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The cytosolic splicing variant of NELL2 inhibits PKCβ1 in glial cells

Da Yong Lee [d], Eunju Kim [a], Young-Sun Lee [a], Hwani Ryu [a], Jae-Yong Park [b,*], Eun Mi Hwang [a,c,*]

[a] Center for Functional Connectomics, Korea Institute of Science and Technology (KIST), Seoul 136-791, Republic of Korea
[b] School of Biosystem and Biomedical Science, College of Health Science, Korea University, Seoul 136-703, Republic of Korea
[c] Neuroscience Program, University of Science and Technology (UST), Daejeon 305-350, Republic of Korea
[d] Stem Cell Research Center, Korea Research Institute of Bioscience and Biotechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea

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ABSTRACT

NELL2 is an abundant glycoprotein containing EGF-like domain in the neural tissues where it has multiple physiological functions by interacting with protein kinase C (PKC). There are two different splicing variant forms of NELL2 identified so far. One is secreted NELL2 (sNELL2) which is a neuron-specific variant and the other is cytosolic NELL2 (cNELL2) which is non-secreted splicing variant of NELL2. Although cNELL2 structure was well characterized, the expression pattern or the cellular function of cNELL2 is not fully determined. In this study, we found that cNELL2 specifically interacts with PKCβ isotypes and inhibits PKCβ1 through direct binding to the N-terminal pseudosubstrate domain of PKCβ1. Here, we also demonstrate that cNELL2 is predominantly expressed and has inhibitory effects on the PKC downstream signaling pathways in astrocytes thereby establishing cNELL2 as an endogenous inhibitor of PKCβ1 in glia.

1. Introduction

NEL-like 2 (NELL2) is a glycoprotein containing six EGF-like repeats which is involved in many of Ca^{2+}-dependent intracellular signaling pathways [1]. NELL2 was first identified as a neuron-specific protein which is produced in neurons and secreted to the extracellular matrix [1,2]. In our previous study, another splicing variant form of NELL2 which is cytosolic, non-secreted form of NELL2, named as cNELL2, was identified. cNELL2 is relatively smaller than secreted NELL2 (sNELL2) lacking the putative signaling peptide sequence (~129 nucleotides in exon 3) and mostly expressed in the cytoplasm rather than the membrane fraction thereby allowing itself to interact with protein kinase C (PKC) in the cytoplasm [3].

Previous report shows that sNELL2 is transiently expressed during the neural development in CNS and predominantly expressed while the sensory and motor neurons are differentiated [4] resulting in the promotion of neuronal differentiation [5] in chick dorsal root ganglion. Besides of the function in the neuronal differentiation, sNELL2 also increases the survival of retinal ganglion cells by interacting with microtubule-actin crosslinking factor 1 (Macf1) which is highly expressed in the neuronal tissues and has a crucial role during the neural development in the retina [6]. In the other hand, sNELL2 is also involved in the sexually dimorphic formation of brain structure during development [7] and the glutamate related neuroendocrine regulation in rat hypothalamus as well [8]. However, the expression pattern or the physiological function of cNELL2 is not yet determined.

cNELL2 contains EGF-like repeats which provide a binding domain for the interaction with PKC [3]. PKC is a multi-functional protein kinase family which consists of over 10 isotypes including PKCα, -β, -γ and -ε. It is well established that conventional PKC isotypes (-α, -β1 and -β2) are activated by diacylglycerol and intracellular Ca^{2+} which allows the translocation of PKC to the membrane fraction [9]. Although the molecular structure of PKC and NELL2 is well established, the mechanism how cNELL2 interacts with PKC still has to be determined. Furthermore, it is not completely known yet which isotype has a binding affinity with cNELL2.

In this study, we demonstrate that only PKCβ isotypes (-β1 and -β2) specifically interact with cNELL2 rather than any other isotypes and also determined that N-terminal pseudosubstrate (PS) domain of PKCβ1 is necessary for the interaction with cNELL2 using in vitro heterologous system. Next, we found that cNELL2 has an inhibitory effect on the activity of PKCβ1 and its downstream signaling pathways. Moreover, we show that cNELL2 is highly expressed in astrocytes demonstrating that cNELL2 functions as an intrinsic inhibitor of PKCβ1 in brain astrocytes.

