

Caspr4 Interaction with LNX2 Modulates the Proliferation and Neuronal Differentiation of Mouse Neural Progenitor Cells

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Contactin-associated protein 4 (Caspr4), also known as contactin-associated protein-like protein (CNTNAP4), is expressed in various regions of the brain. Recent reports suggest that *CNTNAP4* is a susceptibility gene of autism spectrum disorders (ASDs). However, the molecular function of Caspr4 in the brain has yet to be identified. In this study, we show an essential role of Caspr4 in neural progenitor cells (NPCs). Caspr4 is expressed in NPCs in the subventricular zone (SVZ), a neurogenic region in the developing cortex. Knocking down of Caspr4 enhances the proliferation of NPCs derived from the SVZ of embryonic day 14 mouse. Neuronal differentiation is increased by overexpression of Caspr4, but decreased by knocking down of Caspr4 in cultured mouse NPCs. Transfection of the intracellular domain of Caspr4 (C4ICD) rescues the abnormal decreased neuronal differentiation of Caspr4-knocking down NPCs. Ligand of Numb protein X2 (LNX2), a binding partner of Numb, interacts with Caspr4 in a PDZ domain-dependent manner and plays a similar role to Caspr4 in NPCs. Moreover, transfection of LNX2 rescues the decreased neuronal differentiation in Caspr4-knocking down NPCs. In contrast, transfection of C4ICD fails to do so in LNX2-knocking down NPCs. These results indicate that Caspr4 inhibits neuronal differentiation in a LNX-dependent manner. Therefore, this study reveals a novel role of Caspr4 through LNX2 in NPCs, which may link to the pathogenesis of ASDs.

Introduction

THE FUNCTIONAL COMPLEXITY of the mammalian central nervous system is predicated on the ability to devise cell types, including neurons, astrocytes, and oligodendrocytes during development. Neural progenitor cells (NPCs) are a population of cells, which could self-renew and differentiate into neurons and glial cells [1]. These NPCs proliferate, differentiate, migrate, and eventually integrate into the neural network. The abnormalities in any of these processes will

cause dysfunctions of the brain and leads to neurological diseases, such as brain tumors [2,3], schizophrenia [4], depression [5,6], and Alzheimer's disease [7,8].

Autism spectrum disorders (ASDs), which are characterized by impairments in social reciprocity and language development and highly restrictive interests and/or repetitive behaviors, exhibited developmental abnormalities in the hippocampus, the amygdala, and the cerebral cortex of the patients [9]. However, the etiology of ASDs remains unknown. Recent studies indicate that some autism risk genes,

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such as contactin-associated protein 2 (Caspr2) [10], myocyte enhancer factor 2C [11], and phosphatase and tensin homolog on chromosome 10 [12], modulates the proliferation, differentiation, or migration of NPCs. These studies indicate that NPCs play essential roles in the pathogenesis of ASDs.

Contactin-associated protein 4 (Caspr4), also known as contactin-associated protein-like protein (CNTNAP4), is a transmembrane protein member of the neuroligin superfamily involved in neuron–glia interaction and the clustering of K⁺ channels in myelinated axons [13–17]. *CNTNAP4* gene has recently been identified as a novel susceptibility gene of ASDs [18,19]. Caspr4-deficient mice exhibited hypersensitivity in sensory and overgrooming behaviors [20], the phenotypes often observed in mouse models of autism [10,21]. Expression of Caspr4 has been detected in the olfactory bulb, hippocampus, deep cerebellar nuclei, and the substantia nigra [22]. These studies suggest that Caspr4 may play an important role in the brain development. However, the functions of Caspr4 in the brain remain unknown.

