Neuron-Restrictive Silencer Factor Regulates the *N*-Methyl-D-aspartate Receptor 2B Subunit Gene in Basal and Ethanol-Induced Gene Expression in Fetal Cortical Neurons

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ABSTRACT

Neuron-restrictive silencer factor (NRSF) is a transcriptional repressor of multiple neuronal genes. This study addressed the role of NRSF in *N*-methyl-D-aspartate (NMDA) receptor NR2B promoter activity and the molecular mechanisms of ethanol-induced NR2B up-regulation in fetal cortical neurons. The 5′-flanking region of the NR2B gene contains five NRSE-like elements. Functional analysis of the upstream regions of the NR2B gene by transient transfection of neurons revealed that neuron-restrictive silencer element (NRSE) motifs located between base pair −1407 and −2741 represses transcription of the gene. Analysis by electrophoretic mobility shift assay and reporter gene assay identified NRSE2 and 3 as responsible for repressing NR2B gene transcription. The identity of NRSF as the functional binding factor is suggested by the specific binding of in vitro synthesized NRSF or cell lysate to the labeled probes and the specific antibody-induced supershift. Furthermore, whereas mutations of NRSE2 and 3 motifs increased the promoter activity, overexpression of NRSF reduced it significantly. The pattern of NRSF expression during development was investigated and demonstrated that the highest expression is on embryonic day 14 with moderate expression on postnatal day 0, reflecting a possible role of NRSF as a regulator during development. Treatment of cultured cortical neurons with 100 mM ethanol for 5 days caused a significant decrease in the NRSF mRNA and protein levels, NRSF/NRSE binding activity, and an increase in the promoter activity. Therefore, our studies suggest that NRSF is a negative regulator of NR2B expression and may contribute to the ethanol-induced up-regulation of the NR2B gene in fetal cortical neurons.

The neuron-restrictive silencer element (NRSE), also called repressor element-1, has been identified in many genes. Studies indicate that NRSE mediates transcriptional repression of many neuron-specific genes via the neuron-restrictive silencer factor (NRSF) or repressor element silencing transcription factor (Chong et al., 1995; Schoenherr and Anderson, 1995; Wood et al., 1996; Bessis et al., 1997; Palm et al., 1998; Huang et al., 1999; Palm et al., 1999) and suggest that the NRSF/NRSE interaction blocks expression of neural genes in non-neural cells during neurogenesis (Mori et al., 1990, 1992; Kraner et al., 1992; Chong et al., 1995; Schoenherr and Anderson, 1995; Paquette et al., 2000). NRSF is also involved in directing choline acetyltransferase gene expres-

sion specifically in the cholinergic neuron (Lönnerberg et al., 1996). Knock-out of the NRSF gene resulted in derepression of neuron-specific tubulin in a subset of non-neural tissues in the developing embryo and was embryonically lethal (Chen et al., 1998). A similar regulatory effect was seen by in vivo expression of a dominant-negative form of NRSF. NRSF acts as a negative modulator in adult mice by an increase in its levels after kainic acid-induced seizures (Palm et al., 1998), and it acts as both a repressor and an enhancer of gene transcription (Bessis et al., 1997; Chen et al., 1998; Seth and Majzoub, 2001). In addition, a number of splice variants of NRSF/repressor element silencing transcription factor have been identified (Palm et al., 1999; Lee et al., 2000; Tabuchi et al., 2002), and two of these are specifically expressed by neurons (Palm et al., 1998, 1999), thus suggesting a more complicated role for this transcription factor in regulating neuronal genes.

The *N*-methyl-D-aspartate receptor (NMDAR) mediates

ABBREVIATIONS: NRSE, neuron-restrictive silencer element; NRSF, neuron-restrictive silencer factor; NMDAR, *N*-methyl-D-aspartate receptor; NMDA, *N*-methyl-D-aspartate; NR2B, *N*-methyl-D-aspartate receptor R2B subunit; MK-801, dizocilpine maleate; FBS, fetal bovine serum; DIV, days in vitro; EMSA, electrophoretic mobility shift assay; bp, base pair(s); TnT, transcription/translation; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; E, embryonic P, postnatal; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis.

most excitatory neurotransmission in the central nervous system and may be involved in learning and memory formation in the brain, and in the refinement of synaptic connections during development (Kutsuwada et al., 1996). The NMDA receptors are composed of multiple subunits: NMDAR1 (NR1), different NMDAR2 (NR2A-D), and NMDAR3A (NR3A). The NR1 subunit, in association with NR2 subunit, leads to the formation of highly active receptor channels (Nakanishi, 1992). In contrast to NR1, which is expressed constitutively, NMDA receptor R2B (NR2B) expression is highly regulated during development of the brain. Studies have revealed that exposure to drugs such dioxin (Kakeyama et al., 2001), NMDA antagonists MK-801 and activator protein-5 (Follesa and Ticku, 1996), ethanol (Kalluri et al., 1998; Kumari and Ticku, 1998), and heavy metal lead (Lau et al., 2002) altered the expression of the NR2B gene.

Transcription regulation of NMDA receptor components has been described in more detail for the NR1 subunit. The NR1 gene proximal promoter region includes a 5′-untranslated region containing an NRSE site that was found to suppress the transcription in non-neuronal cells (Bai et al., 1998; Okamoto et al., 1999). Coexpression of MEF2C and Sp1 cDNAs in primary neurons or cell lines synergistically activates the NR1 promoter (Krainc et al., 1998). In addition to these, it has been reported that transcription factor SP1 and MAZ bind to a GC-rich region, thereby mediating enhancement of NR1 promoter activity during neuronal differentiation (Okamoto et al., 2002). However, in the NR2A subunit of the NMDA receptor, the RE1/NRSE element is not thought to be necessary for specific NR2A neuron expression, but instead, a potential CRE-like element (TGACATCA) at position −1195 was found to be more important (Desai et al., 2002). Our studies have indicated that long-term ethanol treatment induced stabilization of NR1 receptor subunit mRNA but increased the transcription of the NR2B subunit (Kumari et al., 2003). The transcriptional analysis of NR2B gene in transgenic mice has revealed that an upstream 800-base pair region is sufficient to direct neural-specific transcription (Sasner and Buonanno, 1996). However, specific factors involved in the transcription of NR2B gene have not yet been characterized.

Our previous studies indicated that long-term ethanol treatment induced up-regulation of NR2B binding, function, and gene transcription (Follesa and Ticku, 1996; Hu et al., 1996; Kumari and Ticku, 1998). The previous studies indicating that NRSF was involved in regulating gene expression in neurons led us to investigate its potential role in the NR2B gene transcription. In the current study, with the use of primary cultured cortical neurons we found that NRSF is a negative regulator of NR2B expression and may contribute to the ethanol induced up-regulation of the NR2B gene.

Materials and Methods

Cell Culture. Primary cortical neurons were prepared from C57BL/6 mouse (Harlan, Indianapolis, IN) fetuses as described previously (Hu and Ticku, 1995) with minor modifications. In brief, cortices were isolated from E14 mice; cells were dissociated by trituration and resuspended in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 5% heat-inactivated horse serum, 100 μM L-glutamine, 28 mM D-glucose, and 1× antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO). Cells ($\sim$2.8 × 10^5/cm^2)

were plated onto poly-L-lysine-coated tissue culture dishes and incubated under 5% CO$_2$ at 37°C. On the second day, a mixture of 5-fluoro-2′-deoxyuridine and uridine at a concentration of 20 and 40 μg/ml, respectively, was added into the medium to inhibit non-neuronal cell proliferation. From days in vitro (DIV)3 onwards, cells were exposed to 100 mM ethanol for a total of five consecutive days, whereas control cells were untreated. Ethanol incubations were carried out in an incubator saturated with ethanol, which maintained ethanol concentration at the level added to the medium as determined using an alcohol assay kit (Sigma-Aldrich).

