

Estrogen-dependent Transcription of the *NEL-like 2* (*NELL2*) Gene and Its Role in Protection from Cell Death^{*[S]}

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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 (ER α and ER β) 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)⁴-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

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⁴ The abbreviations used are: EGF, epidermal growth factor; NEL, EGF-like protein; A β , amyloid β -(25–35) peptide; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazole-propionate; ChIP, chromatin immunoprecipitation; dbESTs, expressed sequence tags database; E2, estrogen; E2-BSA, bovine serum albumin-conjugated E2; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; ERE, estrogen-response element; ERK, extracellular signal-regulated kinase; *NELL2*, neural epidermal growth factor-like 2; *NELL2*-P, *NELL2* promoter; RT, reverse transcription; SDN-POA, sexually dimorphic nucleus of the preoptic area; siRNA, small interference RNA; SP1RE, SP1 response element; TSS, transcription start site; TUNEL, terminal

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; CS, 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 *Bam*HI site of a phage vector lambda EMBL3 (Promega, Madison, WI). For screening clones containing the *mNELL2* gene, $\sim 1 \times 10^7$ phage plaques were transferred to Hybond N⁺ membrane (Amersham Biosciences) and hybridized to a [α -³²P]dCTP (50 μ Ci/ μ l, 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 GenBankTM accession number NM_016743). Hybridization was performed overnight at 50 °C in a hybridization buffer containing 45% formamide, 5 \times SSC (1 \times SSC: 8765 g of sodium chloride, 4.41 g of sodium citrate/liter), 1 \times Denhardt solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% bovine serum albumin), 1% SDS, 5% dextran sulfate, and 10 mg salmon sperm DNA (Sigma). After hybridization, the membranes were washed with 1 \times SSC and 0.1 \times 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 GenBankTM accession number NM_016743): sense primer, 5'-CCA CCT ATC TAG GTA CCC CAT CC-3'; antisense primer, 5'-ATG GCT CGA CCG GGG AAC AT-3'. The amplified product was cloned into the pGL3 basic luciferase reporter vector (Promega). The sequence of the cloned DNA was verified by comparing it to that of the mouse genome data base.

Primer Extension—To identify the transcription start site (TSS) of the *mNELL2* gene, primer extension of mRNA samples from the mouse brain was employed. A 36-oligonucleotide long primer (5'-TGA GAA CGT TCT GCG TGA TCC TCG GGC TTG GAG CGG-3') complementary to nucleotides downstream from the translation initiation site within exon 2 of the *mNELL2* gene was end-labeled with [γ -³²P]ATP (Amersham Biosciences) and 1 μ l of T4 polynucleotide kinase (Invitrogen) at 37 °C for 60–90 min. Poly(A)⁺ mRNA from mouse brain was hybridized with the radiolabeled primer in 30 μ l of hybridization buffer (40 mM bis-Tris, pH 6.4, 0.4 M NaCl, 1 mM EDTA, and 50% formamide) at 65 °C for 90 min. After the mixture was slowly cooled down to room temperature, the hybridized mRNA was reverse-transcribed at 42 °C for 1 h using Superscript II Moloney murine leukemia virus reverse transcriptase (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/BL6) 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 TGT GAA CTC GTG-3') designed from the DNA sequence located on exon 4; sense primer 1 (5'-GGT AGA ATC CCC TTG CCT TGC CCT TTG AC-3') located upstream of exon 1; sense primer 2 (5'-CCT TCG GTC TCC CGC GCC TGT C-3') spanning a region located upstream of exon 1 and the 5' end of exon 1; sense primer 3 (5'-CGC CCG ATT TCG AGG GGG-3') corresponding to the 3' end of exon 1; sense primer 4 (5'-CCA TTG TCT CCG CCT TTC CAA C-3') located on intron 1; sense primer 5 (5'-GAT TTC GAG GGG GGA GGG AGA CGA T-3') spanning the 3' end of exon 1 and 5' end of exon 2; sense primer 6 (5'-GAC GAT GGA CTG AGA CGA TGC AC-3') on exon 2; sense primer 7 (5'-CGT TCT GCG TGA TCC TCG GGC TTG-3') located at 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 QuikChangeTM 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'-CCC CCT CGA AAC AGC TGT CGG GC-3'; antisense primer, 5'-GAG GGA GAC GAT GGC AGC TGA CTG AGA C-3'), (b) a primer set for deleting TATA-like sequence (5'-CCC AGC ACC TCT TGG TTT TTA TCT TAA AGA GGG-3' and its complementary sequence (CS)), (c) a set for deleting half-ERE (5'-CCC 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'-CCT TGC CTT GCC CTT CTT GCT GCT GTG TAG-3' and its CS) located at −1047 of the promoter, (e) the SP1RE sequence (5'-GGC TGC GGG CGT GCG GGA GCG ATG CGC GCA GAG-3' and its CS) at −209, (f) the SP1RE sequence (5'-GGC TCG CGC CTC CCG GAG CGG TCT C-3' and its CS) at −430, (g) SP1RE sequence (5'-GAA AAA GAG GCC GCC GCG CCC CGG CTC-3' and the CS) 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

