Estrogen-dependent Transcription of the NEL-like 2 (NELL2) Gene and Its Role in Protection from Cell Death*

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NELL2 (neural tissue-specific epidermal growth factor-like repeat domain-containing protein) is a secreted glycoprotein that is predominantly expressed in neural tissues. We reported previously that NELL2 mRNA abundance in brain is increased by estrogen (E2) treatment and that NELL2 is involved in the E2-dependent organization of a sexually dimorphic nucleus in the preoptic area. In this study we cloned the mouse NELL2 promoter and found it to contain two half-E2 response elements. Electrophoretic mobility shift assays and promoter assays showed that E2 and its receptors (ERα and ERβ) stimulated NELL2 transcription by binding to the two half-E2 response elements. Hippocampal neuroprogenitor HiB5 cells expressing recombinant NELL2 showed increased cell survival under cell death-inducing conditions. Blockade of endogenous synthesis of NELL2 in HiB5 cells abolished the cell survival effect of E2 and resulted in a decrease in phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2). These data suggest that the NELL2 gene is trans-activated by E2 and contributes to mediating the survival promoting effects of E2 via intracellular signaling pathway of ERK.

A gene encoding neural epidermal growth factor (EGF)3-like protein (NEL) was first identified in the chick embryo (1). A subsequent study reported two mammalian homologs of the chick NEL gene, NEL-like 1 (NELL1) and NEL-like 2 (NELL2), isolated from a human fetal brain cDNA library (2). NELL2 has a greater sequence similarity to chicken NEL than NELL1 and is strongly expressed in mammalian brain in an apparent neuron-specific manner (3–5). NELL2 is a secreted, N-glycosylated protein (3). A large fraction of NELL2 is secreted into culture medium from transfected COS7 and HiB5 cells (3, 6) and from in ovo-transfected chicken spinal cord cells (7). The released NELL2 promotes proliferation and differentiation of neural cells (7) and increases survival of in vitro cultured primary cortical and hippocampal neurons (8).

NELL2 is also involved in promoting the neuronal survival required for the formation of a sexually dimorphic nucleus of the preoptic area (SDN-POA) in male rats (6). The volume of the SDN-POA in male rats is much larger than females (for review, see Ref. 9). This has been known to result from the actions of estrogen (E2) on cells of the male SDN-POA (9). E2 exerts multiple effects on developmental processes taking place in the mammalian central nervous system, such as neurogenesis, survival, and differentiation of different neuronal populations (for review, see Ref. 10). A prominent function of E2 in the nervous system is to protect neurons from cell death (for review, see Ref. 11). E2 produced locally by aromatization of circulating testosterone promotes survival of the SDN-POA in neonatal male rats, whereas this effect is not observed in females because fetoneonatal E2 binding protein blocks E2 action in the female brain (12–14). Because blockade of NELL2 synthesis in the neonatal male rat brain resulted in a decreased size of SDN-POA (6), it has been suggested that NELL2 is involved in the E2-induced protection of SDN-POA in male rats. This notion was inferentially supported by the results of a differential display analysis of RNA samples from the hypothalamus of E2-injected female rats indicating that NELL2 is an E2-responsive gene (15).

We now report that (a) E2-its receptor (ER) complexes bind and activate the NELL2 promoter, and (b) E2-induced cell survival is blocked by silencing the NELL2 gene via RNA interference (RNAi) and by overexpression of a mutant NELL2 lacking deoxynucleotidyltransferase dUTP nick-end labeling; VMH, ventromedial nucleus; MAPK, mitogen-activated protein kinase; mNELL2, mouse NELL2; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; C5, complementary sequence.
EGF-like domains. These results suggest that NELL2 is a mediator of E2-induced neuronal survival in the central nervous system.

**EXPERIMENTAL PROCEDURES**

**Cloning of the Mouse NELL2 (mNELL2) Gene 5′-Flanking Region**—A mouse genomic DNA library (Invitrogen) was constructed in *Escherichia coli* LE392 after ligating partial Sau3AI DNA digests of mouse genomic DNA (strain 129/Sv) at the BamHI site of a phage vector lambda EMBL3 (Promega, Madison, WI). For screening clones containing the mNELL2 gene, ~1 × 10⁷ phage plaques were transferred to Hybond N+ membrane (Amersham Biosciences) and hybridized to a [α-³²P]dCTP (50 μCi/μL, Amersham Biosciences)-labeled mNELL2 cDNA probe; the cDNA fragment (a 680-bp fragment spanning nucleotides 21–700 in mNELL2 mRNA) was cloned by PCR amplification based on the deposited DNA sequence (NCBI GenBank™ accession number NM_016743). Hybridization was performed overnight at 50 °C in a hybridization buffer containing 45% formamide, 5× SSC (1 × SSC: 8765 g of sodium chloride, 4.41 g of sodium citrate/liter), 1% SDS, 5% dextran sulfate, and 10 mg salmon sperm DNA (Sigma). After hybridization, the membranes were washed with 1× SSC and 0.1× SDS and exposed to x-ray films (AGFA CP-G plus). This process yielded positive clones.

The NELL2 promoter region containing about 1.2 kb upstream from the translation initiation site was amplified by PCR using the above clone and a primer set based on the mNELL2 genomic DNA sequence (NCBI GenBank™ accession number NM_016743): sense primer, 5′-CCG CTT CTT CTC TGC TTG GAG GGG GGA GGG AGA CGA T-3′; antisense primer, 5′-ATG GCT CGA CCG GGG AAC AAG AT-3′. The amplified product was cloned into the pGL3 basic luciferase reporter vector (Promega). The extended cDNA products were extracted and separated on a 6% denaturing polyacrylamide gel, and the putative site of TSSs was determined by comparison with adjacent sequencing reaction that used the same oligonucleotide primer and a genomic clone containing this region as a template.

