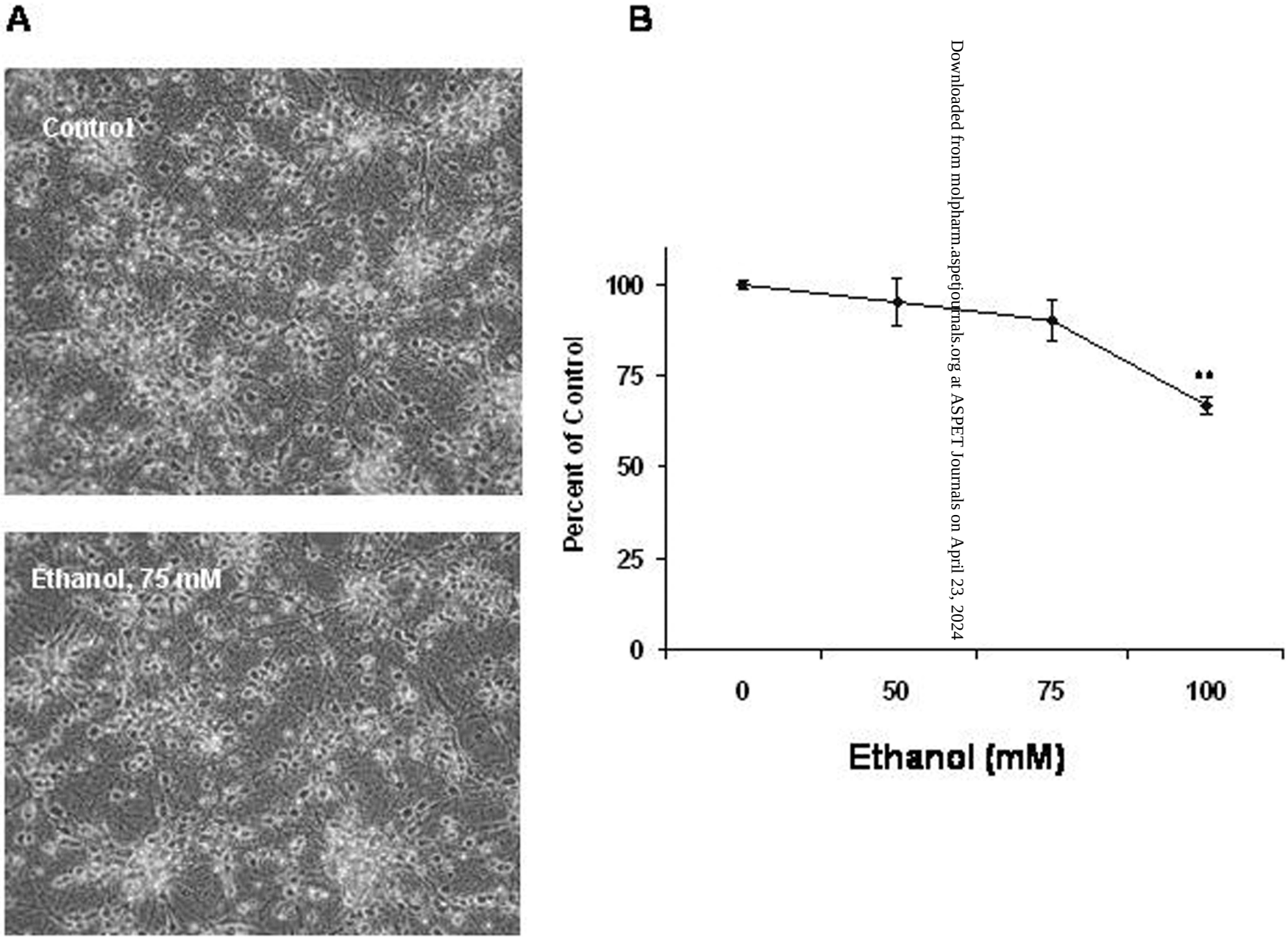


