Molecules and Cells

Transcriptional Regulatory Role of NELL2 in Preproenkephalin Gene Expression

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Preproenkephalin (PPE) is a precursor molecule for multiple endogenous opioid peptides Leu-enkephalin (ENK) and Met-ENK, which are involved in a wide variety of modulatory functions in the nervous system. Despite the functional importance of ENK in the brain, the effect of brain-derived factor(s) on PPE expression is unknown. We report the dual effect of neural epidermal growth factor (EGF)-like-like 2 (NELL2) on PPE gene expression. In cultured NIH3T3 cells, transfection of NELL2 expression vectors induced an inhibition of PPE transcription intracellularly, in parallel with downregulation of protein kinase C signaling pathways and extracellular signal-regulated kinase. Interestingly, these phenomena were reversed when synthetic NELL2 was administered extracellularly. The *in vivo* disruption of NELL2 synthesis resulted in an increase in PPE mRNA level in the rat brain, suggesting that the inhibitory action of intracellular NELL2 predominates the activation effect of extracellular NELL2 on PPE gene expression in the brain. Biochemical and molecular studies with mutant NELL2 structures further demonstrated the critical role of EGF-like repeat domains in NELL2 for regulation of PPE transcription. These are the first results to reveal the spatio-specific role of NELL2 in the homeostatic regulation of PPE gene expression.

Keywords: calcium ion, endoplasmic reticulum, extracellular signal-regulated kinase, NELL2, preproenkephalin, protein kinase C

INTRODUCTION

Enkephalins (ENKs) are endogenous opioid peptides that are involved in a wide variety of physiological systems including cardiovascular system, thirst, feeding, analgesia, and gastrointestinal functions (Bodnar, 2017; Duque-Diaz et al., 2019). In the brain, ENKs are neuromodulators involved in motivation such as drinking, feeding and reproductive behavior and emotional behavior (Bodnar, 2017; Henry et al., 2017). ENKs, including Leu-ENK and Met-ENK, are synthesized from a precursor protein, proenkephalin, which is translated from preproenkephalin (PPE) mRNA (Noda et al., 1982). There is a wide distribution of ENK expression within the brain, with relatively high level observed in the hypothalamic nuclei (Koshimizu et al., 2008). Regulation of PPE gene transcription in the brain is multifactorial and involves neurotransmitters and blood-borne hormones including estrogen (Borsook and Hyman, 1995; Dellovade et al., 1999; Morissette et al., 2008; Quinones-Jenab et al., 1996). In parallel, other investigations have demon-

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strated the involvement of intracellular signaling molecules, such as protein kinase C (PKC) (Borsook and Hyman, 1995). However, little is known about the action of brain-derived neuropeptides on regulation of PPE gene expression.

Here we report that neural epidermal growth factor (EGF)-like-like 2 (NELL2) regulates PPE gene expression in cultured NIH3T3 cells and in the rodent brain. NELL2 was identified as a PKC-interacting molecule through its EGF-like domains (Kuroda and Tanizawa, 1999). In support of this finding, three of six domains in NELL2 share common amino acid residues for Ca^{2+} binding (Handford et al., 1991; Rao et al., 1995), suggesting its function in Ca^{+}-dependent and PKC-mediated cellular events (Kuroda and Tanizawa, 1999; Rao et al., 1995). Therefore, in this context, NELL2 seemed to be an intracellular signaling molecule.

In contrast, multiple investigations have demonstrated that NELL2 is an N-glycosylated secreted protein (Ha et al., 2008; Kuroda and Tanizawa, 1999; Nelson et al., 2004). Once released, NELL2 has been suggested to bind to and activate unknown receptors and subsequently modulate PKC and extracellular signal-regulated kinase (ERK) pathways in target cells (Aihara et al., 2003; Choi et al., 2010; Kim et al., 2014; 2015; Nelson et al., 2004). Recent studies have found that this extracellular NELL2 is a novel ligand for the roundabout guidance receptors Robo 2 and Robo 3 for axon-pathfinding during neural development (Jaworski et al., 2015; Pak et al., 2020; Yamamoto et al., 2019). Therefore, it is likely that NELL2 establishes two independent routes (intracellular and extracellular) to the PKC-ERK signaling pathway.

Similar to PPE, NELL2 expression is relatively high in the hypothalamic nuclei, including the ventromedial hypothalamic nucleus (VMH), arcuate nucleus (ARC), and paraventricular nucleus (PVN) (Jeong et al., 2008a; 2017). Within the hypothalamus, NELL2 is one of the downstream targets of estrogen (Choi et al., 2001; 2010; Jeong et al., 2008b) and is involved in sexual dimorphic brain development and behavior, as well as metabolic regulation (Ha et al., 2008; Jeong et al., 2008b; 2017; Ryu et al., 2011). Furthermore, because PKC is an intracellular target of NELL2 action and is a signaling mediator for PPE gene transcription, NELL2 and PPE seem to be closely correlated in regional and functional aspects.

Thus, we sought to investigate the potential of NELL2 function in the regulation of PPE gene transcription through PKC signaling. Our results demonstrate the dual effect of NELL2 in PPE gene transcription: intracellular overexpression of NELL2 resulted in a decrease in PPE promoter activity following downregulation of PKC and ERK pathways, whereas extracellular NELL2 stimulated ERK signaling and PPE gene transcription. Experiments with mutant NELL2 further demonstrate a key role of the EGF repeat domains in these processes. Together, our results are the first to indicate that both intracellular and extracellular NELL2 contribute to PPE gene transcription by functionally opposing each other in modulating the PKC-ERK pathway.

MATERIALS AND METHODS

Double *in situ* hybridization

Two-month-old male Sprague–Dawley rats and C57BL/6 mice (Hyochang Science, Korea) were used for histochemical analyses (permission number from Animal Care and Use Committee at the University of Ulsan: BJL-21-010). To identify NELL2 expression in the cells expressing PPE, brains of 2-month-old male rats were fixed by transcardiac perfusion of 4% paraformaldehyde-borate buffer, pH 9.5. Thereafter, the brains were postfixed for ~20 h at 4°C in the same fixative containing 10% sucrose, blocked coronally, frozen on dry ice, and stored at −80°C until use. Sections (30 μm) were prepared with a freezing sliding microtome and processed for hybridization as reported previously (Berg-von der Emde et al., 1995).

Hybridization was performed as recommended (Berg-von der Emde et al., 1995) with minor modifications. The probe was prepared by SP6 RNA polymerase-directed *in vitro* transcription with a 333-bp NELL2 cDNA template obtained by polymerase chain reaction (PCR) amplification of the coding region corresponding to nucleotides 548-880 in rat NELL2 mRNA (NCBI GenBank accession No. AY089719). The 252-bp PPE cDNA template was obtained from nucleotides 472-723 in rat PPE mRNA (NCBI GenBank accession No. Y07503). For double-labeled *in situ* hybridization, we used an ^{35}S-UTP-labeled NELL2 cRNA probe and a digoxygenin-UTP-labeled PPE cRNA probe. Following overnight hybridization at 55°C-56°C, the slides were washed and processed for digoxygenin detection as reported (Ha et al., 2008). After dehydration, the slides were dipped in Ilford K5 emulsion (without defatting) for isotopic hybridization and exposed to the emulsion for 3 weeks at 4°C. At this time, the slides were developed, quickly dehydrated, dried, and cover-slipped for microscopic examination.

Immunohistochemistry

Immunohistochemistry procedures in this study followed the descriptions in our previous reports (Jeong et al., 2008a; 2008b; 2017; Kim et al., 2021). Briefly, the brains of mice were collected following 4% paraformaldehyde perfusion and stored at −80°C until use. Coronal cryostat sections of 10 μm thickness were air-dried and incubated for 30 min in phosphate-buffered saline (PBS) and for 1 h in blocking buffer (3% bovine serum albumin, 0.3% Triton X-100 in PBS) at room temperature (RT). The brain sections were incubated overnight at −4°C with rabbit anti-rat NELL2 antibodies (Jeong et al., 2008a; 2008b) diluted in blocking buffer (1:500). After 3 × 10-min washes with slow agitation in PBS, the sections were incubated for 2 h at RT with secondary antibodies (goat anti-rabbit IgG with Alexa Fluor-594: 1:1,000, #ab150084; Abcam, USA). The sections were incubated for 1 h in blocking buffer at RT and incubated overnight at −4°C with primary antibody against PPE diluted in blocking buffer (goat anti-PPE: NBP1-20963, 1:500; Novus Biologicals, USA). The sections were washed 3 × 10-min in PBS and incubated with secondary antibodies (donkey anti-goat IgG with Alexa Fluor-488: #ab150129, 1:1,000; Abcam) for 2 h at RT. Following the series of washing steps, the sections were cover-slipped and subjected to fluorescence microscopy (Axioplan2 Imaging; Carl Zeiss Microimaging, USA).

Plasmids and mutant NELL2 constructs

Transcriptional activity assays were carried out using 5′-flanking sequences of genes cloned into luciferase reporter plasmids. For the PPE gene, we subcloned a 2.7-kb HincII DNA fragment containing nucleotides (nt) −2495 to +207 of the human PPE gene (kindly provided by Dr. L. Kobierski, Harvard Medical School, MA, USA) into pGL2-basic luciferase reporter vector (PPE-Luc). For cFos, we used a construct (a gift from Dr. R. Prywes, Columbia University, NY, USA) containing nt −711 to +45 of human cFos promoter constructed in the luciferase reporter vector (cFos-Luc). Previously, we reported cloning of rat NELL2 cDNA (Kim et al., 2002). NELL2 contains several functional domains: signal peptide, thrombospondin-1 (TSP-N)-like module, five von Willebrand factor C (CR) domains, and six EGF-like domains (Kuroda and Tanizawa, 1999). Using overlapping PCR, we prepared several mutant cDNAs encoding proteins lacking functional domains (Choi et al., 2010): NELL2△EGF-Ca^{2+} (lacking three EGF-like Ca^{2+}-binding domains) and NELL2△EGF (lacking all 6 EGF-like domains). The mutants were cloned into the expression vector pcDNA 3.1-Zeo^{+} (Invitrogen, USA) and confirmed by automatic sequencing.