**Reverse Transcription-PCR Confirmation of the TSS**—Total RNA was isolated from mouse (C57/B6) brain using TRI reagent (Sigma) and reverse-transcribed using oligo(dT) as primer. PCR amplification was performed using 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s preceded by 95 °C for 5 min and followed by 72 °C for 7 min. The PCR primers (indicated in Fig. 1B) used in this experiment were: antisense primer (5′-GGT CAC CAA GAT GTG GAA CTC GTG-3′) designed from the DNA sequence located on exon 4; sense primer 1 (5′-GGT AGA AGA ATC CCC TTG CCT TGC TTG AC-3′) located upstream of exon 1; sense primer 2 (5′-CCT CGC TTC CGC CTC GGC GTG TTG-3′) located on exon 1; sense primer 3 (5′-GAT TTC GGC GGA CGA CGA TGA C-3′) spanning the 3′ end of exon 1 and the 5′ end of exon 1; sense primer 4 (5′-CCA TTG TCT CTC CCA CAA C-3′) located on intron 1; sense primer 5 (5′-GAT TTC GGC GGA CGA CGA TGA C-3′) spanning the 3′ end of exon 1 and 5′ end of exon 2; sense primer 6 (5′-GAC GAT GTA CTA GGA CGA TGC AC-3′) on exon 2; sense primer 7 (5′-CGT CCT CGC TGG TAA GCG GGT TTG-3′) located on the 3′ end of exon 2. The amplified products were separated by agarose gel electrophoresis, isolated, and cloned into pGEM-T easy vectors (Promega), and their DNA sequence was confirmed by sequencing.

**Mutant NELL2 Promoter Construct**—Mutant NELL2 promoters carrying deletion of the TATA-like sequence, intron 1, half-EREs, and SP1 response elements (SP1REs) were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) and specific oligonucleotide primer sets; the intended mutations were confirmed by sequence analysis. The oligodeoxynucleotide primers used were (a) a primer set for deletion of intron 1 (sense primer, 5′-CCCC CTT CGA AAC AGC TGT CGG GC-3′; antisense primer, 5′-GAG GGA GAC GAT GGC GAC TGC TGG AGA C-3′, (b) a primer set for deleting TATA-like sequence (5′-CCCC AGC ACC TCT GCC TGG TTG TTA TCT TAA GAG GGG-3′ and its complementary sequence (CS)), (c) a set for deleting half-ERE (5′-CCCC CGC AGG TCC CCG AGA GCC CGG CTG CGG-3′ and its CS) located at the NELL2 promoter (~223), (d) a primer set for deletion of the other half-ERE sequence (5′-CTG CTG GCC CTT GCC GTT GCT GCT GTG TGA TGC-3′ and its CS) located at ~1047 of the promoter, (e) the SP1RE sequence (5′-GGC TGC GCC CTT GCC GTT GCT GTG TGA TGC-3′ and its CS) located at ~219, (f) the SP1RE sequence (5′-GGT CGG TTC GAC CTC CCG GAG CGG CTC GCT-3′ and its CS) located at ~430, (g) SP1RE sequence (5′-AAA GAG GAC GCC GCC CCG CC CTC-3′ and its CS) located at ~453.

**Cell Culture and Promoter Assays**—The conditionally immortalized rat hippocampal cell line HiB5 was used for this study. Because HiB5 cells were conditionally immortalized by the stable expression of a temperature-sensitive mutant form of the SV40 virus large T antigen (16), they proliferate at the permissive temperature of 33 °C and stop growing at 37 °C. They were cultured in phenol-red free medium (Sigma) supplemented with 10% charcoal-stripped fetal bovine serum (Invitrogen) and antibiotics at 33 °C, 95% O₂, 5% CO₂.

To determine whether E2 regulates NELL2 transcription, HiB5 cells were transiently transfected with mNELL2 promoter
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(NELL2-P)-luciferase reporter constructs (NELL2-pGL3) using Lipofectamine/PLUS (Invitrogen) and rat ERα and ERβ expression vectors (50 ng each) (kindly provided by Dr. Keesook Lee, Chonnam National University, Kwangju, South Korea). After 24 h the cells were treated with E2 or E2-conjugated with bovine serum albumin (E2-BSA, Sigma) that cannot penetrate through the membrane for 24 h and lysed with Cell Culture Lysis Reagent (Promega). Luciferase assays were performed using a luciferase reporter assay kit (Promega). Transfection efficiency of each assay was normalized by cotransfecting the plasmid pCMV-β-gal (Clontech, Palo Alto, CA) at 60 ng/ml.

Real-time PCR—To determine the effect of E2 on the endogenous NELL2 expression, RNA (2 μg), isolated from HiB5 cells treated with E2 (10 nM) with or without ERs, was reverse-transcribed and amplified by a real-time PCR using two sets of primers: NELL2 sense primer, 5′-GGG TAG GTG TGT CCC TTC AT-3′, and antisense primer, 5′-CAG GAC TGC ATA ATG GGA CGG CGG ATC-3′; glyceraldehyde-3-phosphate dehydrogenase sense primer, 5′-GTT ATG GTA TGG GCA TGG ACT GTG GTG CTA-3′, and antisense primer, 5′-TGG GTA AGG AGG TGC GTG TCA-3′. Real-time PCR reactions (20 μl total volume containing 5 pmol of primer, 10 μl of SYBR Green dye (Qiagen, Valencia, CA), and 2 μl of cDNA) were carried out with a DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research Inc., Waltham, MA) for ~40 cycles.

Western Blotting—Protein from HiB5 cells was homogenized in M-PER lysis buffer (Pierce). Extracted protein (15 μg) was separated by SDS-polyacrylamide gel electrophoresis and transferred to a membrane by electrophoretic transfer. The membrane was incubated with rabbit anti-rat NELL2 antibody (17), rabbit anti-extracellular signal-regulated kinase (ERK) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-phospho-p44/42 (p-ERK1/2) antibody (Cell Signaling Technology Inc., Beverly, MA), and rabbit anti-Bax antibody (Santa Cruz). Immunoreactivity was detected with an enhanced chemiluminescence (ECL) kit (Amersham Biosciences).