* Corresponding authors at: Center for Functional Connectomics, Korea Institute of Science and Technology (KIST), Seoul 136-791, Republic of Korea. Fax: +82 2 958 7219 (E.M. Hwang), School of Biosystem and Biomedical Science, College of Health Science, Korea University, Seoul 136-703, Republic of Korea. Fax: +82 2 917 2388 (J.Y. Park).
E-mail addresses: jaeyong68@Korea.ac.kr (J.-Y. Park), emhwang@kist.re.kr (E.M. Hwang).

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2. Materials and methods

2.1. Reagents and antibodies

UTP, GF109203X (*GFX*), and thrombin are obtained by Sigma. Anti-ERK (MK), phospho-ERK (E-4), and GFP (B-2) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag (M2) antibody was purchased from Sigma (St. Louis, MO).

2.2. Plasmid construction

Full-length PKC isoforms were obtained from Dr. Deok-Ryung Kim (Gyeongsang National University, JinJu, Korea). PKCβ1 full clone was used as template for the generation of C1 (by deletion, aa161-671 WT), C2 (by serial deletion, aa1-161 WT and aa30-671 WT), Ki (by deletion, aa1-302 WT), ΔPS (by deletion, aa1-71 WT), and PS (by deletion, aa72-671 WT) using EZchange Site-directed Mutagenesis kit (Enzynomics, Korea). All clones were confirmed by sequence analysis.

2.3. Cell lines and primary cortical astrocyte culture

The CHO and HEK cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 1000 units/ml penicillin–streptomycin and 10% Fetal bovine serum (FBS) under a humidifying atmosphere containing 5% CO_2 at 37 °C. These two cell lines are used for PKC translocation upon UTP stimulation and binding assay between cNELL2 and PKC isotypes. Primary cortical astrocytes were prepared from P0–P3 of C57BL/6 mouse as described [10]. All experimental procedures described were performed in accordance with the institutional guidelines of Korea Institute of Science and Technology (KIST, Seoul, Korea). Cells were grown in DMEM supplemented with 10% horse serum (HS), 10% FBS and 1000 units/ml of penicillin–streptomycin. After 6–7 days, or once the primary astrocyte cultures are confluent, cells were transfected with cNELL2 cDNA and shRNA by electroporation using the neon microporator MP-100 kit (Invitrogen).

2.4. Co-immunoprecipitation and Western blot analysis

HEK293T cells were lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 1% NP-40) containing a protease-inhibitor cocktail. Whole-cell lysates were incubated on ice for 30 min and then cleared at 20,000*g* for 20 min at 4 °C. The supernatants were incubated overnight at 4 °C with anti-Flag (M2; Sigma) antibody, followed by incubation with protein A/G PLUS-agarose beads for 1 h. The proteins were separated by SDS–PAGE using 10% gels and blotted onto PVDF membranes. The blots incubated overnight at 4 °C with anti-GFP (B-2; Santa Cruz Biotechnology) antibody. Blots were then washed and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, followed by washing and detection of immunoreactivity with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

For Erk phosphorylation assay, the cells were treated with 10 μM UTP for indicated times and lysed with RIPA buffer. Protein concentrations of lysates were determined by BCA method. Lysates containing equal amounts of protein (20 μg protein) were subjected to SDS-PAGE using 12% gels and blotted onto PVDF membranes. Membranes were incubated with specific antibodies (anti-phospho-ERK (E-4; Santa Cruz Biotechnology) and anti-Erk (H-72; Santa Cruz Biotechnology)). cNELL2-GFP expressing CHO cells were preincubated with 10 μM GFX, a general PKC inhibitor, for 30 min at 37 °C to inhibit PKCβ1.