Cell culture and assays for luciferase activities

Mouse embryonic fibroblast NIH3T3 cells and hippocampal HT22 cells were grown in Dulbecco's modified Eagle's medium (H-DMEM) containing high glucose and 10% bovine calf serum at 37°C in a humidified atmosphere with 5% CO_2. To determine whether endoplasmic reticulum (ER)-localized NELL2 regulates PPE transcription, NIH3T3 cells were transfected transiently with PPE-Luc and expression vectors containing naïve or mutant NELL2 coding region using Lipofectamine/Plus reagents (Invitrogen). NELL2-KDEL carrying the carboxy-terminal ER retention sequence KDEL (Mei et al., 2017; Munro and Pelham, 1987) was kindly provided by Dr. EM Hwang (Center for Functional Connectomics, Korea Institute of Science and Technology [KIST], Seoul, Korea). To chemically retain NELL2 in the ER, brefeldin A (BFA; Sigma-Aldrich, USA), an inhibitor of ER-Golgi trafficking (Chardin and McCormick, 1999), was added to the culture medium of cells transfected with NELL2 expression vectors. To determine the effect of NELL2 on Ca^{2+}-dependent and PKC-induced PPE transcription, phosphorylation of ERK (pERK), and cFos expression, the NIH3T3 cells transfected with NELL2 expression vectors were treated with phorbol 12-myristate 13-acetate (PMA, #524400; Calbiochem, USA) or with Ca^{2+} ionophore A23187 (#100105; Calbiochem). To further identify the effect of extracellularly released NELL2 on PPE-Luc and pERK, the cells were treated with synthetic human NELL2 (sNELL2) protein (#13132-H08B; Sino Biological, USA). To determine effect of cFos on the NELL2-induced PPE transcription, the NIH3T3 cells transfected with cFos siRNA were treated with sNELL2 protein or co-transfected with NELL2 expression vector. The cFos siRNA duplex used for targeting mouse cFos (accession No. NM_010234.3) was composed of sense (5′-AAG GAA AAA CUG GAG UUU AUU UU-3′) and antisense (5′-AAA AUA AAC UCC AGU UUU UCC UU-3′) sequences. Luciferase assays were performed using a luciferase reporter assay kit (Promega, USA). Transfection efficiency was normalized by co-transfecting the β-galactosidase reporter plasmid (pCMV-β-gal; Clontech, USA) at 60 ng/well.

Blockade of NELL2 synthesis

To examine the effect of NELL2 synthesis blockade on PPE mRNA contents in the rat brain, a phosphorothioate antisense-oligodeoxynucleotide (AS-ODN) (Genotech, Korea) was delivered into the lateral ventricle of 2-month-old male rats. The AS-ODN used (5′-CCG GGA TTC CAT GGC GTG CAT-3′) is complementary to a 21-nt sequence encompassing the NELL2 translation initiation site (Kim et al., 2002; Ryu et al., 2011). As a control, scrambled (SCR) sequence of AS-ODN was used: 5′-TAT CGC ATG CGG GCC TAT GCG-3′. For injection, the ODNs were diluted to a final concentration of 0.5 nmol/μl in artificial cerebrospinal fluid (ACSF). A polyethylene cannula (outer diameter, 1.05 mm; inner diameter, 0.35 mm) was implanted stereotaxically into the lateral ventricle (coordinates: AP = 1.0 mm caudal to bregma; V = 3.6 mm from the dura mater; L = 0.16 mm from the midline). After one week of recovery, ODNs were injected through the cannula. To determine effects, AS-ODN (2 nmol in 4 μl ACSF/rat) was injected with a Hamilton syringe once a day for 2 days. The rats were killed 24 h after the second ODN injection, and tissues (hypothalamus and basal cortical regions surrounding the piriform cortex) were collected for western blot analysis of NELL2 protein and northern blot analysis of PPE mRNA.

Northern blot hybridization

To determine the effect of NELL2 synthesis blockade on PPE mRNA content, we performed northern blot hybridization using hypothalamus and piriform cortex injected with NELL2 AS-ODN. Total RNA was prepared using the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987), and a 20 μg sample of total RNA was separated by electrophoresis in a 1.2% formaldehyde-agarose gel containing ethidium bromide. Following transfer to a nylon membrane for 18 h by diffusion blotting, the membrane was dried and cross-linked by UV illumination. The [^{32}P]dCTP-labeled PPE cDNA probe was prepared using the random primer-labeling method (Choi et al., 2001; Feinberg and Vogelstein, 1983). The labeled probe was separated from unincorporated [^{32}P]dCTP by a Nick column (Amersham-Pharmacia Biotech, UK). Prehybridization was carried out at 42°C for 2 h in a heat-sealed plastic bag with a hybridization buffer. After addition of the ^{32}P-labeled cDNA probe, hybridization was performed at 42°C for 20 h. The membrane was washed twice with 2× SSC and 0.1% SDS at RT for 20 min, followed by a second wash with 0.1× SSC, 0.5% SDS, and 5 mM EDTA (pH 8.0) at 50°C for 5 min. The membrane was dried and exposed to X-ray film at −70°C for 2-4 days.

Real-time PCR

To determine the effect of NELL2 on endogenous PPE expression, RNA (2 μg) was isolated from NIH3T3 cells transfected with control vector or NELL2 expression vector. Total RNA samples were extracted using the Sensi-TriJol™ reagent (#LGR-1117; Lugen Sci, Korea) according to the manufacturer's instructions. cDNA was synthesized using MMLV reverse

transcriptase (#3201; Beams Bio., Korea). After reverse transcription, real-time PCR analyses were performed with BrightGreen 2× qPCR MasterMix-ROX (ABM, Canada) using a StepOnePlus Real-Time PCR System (Applied Biosystems, USA) for ~40 cycles. The target DNA species were amplified by real-time PCR using the following primer sets: PPE sense primer, 5′-CGA CAT CAA TTT CCT GGC GT-3′; PPE antisense primer, 5′-AGA TCC TTG CAG GTC TCC CA-3′; cFos sense primer, 5′-GGG AAT GGT GAA GAC CGT GT-3′; cFos antisense primer, 5′-CCG TTC CCT TCG GAT TCT CC-3′; β-actin sense primer, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′; β-actin antisense primer, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G -3′. Data were normalized for gene expression using β-actin as an internal control. The expression pattern of β-actin in our experimental sets did not differ between groups. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to analyze the relative quantification of gene expression.

Western blot analysis

NIH3T3 cells and hypothalamic fragments were homogenized with lysis buffer (mammalian cell protein extract reagent [M-PER], #78501; tissue protein extraction reagent [T-PER], #78510; Pierce Chemical, USA) containing protease inhibitor cocktail (Roche, Switzerland). Briefly, protein concentration was measured by the Bradford assay (#5000006; Bio-Rad, USA), and 20 μg of proteins from each sample were separated by SDS-PAGE and transferred onto PVDF membranes by electrophoretic transfer. The membrane was blocked with 5% non-fat skim milk in TBS-Tween and incubated with antibodies against NELL2 (1:1,000, #sc-390137; Santa Cruz Biotechnology, USA), pERK (1:1,000, #9101; Cell Signaling Technology, USA), ERK (1:1,000, #sc-153; Santa Cruz Biotechnology), or cFos (1:1,000, #sc-7202; Santa Cruz Biotechnology). The membrane was incubated with HRP-conjugated mouse secondary antibody (1:3,000, #7076; Cell Signaling Technology) or HRP-conjugated rabbit secondary antibody (1:3,000, #7074; Cell Signaling Technology), and the immunoreactive signals were detected by chemiluminescent detection reagent (#34095, Thermo Fisher Scientific, USA). Protein density was normalized using β-tubulin (1:3,000, #sc-5274; Santa Cruz Biotechnology) or β-actin (1:4,000, #A5441; Sigma-Aldrich), and Image J software was used to analyze data.

Intracellular localization of NELL2

To determine subcellular localization of NELL2 in the ER, NIH3T3 cells were transfected with NELL2 expression vector fused with enhanced green fluorescence protein at C-terminal (NELL2-EGFP, kindly provided by Dr. EM Hwang, Center for Functional Connectomics, Korea Institute of Science and Technology, Seoul, Korea). pECFP-ER (Clontech) and pDsRed-Mito (Clontech) were used as fluorescence markers for ER and mitochondria, respectively. Transfected cells were fixed with 4% paraformaldehyde and imaged by Confocal microscopy (A1Rsi; Nikon, Japan).

Statistical analyses

Statistical analyses were performed in GraphPad Prism 9 software (GraphPad Software, USA). All data are expressed as the mean ± SEM. Data were analyzed with one-way and two-way ANOVA followed by multiple comparison. A Student's *t*-test was used to compare the two groups.

RESULTS

NELL2 is expressed in a subset of neurons showing enkephalinergic phenotype

Previously, we found that NELL2 is expressed in many regions of the adult rat brain (Jeong et al., 2008a; 2008b; Kim et al., 2002). In particular, the forebrain regions including the VMH, amygdala, and piriform cortex, with abundant expression of endogenous opioid peptides such as ENK (Harlan et al., 1987; Khachaturian et al., 1983), are the major sites for NELL2-containing neurons (Kim et al., 2002). Therefore, we first determined the colocalization of NELL2 and PPE in these brain regions using *in situ* hybridization. Our results showed abundant NELL2 mRNA signals in the mentioned brain regions containing PPE transcripts (Fig. 1A). To confirm our findings at a single-cell level, we performed VMH-targeted immunohistochemistry and observed that neurons positive for PPE immunoreactivity (ir) also were labeled with NELL2-ir, clearly indicating the co-presence of NELL2 and PPE in the same VMH neurons (Fig. 1B). Moreover, co-expression of NELL2 and PPE was also found in caudate putamen (Cp), amygdala (Amy), and piriform cortex (Pir) (Supplementary Fig. S1). These anatomical results indicate that PPE-expressing cells in the brain could be targets of both intracellular and extracellular NELL2.