(*NELL2*-P)-luciferase reporter constructs (*NELL2*-pGL3) using Lipofectamine/PLUS (Invitrogen) and rat ER α and ER β 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 μ g), 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'-CGG TAG GTG GTT CCC TTC AT-3', and antisense primer, 5'-CAG GAC TGC ATA ATG GGA CG-3'; glyceraldehyde-3-phosphate dehydrogenase sense primer, 5'-GTG ATG GCA TGG ACT GTG GT-3', and antisense primer, 5'-TGG TGA AGG TCG GTG TGA AC-3'. Real-time PCR reactions (20- μ l total volume containing 5 pmol of primer, 10 μ l of SYBR Green dye (Qiagen, Valencia, CA), and 2 μ l 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 μ g) 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 (5×10^6) cells were transfected with 1 μ g each of ER α and ER β 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 - 32 P]ATP and purified over a NICK column (Amersham Biosciences). The binding assay was performed as described (20) with minor modifications using 10 μ g of nuclear protein extracts, 20,000 cpm of probe, and 1 μ g of poly(dI-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'-GGA TCT AGG TCA CTG TGA CCC CGG ATC-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 Agt aca CTG TGA CCC CGG 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 m*NELL2* promoter (-223), (d) a probe containing the other half-ERE sequence (5'-TGC CCT TTG ACC CTT 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'-CTC GCG CCC CGC CCT CCC G-3') at -430 , and (g) the SP1RE sequence (5'-AGG CCG CCC CGC CCG 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 μ g of antibodies against ER α (Affinity BioReagents, Golden, CO) and ER β (Santa Cruz) at 4°C overnight. Immune complexes were collected by reacting with 60 μ l 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 M LiCl. DNA from the protein-DNA cross-links was extracted by incubating the reactions with solution (1% SDS, 0.1 M NaHCO $_3$, 10 μ g RNase, and 0.3 M 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'-CGC CTT 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 5'-ATG GGG TAG AAT CCC CTT GC-3'; antisense, 3'-AGC CCA GAC TGA GAG GCT TT-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 μ m) 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 [35 S]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 ER α -expressing cells in the rat brain. Floating 30- μ m sections were incubated for 18–20 h at 4°C with a mouse monoclonal ER α 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% H_2O_2 . 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 Ca^{2+} -binding EGF-like domains (3). Using overlapping PCR, we prepared two different mutant cDNAs encoding proteins lacking EGF-like domains: *NELL2*- $\Delta\text{EGF-Ca}^{2+}$ (lacking the three Ca^{2+} -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 mutants: sense primer for *NELL2*- ΔEGF , 5'-CTC AGA ACA GCG GCC GCG AAG TCA TAA CC-3'; antisense primer for *NELL2*- ΔEGF , 5'-ATG GAA AGA ACT GCG CGG CCG CAC AG-3'; sense primer for the first round of PCR generation of *NELL2*- $\Delta\text{EGF-Ca}^{2+}$, 5'-AAT GTC CCG GCC GCT TCA CAG TAG GCA TTG-3'; antisense primer for the first round of PCR for *NELL2*- $\Delta\text{EGF-Ca}^{2+}$, 5'-TCG TGT GCG GCC GCA CGG AAC-3'; sense primer for the second round of PCR for *NELL2*- $\Delta\text{EGF-Ca}^{2+}$, 5'-CAA TGT CGC GGC CGC TGT CTC AC-3'; antisense primer for the second round of PCR for *NELL2*- $\Delta\text{EGF-Ca}^{2+}$, 5'-ATG GAA AGA ACT GCG CGG CCG 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 $\mu\text{g}/\text{ml}$) (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) ($\text{A}\beta$) 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 methylthia-