2.5. Live imaging of PKC translocation

The CHO cells were seeded on the coverglass and transiently transfected with GFP-PKCβ1 or cNELL2. The next day, the culture medium was replaced with normal Hepes buffer composed of: 135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM Hepes, 10 mM Glucose, pH 7.3. The fluorescence of the GFP-PKCβ1 was monitored with a confocal laser scanning microscope (Nikon A1) at 488-nm argon excitation with a time interval of 5 s between two scannings. Translocation of GFP-PKCβ1 was triggered by a direct application of 10 μM UTP into the Hepes buffer. Quantitative analysis of relative fluorescence intensity was performed on Nikon A1 software. The relative change in plasma membrane fluorescence intensity was calculated according to a previously reported method [11]. Briefly, from a series of images recorded before and after angiotensin II stimulation, line intensity profiles across each cell were determined. The relative fluorescence intensity on the plasma membrane was calculated by the formula $(I_{mb} - I_{cyt})/I_{cyt}$, where I_{mb} is the amplitude of the fluorescence signal on the plasma membrane and I_{cyt} is the average cytosolic fluorescent intensity.

2.6. RNA extraction and RT-PCR

Total RNA was isolated from cultured astrocytes using RibospinTM kit (GeneAll, Korea) according to the manufacturer's instruction. Ten microgram of total RNA was reverse-transcribed using SuperScript VILO cDNA synthesis kit (Invitrogen), random hexamers (50 pmol), and dNTPs (1 mM) at 42 °C for 1 h. For RT-PCR, NELL2 forward primer (5′-GCTTGGAGCGTCCCCCAGAA-3′), reverse primer (5′-GGCTAAGGAGAGCTTGTGCC-3′), GAPDH forward primer (5′-GTCTTCACCACCATGGAGAA-3′) and reverse primer (5′-GCATGGACTGTGGTCATGAG-3′) were designed. NELL2 fragments were amplified under the following cycle conditions: denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. This cycle was repeated a total of 35 times. GAPDH fragments were also amplified under the same conditions except that 25 cycles were run.

2.7. Immunocytochemistry

Primary cultured astrocytes were transfected with cNell2-GFP using Neon microporator (Invitrogen). Transfected cells were transferred to coverslip in 24 well plates. After 24 h, cells were incubated with serum-free medium (Dulbecco's modified Eagle's medium, GIBCO) for 16 h. Serum-starved astrocytes were exposed to thrombin (5 U/ml, Sigma) for 20 h. After fixation with 4% paraformaldehyde, the cells were soaked in 0.1% Triton X-100 for permeabilization for 15 min at room temperature and then incubated for overnight at 4 °C with Anti-S100β antibody (1:200, Abcam, Cambridge, MA). After washing, AlexaFluor 647-conjugated secondary antibody (1:300, Jackson Laboratory, Bar Harbor, ME) was added and incubated for 1 h at room temperature. The cells were washed and mounted, and then observed under a Nikon A1 confocal microscope.

2.8. Data analyses

Each data was obtained from at least three independent experiments. Statistical differences between 2 groups were calculated by the unpaired Student's *t*-test. For the analysis of data from more than 3 groups, one-way ANOVA with the Tukey's post hoc test was used. *P* values less than 0.05 were considered to be significantly different.

3. Results

3.1. cNELL2 directly interacts with the N-terminal PS domain of PKCβ1

Previously, we characterized the novel non-secreted splicing variant of NELL2, cytosolic NELL2 (cNELL2), which has the function as a cytoplasmic signaling molecule involved in Ca^{2+} mediated PKCβ1 signaling [3]. To determine whether PKCβ1 is the only isotype which has the ability to bind with cNELL2, we adopted HEK293T cell heterologous in vitro cell culture systems where cNELL2 was co-expressed with each different subtypes of PKC. Using co-immunoprecipitation experiment, we found that cNELL2 was mainly co-immunoprecipitated with β subtypes of PKC including β1 and β2 rather than any other subtypes (α, γ or ε) (Fig. 1A and B). We also found that no other subtypes (η, σ, ζ and δ) of PKC was co-immunoprecipitated with cNELL2 (data not shown). To identify the cNELL2-binding subunit in PKCβ1, we generated several different mutant forms of PKCβ1 including C1 domain which has DAG binding site, C2 with Ca^{2+} binding site, kinase domain (Ki), pseudo-substrate (PS) domain which binds to kinase domain so that acts as an autoinhibitory domain of PKC [12], ΔPS, the mutant lacking PS domain, and PS which contains PS domain only (Fig. 1C). Co-immunoprecipitation results show that wild type (WT) and C1 mutant, but not the mutants without PS domain (C2, Ki or ΔPS), have an ability to bind with cNELL2 demonstrating that PS domain is necessary for the interaction between PKCβ1 and cNELL2 (Fig. 1D and E). To confirm that PS domain in PKCβ1 contains cNELL2 binding site, we performed competition assay, the co-immunoprecipitation of PKC and cNELL2 in presence of the excess amount of PS domain and measured the binding ability of GFP-PKCβ1 to Flag-cNELL2. As a result, we found that the overexpression of PS domain interferes the interaction between PKCβ1 and cNELL2 (Fig. 1F). Collectively, these data demonstrate that only β isotypes of PKC, but not any other isotypes, show the specific binding affinity with cNELL2 through the N-terminal PS domain.