ER-localized NELL2 suppresses PPE promoter activity

A major focus of this study is to evaluate the potential function of NELL2 on PPE gene transcription. Therefore, we used *in vitro* approaches with NIH3T3 cells, as they possess endogenous machinery to synthesize ENK (Takahashi et al., 1995), to directly test the influence of NELL2 on PPE promoter activity. First, we tested the expressional efficiency of our PPE promoter-luciferase reporter vector (PPE-Luc) in NIH3T3 cells and found that this construct is transcriptionally active over a wide range of concentrations in cultured cells (Fig. 2A). Then, NIH3T3 cells were co-transfected with PPE-Luc together with different concentrations of NELL2 expression vectors, which resulted in a dose-dependent decrease in PPE-Luc activity (Fig. 2B). These results are likely the reflection of the negative regulatory role of NELL2 in PPE transcription and provide the first evidence indicating NELL2 influence on PPE gene expression.

As NELL2 is a secreted glycoprotein that normally follows the conventional release pathway through ER, we identified intracellular localization of NELL2 in the ER using image analysis of NIH3T3 cells transfected with vectors for NELL2-EGFP and ER marker ECFP-ER (Supplementary Fig. S2A) and determined a portion of NELL2 in the culture medium after transfection of NELL2 expression vector (Supplementary Fig. S2B). To further pinpoint the site of NELL2 action, we used a mutant NELL2 (NELL2-KDEL) that carries an ER retention signal in addition to normal functional motifs (Mei et al., 2017; Munro and Pelham, 1987). In this case, mutant NELL2 will be accumulated within the cytoplasm, more specifically in the ER, of transfected cells without being released (Sup-

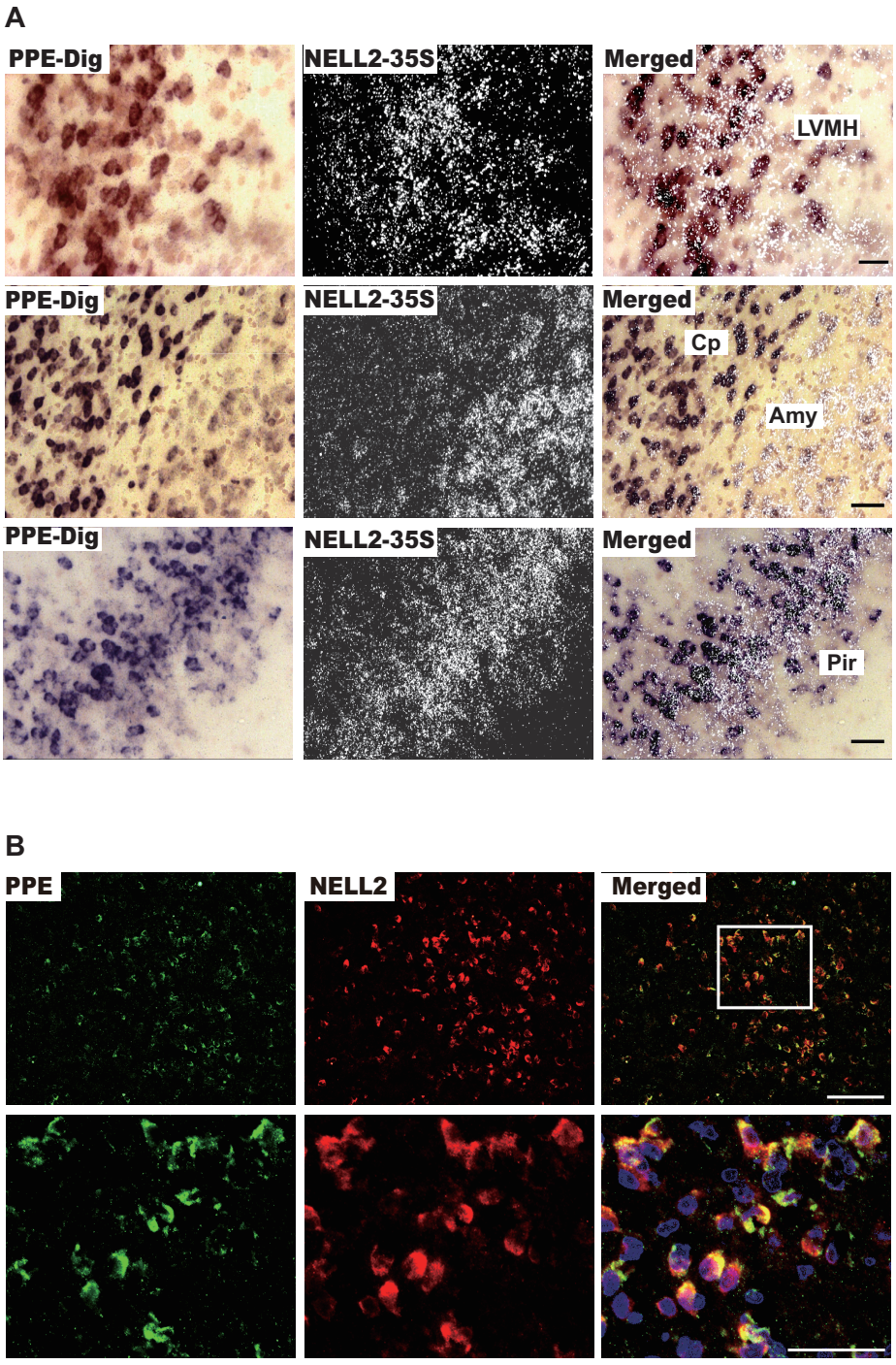

Fig. 1. Localization of NELL2 expression in neurons expressing PPE. (A) To identify NELL2 expression in PPE-positive neurons, we performed double *in situ* hybridization using digoxygenin-labeled (Dig) cRNA probe for PPE and ^{35}S-UTP-labeled (35S) cRNA probe for NELL2. PPE mRNA expression was found in the lateral ventromedial hypothalamic nucleus (LVMH), caudate putamen (Cp), lateral nucleus of the amygdala (Amy), and the piriform cortex (Pir). Silver grains for NELL2 mRNA were found and merged with PPE mRNA signals in LVMH, Amy, and Pir. Scale bars = 10 μm (LVMH and Pir) and 20 μm (Amy). (B) Double immunohistochemistry was applied to coronal brain sections of mice to identify immunoreactive NELL2 proteins (red) in the cells expressing PPE (green). Images shown are of the LVMH of mouse brain. Scale bar = 100 μm. Higher-magnification images from the inset area shown in upper panel. Scale bar = 50 μm.

plementary Fig. S2C). Importantly, transfection of vectors for NELL2-KDEL into the NIH3T3 cells also induced a decrease in PPE-Luc activity (Fig. 2C). In parallel, we performed an independent experiment using BFA, an inhibitor for protein transfer from the ER to Golgi apparatus (Chardin and McCormick, 1999), and observed an inhibitory effect of ER-localized NELL2 on PPE-Luc activity (Fig. 2D, Supplementary Fig. S2D). In line with these results, PPE mRNA level in the cultured cells was decreased with NELL2 expression vector (Fig. 2E). Similar results were found in other cell lines (Supplementary Fig. S3), indicating that these observations are not cell-type-specific. Altogether, these results suggest that ER-localized NELL2 negatively influences PPE transcription.

ER-localized NELL2 inhibits PPE transcription via its EGF-like domains

As mentioned, the NELL2 protein consists of multiple functional domains including EGF-like repeats (Kuroda and