Fig. 1

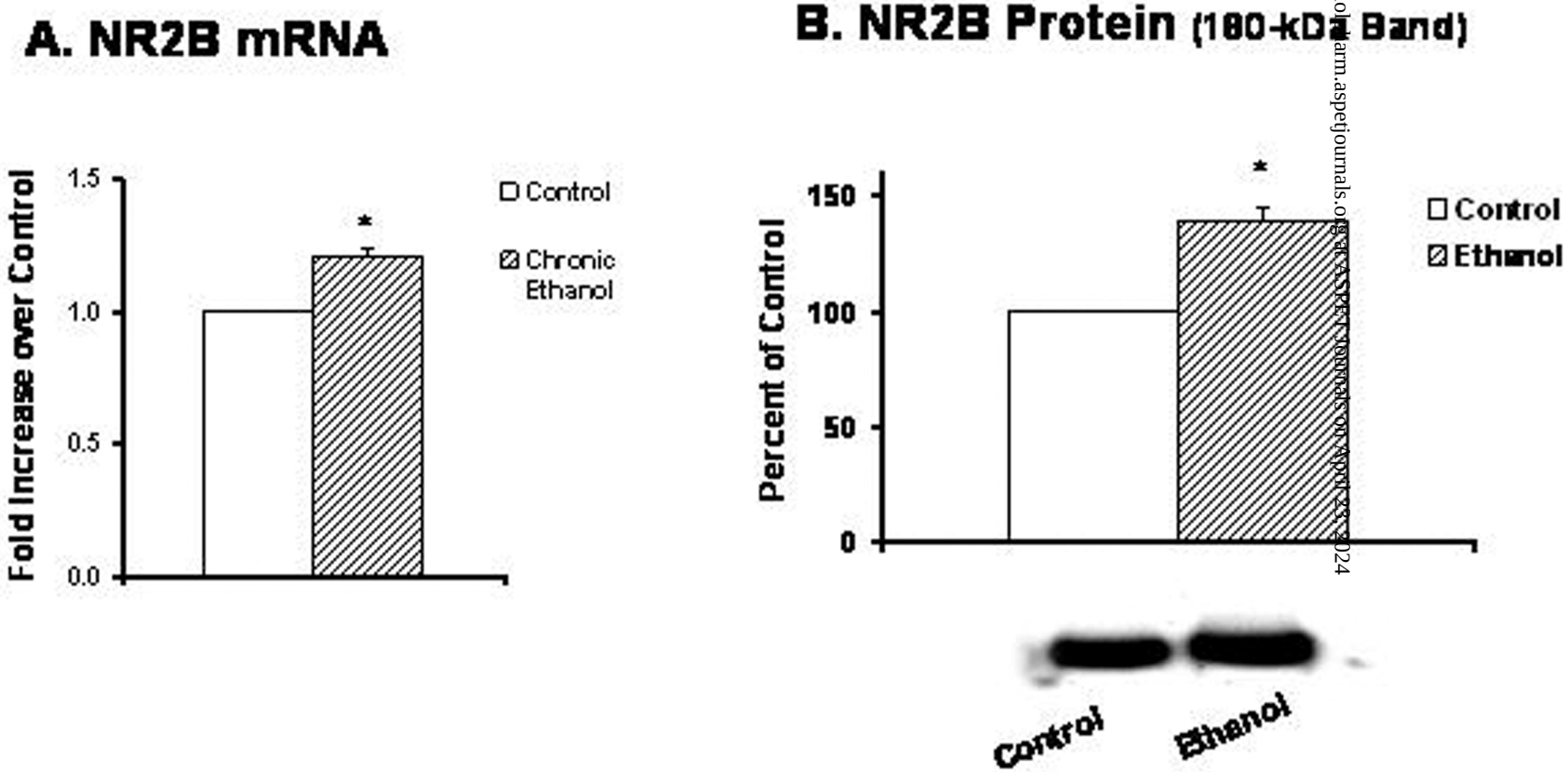