3.2. cNELL2 interferes the surface expression of PKCβ1 resulting in the inhibition of PKC downstream signaling pathway

Next, we sought to determine the molecular function of cNELL2 in Ca^{2+}-mediated PKC signaling pathway. Since it has been well illustrated that PKCβ subtypes are activated by intracellular Ca^{2+} [13] by which PKC is translocated to the cell membrane, we examined whether cNELL2 affects the membrane translocation of PKCβ1 following GPCR-mediated Ca^{2+} transition after UTP treatment [14] in HEK293T cell heterologous system. The treatment of UTP leads to the membrane translocation of PKCβ1 in mCherry-PKCβ1 overexpressed cells without cNELL2 expression (Fig. 2A and D). However, we found that the membrane translocation of PKCβ1 induced by Ca^{2+} (UTP treatment) was interfered by the overexpression of GFP-cNELL2 (Fig. 2B and E). To examine whether direct interaction between PKCβ1 and cNELL2 is necessary for the inhibition of PKCβ1 translocation, we measured the level of PKCβ1 surface expression in HEK293T cells co-expressing GFP-cNELL2 and the mutant form of PKCβ1 lacking PS domain (ΔPS) which is a putative cNELL2 binding site in PKCβ1. We found that cNELL2 was not able to affect the membrane translocation of ΔPS mutant after the Ca^{2+} stimulation by UTP treatment (Fig. 2C). These data demonstrate that cNELL2 inhibits membrane translocation of PKCβ1 through direct binding to the PS domain. To determine whether PKCβ1 downstream signaling pathway is also inhibited by cNELL2, we measured the activity of Erk which is the most well-known downstream molecule of PKC [15–17], after UTP treatment in cNELL2-overexpressing HEK293T cells. We found that the phosphorylation of Erk was significantly increased by the elevation of intracellular Ca^{2+} at 5–10 min after UTP treatment (Fig. 3A).