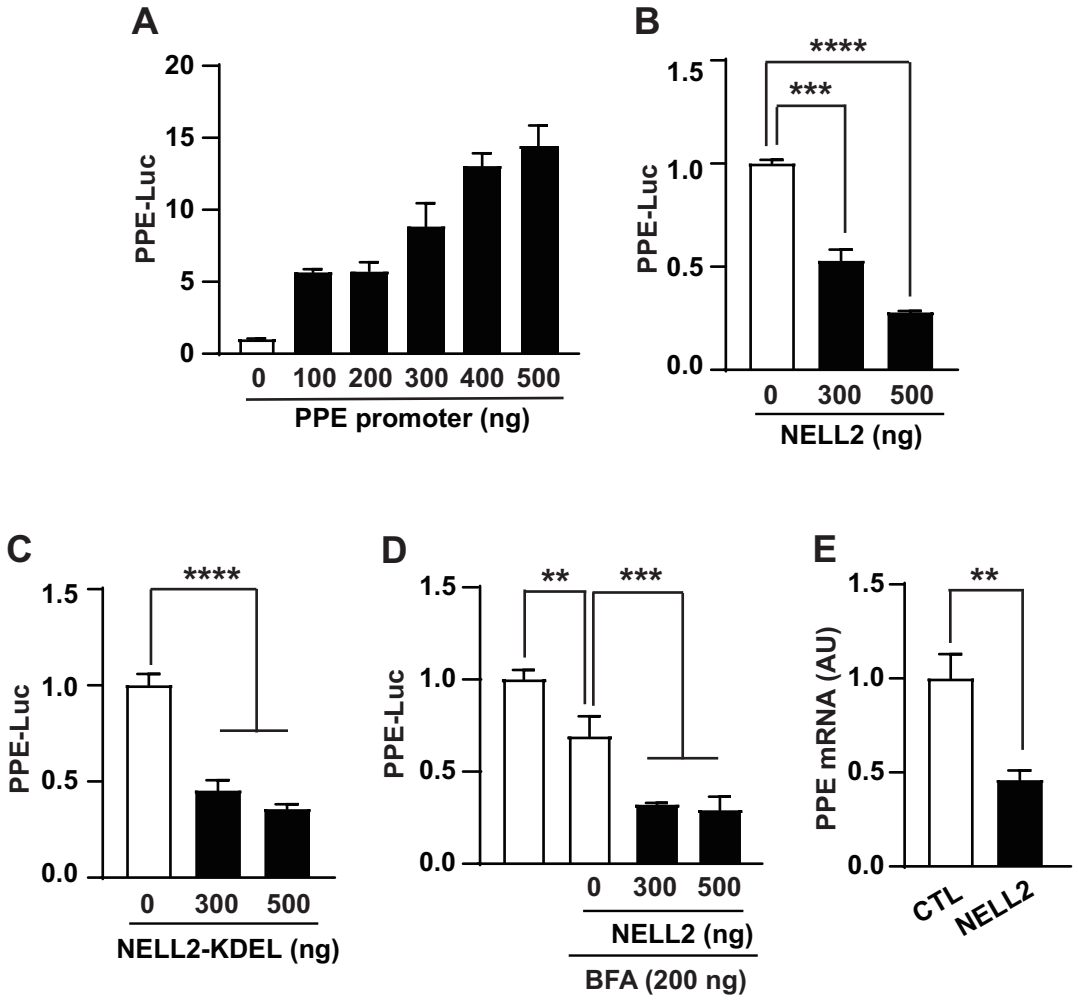

Fig. 2. Effect of ER-localized NELL2 on PPE transcription. (A-D) Luciferase (Luc) reporter vector (pGL2) containing the 5′-flanking region of the PPE gene (PPE-Luc) was transfected into NIH3T3 cells with an expression vector carrying the NELL2-coding region (NELL2) at the concentrations indicated. The cells were harvested for Luc and β-galactosidase assays at 24 h after transfection, and relative Luc activity is shown. (A) A dose-related increase in PPE-Luc activity following transfection of different concentrations of PPE-Luc vectors. (B) Dose-related decrease of PPE-Luc activity by transfected NELL2 expression vector (NELL2). (C) Decrease of PPE-Luc activity by expression vector carrying a NELL2-coding region fused with an ER retention signal at its C-terminal end (NELL2-KDEL). (D) Effect of BFA, an inhibitor of transport from ER to Golgi apparatus, on PPE-Luc activity in cells transfected with the indicated amount of NELL2 expression vector (NELL2). (E) PPE mRNA level was determined using real-time PCR analysis after transfection with NELL2 expression vector. Data are expressed as the mean ± SEM of at least 4 wells per group. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. CTL, control.

Tanizawa, 1999). Therefore, we sought to investigate a potential involvement of NELL2 EGF-like repeats domain on PPE transcription. To do this, we constructed mutant NELL2 expression vectors with differential deletion of functional domains, as indicated in Fig. 3A. The NIH3T3 cells were co-transfected with PPE-Luc and each naïve or mutant NELL2 expression vector. Transfected mutant forms of NELL2 were selectively localized within the cytoplasm of cells (Supplementary Fig. S4). Interestingly, ER-localized naïve NELL2-induced inhibition of PPE-Luc activity was mitigated with vectors for NELL2 mutants lacking the EGF-like domains such as NELL2 △EGF-Ca^{2+} (lacking Ca^{2+}-binding EGF domains) and NELL2△EGF (lacking all EGF domains) (Fig. 3B). These results indicate that the EGF domains are key to regulation of PPE transcription. Additionally, our results suggest a possible involvement of the Ca^{2+}-dependent pathway in ER-localized NELL2-mediated PPE transcription.

ER-localized NELL2 suppresses PPE transcription via the Ca^{2+}-PKC-ERK-cFos pathway

We next sought to address if ER-localized NELL2 regulates PPE transcription in association with Ca^{2+}-dependent intracellular signaling cascades. First, we used the drug A23187, a cation Ca^{2+} ionophore, to induce Ca^{2+} influx into the cytoplasm of cells (Davies and Hallett, 1996; Lages and Weiss, 1995). As expected, NIH3T3 cells exposed to A23187 for 1 h

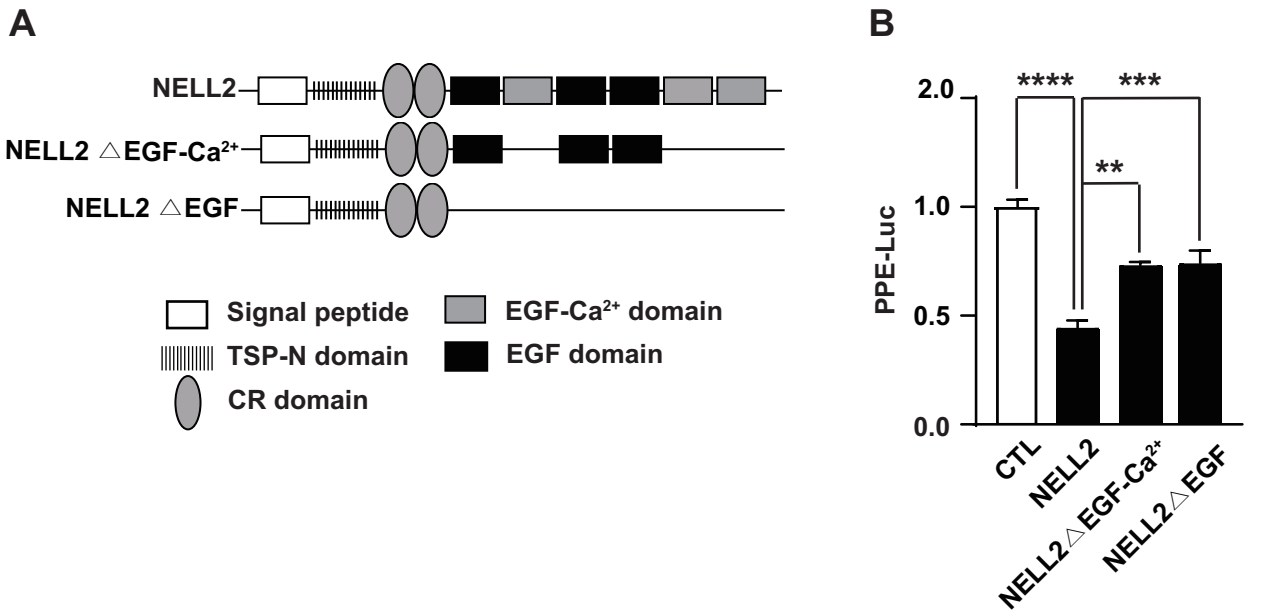

Fig. 3. Effect of NELL2 mutants on PPE transcription. (A) Schematic representation of the three NELL2 constructs including naïve NELL2 and NELL2 mutants lacking different domains: NELL2△EGF-Ca^{2+}=mutant NELL2 lacking Ca^{2+}-binding EGF-like domains; NELL2 △EGF=mutant NELL2 lacking all EGF-like domains. (B) NIH3T3 cells were transfected with PPE-Luc vectors (500 ng) and an equal concentration (300 ng each) of expression vector coding NELL2 or mutant NELL2 constructs as indicated below the figure. The results are expressed as fold change compared to the PPE-Luc activity in the control pcDNA vector-transfected group (CTL). Data are expressed as the mean ± SEM (n = 6). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

showed a significant increase in PPE-Luc activity in the control group without the NELL2 expression vector (Fig. 4A). Importantly, co-transfection of NELL2 expression vectors resulted in strong inhibition of the Ca^{2+} ionophore-induced increase in PPE-Luc activity, while this phenomenon was rescued partially with NELL2△EGF-Ca^{2+} vectors. These results suggest that ER-localized NELL2 affects PPE transcription, at least in part, by modulating Ca^{2+}-dependent signaling.

In parallel, we also tested possible involvement of PKC signaling in NELL2-mediated regulation of PPE-Luc activity. To do this, NIH3T3 cells were treated with PMA, a PKC activating phorbol ester, for 3 h with or without NELL2 expression vectors (Fig. 4B). As expected, PMA treatment stimulated PPE-Luc, while co-transfection of either NELL2 expression vectors or NELL2 expression vectors + BFA inhibited PMA-induced PPE-Luc activity. Given that NELL2 is known to be related to ERK activation (Aihara et al., 2003; Choi et al., 2010; Kim et al., 2014; 2015; Liu et al., 2021), one of the multiple downstream pathways of PKC signaling, we next examined the involvement of ER-localized NELL2 in the activity of ERK, as represented by level of phosphorylated ERK (pERK). In western blot analyses, pERK was undetectable in control NIH3T3 cells, but was induced strongly by PMA treatment (Fig. 4C). Consistently, the level of pERK was downregulated with transfection of NELL2 expression vectors, while it was rescued partially with vectors for NELL2△EGF-Ca^{2+}. Therefore, ER-localized NELL2 is likely linked with the Ca^{2+}-PKC-ERK signaling pathway to regulate PPE transcription.

Previous studies have demonstrated that cFos is one of the final effectors of the PKC-ERK signaling pathways for regulation of gene expression (Kominato et al., 2003; Nakakuki et al., 2010; Oh et al., 2003; Sgambato et al., 1998). Moreover, cFos has been reported to regulate PPE gene expression (reviewed by Puryear et al., 2020). Therefore, we examined ER-localized NELL2-dependent cFos expression (Fig. 4D). NIH3T3 cells treated with PMA showed significant induction of cFos proteins, while this induction was suppressed strongly with the NELL2 expression vectors. PMA-dependent cFos expression was recovered with NELL2△EGF-Ca^{2+}. Following these results, we examined NELL2 effects on cFos activation using the cFos promoter-luciferase reporter (cFos-Luc) vector (Oh et al., 2003). The cFos-Luc was active transcriptionally over a wide range of concentrations in NIH3T3 cells (Fig. 4E) and was decreased by transfection of the NELL2 expression vectors in a dose-dependent manner (Fig. 4F). In Fig. 4G, the patterns of cFos-Luc activity following expression vectors either for naïve NELL2 or NELL2△EGF-Ca^{2+} were similar to those of the Ca^{2+}, PKC, and pERK experiments described above. Altogether, these results from *in vitro* approaches demonstrate the fundamental inhibitory role of ER-localized NELL2 in PPE transcription, through sequential signaling cascades involving the Ca^{2+}-PKC-ERK-cFos pathway.