NE-4C cells, a neuroectodermal progenitor cell line derived from forebrain vesicles of 9-day-old p53-deficient mouse embryos (Schlett and Madarasz, 1997) were the gift of Dr. K. Schlett (Department of Physiology and Neurobiology, Eötvös Loránd University, Budapest, Hungary). NE-4C cells were maintained in minimal essential medium supplemented with 5% FBS, 4 mM glutamine, and 40 μg/ml gentamycin at 37°C in 5% CO$_2$. HeLa cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% FBS and 1× antibiotic-antimycotic solution (Sigma-Aldrich).

Electrophoretic Mobility Shift Assay. Nuclear extracts from DIV7 primary cultured neurons and HeLa cells were prepared. Cells (10 × 10^6) were scraped, washed twice in phosphate-buffered saline, and resuspended in 1 ml of ice-cold buffer A (100 mM KCl, 1.5 mM MgCl$_2$, 10 mM PIPES, pH 7.9, 200 mM sucrose, and 0.5% Nonidet P-40). The cells were allowed to swell on ice for 15 min and then vortexed vigorously for 10 s. The nuclear debris was then pelleted by spinning for 5 min at 12,000g. Crude nuclear pellet was resuspended in the equivalent of one packed cell volume of buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl$_2$, and 0.2 mM EDTA), followed by incubation on ice with vortexing every 5 min for 30 min. The supernatant (nuclear extract) was diluted by adding an equal volume of buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, and 20% glycerol) and divided into aliquots. To buffers A, C, and D were added 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1% protease inhibitor cocktail (Sigma-Aldrich) before use. Protein concentration in the nuclear extracts was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Complementary DNA oligonucleotides NRSE1–5 (Fig. 2B), consensus sequence of the NRSE from SCG10 gene, 5′-AGC TGC AAA GCC ATT TCA GCA CCA CGG AGA GTG CCT CTG C-3′ (Mori et al., 1992) and unrelated control, 5′-CCG TTG ATA TAT CCC AAT GGC-3′ (Lönnerberg et al., 1996), were annealed by heating in 1× NET at 95°C for 5 min and cooling at ambient temperature. Probes were then labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. For gel shift assays, nuclear extract (10 μg of protein) was incubated for 20 min at ambient temperature with 5 × 10^5 cpm of the labeled DNA probe in 20 μl of binding buffer containing 10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 μg/μl bovine serum albumin, and 2 μg of poly-d (I-C). For supershift assays, 1 μg of NRSF antibody was added, and the reaction was continued for 15 min (Li et al., 2002). Polyclonal NRSF antibody was a gift from Dr. Gail Mandel (Department of Biochemistry and Cell Biology, Howard Hughes Medical Institute, State University of New York, Stony Brook, NY). The REEX-1 (NRSF expression vector), also provided by Dr. Gail Mandel, was transcribed and translated in vitro using T3 RNA polymerase and the transcription/translation (TNT)-coupled reticulocyte lysate system (Promega, Madison, WI) as a control for NRSF binding. The product was analyzed by SDS-polyacrylamide gel electrophoresis. After reaction, the samples were loaded on 5% nondenaturing polyacrylamide gels, and electrophoresis carried out at 200 V in 25 mM Tris, pH 8.5, with 190 mM glycerol and 1 mM EDTA. Competition assays were carried out in the same manner, except that the above-mentioned reaction mixture was preincubated with competitor DNA for 10 min at 4°C before addition of the ^{32}P-labeled probe.

Luciferase Vector Construction and Site-Directed Mutagenesis. A set of NR2B promoter regions, differing in the location of their 5′ ends (−100, −400, −800, −1224, −1629, −2104, −2255,

−2483, −3004, and −5319 bp) were prepared by PCR (Fig. 1). The M1 clone (NCBI accession number AF 033356) of the mouse genomic DNA (Klein et al., 1998) was used as template. The forward primers were synthesized corresponding to the upstream sequences of desired promoter regions with an added 5′-flanking KpnI site and the reverse primer corresponding to bp +30 relative to the reported transcription start site of the mouse NR2B gene (Klein et al., 1998) with an added 3′-flanking NcoI site. PCR products were cloned directly into the pCRII vector. Positive clones were digested with KpnI and NcoI, and the fragments were subcloned into the same restriction sites of the promoterless luciferase reporter plasmid pGL3-Basic. Site-directed mutagenesis was carried out by using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutated nucleotides are shown in italics in Figs. 3B and 7B. All constructs and mutants were confirmed by restriction mapping and sequencing. Oligonucleotide synthesis and DNA sequencing were carried out by the Advanced DNA Technology Unit (University of Texas Health Science Center, San Antonio, TX).

Transient Transfection and Luciferase Assay. Primary cultures of cortical neurons were grown in 24-well plates and transfected on DIV5 using a modified calcium phosphate technique (ProFection mammalian transfection system calcium phosphate kit; Promega) according to the recent method described by Desai et al. (2002). All DNA constructs were purified using double CsCl gradient centrifugation. Equimolar amounts (0.4 pmol) of each plasmid DNA were used for transfection and pBluescript added where necessary for mass adjustment. To control for transfection efficiency, 40 ng of a *Renilla reniformis* luciferase vector phRG (Promega) was added per well. Cotransactivation experiments were performed with equimolar amounts (0.2 pmol) of each plasmid DNA for all constructs together with 1 μg of either pcDNA3.1/amp or REEX-1 (NRSF expression cassette in pcDNA3.1/amp backbone). Cells were harvested 48 h after transfection for luciferase activity assayed in a Turner Designs TD-20/20 luminometer using the dual luciferase assay system according to manufacturer's protocol (Promega).

RT-PCR and Real-Time PCR Analysis of NRSF mRNA Expression. Total RNA was prepared from C57BL/6 mice cortex at the following developmental stages: embryonic day 14 (E14), postnatal days 0–90 (P0, P7, P14, P30, and P90) and from primary cultures of DIV7 cortical neurons, HeLa and NE-4C cells by using TRIzol reagent (Invitrogen). All RNA samples were treated with DNase I using the DNA-free kit (Ambion, Austin, TX) according to the manufacturer's protocol, to eliminate endogenous DNA contamination. The RNA samples were quantitated spectrophotometrically and

RNA integrity checked by ethidium bromide staining on denaturing agarose gel electrophoresis. Using 1 μg of total RNA as template, single-stranded cDNAs were synthesized using random hexamers and TaqMan reverse transcription reagent kit (Applied Biosystems, Branchburg, NJ). PCR was performed using 1/10 of cDNA products as template and Eppendorf PCR MasterMix kit (Brinkmann, Westbury, NY) for 30 cycles. Each PCR cycle consisted of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a 5-min extension at 72°C. The following primers were used: mNRSF-S, 5′-GTG CGA ACT CAC ACA GGA GA-3′; and mNRSF-AS, 5′-GGA CAG GTG GGA TGC TTA GA-3′. Real-time PCR was performed using the ABI Prism 7900 sequence detection system and the SYBR Green master mix (Applied Biosystems). A 201-bp NRSF product was generated in a two-step protocol and by using following primer pairs: mNRSF-S, 5′-GTG CGA ACT CAC ACA GGA GA-3′; and mNRSF-AS, 5′-AAG AGG TTT AGG CCC GTT GT-3′. As an internal control, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) transcripts (231 bp) were amplified from each of the same RNA samples using the following primer pairs: GAPDH-S, 5′-CTG CAC CAC CAA CTG CTT AG-3′; and GAPDH-AS, 5′-GAG CTT CCC GTT CAG CTC T-3′. All primers used were designed to cross over one or more intron/exon boundaries. In all cases, PCR products were analyzed on 2% agarose gels to confirm that a single band of the correct molecular weight was obtained. The average change in cycle threshold (C_T) of the target gene was first normalized to the endogenous control GAPDH (ΔC_T) and then calibrated with the calibrator value (E14 or DIV7) ($\Delta\Delta C_T$), the amplification difference among the samples was used for calculating the relative levels of gene expression for NRSF. Finally, data were exported to the samples database as $2^{-\Delta\Delta C_T}$ value. Thus, sample- and treatment-related expression data can be analyzed with standard database tools.