Ligand Numb-protein X2 (LNX2), also known as PDZRN1, is one of the members of the LNX family, which also includes LNX1, LNX3, and LNX4. The LNX family of proteins is of special interest as it has been suggested that they serve as molecular scaffolds that localize PDZ containing proteins, including Numb, a cell fate determinant, to specific subcellular sites [23]. LNX1 protein functions as a RING type E3 ubiquitin ligase and promotes degradation of Numb protein [23,24]. Likewise, LNX2 interacts with Numb and Numblike through a mechanism that involves the phosphotyrosine-binding (PTB) domains of Numb and Numblike and the tetrapeptide, NPAF in LNX2 [23]. Moreover, high levels of expression of LNX2 were reported from embryonic day (E) 12.5 in the brain and were evident in the cortical plate at E15.5 [23]. However, the cellular functions of LNX2 in the brain development are unknown.

In this study, we show that both Caspr4 and LNX2 are expressed in NPCs of the subventricular zone (SVZ), a neurogenic region in the embryonic brain. Moreover, we describe that LNX2 binds to Caspr4 in a PDZ domain-dependent manner. We demonstrate that both Caspr4 and LNX2 promote neuronal differentiation while inhibiting the proliferation of NPCs in vitro. We further identify that Caspr4 enhances neuronal differentiation of NPCs in a LNX2-dependent manner. Therefore, this study reveals a novel function of Caspr4 in modulating the proliferation and differentiation of NPCs through LNX2. This study suggests a role of Caspr4 in cortical development, which may link to the pathogenesis of ASDs.

Materials and Methods

Antibodies

Anti-LNX2 (RP670 from Dr. Kerstin, Ludwig Institute for Cancer Research Stockholm Branch, Karolinska Institute), anti-HA (Upstate), anti-myc (9E10), anti-GAPDH (6C5), anti- γ -tubulin (Sigma), anti-MAP2 (Sigma), anti- β III tubulin (Chemicon; TUJ1), anti-Nestin (Dako), and anti-CaNI (Abcam). Polyclonal antibodies against Caspr4 were generated by immunizing rabbits with glutathione S-transferase (GST)-fusion protein containing the cytoplasmic domain of human Caspr4.

Plasmids and siRNAs

Yeast expression plasmids: For bait vector construction, the 157 bp cDNA corresponding to the cytoplasmic region of human Caspr4 (hC4ICD) was amplified from human spinal cord cDNA by polymerase chain reaction (PCR) using a forward primer 5'-CGGAATTCGCATTTCAG CAGAAAAG-3' with an *Eco*R1 site (in bold) and reverse primer 5'-CGGGATCCT CAGAAGAAGTACTC-3' with a *Bam*H1 site (in bold) and subsequently cloned into the pGBKT7 vector (Clontech). Similarly, human LNX2 (hLNX2) was cloned from a human brain cDNA library (Clontech) by PCR using a forward primer 5'-CGGAATTCATGGGA ACAACAAGTGATGAGATGGTGTC-3' with an *Eco*R1 site and reverse primer 5'-CGGGATCCCTATACAAGGCTGC CAGGCC AAC-3' with a *Bam*H1 site. The PCR product was subcloned into pGADT7 (Clontech). Mouse postnatal day 10 brain cDNA was isolated. Reverse transcription (RT) reaction was performed using the Superscript First-Strand System (Invitrogen) according to the manufacturer's protocol. Full-length Caspr4 was amplified by PCR using 5'-GGGGTACCGC CACCATGAATATGGGATCTGTC-3' with a *Kpn*I site and reverse primer 5'-GGATAGCCCTCGAGGAGGAT CATT GCCAATCAGAAGAAATACTC-3' with a *Xho*I site and subsequently cloned into the pcDNA4/V5-His-A vector (Invitrogen). For transfection of NPCs, mouse Caspr4 and LNX2 were subcloned into the pCDF1-MCS1-EF1-copGFP vector (System Biosciences). phLNX2/pGADT7 was digested by *Eco*R1/*Bam*H1, and the 2.1 kb fragment was cloned into the pCMV-HA vector by *Eco*R1/*Bgl*II with an N-terminal HA tag (Clontech). The mouse LNX2 plasmid (mLNX2/pcDNA3.1/Zeo) and p80-LNX (full-length LNX1 cDNA) were provided by Dr. Kerstin [23]. For myc-tagged expression vector construction, the coding regions of mLNX2 were cloned into pCMV-myc vector by *Eco*R1/*Kpn*I with an N-terminal myc tag (Clontech). All mLNX2 deletions were amplified by PCR and cloned into pCMV-myc vector by *Eco*R1/*Kpn*I with an N-terminal myc tag. Small hairpin RNAs (shRNAs) targeting the coding regions of Caspr4 and LNX2 were designed using the Ambion website. Hairpins were inserted into the backbone pFIV-H1-copGFP shRNA cloning vector (System Biosciences). Validation of shRNA constructs was performed by transfecting HEK293T/17 cells with pCMV-myc-mLNX2 or pcDNA4-mCaspr4 and the shRNA plasmid of interest at a 1:2 ratio. Immunoblotting was performed using anti-myc and anti-Caspr4 antibodies to determine the extent of knockdown. The sequences of the shRNAs were as follows: Caspr4 shRNA#1 (194–214) 5'-GGCTGAATC GAAGAGACGG-3', Caspr4 scrambled (Control) shRNA 5'-TGTATCGCTCGTTCCTACG-3', LNX2 shRNA#2 (468–488) 5'-GCGGCTTCATTT CAAGCTG-3', and LNX2 scrambled (Control) shRNA 5'-CGAGCTCTTCAAGAACCAG-3'. siRNAs were purchased from Genepharma Technology. The sequences of siRNA are as follows: mouse Caspr4: 5'-GG AGCACCUUUCUUGUAATT-3'; mouse LNX2: 5'-GU GACGUGUUGCUGAACAUUTT-3'.