Extracellular NELL2 activates PPE transcription

As mentioned, NELL2 is a secretion protein and can modulate intracellular events of target cells through receptor signaling. Therefore, we sought to examine the effects of extracellular NELL2 on PPE transcription. In this case, sNELL2 was administered into the NIH3T3 cell culture medium. Application of sNELL2 resulted in a rapid increase in pERK (Fig. 5A), which

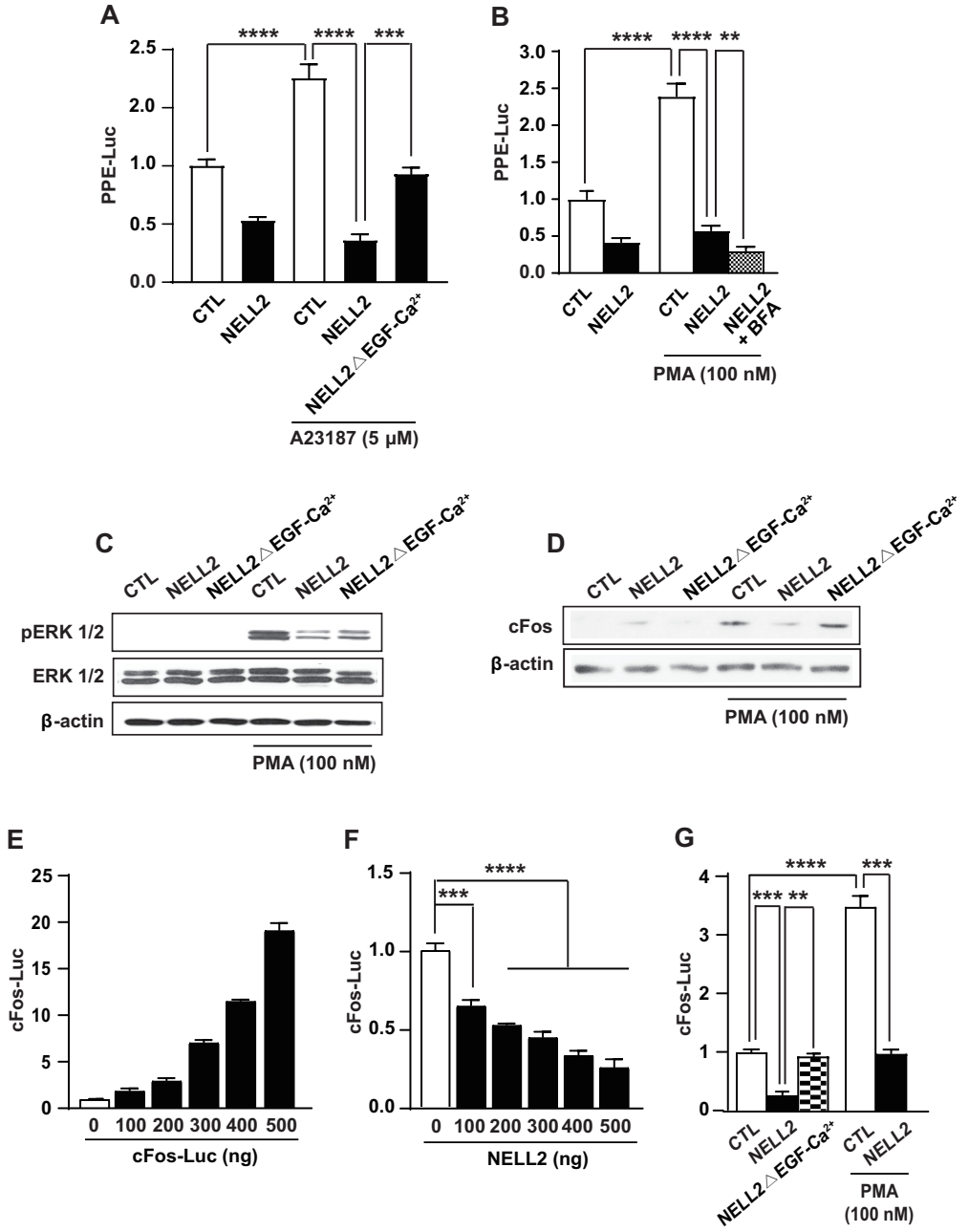

Fig. 4. ER-localized NELL2 action on Ca^{2+} and PKC-activated ERK, cFos, and PPE expression. (A) Effect of transfected NELL2 or NELL2△EGF-Ca^{2+} expression vector on the Ca^{2+} ionophore A23187-induced PPE-Luc activity. Luc activity was determined at 1 h after ionophore treatment. Data are expressed as the mean ± SEM (n = 6). (B) Effect of NELL2 on PMA-induced PPE-Luc activity. Cells were transfected with PPE-Luc vector and NELL2 expression vector with or without treatment of BFA (200 ng) for 24 h, and PMA (100 nM) was applied for 3 h before harvesting cells. Data are expressed as the mean ± SEM (n = 6). (C) Effect of NELL2 on PMA-induced phosphorylation of ERK (pERK) in NIH3T3 cells. Cells transfected with expression vector NELL2 or NELL2△EGF-Ca^{2+} were treated with PMA (100 nM) for 30 min and harvested for western blotting. (D) Effect of NELL2 or NELL2△EGF-Ca^{2+} expression vector on PMA-induced cFos expression in NIH3T3 cells. Proteins extracted from the cells treated with PMA for 3 h were analyzed using western blotting, and the levels of β-actin were used as a loading control. (E-G) Effect of NELL2 on cFos-Luc activity. The cFos-Luc vector was transfected into NIH3T3 cells together with NELL2 or NELL2△EGF-Ca^{2+} expression vector. The cells were harvested for Luc assays at 24 h after transfection. (E) A dose-dependent increase in cFos-Luc activity in cells transfected with different concentrations of cFos-Luc vector. (F) cFos-Luc activity was decreased by NELL2 expression vector in a dose-dependent manner. (G) Effect of NELL2 expression vector on the PMA-induced increase of cFos-Luc activity. NIH3T3 cells were co-transfected with cFos-Luc vector (500 ng) and the expression vector (300 ng). One day after transfection, cells were treated with PMA (100 nM) at 3 h before harvest. CTL, cells transfected with control pcDNA vector. Data are expressed as the mean ± SEM (n = 6). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

is in concert with our previous reports (Choi et al., 2010; Kim et al., 2014; 2015). Subsequently, we revealed an activation role of extracellular sNELL2 on PPE-Luc activity via intracellular ERK signaling, as treatment of cells with U0126, pharmacological inhibitor of ERK, attenuated sNELL2-induced PPE-Luc activity (Fig. 5B). In line with these findings, sNELL2 further induced cFos activation (Figs. 5C and 5D). To determine effect of cFos on the NELL2 signaling pathway, PPE-Luc activity was measured after siRNA-mediated knockdown of cFos expression. cFos siRNA significantly reduced cFos mRNA level in the transfected cells (Fig. 5E), and in turn, resulted in a significant inhibition of the sNELL2-stimulated PPE-Luc activity (Fig. 5F). Moreover, the cFos siRNA inhibited change in the PPE-Luc activity that was caused by ER-localized NELL2 in the control siRNA group. These results demonstrate the dual-faceted actions of NELL2: while ER-localized NELL2 inhibits the PKC-ERK pathway, extracellular NELL2 stimulates the same events to regulate PPE transcription.

In vivo inhibition of NELL2 synthesis increases PPE mRNA content in the brain

Finally, we examined the effect of NELL2 on PPE gene expression in the brain by administration of NELL2-targeted AS-ODN into the lateral ventricle of 2-month-old male rats. This strategy has been used to block the biosynthesis of NELL2 in the brain (Ha et al., 2008; Jeong et al., 2008b; Kim et al., 2002). As shown in Fig. 6A, NELL2 production in the AS-ODN group was significantly reduced compared to that of the group that received scrambled-oligodeoxynucleotide (SCR-ODN) in the hypothalamus. In parallel, PPE mRNA level in the brain was elevated following NELL2 AS-ODN treatment (Figs. 6B and 6C), suggesting inhibitory activity of NELL2 on PPE transcription in the brain.