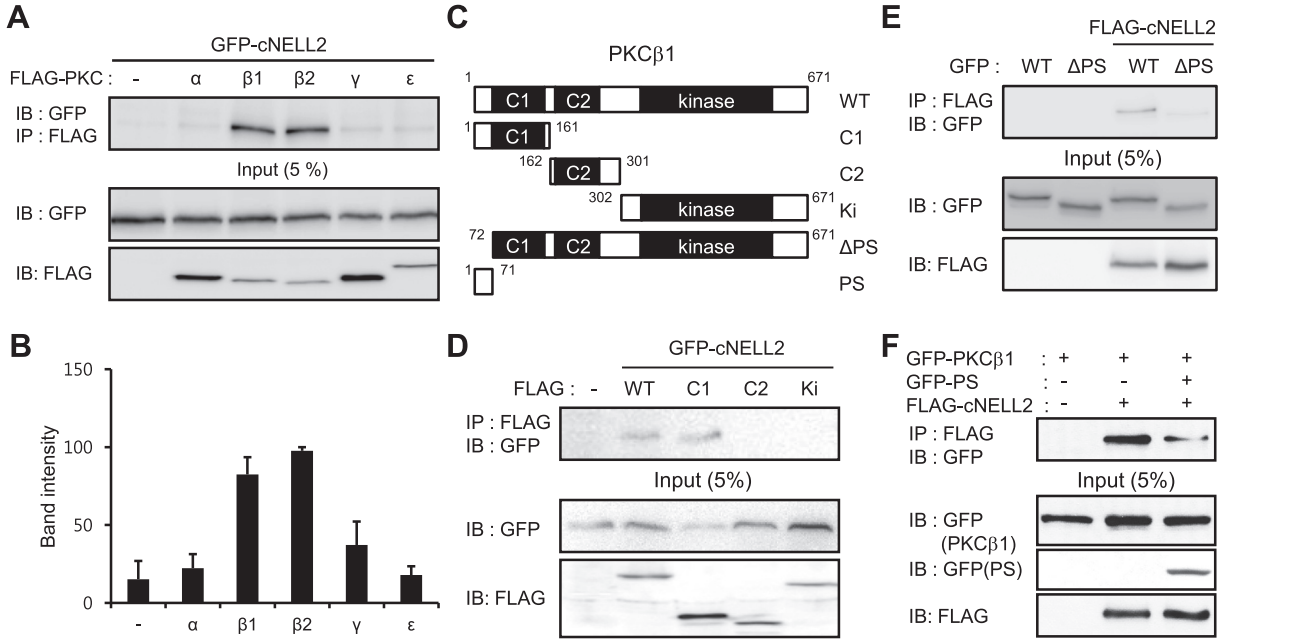

Fig. 1. cNELL2 directly interacts with the N-terminal PS domain of PKCβ1. HEK293T cells were co-transfected with GFP tagged cNELL2 (GFP-cNELL2) and each different subtypes (α, β1, β2, γ and ε) of Flag-tagged PKC (Flag-PKC). (A) GFP-cNELL2 was mainly co-immunoprecipitated with Flag-tagged PKCβ1 and PKCβ2 but not any other subtypes. Normal goat IgG was used as the negative control (−). (B) The signal intensity was measured and graphically represented. Values represent mean ± SEM. (C) Schematic illustrations of PKCβ1 deletion mutants were shown. (D) Co-immunoprecipitation data shows that HEK293T cells expressing Flag-tagged wild type (WT) and C1 mutant of PKCβ1 have an ability to interact with GFP-cNELL2 whereas no co-immunoprecipitation was observed in the cells expressing C2 or Ki mutant of PKCβ1. Normal goat IgG was used as a negative control (−). (E) WT PKCβ1 was co-immunoprecipitated with cNELL2 whereas the mutant form of PKCβ1 lacking PS domain (ΔPS) was not co-immunoprecipitated with cNELL2 in the cells co-expressing FLAG-cNELL2 and GFP-ΔPS. (F) Co-immunoprecipitation data show that the interaction between GFP-PKCβ1 and FLAG-cNELL2 was interfered by the overexpression of GFP-ΔPS in HEK293T cell heterologous system.