Western Blotting. Equal amounts of protein (nuclear extract, ~35 μg) were separated by SDS-PAGE on 4 to 12% NuPAGE Bis-Tris gels and transferred to polyvinylidene difluoride membranes (Invitrogen). Membranes were probed sequentially using different antibodies with a stripping step included to remove the previous signal using Restore Western blot stripping solution (Promega). Anti-NRSF monoclonal antibody 12c11 was the kind gift of Dr. David J. Anderson (Division of Biology, 126-76, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA). The chemiluminescence signal developed using Western lighting plus reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) was captured on X-ray film (BioMax-Light; Eastman Kodak, Rochester, NY), and the band intensity was quantitated using the NIH Image J
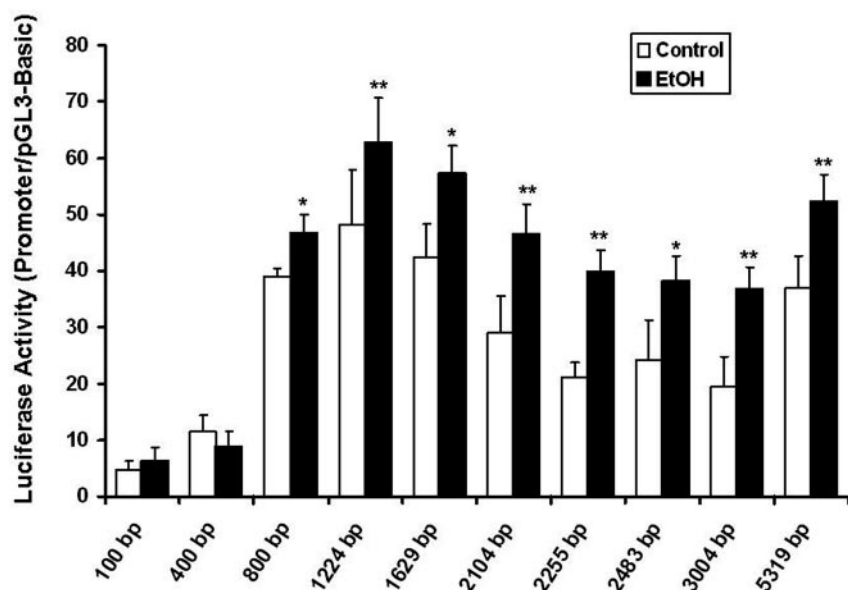
Fig. 1. 5′ Deletion analysis of the NR2B promoter and their response to ethanol treatment. NR2B luciferase reporter constructs, which end from the indicated bases of the promoter upstream and downstream to the base +30 bp relative to the reported transcription start site (bp +1) were transiently transfected into primary cultured cortical neurons on DIV5. Cultured neurons were either exposed to 100 mM ethanol for 5 days or remained in the normal growth media. Luciferase activity was determined 48 h after transfection and reported relative to the baseline activity of the promoterless construct (pGL3-Basic). Values were corrected for transcription efficiency by cotransfection of a *R. reniformis* phRG expression plasmid. Data (mean ± S.E.) shown are from at least five independent experiments. Two-way analysis of variance revealed significant sequence-dependent and ethanol-response effects of deletion ($p < 0.0001$, $p < 0.0001$, respectively); ∗, $p < 0.05$; ∗∗, $p < 0.01$ compared with nonethanol control constructs (post hoc comparisons).

system. Data were normalized to the intensity of actin band and are represented as percent of control basal group.

Results

Analysis of the 5′Flanking Regulatory Region of NR2B Gene. To identify the functional element in the 5′-flanking region of the NR2B gene, 10 constructs that extended from −5319 to +30 relative to the reported transcription start site of the mouse NR2B gene, were prepared by PCR and subcloned into the luciferase reporter plasmid. Transient transfections of primary cultured cortical neurons were carried out with these constructs. The normalized data are shown in Fig. 1. For the basal activity (open columns), the highest level (approximately 48-fold increase over pGL3-Basic control) was observed with the construct −1224 bp. Constructs extending further upstream, between the region −3004 to −1224, exhibited a significant reduction ($p < 0.05$–0.01) in reporter gene activity, and subsequently there was an increased activity between −5319 and −3004 bp. These results suggest the presence of enhancing elements within the −1224 to −400 region, and silencing element in the region between −3004 and −1224 bp.

We next investigated the influence on NR2B promoter activity after long-term ethanol treatment. The above-described constructs were transfected into cultured cortical neurons, and ethanol treatment administered to the cells for five consecutive days. No increase of luciferase activities was detected after ethanol treatment in the constructs −100 and −400 bp. As the length of constructs increase within selected range, ethanol treatment caused higher luciferase activities (filled columns) compared with those in controls. Although increased luciferase activities were observed in the eight constructs, a series of constructs between −2104 and −3004 bp showed decreased basal promoter activities companied by increased ethanol-induced promoter activities, suggesting possible ethanol sensitive negative regulatory element in this region. In the current study, our aim was to determine whether any negative regulative elements were involved in ethanol-induced NR2B up-regulation in cortical neurons.

Identification of NRSE Sites in the 5′-Flanking Region of the NR2B Gene. The potential negative regulatory elements in the −1224 to −3004 bp were further investigated. We used the online transcription factor database MatInspect (version 3.3) (http://www.genomatix.de/cgi-bin/./eldorado/main.pl) to screen for potential silencing elements. The results included five highly conserved putative NRSE binding sites, which were located in the 5′-flanking region between −1407 to −2741 bp (Fig. 2, A and B).