DISCUSSION

In this study, we performed a combination of *in vitro* and *in*

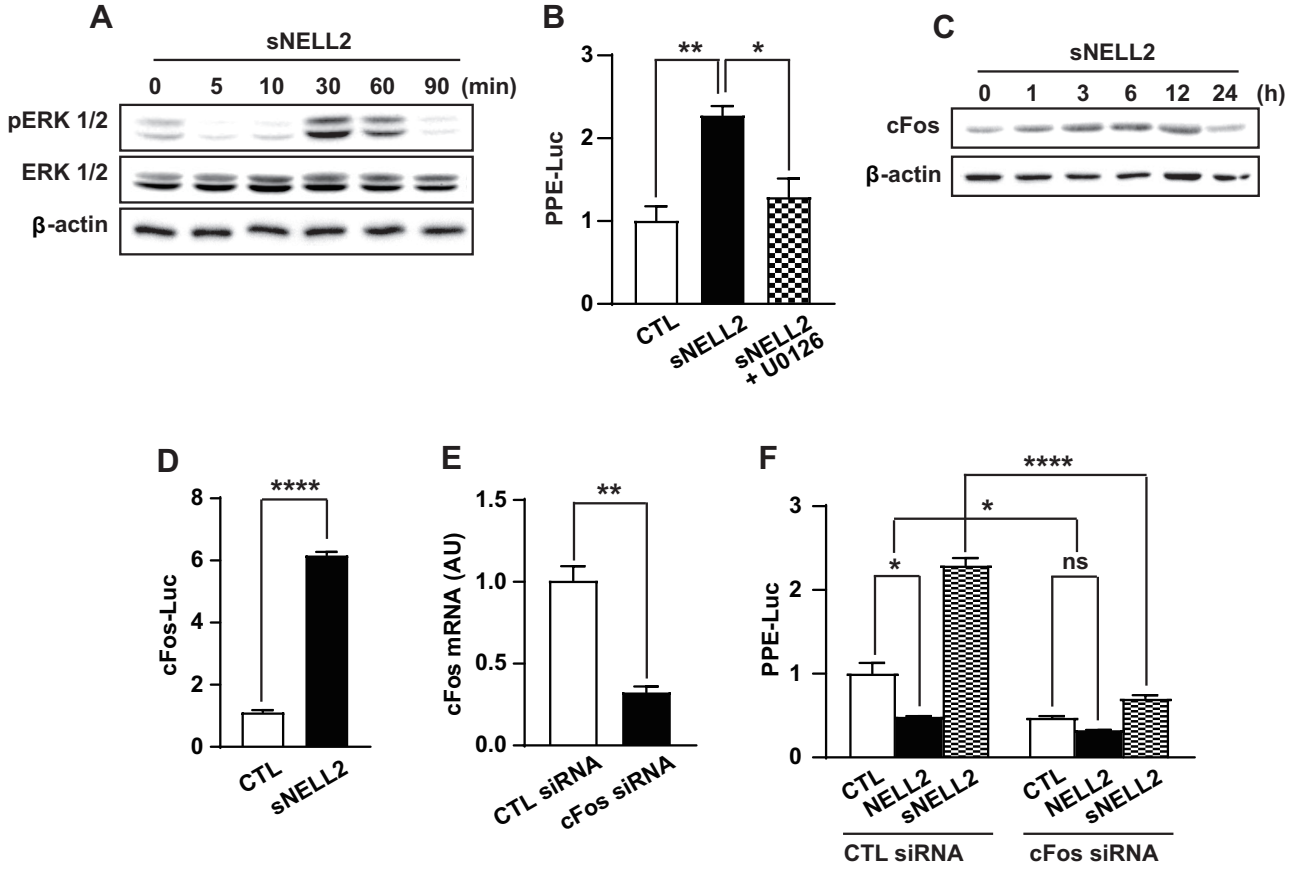

Fig. 5. Effect of extracellular NELL2 on phosphorylation of ERK, cFos and PPE transcription. (A and C) Effect of synthetic NELL2 (sNELL2, 300 ng/ml) added to culture medium on phosphorylation of ERK (pERK) and cFos expression. Cells were harvested at the indicated time after sNELL2 treatment, and protein extracts were analyzed by western blotting. β-Actin was used as a loading control. (B) Change of PPE-Luc activity by treatment with sNELL2 or sNELL2 with U0126, an inhibitor of ERK signaling for 24 h. (D) Increase of cFos-Luc activity by treatment with sNELL2 for 12 h. (E and F) Effect of cFos knockdown on the NELL2-induced PPE-Luc activity. Cells were transfected with control siRNA (CTL siRNA) or siRNA against cFos mRNA (cFos siRNA) at 50 nM concentration. (E) cFos mRNA level was determined by real-time PCR analysis. (F) cFos siRNA caused changes in the PPE-Luc activity that was induced by ER-localized NELL2 (NELL2) or by sNELL2. Data are expressed as the mean ± SEM (n = 6). $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$. CTL, control; ns, not significant.

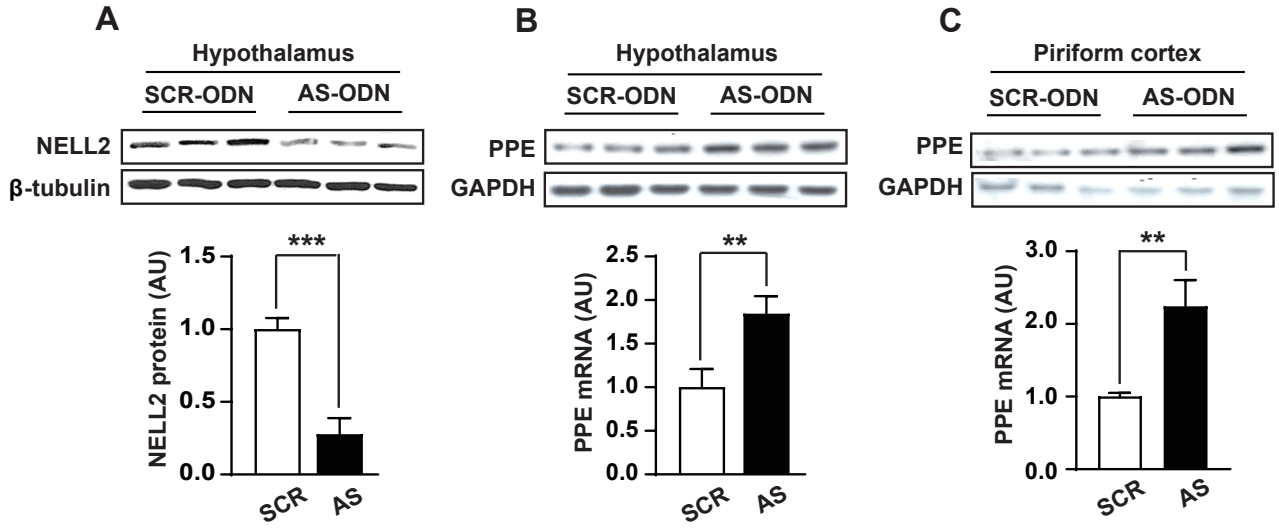

Fig. 6. Effect of *in vivo* inhibition of NELL2 synthesis on PPE mRNA level in the brain. To determine *in vivo* disruption of NELL2 synthesis on PPE expression in the brain, AS-ODN against NELL2 mRNA was icv injected into the lateral ventricle of 2-month-old male rat brain, and NELL2 protein and PPE mRNA expression was measured by western blot and northern blot analyses, respectively. (A) Western blots showing the effectiveness of AS-ODN on the suppression of NELL2 protein content in the hypothalamus. β-Tubulin was used as an internal control. (B and C) Northern blot analyses showing an increase in PPE mRNA level by treatment with AS-ODN in the hypothalamus (B) and piriform cortex (C). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Data are expressed as the mean ± SEM (n = 6). $^{**}P < 0.01$; $^{***}P < 0.001$. SCR-ODN, scrambled ODN; AU, arbitrary units.

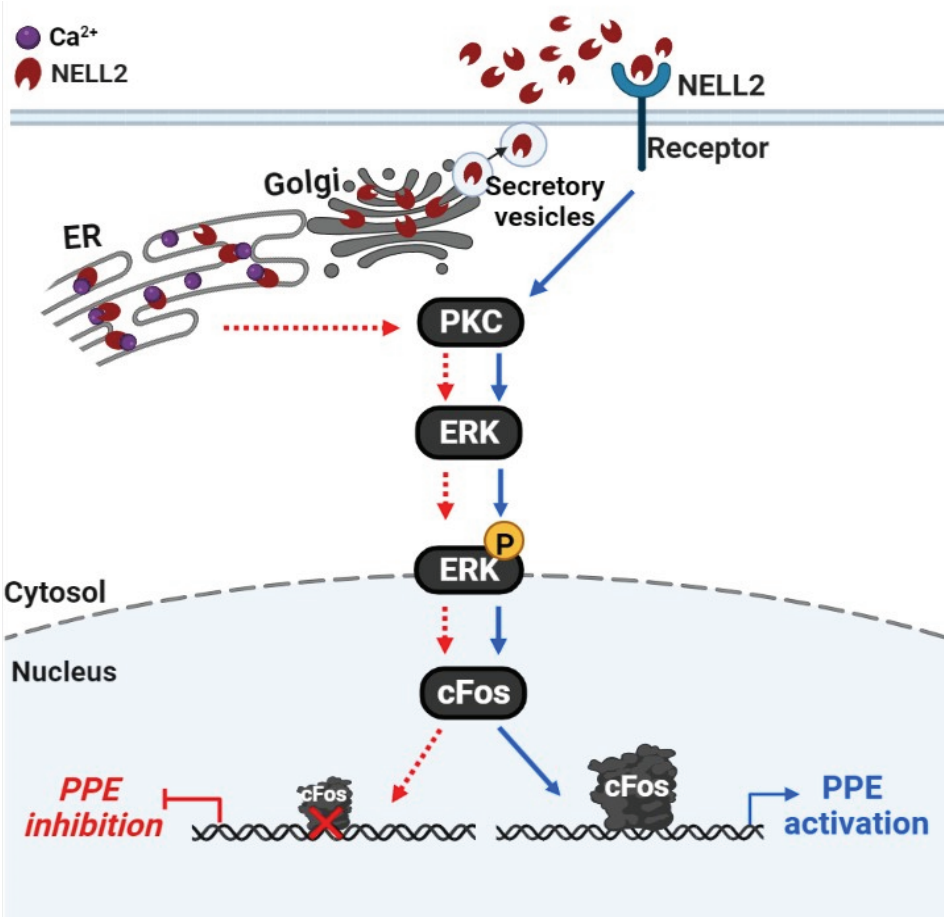

Fig. 7. Schematic summary for role of NELL2 in PPE gene expression. NELL2 is involved in a functionally opposing dual-faced mechanism in PPE gene transcription. ER-localized NELL2 inhibits Ca^{+}-mediated and PKC-ERK-cFos-dependent signaling and suppresses PPE gene transcription during its release process. Once released, NELL2 binds its unknown receptor and stimulates PKC-ERK-cFos signaling pathway and subsequently activates PPE transcription. Dotted lines represent inhibitory action of ER-localized NELL2 and solid lines represent stimulatory action of released NELL2.

vivo experiments to determine the regulatory role of NELL2 in PPE gene expression. Our results showed that 1) hypothalamic neurons expressing PPE also expressed NELL2, suggesting its possible autocrine and/or paracrine action on PPE cells. 2) ER-localized NELL2 inhibited PPE gene transcription through suppression of the intracellular PKC-ERK signaling pathway. 3) Extracellular NELL2 stimulated PKC-ERK signaling and subsequently activated PPE transcription. 4) The *in vivo* disruption of NELL2 biosynthesis in the brain resulted in an increase in PPE mRNA levels. These results demonstrated for the first time that NELL2, a recently discovered neuromodulator and guide for axon-pathfinding, is involved in PPE gene transcription via two routes (Fig. 7).