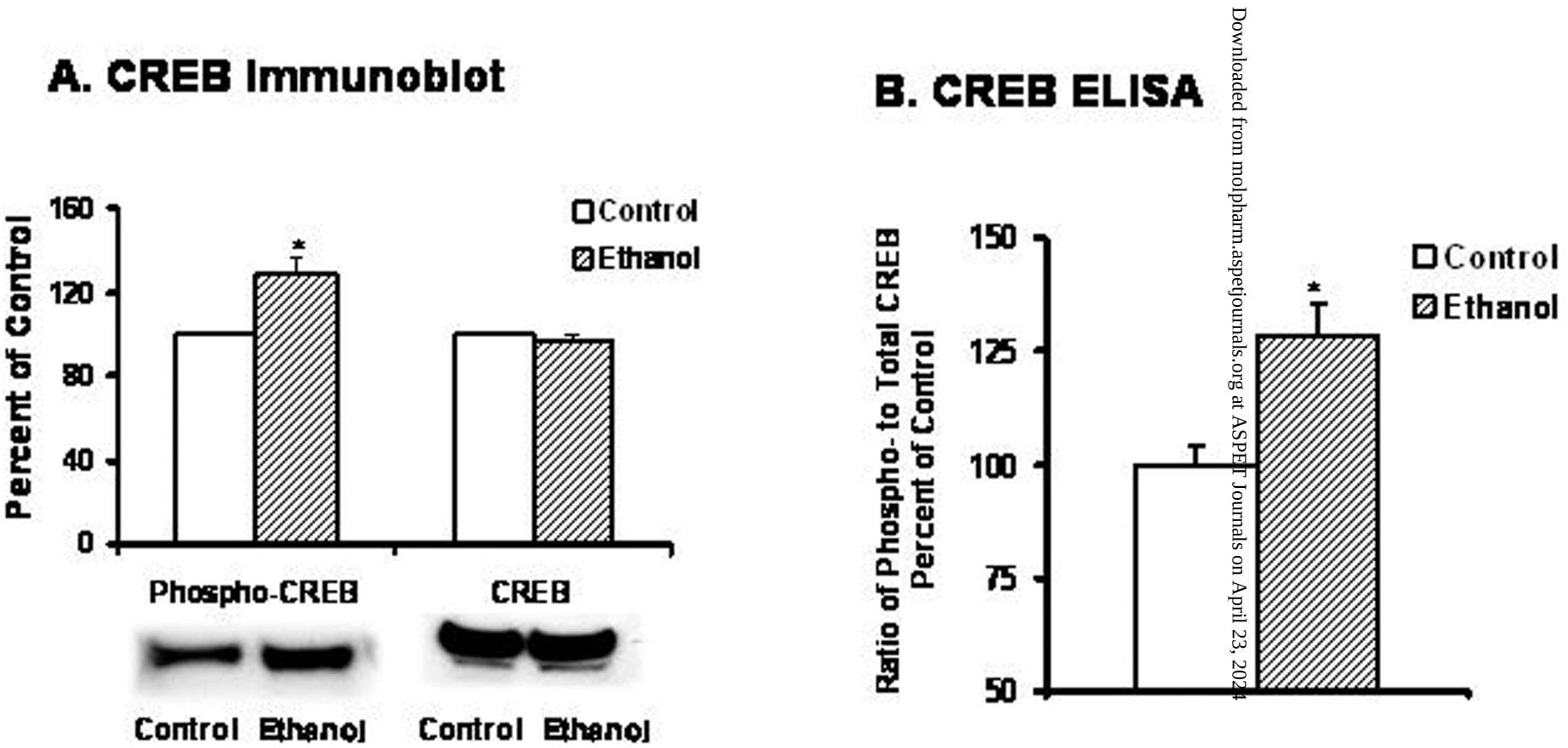