Electrophoretic Mobility Shift Assays (EMSAs)—HiB5 (5 × 106) cells were transfected with 1 μg each of ERα and ERβ expression vectors and cultured in the presence of 10 nM E2. After 24 h of incubation, nuclear protein extracts were prepared according to the method of Andrews and Faller (18) utilizing protease inhibitors (19). The double-stranded oligodeoxynucleotide probes used for the assays were end-labeled with [γ-32P]ATP and purified over a NICK column (Amersham Biosciences). The binding assay was performed as described (20) with minor modifications using 10 μg of nuclear protein extracts, 20,000 cpm of probe, and 1 μg of poly(dl-dC). The reaction mixtures were separated by electrophoresis on a 6% non-denaturing polyacrylamide gel. The gels were then dried and exposed to film at ~85°C.

The oligodeoxynucleotide probes employed were (a) a positive control probe (5′-GAG AGC CTG ACC CGG CTG C-3′) containing the palindromic ERE and flanking sequence adopted from the promoter of the vitellogenin gene (21), (b) a negative control probe (5′-GGA TCT AAT TCT GGG CCG ATC-3′) containing a mutation in one arm of the palindromic sequence of the positive control probe (21), (c) a probe containing the half-ERE sequence (5′-GAG AGC CTG ACC CGG CTG C-3′) located at the mNELL2 promoter (~223), (d) a probe containing the other half-ERE sequence (5′-TGC CCT TTG ACC CTG GCT G-3′) located at ~1047 of the promoter, (e) the SP1RE sequence (5′-CTG CGG CCC GCC GCG TGC G-3′) at ~209, (f) the SP1RE sequence (5′-TCG CCC CCC CCC CCT CCC G-3′) at ~430, and (g) the SP1RE sequence (5′-AGG CCG CCC CCC GCG CGC C-3′) at ~453.

Chromatin Immunoprecipitation (ChIP) Assay—After lysis of the HiB5 cells transfected with expression vectors for ERs, nuclei were extracted and resuspended with nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors). Chromatin was sheared by sonication and diluted 5-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl, and protease inhibitors). The reactions were incubated with 5 μg of antibodies against ERα (Affinity BioReagents, Golden, CO) and ERβ (Santa Cruz) at 4°C overnight. Immune complexes were collected by reacting with 60 μl of the salmon sperm DNA/protein A-agarose for 1 h at 4°C and then washed consecutively for 5 min each with buffers (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1) containing different concentration of salts (150–500 mM) and 0.25 mM LiCl. DNA from the protein-DNA cross-links was extracted by incubating the reactions with solution (1% SDS, 0.1 mM NaHCO3, 10 μg RNase, and 0.3 mM NaCl) at 65°C for 4 h and was further purified with phenol/chloroform. PCR amplification was performed using 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, preceded by 94°C for 5 min, and followed by 72°C for 10 min. Primer sets used for the PCR amplification were a primer set for half-ERE at ~233 (sense primer, 5′-CGG CCT TCC AAC AGG TTC-3′; antisense primer, 3′-AAC TTG GAG CCG CCT CTG-5′) and a primer set for the other half-ERE at ~1047 (sense primer, 5′-ATG GGG TAG AAT CCC CCT GC-3′; antisense, 3′-AGG CCA GAC TGG GAG CCT GTT-5′).

In Situ Hybridization and Immunohistochemistry—Localization of NELL2 mRNA in the female rat hypothalamus was carried out after an in situ hybridization procedure (22) reported in detail elsewhere (23). In brief, the brains were transcardially perfused with 4% paraformaldehyde in borate buffer, pH 9.5. The sections (25 μm) were prepared using a freezing sliding microtome, mounted on glass slides, and dried overnight under vacuum. The next day the sections were prehybridized, hybridized with [35S]UTP-labeled NELL2 cRNA probe, and washed as reported (22, 23). After dehydration in graded alcohols, the slides were dipped in Ilford K5 emulsion and developed after 3 weeks of exposure. Control sections were hybridized with a NELL2 sense RNA probe. The probes were prepared by SP6 RNA polymerase-directed in vitro transcription of a 333-bp rat NELL2 cDNA template obtained by PCR amplification of a portion of the NELL2 mRNA-coding region corresponding to nucleotides 548–880 in rat NELL2 mRNA (23).

A combined immunohistochemistry/in situ hybridization procedure was used to determine whether NELL2 is expressed in the ERα-expressing cells in the rat brain. Floating 30-μm sections were incubated for 18–20 h at 4°C with a mouse monoclonal ERα antibody (clone 1D5 diluted 1:100, LabVision...
Neomarkers, Fremont, CA) and incubated with a horse anti-rabbit γ-globulin (1:250, Vector Laboratories, Burlingame, CA) for 1 h at room temperature and then with AB complex (Vector Laboratories) for 1 h. The immunohistochemistry reaction was then developed to a brown color with 3,3′-diaminobenzidine- 
HCl and 0.005% H₂O₂. After washing, the sections were mounted onto glass slides and dried overnight before hybridization with the radiolabeled NELL2 cRNA probe. After an overnight hybridization at 55–56 °C, the slides were washed. After dehydration, the slides were dipped in Ilford K5 emulsion and exposed for 3 weeks at 4 °C. At this time the slides were developed, quickly dehydrated, dried, and coverslipped for microscopic examination.