The most striking result in this study is that NELL2 is involved in a functionally opposing dual-faced mechanism in PPE gene transcription: while ER-localized NELL2 inhibited Ca^{+}-mediated and PKC-ERK-dependent signaling and suppressed PPE gene transcription, but this inhibitory action was reversed when NELL2 signaling was initiated extracellularly. In line with these observations, previous investigations have recognized dual-faceted NELL2 actions in axonal growth and synaptogenesis. For example, in our recent report, we showed that NELL2 has a key role in neurite and axonal growth as well as in the morphological development of neurons (Kim et al., 2020). In an *ex vivo* experiment with primary embryonic cells, the cells developed rapid axonal growth when they were transfected with NELL2 overexpression vectors. Importantly, this phenomenon was mediated through Robo receptor 2/3-independent intracellular ERK signaling. On the other hand, other studies have demonstrated the chemorepellent action of extracellular NELL2 when coupled with Robo receptor signaling in axonal guidance (Jaworski et al., 2015; Pak et al., 2020; Yamamoto et al., 2019). These studies together indicate that NELL2 could be either an activator of axonal growth or an inhibitor of axonal pathfinding during neural development. The underlying mechanism that determines NELL2 fate is unclear. However, existing evidence suggests that it could depend on the site of NELL2 action initiation (e.g., intracellular vs extracellular) or cell type-specific receptors for NELL2 (e.g., Robo2/3 vs unknown). A recent study demonstrated the existence of different receptor and signaling systems for NELL2 action, such as testicular germ cell-derived NELL2 acts on male fertility by binding to orphan receptor tyrosine kinase-c-ros oncogene 1 (ROS1) (Kiyozumi et al., 2020). In this study, we identified NELL2 expression in the PPE neurons of VMH but found no immunoreactive Robo2/3 in these PPE neurons (data not shown). Therefore, NELL2 might not exert its extracellular effect on PPE gene expression through Robo2/3 but through different unknown receptor(s) in the VMH. Further studies are required to uncover NELL2 receptor signaling in the hypothalamus.

Regarding NELL2 signaling, the important question is how the secretion factor NELL2 drives intracellular signaling cascades before being released from NELL2-producing cells. Secretory proteins, including NELL2, contain a signal peptide that enables release from the cells. However, we have reported the presence of a splice variant form of NELL2 that has a partially deleted signal peptide (Hwang et al., 2007; Lee et al., 2014). Instead of being released, this NELL2 variant accumulates in the cytoplasm. Importantly, this cytosolic NELL2 was able to interact with PKC and negatively regulate PKC signaling (Lee et al., 2014), which resembles ER-localized NELL2 actions in the current study. However, it is not likely that the ER-localized NELL2 in this study mimics the actions of cytosolic NELL2, because cytosolic NELL2 is a product of the RNA-splice variant missing the functional signal peptide, while the ER-localized NELL2 in this study contains an intact signal peptide and is processed through the ER-Golgi apparatus secretary pathway. The other possibility is that NELL2 exerts an effect on intracellular signaling at the ER before being released. Secreting proteins visit the ER during the conventional release process. We previously designed a reporter vector to visualize intracellular localization of NELL2 (NELL2-EGFP) and observed that NELL2 travels through the ER and Golgi apparatus (Ha et al., 2013). This result suggests that NELL2 follows the conventional release pathway. Keeping this in mind, we considered that NELL2 might have prolonged residence in the ER, induced by BFA (NELL2/BFA), an inhibitor of ER-Golgi transfer (Chardin and McCormick, 1999), or using a NELL2 expression vector fused with the ER retention signal KDEL (NELL2-KDEL) (Mei et al., 2017; Munro and Pelham, 1987). Under these circumstances, PPE gene transcription was inhibited at a similar level to that of the ER-localized naïve NELL2. This observation suggests that the ER and not the cytosol could be the site where ER-localized NELL2 initiates PKC-ERK signaling for regulation of PPE gene expression.

In the brain, multiple factors, including sex hormones (Le Saux and Di Paolo, 2005; Spool et al., 2016), nutrients (Hawkins et al., 2001), and neurotransmitters (Morin et al., 2016), participate in the regulation of PPE synthesis. Within PPE cells, investigations identified a key role for intracellular cAMP- and PKC-dependent pathways for PPE transcription (Borsook and Hyman, 1995). Here, we provide evidence that NELL2 is a novel player regulating PPE transcription. As already mentioned, NELL2 possesses several functional domains. Among these domains, six EGF-like repeat domains have been identified as key domains for interaction with PKC (Kuroda and Tanizawa, 1999). In line with this, our results show that NELL2, through its EGF-like repeat domains, modulates the Ca^{2+}-dependent PKC-ERK-cFos pathway to regulate PPE transcription. In consideration of this, we investigated the effects of mutant NELL2 lacking Ca^{2+}-binding EGF domains on PKC-ERK signaling in PPE gene regulation. Interestingly, mutant NELL2 partially lost the inhibitory nature of the naïve NELL2 on PPE-Luc activity, pERK, and cFos expression. Together, these results indicate that the Ca^{2+}-binding EGF domains are required to achieve the inhibitory NELL2 effect on Ca^{2+}-dependent PKC-ERK signaling for PPE expression and suggest that NELL2 plays a Ca^{2+} buffering role in the ER. Further studies are necessary to clarify how NELL2 initiates Ca^{2+}-dependent PKC activation in the ER.

In summary, the present findings identify NELL2 as a novel component involved in regulation of PPE gene transcription through the Ca^{2+}-dependent PKC-ERK-cFos pathway. Furthermore, the direction of NELL2 actions on PPE gene transcription is spatio-dependent.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS
C.M.H., D.H.K., J.W.P., J.K.J., and B.J.L. conceived and designed the research. C.M.H., D.H.K., T.H.L., H.R.K., J.C., Y.K., D.K., S.R.O., and J.K.J. performed experiments. H.R.K., D.H.K., T.H.L., C.M.H., J.C., S.R.O., J.K.J., and B.J.L. analyzed data. C.M.H., D.H.K., S.R.O., J.K.J., and B.J.L. wrote the manuscript. All authors contributed to overall data interpretation, provided intellectual input, and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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REFERENCES

Aihara, K., Kuroda, S., Kanayama, N., Matsuyama, S., Tanizawa, K., and Horie, M. (2003). A neuron-specific EGF family protein, NELL2, promotes survival of neurons through mitogen-activated protein kinases. Brain Res. Mol. Brain Res. *116*, 86-93.

Berg-von der Emde, K., Dees, W.L., Hiney, J.K., Hill, D.F., Dissen, G.A., Costa, M.E., Moholt-Siebert, M., and Ojeda, S.R. (1995). Neurotrophins and the neuroendocrine brain: different neurotrophins sustain anatomically and functionally segregated subsets of hypothalamic dopaminergic neurons. J. Neurosci. *15*, 4223-4237.

Bodnar, R.J. (2017). Endogenous opiates and behavior: 2015. Peptides *88*, 126-188.

Borsook, D. and Hyman, S.E. (1995). Proenkephalin gene regulation in the neuroendocrine hypothalamus: a model of gene regulation in the CNS. Am. J. Physiol. *269*(3 Pt 1), E393-E408.

Chardin, P. and McCormick, F. (1999). Brefeldin A: the advantage of being uncompetitive. Cell *97*, 153-155.

Choi, E.J., Ha, C.M., Choi, J., Kang, S.S., Choi, W.S., Park, S.K., Kim, K., and Lee, B.J. (2001). Low-density cDNA array-coupled to PCR differential display identifies new estrogen-responsive genes during the postnatal differentiation of the rat hypothalamus. Brain Res. Mol. Brain Res. *97*, 115-128.

Choi, E.J., Kim, D.H., Kim, J.G., Kim, D.Y., Kim, J.D., Seol, O.J., Jeong, C.S., Park, J.W., Choi, M.Y., Kang, S.G., et al. (2010). Estrogen-dependent transcription of the NEL-like 2 (NELL2) gene and its role in protection from cell death. J. Biol. Chem. *285*, 25074-25084.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156-159.

Davies, E.V. and Hallett, M.B. (1996). Near membrane Ca^{2+} changes resulting from store release in neutrophils: detection by FFP-18. Cell Calcium *19*, 355-362.

Dellovade, T.L., Kia, H.K., Zhu, Y.S., and Pfaff, D.W. (1999). Thyroid hormone coadministration inhibits the estrogen-stimulated elevation of preproenkephalin mRNA in female rat hypothalamic neurons. Neuroendocrinology *70*, 168-174.

Duque-Diaz, E., Alvarez-Ojeda, O., and Covenas, R. (2019). Enkephalins and ACTH in the mammalian nervous system. Vitam. Horm. *111*, 147-193.

Feinberg, A.P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6-13.

Ha, C.M., Choi, J., Choi, E.J., Costa, M.E., Lee, B.J., and Ojeda, S.R. (2008). NELL2, a neuron-specific EGF-like protein, is selectively expressed in glutamatergic neurons and contributes to the glutamatergic control of GnRH neurons at puberty. Neuroendocrinology *88*, 199-211.

Ha, C.M., Hwang, E.M., Kim, E., Lee, D.Y., Chang, S., Lee, B.J., Hong, S.G., and Park, J.Y. (2013). The molecular mechanism of NELL2 movement and secretion in hippocampal progenitor HiB5 cells. Mol. Cells *36*, 527-533.

Handford, P.A., Mayhew, M., Baron, M., Winship, P.R., Campbell, I.D., and Brownlee, G.G. (1991). Key residues involved in calcium-binding motifs in EGF-like domains. Nature *351*, 164-167.

Harlan, R.E., Shivers, B.D., Romano, G.J., Howells, R.D., and Pfaff, D.W. (1987). Localization of preproenkephalin mRNA in the rat brain and spinal cord by in situ hybridization. J. Comp. Neurol. *258*, 159-184.

Hawkins, P., Hanson, M.A., and Matthews, S.G. (2001). Maternal undernutrition in early gestation alters molecular regulation of the hypothalamic-pituitary-adrenal axis in the ovine fetus. J. Neuroendocrinol. *13*, 855-861.

Henry, M.S., Gendron, L., Tremblay, M.E., and Drolet, G. (2017). Enkephalins: endogenous analgesics with an emerging role in stress resilience. Neural Plast. *2017*, 1546125.