To determine whether the five putative NRSE sites will bind to NRSF, we performed EMSA using ^{32}P-labeled double-stranded oligonucleotides corresponding to the five sites in the NR2B promoter region (Fig. 2B). The nuclear extract was derived from the cultured primary cortical neurons. We used NRSE motif from the SCG10 gene as a positive control, which has been shown to bind NRSF in HeLa cells (Schoenherr and Anderson, 1995). As can be seen in Fig. 3, the DNA-protein complexes were formed between the NR2B-derived DNA probes 2, 3, and 4 and the nuclear extract from the neuronal cells, and they migrated to the same position as the complexes formed with the in vitro synthesized NRSF, and as the DNA-protein binding complex formed with the consensus SCG10 probe. We confirmed specificity of NRSE/NRSF complexes by using gel shift assay for each of the five binding sites. The supershift bands were detected only in NRSE2 and NRSE3, suggestive of specific binding. In contrast, no detectable band was found with NRSE 1 and 5, the assumed position, except there were some unknown bands migrating faster than control. Although it is possible that NRSF binds to the DNA motif and migrates faster in the electric field because it might be adopting a more "tight" conformation. However, the results from supershift assay do not support it. In NRSE4, there were bands in the assumed positions; however, excess unlabeled probe did not seem to compete for the binding (data not shown), and no shift band was observed, suggesting the bands might be derived from nonspecific binding. The components of the complex were not directly addressed in this study. We also saw that in Fig. 3, all five probes (representing five different NRSE sites) can bind with TnT control NRSF protein indicating all NRSE motif have the capacity to bind NRSF. The reason might be because in neurons, NRSF is present in relative lower concentration; and if NRSE1, 4, and 5 bind with lower affinity to NRSF, then the binding activity may not be sufficient to be detected.
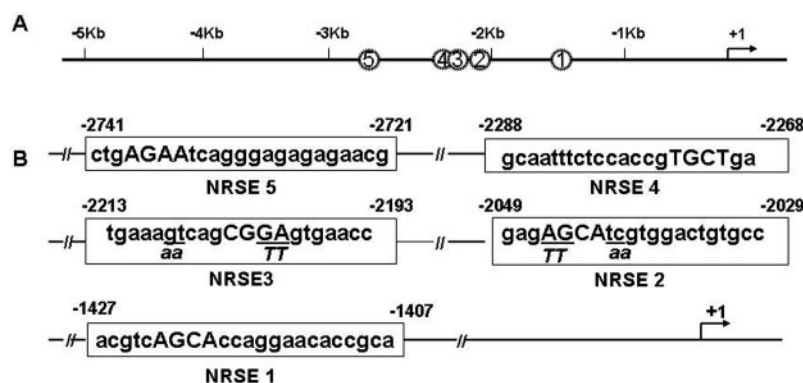
Fig. 2. Location of five putative NRSE *cis*-elements in the 5′-flanking region of the NR2B gene. A, schematic diagram of locations of five putative NRSE *cis*-elements in the promoter region of the mouse NR2B gene: one NRSE binding site (NRSE1) is located between −1 and −2 kb, and four NRSEs (NRSE2–5) closely clustered between −2 and −3 kb. The numbers 1–5 with circles represent NRSEs 1–5 motifs. B, nucleotides of five putative *cis*-elements NRSE 1–5 are boxed and the locations relative to the reported transcription start site are separately numbered. The core consensus sequences are in the upper case. Nucleotides of the particular sequences that were mutated in this study are underlined and replaced nucleotides shown in italic below the wild-type sequences. Oligonucleotides corresponding to the complete sequence shown in each box were also used as probes in EMSA as well as primers in site-directed mutagenesis of reporter gene constructs. The bent arrows indicate the reported transcription start site.

We further confirmed specificity of NRSE2/NRSF and NRSE3/NRSF complexes by using gel shift and competition assay (Fig. 4A). Again, the SCG10 probe and synthesized NRSF were used as controls of the consensus sequence and the full length of the NRSF protein, respectively. A pattern characteristic of NRSF binding to its cognate element NRSE2 and 3 was observed. Effective competition was obtained with graded excesses unlabeled probe of the NRSE2 and 3 wild-type but not with the site-direct mutated NRSE2 Mt1 and NRSE3 MT1 and MT2 (Fig. 4B, see sequences). These results indicate that the nucleotide AG in the core sequence AGCA is critical for the specific binding of NRSF in NRSE2, whereas in NRSE3 the intact sequence gtcagCGGA is required. Specificity was also confirmed by greatly decreased band intensity and the presence of supershifted bands in both NRSE2 and 3 protein complexes when NRSF-specific antibody was used in the reactions, but no such band was observed when IgG was used. An oligonucleotide with a sequence unrelated to NRSEs did not compete for the binding.

Functional Role of the NRSEs in the Promoter Region of the NR2B Gene. After demonstrating sequence-specific binding of NRSF to the NRSEs, we then investigated the possible function role of individual NRSE binding sites in transcriptional repression. According to the location of NRSEs, a series of 5′-deletions of NR2B constructs extending upstream to bp -3004, -2255, -2104, -1629, and -1224, respectively, and downstream to bp $+30$ were used for transient transfections of cultured cortical neurons. The normalized data are shown in Fig. 5B. Consistent with the results from EMSA (Fig. 3), deletion of NRSE2 and 3 did increase the luciferase activity significantly, whereas promoter activity was not substantially influenced by the deletion of NRSE1, indicating the functional importance of NRSE2 and 3. Deletion of NRSE4 and 5 did not show major differences. Together with EMSA studies, the results suggest that NRSE2 and NRSE3 may act as predominant sites in cortical neurons, and NRSE2 may be the most important site in the regulation of the NR2B transcription.

Mutation of NRSE Binding Sites Increases NR2B Promoter Activity, whereas Overexpression of NRSF Decreases the Activity. To confirm that NRSF-mediated repression was attributed specifically to the NRSE, we introduced site-directed mutations to NRSE2 and 3 sites in the -3004-bp construct. We replaced an adenine (A) and a guanine (G) base by two thymidine (T) bases in both NRSE2 and 3 core sequences (see NRSE2 Mt1 and NRSE3 MT1 in Fig. 4B), which have been used as probes for EMSA competition test and shown lost NRSF binding ability. These mutants were transfected into cultured neurons. As expected, the mutated constructs displayed a significant increased promoter activity. In NRSE2 mutant, promoter activity increased by approximately 2-fold; in NRSE3 mutant, by 1.3-fold; and in the combination mutant, by 2.2-fold (Fig. 6A). Although ethanol treatment was administered to the cells for five consecutive days, mutated constructs did not respond to ethanol significantly. These data indicate that both NRSE2 and 3 sites are main positions involved in the role of repressing NR2B promoter activity and that the repression is specifically mediated by the NRSEs, which at least partially mediated ethanol-induced up-regulation of NR2B.

We also performed cotransfection experiments. The promoterless luciferase expression vector pGL3-Basic or the NR2B luciferase constructs -2104, -3004, and -3004bpMt1 were cotransfected into cultured neurons together with pcDNA3 vector or NRSF expression plasmid pcDNA3-REEX1 (Fig. 6B). There was an $\sim$40% decrease in luciferase activity in cotransfection with NRSF expression plasmid in either of -2104- or -3004-bp constructs, whereas little change of luciferase activity was detected in cotransfection with control vector pcDNA3.1. In contrast, promoter activity of the -3004-bp mutant was not attenuated, providing further evidence that NRSF confers repression through the NRSE in the NR2B promoter.

NRSF Expression during Mouse Development. To determine NRSF expression at different stages of mouse development, we investigated NRSF mRNA abundance in mouse
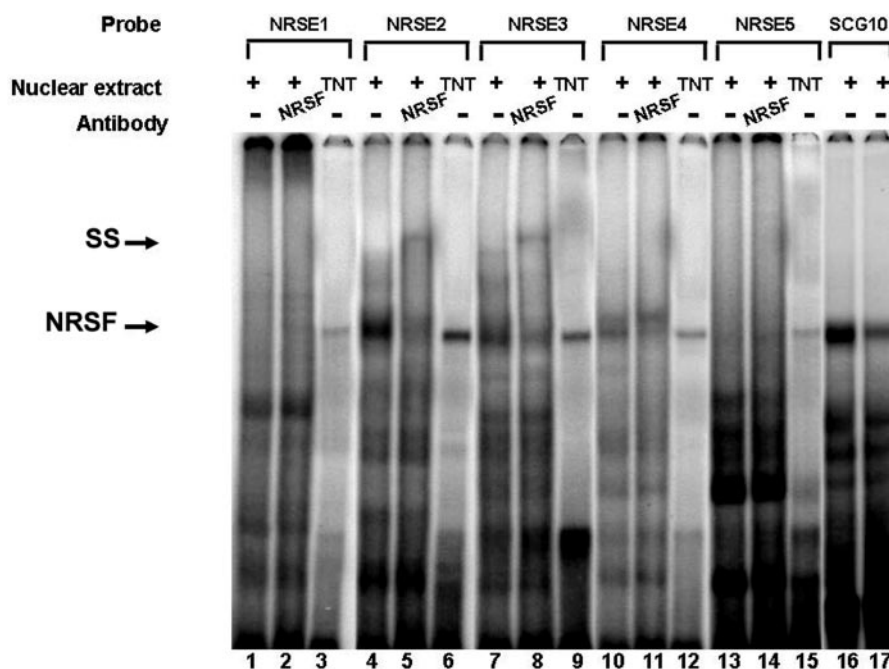