Fig. 2



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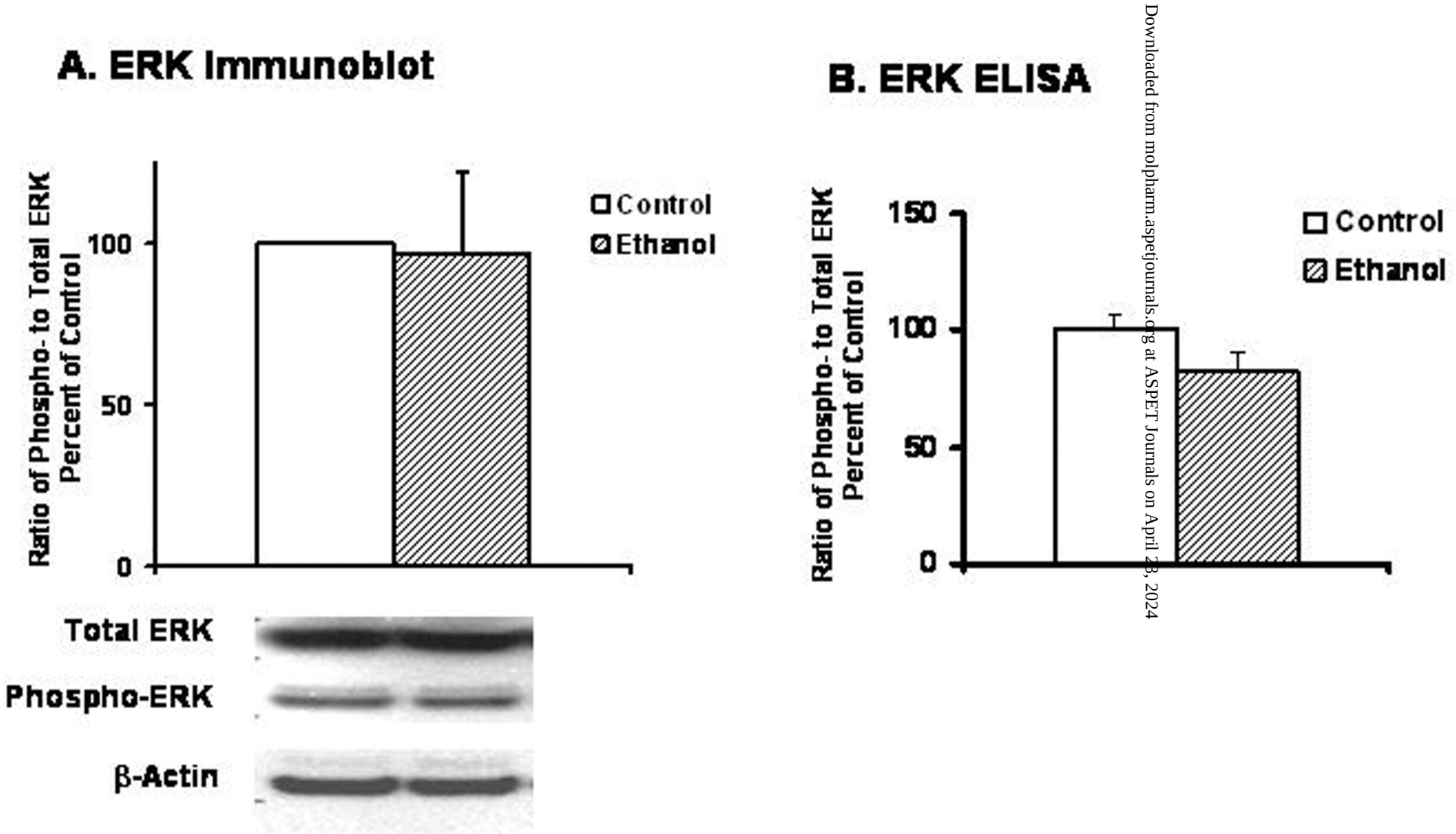