Hwang, E.M., Kim, D.G., Lee, B.J., Choi, J., Kim, E., Park, N., Kang, D., Han, J., Choi, W.S., Hong, S.G., et al. (2007). Alternative splicing generates a novel non-secretable cytosolic isoform of NELL2. Biochem. Biophys. Res. Commun. *353*, 805-811.

Jaworski, A., Tom, I., Tong, R.K., Gildea, H.K., Koch, A.W., Gonzalez, L.C., and Tessier-Lavigne, M. (2015). Operational redundancy in axon guidance through the multifunctional receptor Robo3 and its ligand NELL2. Science *350*, 961-965.

Jeong, J.K., Kim, H.R., Hwang, S.M., Park, J.W., and Lee, B.J. (2008a). Region- and neuronal phenotype-specific expression of NELL2 in the adult rat brain. Mol. Cells *26*, 186-192.

Jeong, J.K., Kim, J.G., Kim, H.R., Lee, T.H., Park, J.W., and Lee, B.J. (2017). A role of central NELL2 in the regulation of feeding behavior in rats. Mol. Cells *40*, 186-194.

Jeong, J.K., Ryu, B.J., Choi, J., Kim, D.H., Choi, E.J., Park, J.W., Park, J.J., and Lee, B.J. (2008b). NELL2 participates in formation of the sexually dimorphic nucleus of the pre-optic area in rats. J. Neurochem. *106*, 1604-1613.

Khachaturian, H., Alessi, N.E., Munfakh, N., and Watson, S.J. (1983). Ontogeny of opioid and related peptides in the rat cns and pituitary: an

immunocytochemical study. Life Sci. *33* Suppl 1, 61-64.

Kim, D.H., Kim, H.R., Choi, E.J., Kim, D.Y., Kim, K.K., Kim, B.S., Park, J.W., and Lee, B.J. (2014). Neural epidermal growth factor-like like protein 2 (NELL2) promotes aggregation of embryonic carcinoma P19 cells by inducing N-cadherin expression. PLoS One *9*, e85898.

Kim, D.H., Kim, K.K., Lee, T.H., Eom, H., Kim, J.W., Park, J.W., Jeong, J.K., and Lee, B.J. (2021). Transcription factor TonEBP stimulates hyperosmolality-dependent arginine vasopressin gene expression in the mouse hypothalamus. Front. Endocrinol. (Lausanne) *12*, 627343.

Kim, D.Y., Kim, H.R., Kim, K.K., Park, J.W., and Lee, B.J. (2015). NELL2 function in the protection of cells against endoplasmic reticulum stress. Mol. Cells *38*, 145-150.

Kim, H., Ha, C.M., Choi, J., Choi, E.J., Jeon, J., Kim, C., Park, S.K., Kang, S.S., Kim, K., and Lee, B.J. (2002). Ontogeny and the possible function of a novel epidermal growth factor-like repeat domain-containing protein, NELL2, in the rat brain. J. Neurochem. *83*, 1389-1400.

Kim, H.R., Kim, D.H., An, J.Y., Kang, D., Park, J.W., Hwang, E.M., Seo, E.J., Jang, I.H., Ha, C.M., and Lee, B.J. (2020). NELL2 function in axon development of hippocampal neurons. Mol. Cells *43*, 581-589.

Kiyozumi, D., Noda, T., Yamaguchi, R., Tobita, T., Matsumura, T., Shimada, K., Kodani, M., Kohda, T., Fujihara, Y., Ozawa, M., et al. (2020). NELL2-mediated lumicrine signaling through OVCH2 is required for male fertility. Science *368*, 1132-1135.

Kominato, Y., Tachibana, T., Dai, Y., Tsujino, H., Maruo, S., and Noguchi, K. (2003). Changes in phosphorylation of ERK and Fos expression in dorsal horn neurons following noxious stimulation in a rat model of neuritis of the nerve root. Brain Res. *967*, 89-97.

Koshimizu, Y., Wu, S.X., Unzai, T., Hioki, H., Sonomura, T., Nakamura, K.C., Fujiyama, F., and Kaneko, T. (2008). Paucity of enkephalin production in neostriatal striosomal neurons: analysis with preproenkephalin-green fluorescent protein transgenic mice. Eur. J. Neurosci. *28*, 2053-2064.

Kuroda, S. and Tanizawa, K. (1999). Involvement of epidermal growth factor-like domain of NELL proteins in the novel protein-protein interaction with protein kinase C. Biochem. Biophys. Res. Commun. *265*, 752-757.

Lages, B. and Weiss, H.J. (1995). Original article: comparison of A23187 vs ionomycin-induced responses and cytosolic calcium increases in aequorin-loaded human platelets. Evidence for ionophore-specific differences in intracellular calcium release. Platelets *6*, 359-365.

Le Saux, M. and Di Paolo, T. (2005). Chronic estrogenic drug treatment increases preproenkephalin mRNA levels in the rat striatum and nucleus accumbens. Psychoneuroendocrinology *30*, 251-260.

Lee D.Y., Kim, E., Lee, Y.S., Ryu, H., Park, J.Y., and Hwang, E.M. (2014). The cytosolic splicing variant of NELL2 inhibits PKCβ1 in glial cells. Biochem. Biophys. Res. Commun. *454*, 459-464.

Liu, J., Liu, D., Zhang, X., Li, Y., Fu, X., He, W., Li, M., Chen, P., Zeng, G., DiSanto, M.E., et al. (2021). NELL2 modulates cell proliferation and apoptosis via ERK pathway in the development of benign prostatic hyperplasia. Clin. Sci. (Lond.) *135*, 1591-1608.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. Methods *25*, 402-408.

Mei, M., Zhai, C., Li, X., Zhou, Y., Peng, W., Ma, L., Wang, Q., Iverson, B.L., Zhang, G., and Yi, L. (2017). Characterization of aromatic residue-controlled protein retention in the endoplasmic reticulum of Saccharomyces cerevisiae. J. Biol. Chem. *292*, 20707-20719.

Morin, N., Morissette, M., Gregoire, L., and Di Paolo, T. (2016). mGlu5, dopamine D2 and adenosine A2A receptors in L-DOPA-induced dyskinesias. Curr. Neuropharmacol. *14*, 481-493.

Morissette, M., Le Saux, M., D'Astous, M., Jourdain, S., Al Sweidi, S., Morin, N., Estrada-Camarena, E., Mendez, P., Garcia-Segura, L.M., and Di Paolo, T. (2008). Contribution of estrogen receptors alpha and beta to the effects of estradiol in the brain. J. Steroid Biochem. Mol. Biol. *108*, 327-338.

Munro, S. and Pelham, H.R. (1987). A C-terminal signal prevents secretion of luminal ER proteins. Cell *48*, 899-907.

Nakakuki, T., Birtwistle, M.R., Saeki, Y., Yumoto, N., Ide, K., Nagashima, T., Brusch, L., Ogunnaike, B.A., Okada-Hatakeyama, M., and Kholodenko, B.N. (2010). Ligand-specific c-Fos expression emerges from the spatiotemporal control of ErbB network dynamics. Cell *141*, 884-896.

Nelson, B.R., Claes, K., Todd, V., Chaverra, M., and Lefcort, F. (2004). NELL2 promotes motor and sensory neuron differentiation and stimulates mitogenesis in DRG in vivo. Dev. Biol. *270*, 322-335.

Noda, M., Teranishi, Y., Takahashi, H., Toyosato, M., Notake, M., Nakanishi, S., and Numa, S. (1982). Isolation and structural organization of the human preproenkephalin gene. Nature *297*, 431-434.

Oh, D.Y., Wang, L., Ahn, R.S., Park, J.Y., Seong, J.Y., and Kwon, H.B. (2003). Differential G protein coupling preference of mammalian and nonmammalian gonadotropin-releasing hormone receptors. Mol. Cell. Endocrinol. *205*, 89-98.

Pak, J.S., DeLoughery, Z.J., Wang, J., Acharya, N., Park, Y., Jaworski, A., and Ozkan, E. (2020). NELL2-Robo3 complex structure reveals mechanisms of receptor activation for axon guidance. Nat. Commun. *11*, 1489.

Puryear, C.B., Brooks, J., Tan, L., Smith, K., Li, Y., Cunningham, J., Todtenkopf, M.S., Dean, R.L., and Sanchez, C. (2020). Opioid receptor modulation of neural circuits in depression: what can be learned from preclinical data? Neurosci. Biobehav. Rev. *108*, 658-678.

Quinones-Jenab, V., Ogawa, S., Jenab, S., and Pfaff, D.W. (1996). Estrogen regulation of preproenkephalin messenger RNA in the forebrain of female mice. J. Chem. Neuroanat. *12*, 29-36.

Rao, Z., Handford, P., Mayhew, M., Knott, V., Brownlee, G.G., and Stuart, D. (1995). The structure of a Ca(2+)-binding epidermal growth factor-like domain: its role in protein-protein interactions. Cell *82*, 131-141.

Ryu, B.J., Kim, H.R., Jeong, J.K., and Lee, B.J. (2011). Regulation of the female rat estrous cycle by a neural cell-specific epidermal growth factor-like repeat domain containing protein, NELL2. Mol. Cells *32*, 203-207.

Sgambato, V., Pages, C., Rogard, M., Besson, M.J., and Caboche, J. (1998). Extracellular signal-regulated kinase (ERK) controls immediate early gene induction on corticostriatal stimulation. J. Neurosci. *18*, 8814-8825.

Spool, J.A., Stevenson, S.A., Angyal, C.S., and Riters, L.V. (2016). Contributions of testosterone and territory ownership to sexually-motivated behaviors and mRNA expression in the medial preoptic area of male European starlings. Horm. Behav. *86*, 36-44.

Takahashi, K., Fujita, T., and Takeuchi, T. (1995). Production of bioactive enkephalin from the nonendocrine cell lines COS-7, NIH3T3, Ltk-, and C2C12. Peptides *16*, 933-938.

Yamamoto, N., Kashiwagi, M., Ishihara, M., Kojima, T., Maturana, A.D., Kuroda, S., and Niimi, T. (2019). Robo2 contains a cryptic binding site for neural EGFL-like (NELL) protein 1/2. J. Biol. Chem. *294*, 4693-4703.