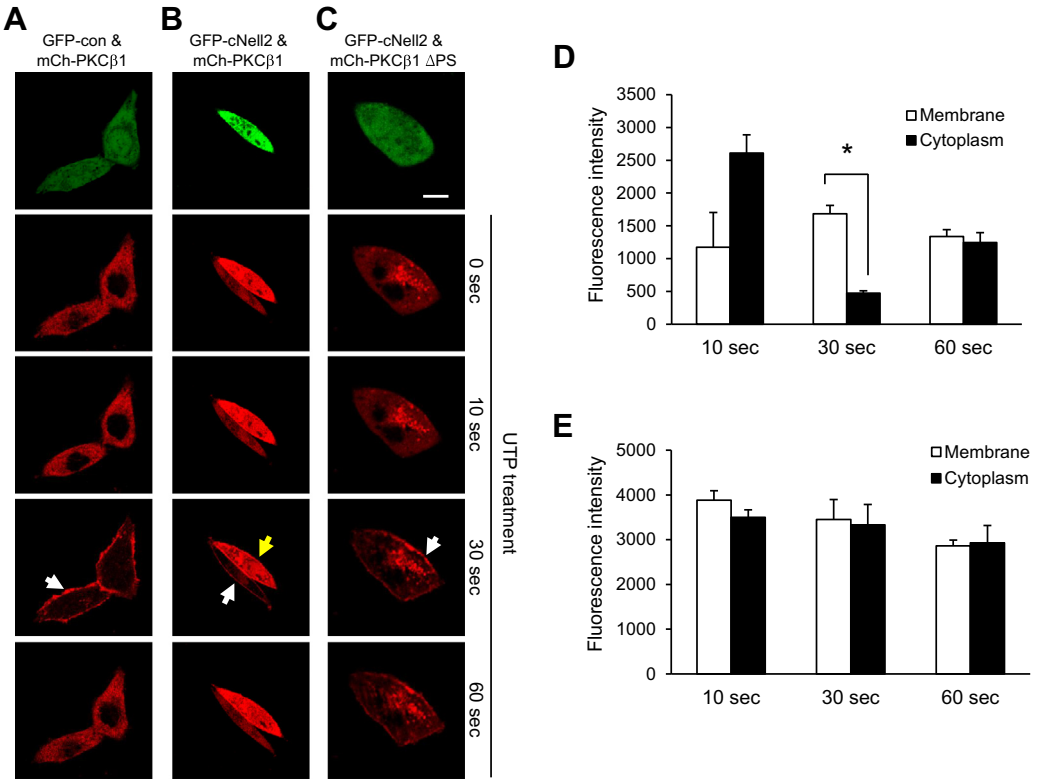

Fig. 2. cNELL2 acts as an inhibitor of PKCβ1 by interfering its translocation to the cell membrane. (A) Confocal images show that UTP (10 μM) treatment increases the surface expression (white arrow) of PKCβ1 in CHO cells overexpressing mCherry-PKCβ1 (mChPKCβ1; red) and GFP containing control vector (GFP-con). (B) The surface expression of PKCβ1 was not changed in the cell overexpressing GFP-cNELL2 (yellow arrow) whereas marked membrane translocation of PKCβ1 was observed when the cell does not express cNELL2 (white arrow) at 30 s after UTP treatment. (C) cNELL2 overexpression was not able to interfere the PKCβ1 translocation of PKCβ1 mutant lacking PS domain (mCh-PKCβ1ΔPS) to the membrane (white arrow). (D) Relative fluorescence intensity of cytoplasm and membrane fraction was measured in the cell expressing only cNELL2-GFP but not mCh-PKCβ1 (white arrow in panel B). (E) Relative fluorescence intensity of cytoplasm and membrane fraction was measured in the cell expressing both GFP-cNELL2 and mCh-PKCβ1 (yellow arrow in panel B). Scale bar, 10 μm. Values represent mean ± SEM. $^{*}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