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 fluorescence 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 GenBankTM accession number NM_016743.1), the existence of an exon 1 has also been reported (GenBankTM 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

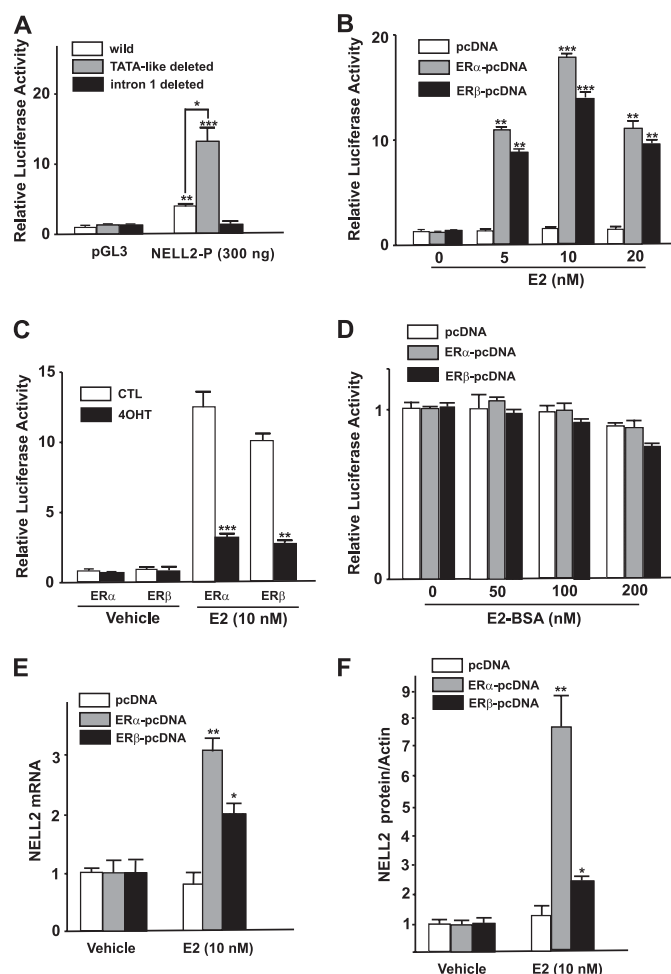


FIGURE 2. Promoter assays assessing E2 action on *NELL2* transcription. Luciferase reporter constructs (pGL3) containing the 5'-flanking region of the *mNELL2* gene (NELL2-P) were transfected into HiB5 cells with or without expression vectors carrying the rat ER α and ER β -coding region and treated with E2 at the indicated concentrations. The cells were harvested for luciferase and β -galactosidase assays 24–30 h after transfection. **A**, shown is the effect of deleting the TATA-like sequence and intron 1, respectively, on the transcriptional activity of the *NELL2* promoter. **B**, transactivation of the *NELL2* promoter by different concentrations of E2 in the presence of expression vectors (50 ng each) encoding ER α (ER α -pcDNA) and ER β (ER β -pcDNA) is shown. **C**, shown is the effect of the ER antagonist, 4-hydroxytamoxifen (4OHT, 50 nM), on the E2-ER-induced transcriptional activation of the *NELL2* promoter. CTL, control. **D**, the absence of transactivation effect of E2-BSA on the *NELL2* promoter is shown. **E** and **F**, shown is the effect of E2-ERs on the HiB5 cell endogenous expression of *NELL2* mRNA (**E**), determined by real-time PCR, and protein (**F**), analyzed by Western blotting, indicated as arbitrary units. β -Actin was used as an internal control for Western blotting. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control (pGL3 for **A**, 0 nM for **B**, CTL for **C**, and vehicle for **E** and **F**). Results are the means \pm S.E. of at least six (**A–C**) and four (**D–F**) wells per group.

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 NELL2-P (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 ER α and ER β 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 ER α and ER β .