Mutant NELL2 Protein Constructs—NELL2 contains several different functional domains: a signal peptide, a thrombospondin-1-like module, five von Willebrand factor C domains, and six EGF-like domains, three of which are Ca²⁺-binding EGF-like domains (3). Using overlapping PCR, we prepared two different mutant cDNAs encoding proteins lacking EGF-like domains: NELL2-ΔEGF-Ca²⁺ (lacking the three Ca²⁺-binding EGF-like domains) and NELL2-ΔEGF (lacking all 6 EGF-like domains). Primer sets carrying a digestion site by NotI were used for generation of NELL2-ΔEGF. Using Zeocin (400 g/ml) (Invitrogen) and screened for NELL2 containing exon 1 (primers 2 and 3) and exon 2 (primers 5, 6, 7) and anti-sense primer at exon 4 (Fig. 1). Sequence Tags data base) was performed using the nucleotide sequence of exons 1 and 2 of mNELL2 (GenBank™ accession number NM_016743.2). Primer sets carrying a digestion site by NotI were used for generation of mutants: sense primer for NELL2-ΔEGF, 5′-CTC AGA ACA GCG GCC GGC GAG TCA TAA CC-3′; antisense primer for NELL2-ΔEGF, 5′-ATG GAA ACT GCG CGC GCC GAC AG-3′; sense primer for the first round of PCR generation of NELL2-ΔEGF-Ca²⁺, 5′-ATG GAA ACT GCG CGC GCC GAC AG-3′; antisense primer for the first round of PCR for NELL2-ΔEGF-Ca²⁺, 5′-TCG TGT GGC GCC GCA CGG AAC-3′; sense primer for the second round of PCR for NELL2-ΔEGF-Ca²⁺, 5′-CAA TGT CGC GCC GCC GCC GCC GCC GCC GGC CAC AC-3′; antisense primer for the second round of PCR for NELL2-ΔEGF-Ca²⁺, 5′-ATG GAA ACT GCG CGC GCC GCG CAC AG-3′. The PCR products were digested with NotI and finally cloned into the expression vector pcDNA 3.1-zeo (Invitrogen), and intended mutations were confirmed by DNA sequencing.

Stable Transfection of NELL2 and NELL2 Mutants—To determine the effect of NELL2 on the cell survival, we used HiB5 cells stably transfected with NELL2 or NELL2 mutant expression vectors. NELL2-transfected cells efficiently release NELL2 into the culture medium (6). As a control, other cells were transfected with the pcDNA 3.1-zeo vector. The cells constitutively expressing the transfected vectors were selected using Zeocin (400 µg/ml) (Invitrogen) and screened for NELL2 expression by RT-PCR.

Viability Assays—HiB5 cells (5000 cells/well) expressing NELL2 or NELL2 mutants were plated in 96-well plates containing 100 µl of phenol-red free Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal-stripped fetal bovine serum. The cells were exposed to 10 nm E2 for 24 h. Thereafter, the medium was changed to Dulbecco’s modified Eagle’s medium supplemented with 2% charcoal-stripped fetal bovine serum followed by an addition of the same amount of E2. Thirty minutes later, 10 µM amyloid β (25–35) (Aβ) and 5 mM α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) (both reagents, Sigma) were added. After 20 h of incubation, cell viability was assessed by using a modified methylthiazo-

zolyltetrazolium (MTT) assay (24). After incubating 20 h with the appropriate reagents, 10 µl methylthiazolyltetrazolium (5 mg/ml stock) was added and incubated for 4–5 h. At this time, 100 µl solubilization solution (50% dimethylformamide, 20% SDS, pH 4.8) was added to each well. Absorption readings were performed at 570 nm using a spectrophotometer.

Terminal Deoxynucleotidyltransferase dUTP Nick-end Labeling (TUNEL) Assays—For detection of apoptotic cells, TUNEL assays were performed using the Fluorescent In Situ Cell Death kit, TMR red (Roche Diagnostics). After treatment with E2 and neurotoxins as indicated above, the HiB5 cells (5000 cells/well) were fixed in a 4% paraformaldehyde for 10 min at 4 °C and treated with 0.1% Triton X-100 in an ice-cold 0.1% sodium citrate for 5 min. The cells were treated with 50 µl of TUNEL reaction mixtures for 60 min at 37 °C in a humidified atmosphere. Samples were analyzed by confocal fluorescent microscopy.

Statistical Analysis—The results were analyzed with a one-way analysis of variance followed by the Student Neuman-Keuls multiple comparison test for unequal replications. Student’s t test was used to compare two groups.

RESULTS

TSSs of NELL2 Gene—The TSS of the NELL2 gene has not been experimentally determined. Although it is clear that the ATG site is located in exon 2 (NCBI GenBank™ accession number NM_016743.1), the existence of an exon 1 has also been reported (GenBank™ accession number NM_016743.2). To determine the location of the TSS, we first performed primer extension studies using mRNA extracted from the mouse brain and an antisense primer complementary to a sequence in exon 2. One strong band and several weak bands were detected (Fig. 1A). Sequence analysis determined that the last nucleotide of the strongest and longest product was a G (Fig. 1A), which corresponds to the first nucleotide of exon 2 (Fig. 1D). A blast search of the mouse dbESTs (Expressed Sequence Tags data base) was performed using the nucleotide sequence of exons 1 and 2 of mNELL2 as a query to identify potential upstream TSSs. Although two clones correspond exactly to the 5′ end of exon 1 (BU612839, AU080563), 17 clones match with the 5′ end of exon 2, suggesting that the 5′ end of exon 2 is the major TSS of mNELL2.

To confirm the TSSs determined by the primer extension and blast search, RT-PCR was performed on RNA isolated from mouse brain by using combinations of sense primers corresponding to regions around 5′ end of exons 1 and 2 and antisense primer at exon 4 (Fig. 1B). The resulting products consisted of cDNA fragments generated by sense primers containing exon 1 (primers 2 and 3) and exon 2 (primers 5, 6, and 7) sequences but not by sense primers located upstream of exon 1 (primer 1) and within intron 1 (primer 4) (Fig. 1C). DNA sequencing revealed that the 5′ end of exons 1 and 2 exactly coincides with the 5′ end of cDNA products generated by primer 2 (spanning part of sequence upstream from exon 1 and the 5′ end of exon 1), and primer 5, spanning the 3′ end of exon 1 and the 5′ end of exon 2. Thus, mNELL2 appears to have two alternative TSSs located at the 5′ end of exons 1 and 2. However, the paucity of ESTs containing exon 1 and the relatively
weaker PCR products produced by amplifications using primers 2 and 3 suggest that the TSS at exon 1 is much less frequently used than the one at exon 2.