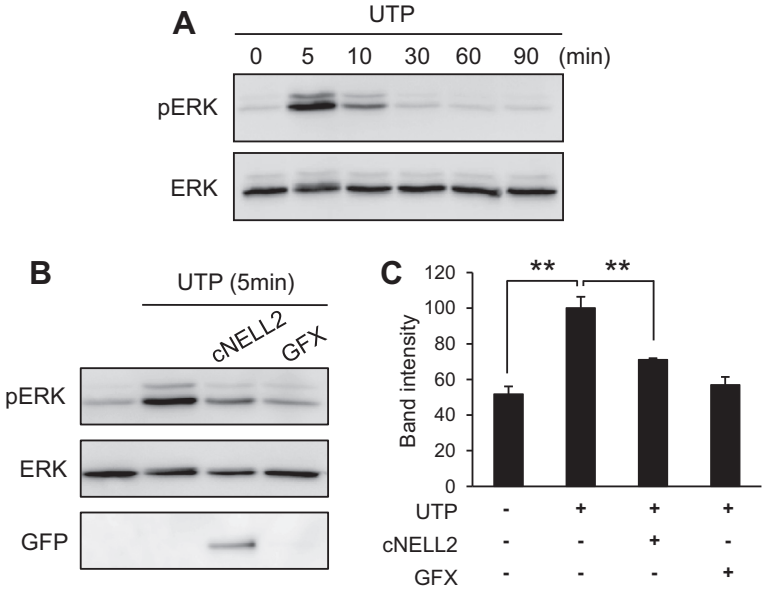

Fig. 3. Erk activation induced by the elevation of intracellular Ca^{2+} was inhibited by cNELL2 expression. (A) Erk phosphorylation (pERK) was increased at 5–10 min after UTP (10 μM) treatment. (B) Erk phosphorylation induced by UTP treatment was blocked in cNELL2-GFP overexpressing HEK293T cells. Negative control group treated with GFX (10 μM), a PKC inhibitor, shows reduced Erk phosphorylation to the untreated control level. (C) Intensity of phospho-Erk in panel B was measured. The value was normalized with total Erk (Erk) signals. Values represent mean ± SEM. $^{**}p \leqslant 0.01$.

However, the Erk phosphorylation increased by UTP treatment was restored in GFP-cNELL2 overexpressing cells (Fig. 3B and C). Collectively, these data show that cNELL2 inhibits PKCβ1 and its signaling pathway through the interaction with PS domain of PKCβ1 in vitro HEK293T cell heterologous system.

3.3. cNell2 is endogenously expressed in astrocytes showing inhibitory effects on the function of PKCβ1

Since sNELL2 has known as an isotype expressed specifically in neurons [2,8], we examined whether cNELL2 also has a similar expression pattern in the brain. To compare the level of cNELL2 expression between glia and neurons in neural tissue, we performed reverse-transcription PCR (RT-PCR) with primary cultured mouse hippocampal neurons and cortical astrocytes. In contrast to neuron-specific sNELL2, cNELL2 mRNA expression level was ~2-fold higher in cultured astrocytes compared to the mouse hippocampal neurons (Fig. 4A). Next, we sought to determine the function of cNELL2 on downstream signaling pathway of PKCβ1 in astrocytes. We measured the level of S100β which is known to be induced by the activation of Erk, one of the PKC downstream signaling molecules [18], in cNELL2 overexpressing cortical astrocytes after the treatment with thrombin, a stimulator of intracellular Ca^{2+} signaling and Erk pathway. We found that thrombin-induced expression of S100β was completely blocked by cNELL2 overexpression (Fig. 4B and C). Collectively, these data demonstrate that cNELL2 is endogenously expressed in cortical astrocytes showing inhibitory effect on PKCβ1 and its downstream Erk pathway.

4. Discussion

In this study, we establish cNELL2 as an endogenous inhibitor of PKCβ1 through the series of molecular and cellular experiments. Current study demonstrates that PKCβ1 and its downstream signaling pathways are inhibited by cNELL2 through its direct binding to the PS domain of PKCβ1. PS domain is a regulatory domain containing basic residues which allows PKC to bind to the acidic lipid in the plasma membrane leading to the membrane translocation and the stabilization of PKC active form [19]. In this regard, our finding which shows that cNELL2 inhibits PKCβ1 membrane translocation may suggest a possible mechanism of PKC inhibition mediated by cNELL2, an endogenous PKC inhibitor. Direct cNELL2 binding to the PS domain could interfere the interaction between PS domain and the acidic lipids in the cell membrane resulting in the inhibition of the membrane translocation and the activation of PKC.

Regarding the role of PKC in diseases, it's been well known that abnormalities in PKC activity are important in the pathophysiology of diabetes. Clinically, Ruboxistaurin, a PKCβ inhibitor, is already being used as a therapeutic drug for the patients with diabetes [20,21]. Similarly, the modulation of PKC activity could provide an insight to find a new therapeutic way for the brain diseases as well, especially for Alzheimer's disease where PKCβ1 is known to have an important pathophysiological role. The previous reports show that the activity (the membrane translocation) of PKCβ1 was highly increased in old aged AD mouse models [22]. Additionally, the protein synthesis and the mRNA expression of COX-2, which is often elevated in AD, were induced by Aβ (25–35) in a PKC-dependent manner in midbrain astrocytes [23]. In this regard,