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 ER α (Fig. 3A) or ER β (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 –453 showed almost no shifted band, whereas SP1REs at –209 and –430 had shifted bands with ER β (Fig. 3B), suggesting that E2-ER β may indirectly interact with these SP1REs through binding to SP1 (26–28). Preincubation of the nuclear extracts with ER α antibody delayed migration of the protein-DNA complex generated with a probe containing half-EREs at –223 and –1047 (data not shown), indicating that ER α 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 ER α and ER β 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 ER α and ER β

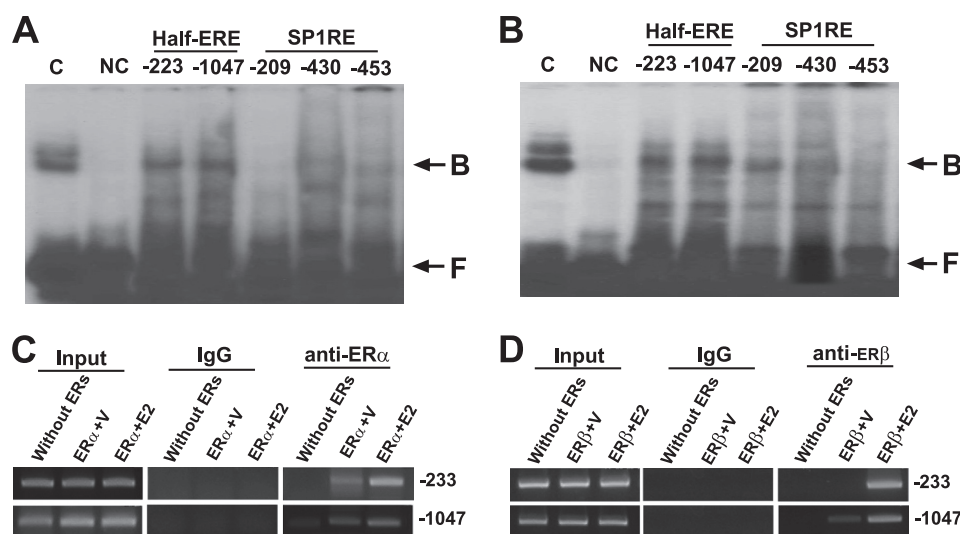


FIGURE 3. EMSAs and ChIP assays. EMSAs were performed using double-stranded oligomer probes containing the putative half-EREs and SP1REs found in the *mNELL2* gene 5' flanking sequence (see "Experimental Procedures"). **A** and **B**, autoradiograms show binding of half-EREs and SP1REs derived from the *mNELL2* promoter to nuclear extracts (10 μ g) prepared from HiB5 cells transfected with ER α (**A**) and ER β (**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 ER α (**C**) and ER β (**D**) antibodies. HiB5 cells were transfected with expression vectors for the ER α (**C**) and ER β (**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-ER α and E2-ER β 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-ER α (Fig. 4A) and E2-ER β (Fig. 4B). Deletion of the proximal half-ERE (at -223), located in intron 1, obliterated E2-ER β -induced promoter activation (Fig. 4B), suggesting that this is a critical site mediating the effect of E2-ER β on *NELL2* gene transcription. The -223 site also appears to be the most important for E2-ER α 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 via 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-ER β (Fig. 4B) but not that by E2-ER α (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.