Fig. 3. NRSF-binding patterns to five putative NRSE motifs of the NR2B promoter region. EMSA was carried out with the nuclear extracts (10 μg/lane) prepared from cultured cortical neurons. ^{32}P-Labeled oligonucleotides representing NRSE1–5 (lanes 1–15) as well as SCG10 motif (lanes 16 and 17) were incubated with cortical neuronal nuclear extract (lanes 1 and 2, 4 and 5, 7 and 8, 10 and 11, and 13 and 14) or synthesized full-length NRSF protein produced by TnT (2 μl, lanes 3, 6, 9, 12, and 15) for 20 min. Four micrograms of anti-NRSF antibody (lanes 2, 5, 8, 11, and 14) were added to the reaction for identification of NRSF-specific band. The DNA-protein complexes were separated on 5% nondenaturing polyacrylamide gels. NRSF plus arrow indicates the specific complex, whereas SS plus arrow indicates the supershifted complex.

cortical tissue by using RT PCR and quantitative real-time RT-PCR. Samples were obtained from three individual brains for each age group. We detected NRSF mRNA expression in mouse cortex of all the developmental stages at E14, P0, P7, P14, P30, and P90 (Fig. 7, A and B). The highest expression was seen on E14 (the earliest time point measured), which decreased rapidly until P7; there is a slightly increased around P14; and then expression fell again gradually. The NRSF mRNA expression was also detected in the adult cortex but only 10% as much as that in E14. In addition, the level of NRSF expression in DIV7 cultured cortical neurons was similar to that in P0 (data not shown). Because non-neuronal cells and progenitors have been reported to express relatively high levels of NRSF, we compared the relative expression of NRSF in neurons with that in HeLa and NE-4C cells. NRSF mRNA in DIV7 showed 6-fold lower than HeLa and approximately 2-fold lower than NE-4C cells (Fig. 7C). These results indicated that during the development NRSF expression goes down gradually, but it is still in the moderate level during the embryonic stage.

Ethanol Exposure Decreased NRSF Expression and Complex Formation in Primary Cultured Cortical Neurons. Our previous studies reported that long-term ethanol treatment up-regulates NR2B gene expression. In this study, we observed that 5 days of ethanol treatment induced significant increased luciferase activity between −2104- and −3004-bp constructs (Fig. 1), which contain NRSE sites, but no significant increase for those in NRSE-mutated constructs (Fig. 6A). These results suggested NRSF/NRSE-mediated activity is at least partially responsible for the up-regulation of NR2B. To understand the possible mechanisms by which ethanol may act through NRSEs, we examined the alterna-

tive effect on NRSF expression and DNA-protein complex formation after long-term ethanol treatment. Using real-time RT-PCR method, we compared relative expression levels of NRSF mRNA in control and long-term ethanol treated cultured neurons (Fig. 8A). The GAPDH equivalents calculated for each sample were used to normalize NRSF expression and reduce the impact of systematic errors in RNA quantification, variability in RNA integrity between samples, and
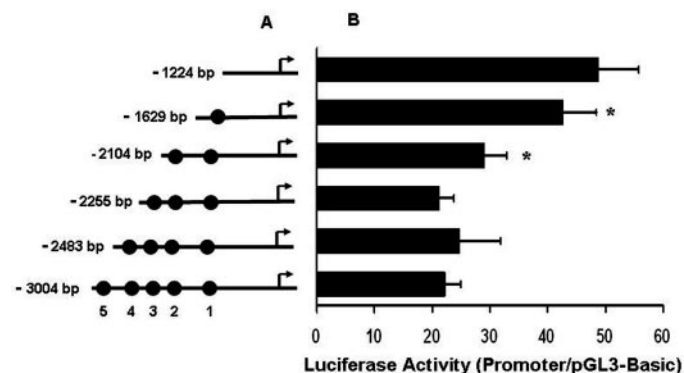
Fig. 5. Effect of NRSEs on the NR2B promoter function. A, schematic representation of a series of luciferase reporter constructs used in this analysis. Numbers 1 to 5 represent NRSE1–5, respectively. Each construct ended upstream at the indicated bases and downstream to the base of +30 bp. B, derepression of NR2B luciferase reporter activity is shown. Primary cultured neurons were transfected with the indicated constructs. Determination of luciferase activity and expression of results were as same as that in Fig. 1. One-way analysis of variance revealed significant derepression effects of deletion ($F = 36.42$; $p = 0.0079$; $N = 4$); ∗, $p < 0.05$ compared with the adjacent construct where the particular NRSE sites remains undeleted (Newman-Keuls post hoc comparisons). Some of these data come from same experiments with the control data in Fig. 1.
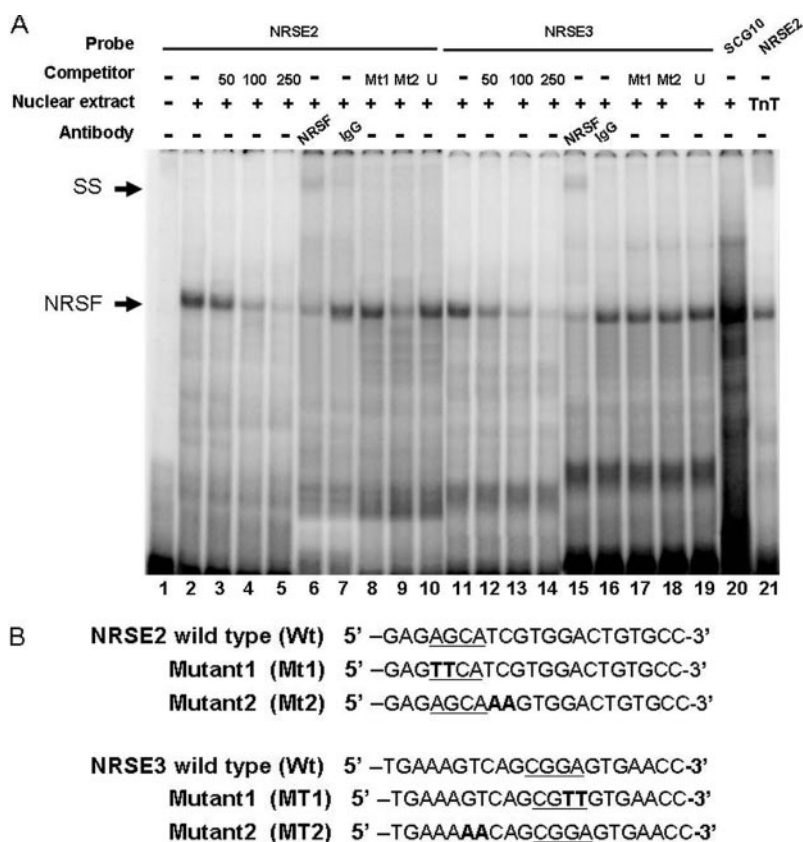
Fig. 4. NRSE2 and 3 binding sites are bound specifically by NRSF. A, gel-shift and competition assay were performed using nuclear extracts (10 μg of protein) from cultured cortical neurons. Oligonucleotides NRSE2 and 3 were labeled with ³²P as probes. Samples were incubated without (lane 1) or with nuclear extract (lanes 2–20). The NRSE2 probe was incubated with nuclear extract (lanes 2–10). The wild-type (lanes 3–5, with graded excesses unlabeled probe: 50×, 100×, and 250×) mutated oligonucleotides [Mt1 (lane 8), Mt2 (lane 9) of NRSE2 unlabeled probes, and unrelated oligonucleotides (U, lane 10)] were used for competition analysis. The NRSE3 probe was also incubated with nuclear extract (lanes 11–20). The wild-type (lanes 12–14, with graded excesses unlabeled probe: 50×, 100×, and 250×) mutated oligonucleotides [MT1 (lane 17), MT2 (lane 18) of NRSE3 unlabeled probes, and unrelated oligonucleotides (U, lane 19)] were used for competition analysis. Where indicated 1 μg of anti-NRSF (lanes 6 and 15) and 1 μg of IgG (lanes 7 and 16) were added to the reaction. As a control, ³²P-labeled SCG10 gene NRSE sequence (lane 20) was incubated with nuclear extract. DNA-protein complexes were separated on a 5% polyacrylamide gel. NRSF plus arrow indicates the specific complex, whereas SS plus arrow indicates the supershifted complex. Full-length NRSF produced by in vitro transcription and translation was also used for identification of NRSF-derived band (lane 21). B, sequence of wild-type and mutated NRSE oligonucleotides used in EMSA studies is shown. Core NRSF-binding sequences are underlined, and mutated nucleotides are shown in bold.