Yeast two-hybrid screening

The yeast two-hybrid analysis was performed according to the manufacturer's instructions (Clontech; Matchmaker Two-Hybrid System 3). A human brain cDNA library was

purchased from Clontech. In total, 3.5×10^6 transformants (AH109; Clontech) were screened. Thirty-three clones were obtained on synthetic dropout (SD) selection plates lacking the amino acids Ade, His, Trp, and Leu, containing X-a-Gal. These positive colonies were blue, indicating that the MEL1 reporter, α -galactosidase, was activated. These clones were introduced into *Escherichia coli*. And TOP10 and the protein coding sequence of prey plasmids were analyzed by sequencing and searching the BLAST database.

Mammalian cell lines, culture conditions, and transfection

HEK293T/17, COS7, and CHO cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin and streptomycin (all from Invitrogen). Cells were cultured at 37°C in a CO₂-humidified incubator. LipofectAMINE 2000 reagent (Invitrogen) and Effectene reagent (QIAGEN) were used for plasmid transfection into cells using conditions that were recommended by the manufacturer.

Mice

C57BL/6J mice were bred in the specific pathogen free condition. All mice used in this study were handled according to the protocols approved by the Institutional Animal Care and Use Committee of the Soochow University.

NPCs culturing, transfection, proliferation, and differentiation

NPCs were isolated from the embryonic day 14 (E14) C57BL/6J mouse SVZ, as described previously [8]. Briefly, NPCs were cultured in the DMEM-F12 (Gibco) containing N₂-supplements (GIBCO), 20 ng/mL basic fibroblast growth factor (bFGF) (Sigma), and 20 ng/mL epidermal growth factor (EGF; Sigma). Nucleofector II (Amaxa Biosystems) was used for plasmid transfection into NPCs. For in vitro differentiation assays, 2 days after transfection, the medium was changed to 0.5% bovine serum-containing medium without EGF and bFGF. Under these conditions, the cells differentiated into neurons or glial cells over 3–5 days. For proliferation assay, NPCs were cultured for 4–5 h in the NPC culture medium containing 10 μ M 5-bromo-2-deoxyuridine (BrdU). All NPCs for differentiation and proliferation assay were isolated from the E14 mouse SVZ.