ER α Protein and *NELL2* mRNA Are Coexpressed in the Rat Hippocampus and Hypothalamus—Combined *in situ* hybridization/immunohistochemistry was performed to determine whether cells synthesizing ER α protein in the rat brain also express *NELL2* mRNA. The hippocampus and hypothalamus were selected as examples, because they are well known to contain ER α -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 ER α 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 ER α (Fig. 5D). A higher magnification view of this region shows that ER α -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 ER α , 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 A β (Fig. 6B) and AMPA (Fig. 6C). However, mutant *NELL2* lacking either the Ca²⁺ binding EGF domains (*NELL2*- Δ EGF-Ca²⁺) or all six EGF-like domains (*NELL2*- Δ EGF) 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 A β (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*- Δ EGF-Ca²⁺ or *NELL2*- Δ EGF (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

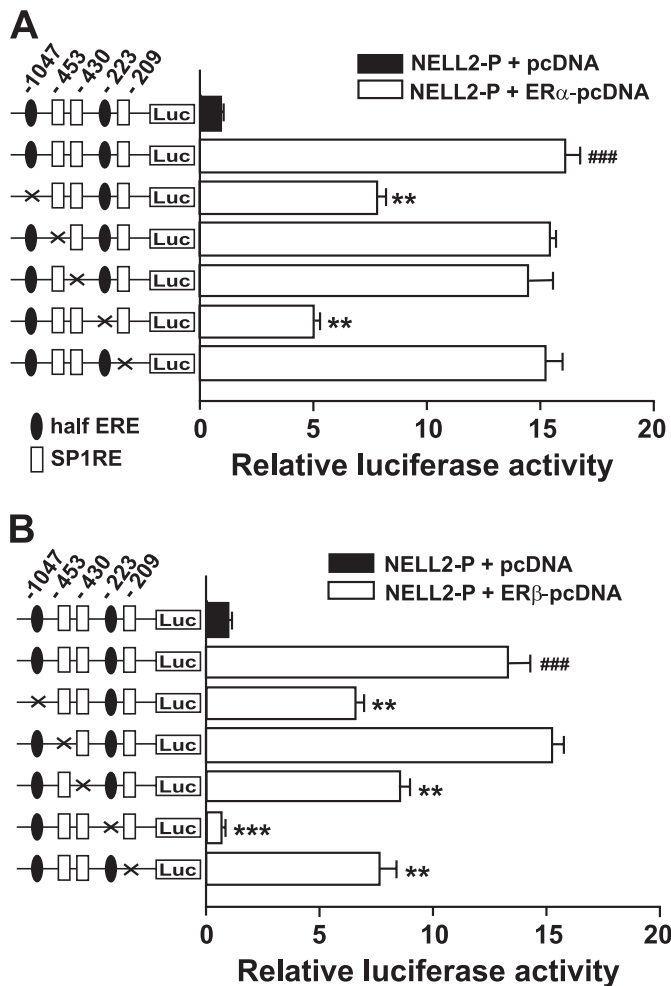


FIGURE 4. Effect of site-specific deletion of half-EREs on the transactivation of the *NELL2* promoter by E2-ERs. Single mutants carrying individual deletions of the half-EREs and SP1REs at -1047 , -453 , -430 , -223 , and -209 were cotransfected with 50 ng each of ER α (ER α -pcDNA, **A**) or ER β (ER β -pcDNA, **B**) expression vectors and treated with E2 (10 nM). The cells were harvested for luciferase and β -galactosidase assays 30 h after transfection. The positions of the deleted binding sites are indicated. The data are the means \pm S.E. of nine wells per construct. ###, $p < 0.001$ versus NELL2-p + pcDNA; **, $p < 0.01$; ***, $p < 0.001$ versus wild NELL2-p + ER α -pcDNA or ER β -pcDNA.