Organization of the mNELL2 Gene and Binding Motifs for Transcription Factors in the mNELL2 Promoter—Organization of the mNELL2 gene was determined based on information obtained from genomic DNA sequences. A blast search (www.ncbi.nlm.nih.gov) of the mouse genomic DNA (NCBI GenBank™ accession number NM_016743) was performed using a mouse cDNA complementary to the NELL2 mRNA coding region as the query. We determined that the mNELL2 gene comprises 21 exons, with sizes ranging from 75 to 707 bp in length and a total gene size of 307 kb in chromosome 15 (supplemental Data 1 and 2). Exon 1 in the mNELL2 gene spans 231 bp and contains the 5′-untranslated region. Exon 2 (87 bp long) contains the ATG translation initiation site (Fig. 1D) followed by a signal sequence. The exon-intron junctions contain the consensus 5′ splice donor site, GT, and the 3′ splice acceptor, AG, except the intron 1 (supplemental Data 2).

Sizes of the introns vary from 98 bp to 54,478 bp in length.

To find motifs for DNA-binding proteins in the 5′-flanking region of the mNELL2 gene, the cloned sequence (NCBI GenBank™ accession number GU290311) was analyzed with the aid of a search program (available online at TESS (Transcription Element Search System)) and shown in Fig. 1D. The two identified TSSs are located at the 5′ end of exons 1 and 2, respectively. A TATA-like sequence is located immediately upstream from the TSS at exon 2. The 5′-flanking sequence of the mNELL2 gene contains several consensus motifs for transcription factors: retinoic acid receptor (retinoid X receptor/retinoic acid receptor), progesterone receptor, and glucocorticoid receptor. The mNELL2 promoter has no palindromic ERE. However, it contains 2 widely spaced half-EREs at position 223 and 1047. Both half-EREs have a well conserved 5′-TGACC-3′ sequence (25). In addition, the promoter region has seven well conserved SP1REs (5′-CCCGCC-3′ or 5′-GGGCGG-3′).

Interestingly, intron 1 contains several important binding domains for transcription factors such as a half-ERE and SP1REs.
regulating transcription mediated by this site. Consistent with this notion, deletion of intron 1 abolished the basal transcriptional activity of the NELL2 gene (Fig. 2A), suggesting that intron 1 is required for maintaining the mNELL2 gene transcriptionally active. The effect of deleting TATA-like sequence and intron 1 was consistent at different concentrations of E2 (data not shown).

Effect of Estrogen and Its Receptor on the NELL2 Transcription—We previously found that E2 increased the NELL2 mRNA content in the hypothalamus of female rats (15). To determine whether E2 directly regulates transcription of the NELL2 gene, we performed NELL2 promoter assays in HiB5 cells. E2 strongly promoted NELL2 transcription after transfection of the cells with Eρα and Eρβ expression vectors (Fig. 2B). The transcriptional activation effect of E2 was most prominent at the 10 nM concentration; at 20 nM, the effect decreased slightly. The specificity of this E2-ER effect on NELL2 transcription was assessed using the ER antagonist, 4-hydroxytamoxifen. Treatment with 4-hydroxytamoxifen markedly reduced the effect of E2-ER on NELL2 gene transcription (Fig. 2C), further suggesting that the trans-activational effect of E2 is specifically exerted via binding to ERs. To verify possible non-genomic action of E2, we performed NELL2 promoter assays with E2-BSA. E2-BSA did not affect transcriptional activity of NELL2 promoter (Fig. 2D), indicating that E2 exerts its effect on NELL2 transcription through genomic action by binding with ERs.

To determine E2 action on the endogenous NELL2 expression, we carried out real-time PCR and Western blot analysis of mRNA and protein extracts from the HiB5 cells treated with E2 together with or without ERs. E2 strongly increased endogenous mRNA (Fig. 2E) as well as protein (Fig. 2F) levels of NELL2 in the presence of Eρα and Eρβ.

ER Binds to Half-ERE Domains in the 5′-Flanking Region of NELL2 Gene—As stated above, the mNELL2 promoter has no palindromic ERE. Instead, it contains two half-EREs. To determine whether ER is recognized by these domains in the NELL2 promoter, we subjected the two sequences of the mNELL2 promoter (located at −223 and −1047) containing the half-ERE 5′-TGACC-3′ core motif and flanking region to EMSA analysis. Both half-EREs showed shifted bands when exposed to nuclear extracts from the HiB5 cells transfected with either Eρα (Fig. 3A) or Eρβ (Fig. 3B) expression vectors and treated with E2. A positive control (labeled C) using the palindromic ERE showed shifted bands of an apparently similar size. In contrast, a mutated ERE (labeled NC) did not generate shifted bands.

Because SP1RE has also been reported to indirectly mediate E2 action on target genes (26–28), EMSAs using probes containing three SP1REs were also performed to identify indirect binding of E2-ERs to these sequences. SP1RE at −593 showed almost no shifted band, whereas SP1REs at −430 had shifted bands with Eρβ (Fig. 3B), suggesting that E2-ERβ may indirectly interact with these SP1REs through binding to SP1 (26–28). Preincubation of the nuclear extracts with Eρα antibody delayed migration of the protein-DNA complex generated with a probe containing half-EREs at −223 and −1047 (data not shown), indicating that Eρα is indeed part of this complex.