Reverse transcription–polymerase chain reaction, quantitative PCR, and western blot analysis

Total cellular and tissue RNAs were extracted using the RNeasy Mini Kit (QIAGEN). RT reactions were performed with the Superscript First-Strand System (Invitrogen) according to the manufacturer's protocol. PCR reactions were performed with Taq DNA polymerase (i-DNA Biotechnology). The primers for reverse transcription–polymerase chain reaction (RT-PCR) and quantitative PCR were designed on the basis of mouse sequences. *Hes1*: 5'-AAAGCCTATCATGGAGAAGAGGCG-3' (Forward), 5'-GGAATGC CGGGAGCTATCTTTCTT-3' (Reverse); *Hes5*: 5'-CGCATCAACAGCAGCATAGAG-3' (Forward), 5'-TGGAAGTGGT

AAAGCAGCTTC-3' (Reverse); *Caspr4*: 5'-GAGCACCTTT CATTGTAACCTG-3' (Forward), 5'-CATTGGTCCATTGCTT CA-3' (Reverse); *LNX2*: 5'-CTGCTGGACAAACTGCTGG -3' (Forward), 5'-CGGGTTAGAGCGGTGGAT-3' (Reverse).

For western blotting, sample proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane. The membranes were then blocked with 5% dry milk in TBST plus 0.05% Tween 20; the blots were incubated with the primary antibodies in TBST containing 5% dry milk overnight at 4°C and for 1 h at room temperature. The membranes were washed with TBST and incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (IgG) or donkey anti-rabbit IgG (Amersham Biosciences) for 2 h at room temperature. ECL Plus or ECL Advance Western Blotting Detection Reagents (Amersham Biosciences) were used to visualize the immunoreactive proteins. The densities of the bands were analyzed by Image Lab.

Expression of GST fusion proteins

To generate a GST fusion protein containing the cytoplasmic tail of human Caspr4 (pGST/C4ICD), pGADT7/hC4ICD was digested with *EcoR1/Sal1* and cloned into pGEX-4T1 (Amersham Biosciences). In addition, to generate Caspr4ICD with the PDZ binding motif (amino acids, EYFF) deleted, pGST/C4ICD Δ EYFF was constructed by the standard PCR amplification. GST fusion constructs were expressed in *E. coli* BL21 (DE3) pLysS competent cells using conditions recommended by the manufacturer (Amersham Biosciences). Equal amounts of purified GST fusion proteins were separated on a 10% SDS-PAGE gel and visualized by staining with Coomassie brilliant blue.

GST-pull down assay

Human LNX2-HA fusion protein was incubated with C4ICD fused with GST-protein at 4°C overnight and then added to glutathione–Sephrose 4B at 4°C for 2 h. After several washes in lysis buffer, the protein was eluted under glutathione. The samples were separated on a 10% SDS-PAGE gel and subjected to western analysis with a monoclonal anti-HA antibody.

Immunoprecipitation analysis

For immunoprecipitation (IP) analysis, cell lysates were extracted by incubating cells in 1 mL of Tris–NaCl–EDTA (TNE) lysis buffer [10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonident P, 5 mM ethylenediaminetetraacetic acid (EDTA), complete protease inhibitor] on ice for 15 min. Cell lysates were centrifuged for 20 min at 4°C. The lysates were precleared with protein A beads (Roche Diagnostics) for 30 min and incubated with anti-myc antibody overnight, followed by further incubation with protein A for 3 h. After four washes in TNE buffer, IP samples were analyzed by SDS-PAGE and immunoblotting with anti-Caspr4 antibody.