experimenter error. Long-term ethanol treatment significantly decreased (*, $p < 0.05$) the expression of NRSF mRNA but not the expression of GAPDH mRNA levels. We observed a decrease of approximately 28% in NRSF expression after long-term ethanol exposure. These results were also supported by Western blot analysis of nuclear protein (Fig. 8, B and C). Long-term ethanol exposure caused approximately 43% reduction in the NRSF protein levels.

To determine whether ethanol treatment induced NRSF reduction contributed to the DNA-protein complex formations, EMSA was carried out using nuclear extracts prepared from cultured neurons with or without ethanol treatment. The binding activity of NRSE2 and NRSE3 probes was decreased with 5-day ethanol treatment (Fig. 8D). Together, the observations suggested that ethanol treatment significantly decreases NRSF expression as well as DNA-protein complex binding, which might result in derepression of NRSF on NR2B gene transcription.

Discussion

NRSF, a zinc finger protein, is a repressor that silences neuronal genes by binding to the NRSE motif found in many neuronal-specific genes at a variety of promoter locations. In the present study, by using deletion analysis, we have located the majority of the silencer activity in the promoter region between −2104 to −3004 bp and identified that NRSF can specifically repress NR2B gene expression in DIV7 primary cultured cortical neurons. This silencing activity is consistent with the previous reports on other neuron-specific genes, including SCG10 (Mori et al., 1990), type II sodium channel (Kraner et al., 1992; Mori et al., 1992), synapsin I (Li et al., 1993), brain-derived neurotrophic factor (Timmusk et al., 1999), and choline acetyltransferase gene (Lönnerberg et al., 1996). Moreover, this study has shown that NRSF expression was reduced after long-term ethanol treatment, which resulted in reduced DNA-protein binding activity. Therefore, we propose that, in addition to its role reported by previous studies, NRSF is also involved in quantitative modulating of the expression level of NR2B gene during development, in response to stimuli from drug abuse such as ethanol.

NRSF was reported to express substantially in non-neural tissues and undifferentiated neural precursors and repressed a subset of neuron-specific genes in those cells, whereas in the neuronal cell line nearly no expression was detected (Chong et al., 1995; Schoenherr and Anderson, 1995; Schoenherr et al.,
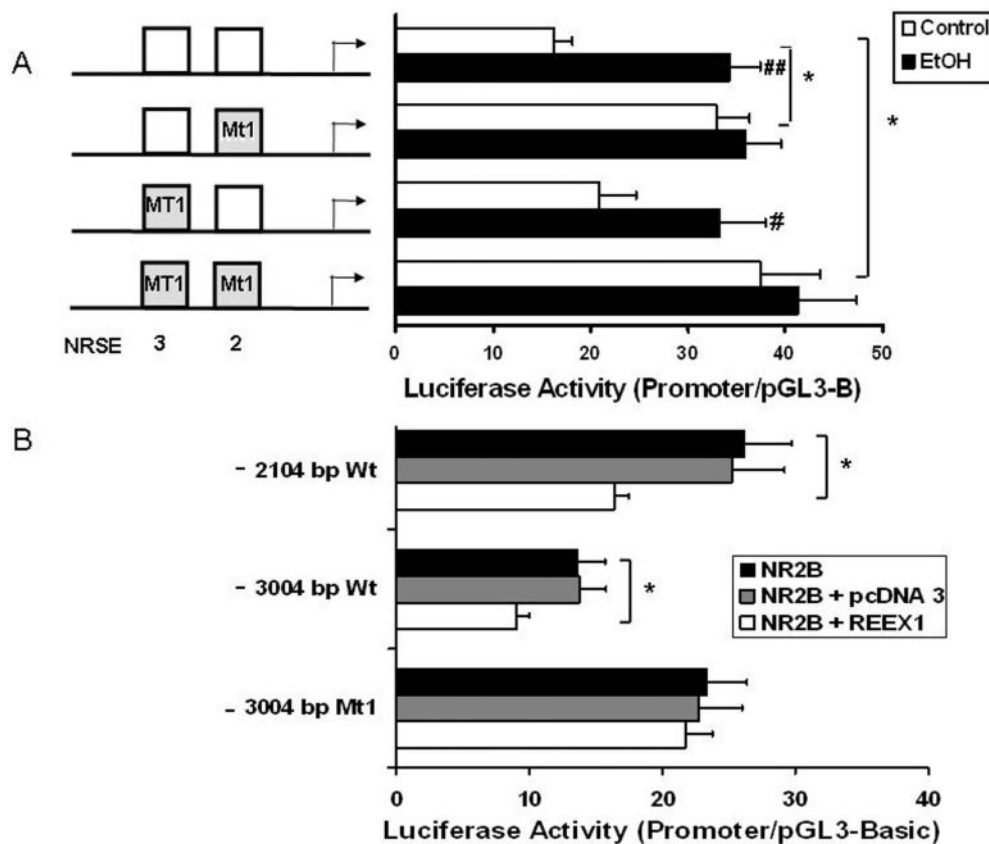
Fig. 6. Mutation of the NRSE2 and 3 binding sites increased NR2B luciferase reporter construct activity, and overexpressed NRSF repressed NR2B luciferase reporter activity. A, mutational analysis of the NRSE2 and 3 sites of NR2B luciferase reporter constructs is shown. The NRSE2 and 3 sites (open boxes) present in the construct −3004 bp were mutated (gray boxes) singly or in combination. Cultured neurons were either exposed to 100 mM ethanol for 5 days or remained in the normal growth media. They were transiently transfected into neurons and assayed for luciferase activities 48 h after transfection. Determination of luciferase activity and expression of results were performed as in Fig. 1. Two-way ANOVA revealed a significant sequence-dependent repression and ethanol-response effect ($p < 0.01$, $p < 0.01$, respectively; $N = 4$); *, $p < 0.05$ compared with the wild-type NR2B constructs −3004 bp (post hoc comparisons); #, $p < 0.05$; ##, $p < 0.001$ compared with nonethanol control constructs (post hoc comparisons). B, NR2B luciferase constructs were cotransfected with pcDNA3 vector or NRSF expression plasmid pcDNA3-REEX1 into primary cultured neurons. Determination of luciferase activity and expression of results were performed as in Fig. 1. One-way analysis of variance revealed significant repression effects of NRSF on constructs −2104 bp ($F = 30.22$; $p = 0.009$; $N = 4$) and −3004 bp ($F = 16.64$; $p = 0.024$; $N = 4$) but not on constructs −3004 bp Mt1; *, $p < 0.05$ compared with the NR2B constructs (Newman-Keuls post hoc comparisons).