Fig. 4

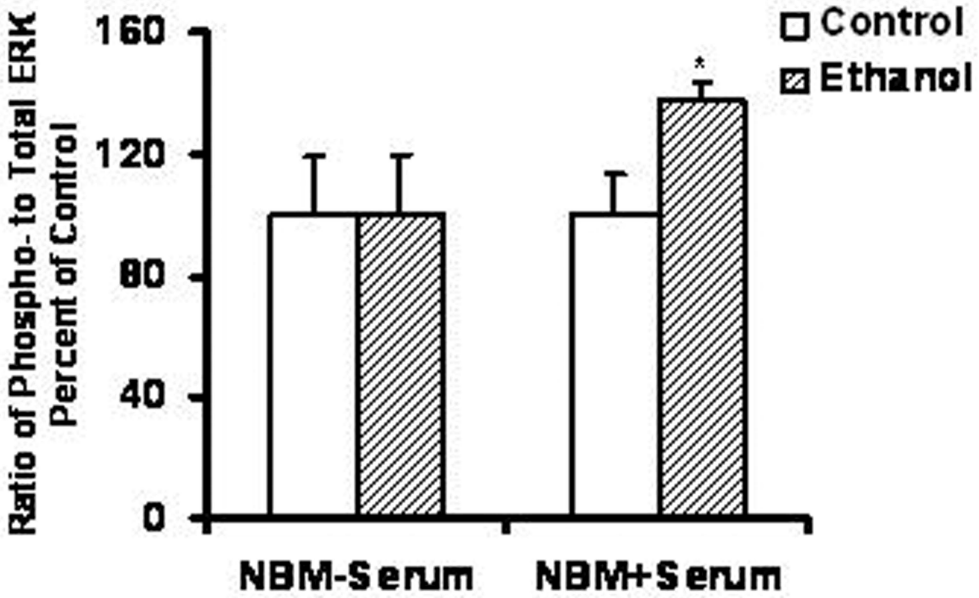

Fig. 5