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

Effect of *NELL2* on $A\beta$ -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 $A\beta$ 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 $A\beta$ -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

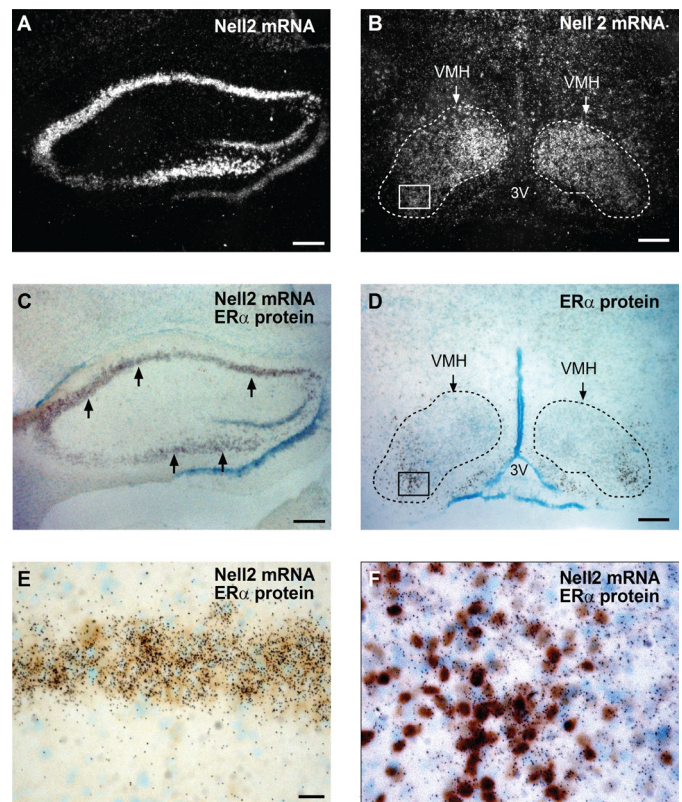


FIGURE 5. Localization of *NELL2* mRNA and ER α protein in the rat hippocampus and hypothalamus. Coronal sections of the female rat brain were first incubated with an ER α antibody, and the reaction was developed to a brown color. Thereafter, the sections were hybridized with a 35 S-labeled *NELL2* cRNA 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 ER α protein (brown) in the hippocampus. Arrows point to examples of colocalization in the hippocampal layers CA1, CA2, and CA3. **D**, localization of ER α protein in the ventrolateral portion of the VMH is shown. **E**, a higher magnification image highlights the colocalization of *NELL2* mRNA and ER α protein in the CA1 layer of the hippocampus. **F**, a high magnification image illustrates the colocalization of *NELL2* mRNA and ER α protein in the ventrolateral region of the VMH (boxed region in the panel **D**). Bars, 250 μ m (**A–D**) and 20 μ m (**E** and **F**). 3V, third ventricle.

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 $A\beta$ (Fig. 7B). These results suggest that normal synthesis and action of *NELL2* is critical for E2-induced cell survival.

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 $A\beta$ -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 the cell survival effect of *NELL2* is disclosed by its action on a signaling pathway of ERK and Bax.