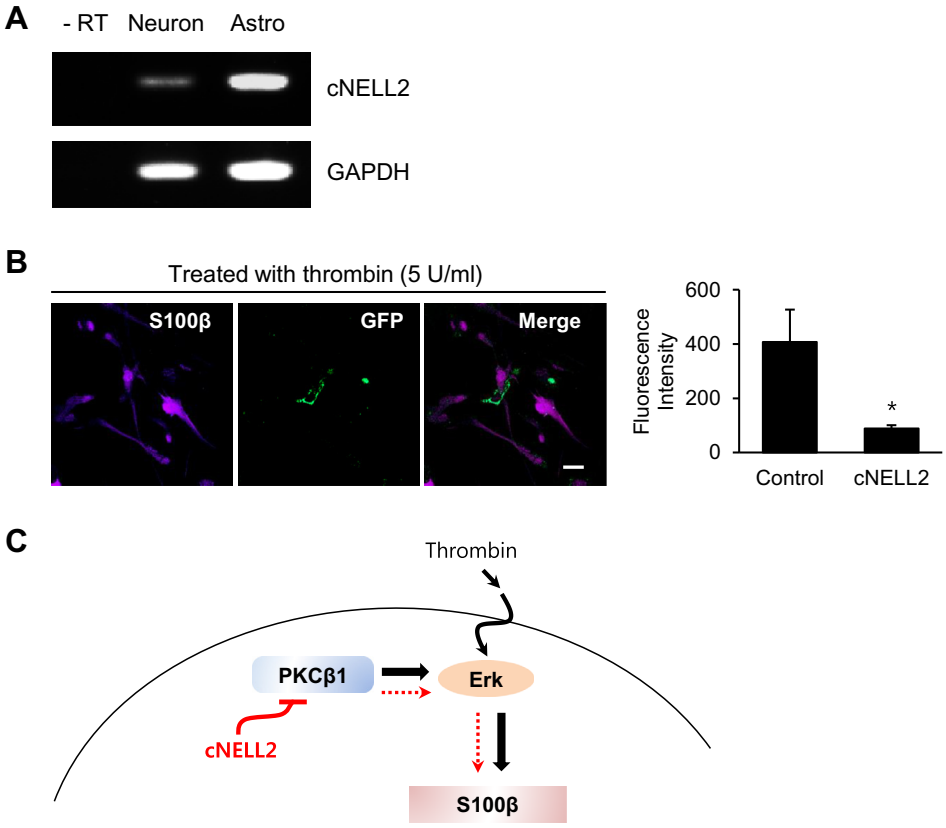

Fig. 4. cNELL2 is highly expressed in astrocytes showing the inhibitory effect on PKCβ1 downstream signaling pathway. (A) RT-PCR results show that cNELL2 has higher mRNA expression in primary cultured astrocytes (Astro) compared to cultured hippocampal neurons (Neuron). GAPDH was used as an internal control. (B) Immunocytochemistry data show that thrombin (5 U/ml)-induced S100β expression was blocked by GFP-cNELL2 overexpression in primary astrocytes. Scale bar, 20 μm. (C) Schematic diagram shows the inhibitory effects of cNELL2 on PKCβ1 and its downstream signaling molecules.

our finding which shows that cNELL2 is specifically expressed in CNS raises the possibility that this endogenous PKC inhibitor, cNELL2 could be another therapeutic target for Alzheimer's disease.

Especially, Hüll and the colleagues mainly focused on the effects of Aβ on the function of astrocytes in their study [23]. In many cases of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, accumulation of reactive astrocytes was often detected in the postmortem brain tissues [24] demonstrating that astrocytes are closely related to the brain pathology. As shown in the previous studies, PKC is one of the key signaling molecules which determine the normal physiological function of astrocytes such as volume-regulated anion channel activity and glutamate release [25], and induce the morphological changes of astrocytes during thrombin-induced astrogliosis [26] as well. These previous observations raise the possibility that cNELL2 could be involved in multiple physiological functions in astrocytes through its inhibitory effect on PKCβ activation in normal and the pathological conditions as well. This idea can be supported by our current result which shows that cNELL2 expression is much higher in cultured astrocytes compared to the hippocampal brain tissues mainly enriched with neurons and that cNELL2 blocks thrombin-induced activation of downstream of PKCβ in primary astrocytes. Based on these current results, our future study will be focused on investigating the role of cNELL2 in brain astrocytes during the process of neurodegenerative diseases.

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