To further determine in vivo interaction of ERs with NELL2 promoter containing the two half-ERE sequences, ChIP assays were performed using Eρα and Eρβ antibodies, and the precipitated DNA was amplified using PCR primer sets specific to the promoter regions containing the half-EREs (Fig. 3, C and D); one primer set amplified a 216-bp fragment of the mNELL2 promoter region from −381 to −166 encompassing the half-ERE at −233; the other primer set amplified a 195-bp fragment corresponding to −1076 to −882 region of the NELL2 promoter with the half-ERE at −1047. As shown in Fig. 3, C and D, NELL2 promoter was immunoprecipitated with Eρα and Eρβ. The specificity of this E2-ER effect on NELL2 transcription was assessed using the ER antagonist, 4-hydroxytamoxifen. Treatment with 4-hydroxytamoxifen markedly reduced the effect of E2-ER on NELL2 gene transcription (Fig. 2C), further suggesting that the trans-activational effect of E2 is specifically exerted via binding to ERs. To verify possible non-genomic action of E2, we performed NELL2 promoter assays with E2-BSA. E2-BSA did not affect transcriptional activity of NELL2 promoter (Fig. 2D), indicating that E2 exerts its effect on NELL2 transcription through genomic action by binding with ERs.
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**FIGURE 3. EMSAs and ChiP assays.** EMSAs were performed using double-stranded oligomer probes containing the putative half-EREs and SP1REs found in the mNELL2 gene 5’ flanking sequence (see “Experimental Procedures”). A and B, autoradiograms show binding of half-EREs and SP1REs derived from the mNELL2 promoter to nuclear extracts (10 μg) prepared from HiB5 cells transfected with ERα (A) and ERβ (B) and treated with E2 (10 nM). Numbers on top of the gel images indicate the site in the NELL2 promoter from where the oligomer probes were designed. B, protein-bound DNA; F, free DNA; C, control palindromic ERE; NC, negative control of mutant ERE. C and D, shown are ChiP assays of mNELL2 promoter DNA using ERα (C) and ERβ (D) antibodies. HiB5 cells were transfected with expression vectors for the ERs (C and ERβ (D), and the immunoprecipitated DNA was PCR-amplified with primers for two NELL2 promoter fragments including the two half-ERE sequences (at −233 and −1047). Input represents the DNA extracted from the HiB5 cells before immunoprecipitation. Normal IgG from mouse (C) and rabbit (D) was included in the assay as negative controls. V = vehicle.

Antibodies. The results revealed that PCR fragments containing the half-ERE sequences at −233 and −1047 were markedly increased in DNA samples from E2-treated HiB5 cells compared with DNA from vehicle-treated cells. No detectable band was observed in the control IgG precipitations, and very faint bands were found in the precipitations from cells without transfection of ERs.

**E2-dependent Activation of NELL2 Transcription Decreases after Deletion of Half-EREs**—To determine whether the two half-EREs found in NELL2 promoter region are required for transactivation of the NELL2 promoter by E2-ERα and E2-ERβ complexes, we deleted each site by the site-directed mutagenesis and examined the ability of E2-ERs to transactivate the mutated promoter in HiB5 cells. As shown in Fig. 4, deletion of each half-ERE resulted in a significant decrease of promoter activity induced by E2-ERα (Fig. 4A) and E2-ERβ (Fig. 4B). Deletion of the proximal half-ERE (at −223), located in intron 1, obliterated E2-ERβ-induced promoter activation (Fig. 4B), suggesting that this is a critical site mediating the effect of E2-ERβ on NELL2 gene transcription. The −223 site appears to be the most important for E2-ERα action (Fig. 4A). These results also suggest that intron 1 is critical not only for maintenance of basal transcription (see Fig. 2A) but also required for E2-ER-mediated transcriptional regulation of the mNELL2 gene.

Because SP1REs are also involved in mediating E2-dependent transcriptional regulation in an interaction between E2-ER and SP1 bound to SP1RE (26–28), we determined if deletion of 3 SP1REs (at −209, −430, and −453) affects E2-ER-dependent NELL2 gene transcription. Interestingly, single deletions of SP1RE at −209 and −430 strongly suppressed activation of NELL2 transcription by E2-ERβ (Fig. 4B) but not that by E2-ERα (Fig. 4A). Thus, certain SP1REs could be indirectly involved in the regulation of NELL2 transcription by E2-ER, but this action may be different between the two ERs.

**ERα Protein and NELL2 mRNA Are Coexpressed in the Rat Hippocampus and Hypothalamus**—Combined in situ hybridization/immunohistochemistry was performed to determine whether cells synthesizing ERα protein in the rat brain also express NELL2 mRNA. The hippocampus and hypothalamus were selected as examples, because they are well known to contain ERα-expressing neurons. As shown in Fig. 5, NELL2 mRNA was expressed at high levels in both the rat hippocampus and the ventromedial nucleus (VMH) of the hypothalamus. In the hippocampus, most NELL2-expressing cells (Fig. 5A) also showed ERα immunostaining (Fig. 5C), a colocalization best illustrated at a higher magnification (Fig. 5E). In the hypothalamus, cells of the VMH were rich in NELL2 mRNA transcripts (Fig. 5B), and cells of the ventrolateral VMH region were also positive for ERα (Fig. 5D). A higher magnification view of this region shows that ERα-stained cells also contain silver grains, indicating the presence of NELL2 mRNA transcripts (Fig. 5F). These results indicate that there are cells in the brain co-expressing both NELL2 and ERα, thus providing the morphological substrate for a functional interaction between these two molecules.