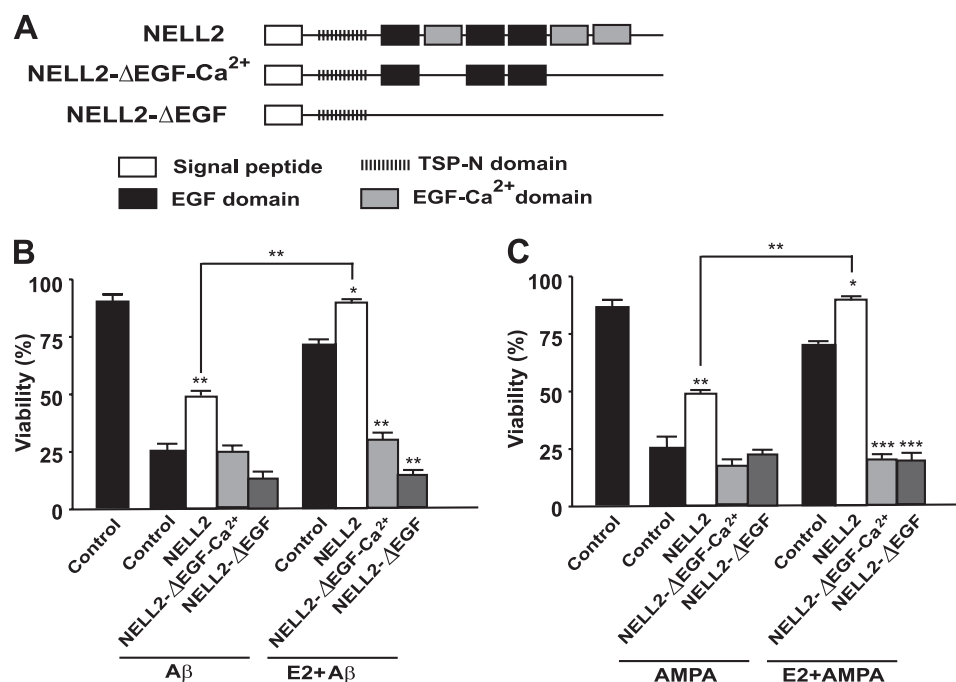


FIGURE 6. Effect of *NELL2* on the survival of HiB5 cells. The cells stably expressing wild-type *NELL2* or mutant *NELL2* forms were plated at a density of 1×10^6 cells per well for 24 h and pretreated with E2 or vehicle for 24 h. The cells were then treated with neurotoxins for 20 h, and methylthiazolyltetrazolium (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 Ca²⁺ binding domains (*NELL2*-ΔEGF-Ca²⁺) 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 neuroprogenitor 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 m*NELL2* 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 prothymosin 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-dependent activation 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.

Sequence analysis and activity assays of the m*NELL2* promoter revealed that the promoter contains neither a functional TATA box nor a CAAT box. Instead, the *NELL2* promoter appears to be GC-rich, a feature shared with other GC-rich promoters that contain several SP1REs and multiple TSSs spread over a large region (32). The majority of the genes lacking a TATA box exhibit multiple TSSs, which is a typical feature of housekeeping genes (33) that also contain SP1REs upstream from the TSSs (34). However, some members of this class do not have housekeeping functions but instead appear to be involved in regulation of growth and differentiation, such as the genes encoding c-Ki-ras (35), the EGF receptor (36), and nerve growth factor receptor (34). *NELL2* is also believed to be involved in regulating proliferation and differentiation processes (5, 7), and thus, it may share similar mechanisms of transcriptional control with the aforementioned genes (34). It has been suggested that SP1 may control the accuracy of transcription initiation in TATA-less genes (37–39). Seven conserved