IP analysis from cells and brain lysates. NPCs and brain tissues were lysed in RIPA buffer (50 mM Tris–HCl, pH 9.0, 1% sodium deoxycholate, 2.5 mM EDTA-free complete protease inhibitor). After centrifugation at 16,000 g for 1 h at 4°C, the supernatant was used as the cell lysate. The lysate was precleared with protein A beads for 30 min. After

centrifugation at 16,000 *g*, the supernatant was collected and this lysate was used for the IP analysis. Polyclonal anti-Caspr4 antibody was added to the mouse brain lysate and rotated overnight at 4°C. Protein A beads were added to the mixture and rotated for 3 h. After the precipitates had been washed five times in RIPA buffer, the IP samples were analyzed by SDS-PAGE and immunoblotting.

Immunofluorescence staining

C57BL/6 mice pregnant with E14 fetuses were perfused with phosphate-buffered saline (PBS) under anesthesia, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains of fetuses were removed, postfixed in the same fixative, and then immersed in 30% sucrose at 4°C. Serial 16- μ m-thick coronal sections were cut on a freezing microtome. Sections were blocked with 10% normal goat serum in PBS and then incubated with primary antibody in the blocking solution overnight at 4°C. Sections were rinsed three times in PBS with 0.3% Triton X-100 and incubated with appropriate secondary Alexa Fluor-conjugated antibodies for 1 h at room temperature. The sections were then mounted with a mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories) after being rinsed three times with PBS with 0.3% Triton X-100.

Immunocytochemistry on cultured cells and immunostaining on tissue sections were performed, as previously described [8]. For quantification of immunofluorescence, images of fields of cultured cells were captured by digital photomicrography under a $\times 20$ objective over the entire surface of each coverslip. All labeled cells were then counted in each photomicrograph. The proportion of neurons was quantified as the numbers of Tuj1⁺EGFP⁺ cells divided by the total number of EGFP⁺ cells in the same fields.

Statistical analyses

Each experiment was repeated more than three times. The statistical analyses were performed by one-way analysis of variance or Student's *t*-test using SPSS software, as appropriate. In all the graphs, the error bars indicate the standard error of the mean. Significance was accepted at $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Results

Expression of Caspr4 in the neurogenic ventricular zone and NPCs

We first investigated the expression profile of Caspr4 in the cortex during development. Western blot analysis showed that expression of Caspr4 in the cortex started at E12 and increased at E14, E16, E18, and peaked at P0 relatively, during the cortex development (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/scd). A similar expression profile of Caspr4 was detected in the SVZ of E12, E14, and E18 mouse brains (Supplementary Fig. S1B). These results suggest that Caspr4 might play a role in the cortex development. NPCs in the SVZ play essential roles in the cortex development [25]. We thus performed immunofluorescence staining with antibodies against Caspr4 and Nestin (Fig. 1A) or Sox2 (Fig. 1B), two markers of NPCs, in the SVZ of E14

(Fig. 1A, B), P0 (Supplementary Fig. S2A), and adult mice (Supplementary Fig. S2C). The results showed that Caspr4 was expressed in Nestin⁺ (Fig. 1A) or Sox2⁺ cells (Fig. 1B) in the SVZ. Next, we performed immunofluorescence staining using antibodies against Caspr4 and Nestin in cultured NPCs derived from E14 mouse SVZ. Caspr4 immunoreactivity was detected in cultured NPCs, which exhibited Nestin⁺ staining (Fig. 1C). In addition, immunoreactivity of Caspr4 was also detected in β III-tubulin⁺ (Tuj1, a maker of neuron) cells in the cerebral cortex (Supplementary Fig. S3A) and glial fibrillary acidic protein⁺ (GFAP, a maker of astrocyte) cells in the corpus callosum (Supplementary Fig. S3E), indicating that Caspr4 is expressed in cortical neurons and astrocytes. The specificity of Caspr4 antibody was validated by coexpression of Caspr4 and myc in HEK293 cells transfected with the plasmid expressing Caspr4 fused with myc at its carboxyl terminus (Supplementary Fig. S4A) and by the lack of immunoreactivity of Caspr4 in the brain sections stained with non-immunoglobulin (Supplementary Fig. S4B). Moreover, Caspr mRNA (Fig. 1D) and protein (Fig. 1E) were detected in cultured NPCs derived from E14 mouse SVZ and hippocampus by RT-PCR and western blot, respectively. These results demonstrate that Caspr4 is expressed by NPCs in the SVZ of the embryonic mouse brain.