**Effect of NELL2 on the Survival of Neuroprogenitor HiB5 Cells**—An effect of NELL2 on cell survival was examined using methylthiazolyltetrazolium assays and HiB5 cells stably transfected with wild-type NELL2 or mutant NELL2 expression vectors (Fig. 6A) and treated with neural cell death-inducing agents in combination with E2. Wild-type NELL2 showed a strong neuroprotective effect on HiB5 cells treated with Aβ (Fig. 6B) and AMPA (Fig. 6C). However, mutant NELL2 lacking either the Ca2+ binding EGF domains (NELL2–ΔEGF–Ca2+) or all six EGF-like domains (NELL2–ΔEGF) did not promote cell survival, indicating that these domains are essential for NELL2-dependent signaling to facilitate cell survival.

E2 (10 nM) treatment alone markedly promoted survival of cells treated with Aβ (Fig. 6B) and AMPA (Fig. 6C). Cells expressing wild-type NELL2 responded to E2 treatment with a further increase in survival rate, but E2 failed to induce cell survival in the presence of either NELL2–ΔEGF–Ca2+ or NELL2–ΔEGF (Fig. 6, B and C). These results suggest that the survival promoting effect of E2 on HiB5 cells requires NELL2 intermediacy and that a defective NELL2 protein blocks the
without E2 (Fig. 7). Neither of the two mutant forms of NELL2 tested was able to rescue the cells from Aβ-induced apoptotic death. These results indicate that, as previously suggested (6), the survival-promoting effect of NELL2 is due to an anti-apoptotic effect. They also support the conclusion that NELL2 is required for E2 to protect cells from apoptosis.

To confirm the involvement of NELL2 in E2-induced cell survival, we performed one more set of TUNEL assays on HiB5 cells that was treated with E2 after knocking down endogenous NELL2 mRNA and ERα protein in the hippocampus and hypothalamus. Coronal sections of the female rat brain were first incubated with an ERα antibody, and the reaction was developed to a brown color. Thereafter, the sections were hybridized with a 35S-labeled NELL2 cRNA probe (white grains). A, the dark field image shows an abundance of NELL2 mRNA-containing cells in the hippocampus including CA1 to CA3 layers and dentate gyrus. B, the dark field image shows that most cells of the VMH contain NELL2 mRNA transcripts. C, shown is expression of NELL2 mRNA (black grains) and ERα protein (brown) in the hippocampus. Arrows point to examples of colocalization in the hippocampal layers CA1, CA2, and CA3. D, localization of ERα protein in the ventrolateral portion of the VMH is shown. E, a higher magnification image highlights the colocalization of NELL2 mRNA and ERα protein in the CA1 layer of the hippocampus. F, a high magnification image illustrates the colocalization of NELL2 mRNA and ERα protein in the ventrolateral region of the VMH (boxed region in the panel D). Bars, 250 μm (A–D) and 20 μm (E and F). 3V, third ventricle.

**FIGURE 5.** Localization of NELL2 mRNA and ERα protein in the rat hippocampus and hypothalamus.

Survival promoting action of E2, likely by antagonizing the effect of endogenous wild-type NELL2.

**Effect of NELL2 on Aβ-induced Apoptosis**—To determine whether the effect of NELL2 on the survival of HiB5 cells is due to an anti-apoptotic effect, we performed TUNEL assays. The number of TUNEL-positive cells showing a red coloration indicative of apoptotic nuclear condensation (Supplemental Data 3) increased after Aβ treatment (Fig. 7A). This increase was reduced in cultures expressing wild-type NELL2 with or without E2 (Fig. 7A). Neither of the two mutant forms of NELL2 tested was able to rescue the cells from Aβ-induced apoptotic death. These results indicate that, as previously suggested (6), the survival-promoting effect of NELL2 is due to an anti-apoptotic effect. They also support the conclusion that NELL2 is required for E2 to protect cells from apoptosis.

To identify intracellular signaling molecules involved in the cell survival effect of NELL2, we performed Western blot analysis using the same experimental settings as Fig. 7B. Western blot analysis revealed that E2 increased phosphorylation of ERK1/2 in the Aβ-treated HiB5 cells (Fig. 7C), as previously reported in other systems (for a review, see Ref. 11 and 29), whereas it decreased pro-apoptotic protein Bax (Fig. 7D). Interestingly, NELL2 siRNA exerted an effect exactly opposite to E2 action on the phosphorylation of ERK and Bax expression. The increase of Bax by NELL2 siRNA further supports anti-apoptotic effect of NELL2. These results suggest that normal synthesis and action of NELL2 is critical for E2-induced cell survival.

To confirm the involvement of NELL2 in E2-induced cell survival, we performed one more set of TUNEL assays on HiB5 cells that was treated with E2 after knocking down endogenous synthesis of NELL2 with a NELL2 siRNA. Transfecting the cells with NELL2 siRNA resulted in a more than 90% loss of endogenous NELL2 mRNA and significantly inhibited the anti-apoptotic effect of E2 while enhancing the apoptotic effect of Aβ (Fig. 7B). These results suggest that normal synthesis and action of NELL2 is critical for E2-induced cell survival.
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**FIGURE 6. Effect of NELL2 on the survival of HiB5 cells.** The cells stably expressing wild-type NELL2 or mutant NELL2 forms were plated at a density of 1 × 10^6 cells per well for 24 h and pretreated with E2 or vehicle for 24 h. The cells were then treated with neurotoxins for 20 h, and methylthiazolyltetrazolium (MTT) assays were performed. Results were calculated and presented as % of viable cells with regard to the total numbers of cells. A, schematic drawings show NELL2 and mutant NELL2 lacking three EGF-like Ca^{2+} binding domains (NELL2-ΔEGF-Ca^{2+}) and mutant NELL2 lacking all 6 EGF-like domains (NELL2-ΔEGF). TSP-N, N-terminal thrombospondin B, shown is cell viability after treatment with Aβ peptide (10 μM) in the presence or absence of E2 pretreatment. C, viability of the cells treated with AMPA (5 mM) in the presence or absence of E2 pretreatment. Bars represent the means ± S.E. (n = 6), *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus control group for each treatment.