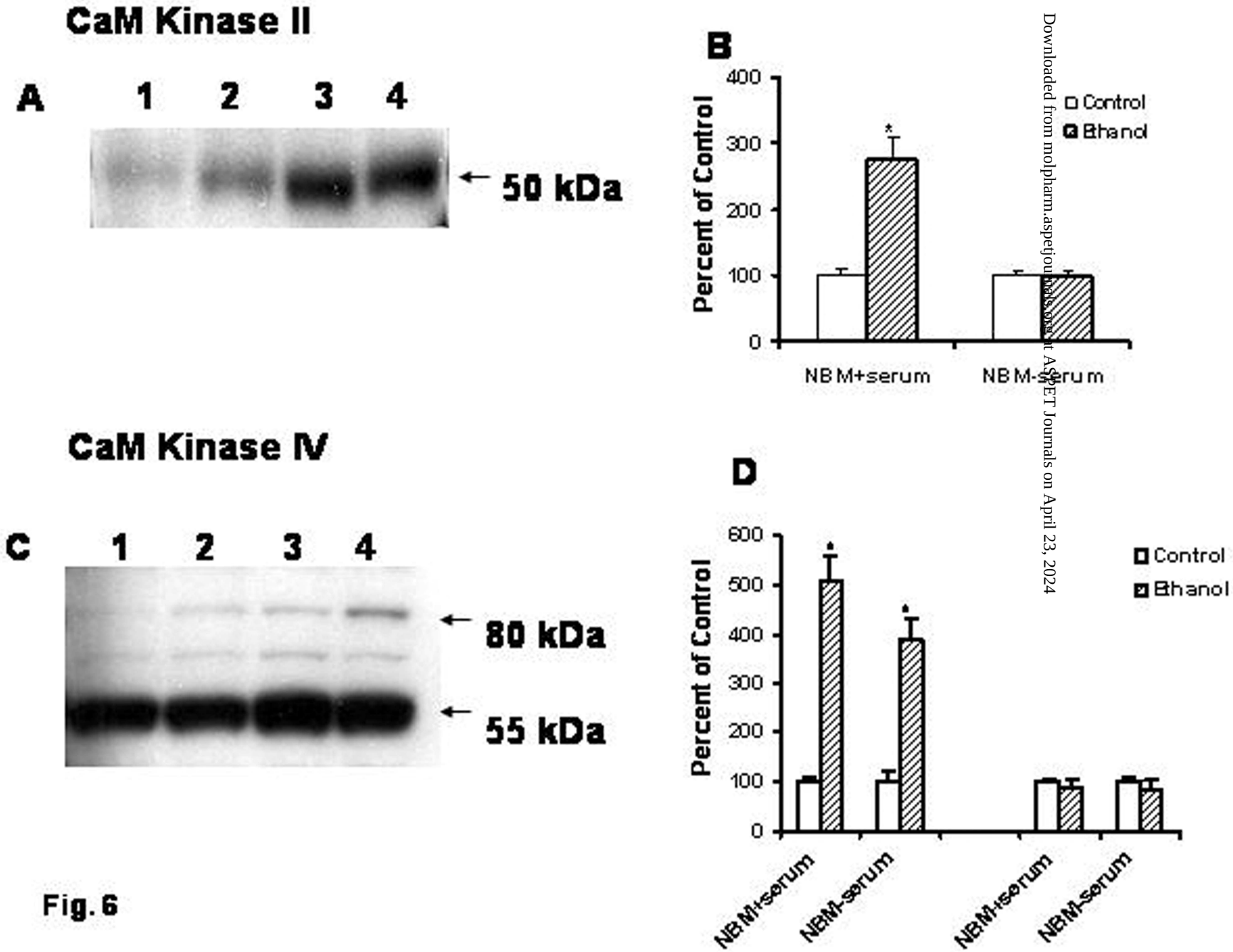

Fig. 6

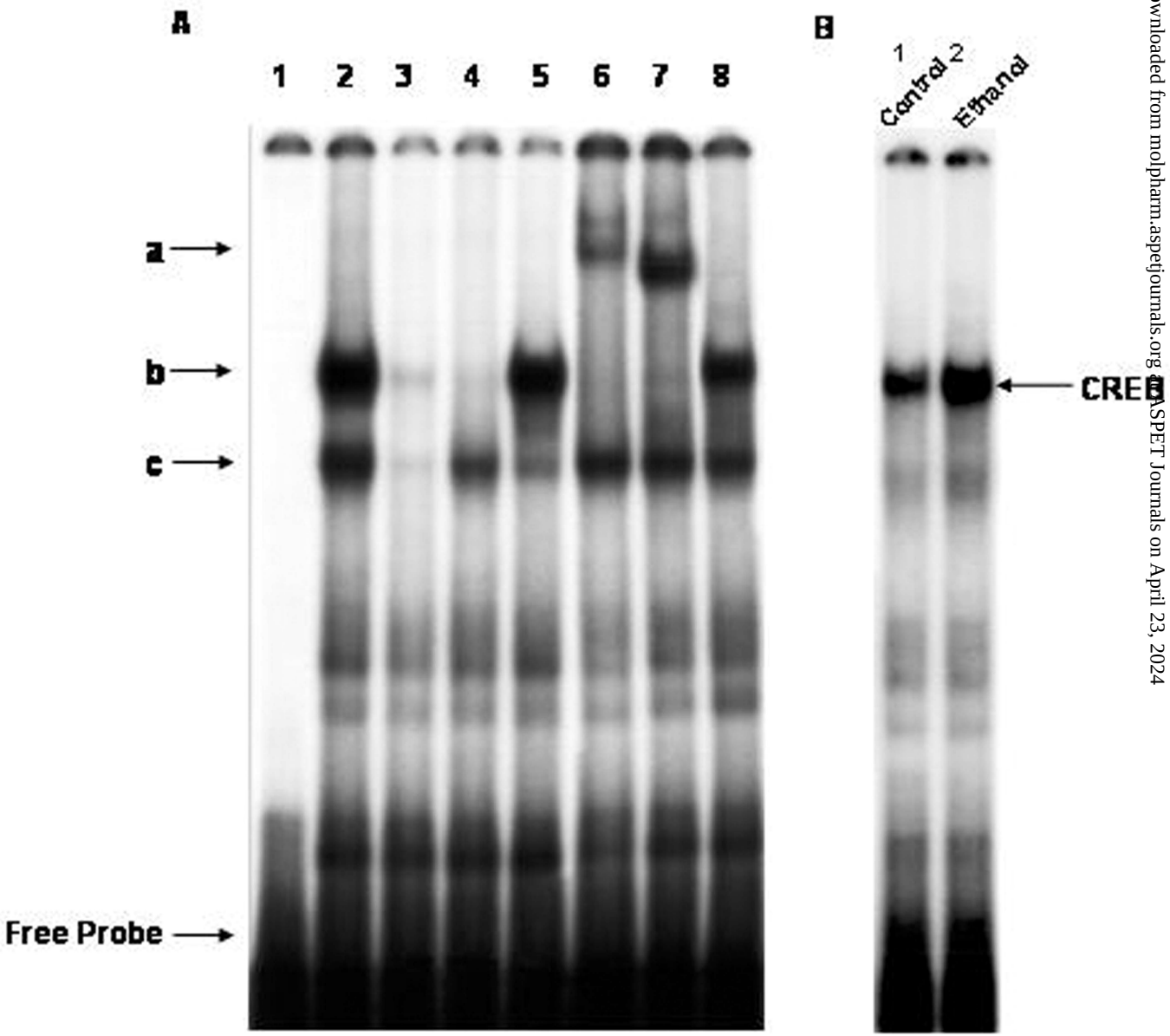