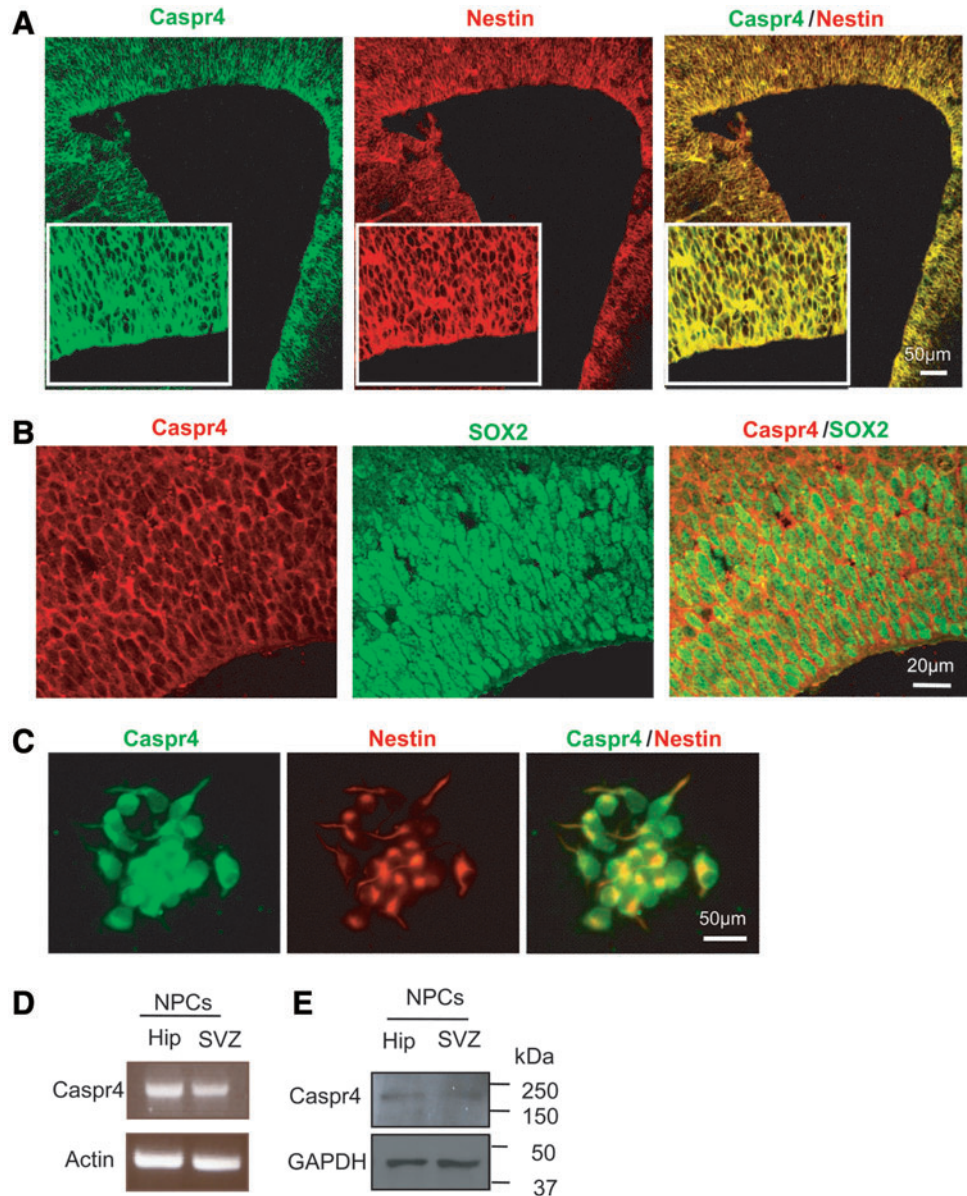
Caspr4 inhibits the proliferation of NPCs