1996). It has been proposed that absence of NRSF activity in neuronal cells is caused by a lack of NRSF expression. However, in studies on central nervous system tissues in vivo, it was observed that NRSF was expressed in low level in E11.5 embryo hindbrain and forebrain (Chong et al., 1995). More recently, NRSF expression pattern was described in rat brain tissue (Palm et al., 1998), showing that the levels of rat NRSF mRNA expression are highest at the embryonic stages and decreased continually with age. In adult brain, in situ hybridization analysis revealed neuronal expression of rat NRSF mRNA, with the highest expression levels in the neurons of hippocampus, pons/medulla, and midbrain.

However, the expression level of NRSF in cortical neurons during development is unknown. In this study, by using more sensitive RT-PCR and real-time PCR analysis, we demonstrated that NRSF mRNA was expressed in the developing mouse cortex, as well as in the adult cortex. The NRSF mRNA expression was on highest levels in E14 and then declined progressively with age, with the lowest level in adult cortex, where the relative expression level was only 10% of that in E14. This is consistent with a previously published study in rats (Palm et al., 1998). In DIV7 cultured cortical neurons, the level of expression of NRSF is similar to that in P0. We compared the relative expression level of NRSF in DIV7 cultured cortical neurons with non-neuronal cells, HeLa, as well as a neural progenitor cell line NE-4C cells. The results showed HeLa cells have 6-fold and NE-4C cells have 2-fold higher NRSF mRNA than DIV7 neurons. Although NRSF is expressed in relatively higher levels and represses gene expression in non-neuronal cells, in the present study, we found it also expressed in a moderate level and acted as a repressor in embryonic neurons. This leads us to postulate that NRSF expression in embryonic neurons could reflect a role of regulating gene expression in the developing brain that may remain until late stages of development.

Expression of the NR2B subunit is neural-specific and differentially regulated. The NR2B mRNA is already detectable at E14 rat embryos and the level increases in cortical

neurons during fetal development. It increases rapidly and reaches peak level at the age between P0 and P7, and then, there is a decline after this time point (Monyer et al., 1994). The unpublished data in our laboratory obtained from primary cultured neurons showed a similar expression pattern. In contrast to the expression of NR2B in neurons, the NRSF decreases coincided with a NR2B progression increase in fetus. This added evidence that down-regulation of NRSF expression during development is necessary for allowing a neural gene such as NR2B to express specifically in neural tissue. The fact that NR2B subunit expression occurs at early embryonic stage suggests that it is functionally required during development. The mechanism of ethanol regulation of the NR2B expression is unknown. A recent report has demonstrated that Tbr-1/CASK/CINAP protein complex is involved in the expression of NR2B gene (Wang et al., 2004); however, its sensitivity to ethanol is unclear. Our laboratory has recently demonstrated that cAMP response element-binding protein and its signaling pathway may be involved in ethanol-induced NR2B transcription up-regulation (Rani et al., 2005).

Previous studies have indicated that NRSF may act in a concentration-dependent manner, and a certain level of NRSF may be required for DNA-protein complex formation and efficient suppression of transcription (Chong et al., 1995; Schoenherr and Anderson, 1995; Lönnerberg et al., 1996). EMSA analysis in this study demonstrated that NRSE2 and 3 have been observed the specific binding, but we failed to find detectable band with NRSE1 and 5 when using nuclear extract from DIV7 cultured neurons. However, we did obtain bands for all five NRSE motifs when using nuclear extract from HeLa cells and TnT NRSF protein (data not shown), although NRSE1, -4, and -5 bands were in much lower density compared with the NRSE2 and -3. The reason may be because in DIV7 cultured neurons, NRSF was found in relative lower concentration; whereas NRSE1 and 5 have a relative lower affinity to the NRSF so that the binding activity may be too weak to be detected. There is also a correlation
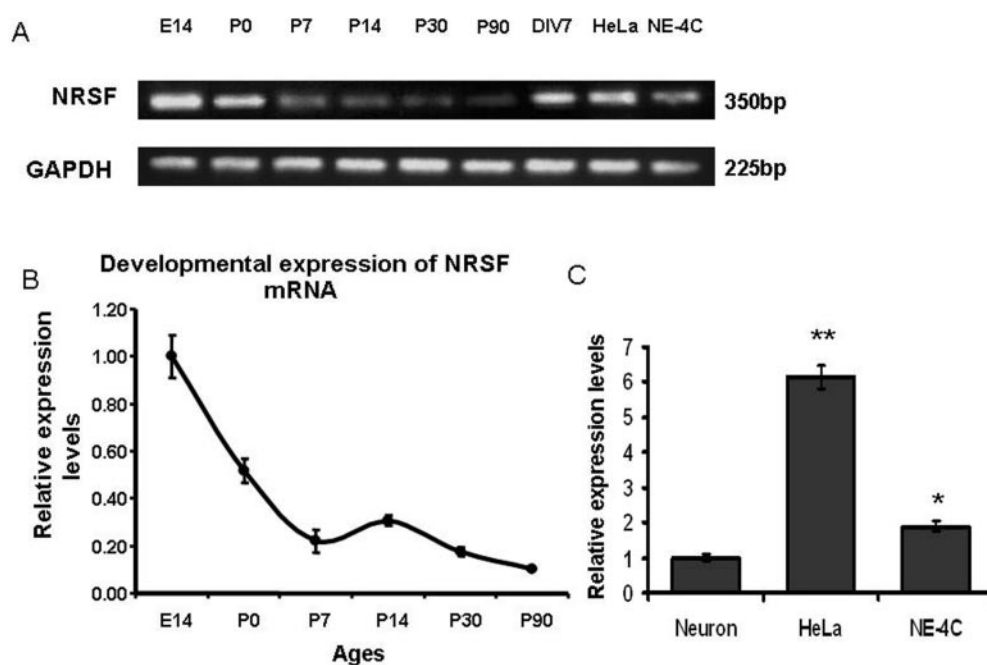

Fig. 7. RT-PCR and real-time PCR analysis of the developmental expression of NRSF. A, ethidium bromide stains of RT-PCR transcripts on 1.5% agarose gels. Total RNA was isolated from different developmental age cortical tissues and DIV7 cultured neurons. Each lane contains one-fifth of the RT-PCR reaction. Note that RT-PCR analyses that were performed are not quantitative and shown only the presence NRSF transcripts. B, relative expression of NRSF determined by quantitative PCR is shown. cDNAs synthesized from different developmental age cortical tissues were used for template in real-time PCR reaction. C, quantitative PCR analysis in DIV7 cultured neurons was also compared with that in HeLa and NE-4C cells. Data are given as mean relative expression level $\pm$ S.E. of three to five independent experiments. One-way ANOVA revealed significant different levels of NRSF among the different cells ($F = 12.36$; $p < 0.01$); *, $p < 0.05$; **, $p < 0.01$ compared with that in neuronal cells (Newman-Keuls post hoc comparisons).

between the transactivation activity of *cis*-elements and their affinity for the transcription factor as reported previously (Li et al., 2002). To support this point, we investigated the possible correlation between the promoter activity mediated by NRSE2 and NRSE3 sites and their affinity to NRSF. NRSE2 turns out to have a higher binding affinity to NRSF and therefore showed more suppressing activity in the neurons.