Fig. 7

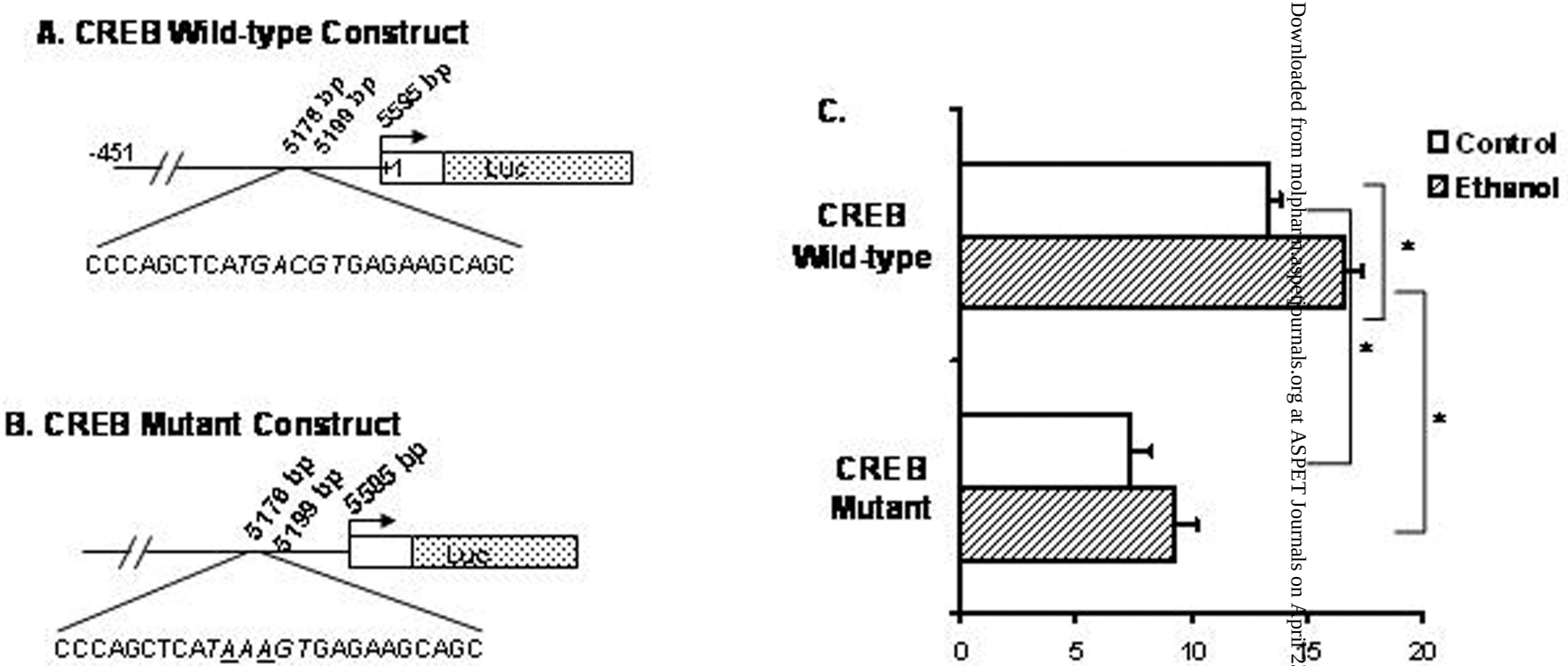

Fig. 8