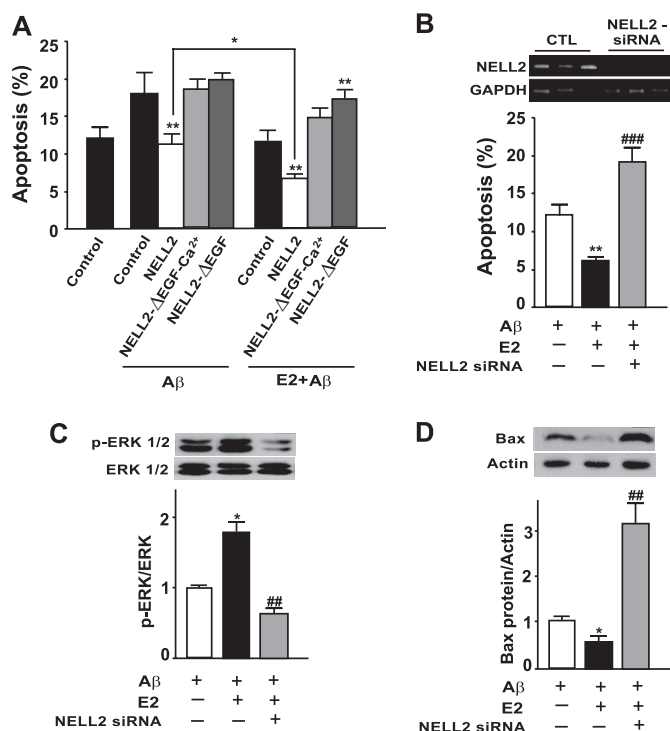


FIGURE 7. *NELL2* decreases apoptosis of HiB5 cells. HiB5 cells stably expressing wild-type *NELL2* or the *NELL2* mutants indicated in the figure were treated with E2 at a concentration of 10 nM for 24 h followed by exposure to 10 μ M A β for 20 h. Cells were stained using the TUNEL method. **A**, quantitative analysis of the results showing differences in the number of TUNEL-positive cells between treatment groups ($n = 6$). **, $p < 0.01$ versus control for each treatment group. **B**, blockade of endogenous *NELL2* synthesis via RNAi prevents E2-dependent protection from A β -induced apoptosis. The cells were transfected with *NELL2* siRNA and then treated with E2 for 24 h followed by treatment with A β for 20 h. The gel on top of the graph illustrates the results of a representative experiment assessing the content of *NELL2* mRNA by RT-PCR. Results are shown as the percentage (%) of apoptotic cells ($n = 8$ /group). CTL, control. GAPDH, glyceraldehyde phosphate dehydrogenase. **, $p < 0.01$ versus A β ; ###, $p < 0.001$ versus A β + E2. **C** and **D**, shown is the effect of *NELL2* siRNA on the phosphorylation of ERK1/2 (**C**) and content of Bax (**D**) in the HiB5 cells treated with A β and E2. Proteins extracted from the cells were subjected to Western blotting. Autoradiograms on top show representative results of repeated experiments ($n = 4$). Data were calculated in comparison with bands generated by total ERK proteins (**C**) and β -actin (**D**) and are indicated as arbitrary units. *, $p < 0.05$ versus A β ; ##, $p < 0.01$ versus A β + E2.

SP1REs are found upstream from the TSS located at the 5' end of exon 2 in m*NELL2* gene. Despite these features, the m*NELL2* promoter also contains putative binding sites for transcriptional regulators such as retinoic acid receptor/retinoid X receptor and progesterone receptor/glucocorticoid receptor, suggesting that it may be subjected to inducible control by factors binding to these sites.

Our transcription assays demonstrated that deletion of intron 1 of *NELL2* gene resulted in loss of basal promoter activity, suggesting that intron 1 is essential for normal *NELL2* transcription. In addition to the promoter region, the intron 1 contains a half-ERE at -223 and several SP1REs. Deletion of these sequences resulted in a decrease of E2-ER-dependent stimulation of *NELL2* transcription, supporting the notion that intron 1 is also important for the transcriptional regulation of *NELL2* gene by E2. Such an involvement of intronic regions in transcriptional regulation has also been reported for several other genes, including those encoding apolipoprotein B (40), renin (41), and vasopressin V_{1b} receptor (42).

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 A β 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 ER α 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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