Increasing evidence has indicated that negative regulatory elements are present in many neuronal specific genes (Desmarais et al., 1992; Lönnerberg et al., 1996; Quinn et al., 2002). It is possible that NRSF not only acts as an on-off switch for neuronal-specific gene expression but also fulfills the role of a molecular regulator of the neural gene expres-

sion in the developing brain. Therefore, misexpression of these genes could have profound effects during development. In the present study, by transfecting NR2B luciferase reporter constructs into cultured neurons, we demonstrated that ethanol exposure does affect NR2B expression through NRSF regulation. Although −1224-bp construct also showed increased luciferase activity after ethanol treatment, which may be caused by other ethanol response factors, constructs −2104 and −3004 bp showed higher response to ethanol, indicating definitely presence of ethanol-sensitive elements in this region. To understand the molecular processes that ethanol could trigger, we investigated the alteration of NRSF mRNA expression and binding activity after long-term etha-
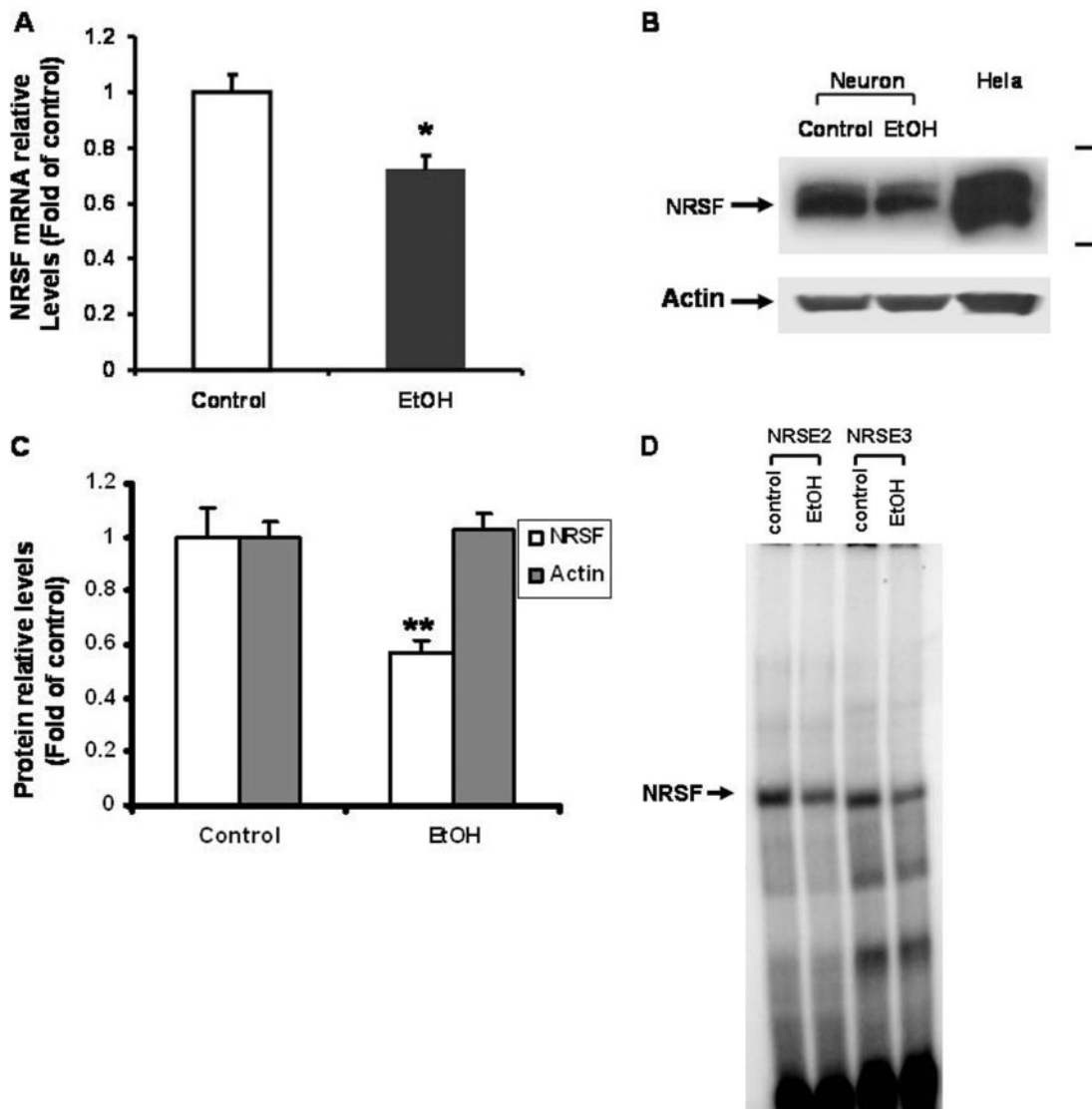
Fig. 8. Effect of ethanol treatment on NRSF expression and complexes binding. A, quantitative PCR was used to characterize alternated expression of NRSF in cultured cortical neuron. Total RNA isolated from DIV7 cultured cortical neuron with and without ethanol treatment (100 mM; 5 days) was reverse-transcribed and analyzed by real time PCR. Data are given as mean relative expression levels ± S.E. ($N = 5$). An asterisk indicates that mRNA relative expression level was significantly different from the control for a given message ($p < 0.05$, Student's t test). B, representative Western blots for NRSF protein level is shown. Equal amounts of protein samples (~35 μg) from cultured neuron nuclear extracts with or without ethanol treatment were separated by SDS-PAGE and analyzed by immunoblotting with NRSF antibody. The NRSF protein is indicated by NRSF plus arrow. The slow-migrating bands probably correspond to a hyperphosphorylated form of the protein (Li et al., 2002). The two bars indicated molecular mass markers of 191 and 97 kDa. C, quantitative analysis compared the expression of NRSF protein level of control cells with ethanol-treated cells in Western blots ($N = 4$ per group). ∗∗, $p < 0.001$ versus control (Student's t test). D, representative EMSA showing protein binding to the NRSEs oligonucleotides in nuclear extracts of cortical cultured neurons after ethanol treatment. EMSAs were performed using nuclear extracts from DIV7 cultured neurons (10 μg of protein) with (lanes 1 and 3) and without (lanes 2 and 4) 5-day ethanol treatment. ³²P-Labeled NRSE2 and NRSE3 were used as the probes. DNA-protein complexes were separated on a 5% polyacrylamide gel. NRSF plus arrow indicates the specific complex.

nol treatment. Contrasting with the results of increased NR2B promoter luciferase activity, long-term ethanol exposure significantly decreased NRSF mRNA expression and binding activity, which may lead to derepression on the NR2B gene expression. Therefore, our results suggest that ethanol treatment may increase the NR2B level through NRSEs, albeit this does not rule out the possibility that there are some other elements coregulating NR2B gene transcription.

NMDA receptor is thought to be of particular importance in mediating the effects of ethanol in the mammalian brain. The association between ethanol-induced dependence, alcohol-withdrawal cytotoxicity, and NMDA receptor NR2B subunit has been established by numerous studies (Kumari and Ticku, 1998; Floyd et al., 2003; Schumann et al., 2003; Nagy et al., 2004). Neurobiological effects of alcoholism, such as intoxication, withdrawal seizures, delirium tremens, Wernicke-Korsakoff syndrome, and fetal alcohol syndrome, can be understood as a spectrum of consequences of ethanol's effect on the glutamatergic system. Hence, a host of findings support the hypothesis that the mechanism of action of ethanol may involve interference with glutamatergic neurotransmission, especially through the NMDA receptor (Tsai et al., 1995). Our findings should increase understanding of the biological mechanism of ethanol abuse and dependence and should aid in the development of more effective pharmacological interventions.

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