**DISCUSSION**

We have previously reported that hypothalamic NELL2 mRNA abundance is increased by E2 treatment (15) and that NELL2 is involved in the E2-dependent formation of the SDN-POA in male rats (6). In the present study we show that E2 and its receptors, ERα and ERβ, trans-activate the NELL2 gene and that NELL2 mediates the protective effect of E2 on cultured neuronoprogenitor cells against cell death-inducing treatments.

A stimulatory effect of E2 on NELL2 transcription appears to be at odds with the decrease in NELL2 mRNA that occurs at the time of female puberty (23). NELL2 mRNA abundance increases in the hypothalamus during the juvenile period, when circulating E2 levels are low and decreases, instead of increasing, at the time of puberty, when circulating E2 levels are elevated. It is possible that the juvenile increase in NELL2 synthesis is independent from changes in E2 secretion and that the stimulatory effect of high (pubertal) E2 levels is counteracted by a repressive E2-independent mechanism set in motion at the time of puberty. Further investigation is obviously required to clarify this phenomenon.

Sequence analysis revealed that the mNELL2 promoter does not have classical palindromic ERE(s). Instead, it contains two half-EREs and seven SP1REs. Both half-EREs strongly bind ERα and ERβ in EMSA, suggesting that these domains physically interact with ERα and ERβ. This interaction was further confirmed by supershift assays and ChIP assays. Deletion of each of the two half-EREs resulted in a significant decrease of E2-ER-dependent promoter activation. Thus, both the half-EREs are required for full E2-ER-dependent transactivation of NELL2. Earlier studies have demonstrated that E2-ER complexes regulate target gene promoters containing half-EREs (30, 31). Gel shift assays showed that ER specifically bound two conserved half-EREs in the pro-thymosin α gene (30) and several half-EREs in the Na^{+}/H^{+} exchanger regulatory factor gene (31). The half-EREs in these genes were critical for the ERα- and ERβ-dependent transcriptional regulation, although binding affinities of these half-EREs to ER are much less than that of the canonical palindromic ERE. EMSA and ChIP assays and promoter analyses in our study revealed that E2-ERs regulate transcription of the NELL2 gene also by directly binding to half-EREs.

Interestingly, deletion of some SP1REs differentially affected the ERα and ERβ dependence of NELL2 transcription; deletion of SP1RE at −209 and −430 decreased ERβ-dependent-, but not the ERα-dependence of NELL2 transcription. The mechanism underlying this difference remains to be ascertained. The decrease of ERβ-dependent activation of NELL2 transcription by deletion of SP1RE at −209 and −430 suggests that the ERβ likely interacts with SP1 bound to these SP1REs. E2-ER action through interaction with SP1-SP1RE complexes has been reported to occur in several different genes and shown not to involve direct ER binding to SP1RE (26–28). Instead, ER appears to bind to SP1 and enhance SP1 binding to SP1REs.
E2 is an important steroid hormone that exhibits multiple effects on various tissues including the nervous system. E2 exerts neuroprotective action in various model systems where it reduces neuronal damage caused by serum deprivation (43, 44), β-amyloid treatment (45, 46), and exposure to glutamate (46, 47). In the present study we found that E2 protects HiB5 cells against toxicity induced by AMPA and Aβ peptide. Earlier studies suggested that the neuroprotection exerted by E2 may be mediated by rapid intracellular signaling events in addition to ERE-mediated transcriptional regulation (for review, see Ref. 11 and 29). For example, activation of the MAPK pathway is necessary for E2 to exert its neuroprotective effects, because blockade of this pathway with a mitogen/extracellular-signal regulated kinase kinase-1 (MEK-1) inhibitor prevented E2-dependent neuroprotection (48). MAPK activation was induced by E2 even in cells transfected with a mutated Erα lacking the ability to mediate ERE-dependent transcription, suggesting that MAPK-mediated neuroprotective action of E2 does not require E2-ER-dependent transcription (48).

Aihara et al. (8) reported that recombinant rat NELL2 promoted survival of primary cultured hippocampal and cortical neurons via the MAPK-dependent pathway. These authors found that NELL2-induced neuronal survival is partly due to inactivation of ERK and activation of c-Jun N-terminal kinase (JNK). In the present study, however, a decreased availability of NELL2 by siRNA induced a decrease of ERK phosphorylation and increase of Bax expression and, in turn, resulted in an increase of apoptosis. Discrepancy between the two studies may be due to differences of cells and treatments.

In this study we showed that E2-ERs activate NELL2 transcription and that NELL2 promotes neuroprogenitor HiB5 cell survival. Therefore, E2-induced survival of HiB5 cells may be at least in part mediated by ER-mediated trans-activation of NELL2. E2 is involved in phosphorylation of ERK through membrane receptors (11, 29), and ERK signaling induces a decrease of Bax expression (for review, see Ref. 49). Thus, our results, showing that NELL2 siRNA induced changes in ERK phosphorylation and Bax expression in the presence of E2 suggest that NELL2 may partly be also responsible to the non-genomic E2 action on ERK and Bax signaling for cell survival.

Because NELL2 is a released protein (3) and a larger portion of NELL2 was found in the culture medium than in cellular extracts of HiB5 cells overexpressing NELL2 (6), it seems plausible that the survival promoting effect of transfected NELL2 in HiB5 cells might require a paracrine effect of NELL2 released to the culture medium. Whether NELL2 is recognized by a specific receptor is not known.

The survival effect of E2 was inhibited not only by siRNA-mediated blockade of NELL2 synthesis but also by NELL2 mutants lacking EGF-like domains, suggesting that these domains are required for the survival effect of E2. It also appears that NELL2 mutants lacking EGF-like domains act as antagonists of NELL2, although the mechanism underlying this effect is not known.

In summary, our results indicate that the NELL2 gene is trans-activated by E2 and that NELL2 mediates the survival promoting effect of E2 on HiB5 neuroprogenitor cells. The
detailed cellular mechanism underlying this effect remains to be further identified.

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