We next investigated whether Caspr4 might play a role in modulating the proliferation of NPCs. NPCs derived from E14 mouse SVZ were transfected with Caspr4 small interference RNA (Caspr4 siRNA), which could downregulate the endogenous expression of Caspr4 in NPCs (Supplementary Fig. S5A) or the control scrambled siRNA (NC, Fig. 2A), respectively. The transfected cells were subjected to BrdU incorporation analysis. Quantification showed that the number of BrdU⁺ cells was increased in Caspr4 siRNA-transfected NPCs compared to the control NPCs (Fig. 2A). In contrast, apoptosis in Caspr4 siRNA-transfected NPCs showed no difference from the control cells (data not shown). These results demonstrate that Caspr4 inhibits the proliferation of NPCs in vitro.

Caspr4 promotes neuronal differentiation of NPCs

We further investigated whether Caspr4 might play a role in regulating neuronal differentiation of NPCs. Caspr4 cDNA was transfected together with copepod green fluorescent protein (copGFP) in the same vector but under control of another promoter into cultured NPCs (Caspr4, Fig. 2B). The control cells were transfected with the empty vector expressing copGFP alone (GFP, Fig. 2B). The transfected cells were immunostained for β III-tubulin, a marker of neurons, and GFP after being cultured under differentiation condition for 3 days. Quantification analysis showed an increased number of β III-tubulin⁺ cells in Caspr4-transfected cells compared to the control cells (Fig. 2B). These data suggest that Caspr4 promotes neuronal differentiation of NPCs in vitro. To further test this notion, we transfected short hairpin RNA (shRNA) of Caspr4 in a copGFP expressing vector, which could downregulate the expression of Caspr4 in transfected HEK293T cells (Supplementary Fig. S5B), to cultured NPCs and immunostained them for β III-tubulin and GFP after

FIG. 1. Contactin-associated protein 4 (Caspr4) is expressed in embryonic neural progenitor cells (NPCs). **(A)** Double immunostaining for Nestin and Caspr4 in the subventricular zone (SVZ) of embryonic day 14 (E14) mouse. Scale bar: 50 μ m. **(B)** Double immunostaining for Caspr4 and Sox2 in the SVZ of E14 mouse. Scale bar: 20 μ m. **(C)** NPCs isolated from the E14 mouse SVZ were double-stained for Caspr4 and Nestin. Scale bar: 50 μ m. **(D)** Reverse transcription–polymerase chain reaction (RT-PCR) analysis of the expression of Caspr mRNA in the cultured NPCs derived from E14 SVZ and hippocampus (Hip). Actin was detected as the loading control. **(E)** Western blot analysis of the expression of Caspr protein in the cultured NPCs derived from E14 SVZ and hippocampus (Hip). GAPDH was detected as the loading control. Color images available online at www.liebertpub.com/scd



3 days in vitro differentiation (Fig. 2C). The number of β III-tubulin⁺ cells was significantly decreased in the NPCs transfected with the Caspr4 shRNA compared with that of the control group transfected with scrambled shRNA (Fig. 2C). Caspr4 is a type I transmembrane protein that contains a large extracellular and a short intracellular domain [26]. The intracellular domain of Caspr4 (C4ICD) contains a PDZ binding motif, through which it may interact with different PDZ domain-containing proteins known to participate in membrane protein trafficking [27], the establishment of specialized membrane domains [28], clustering of transmembrane receptors [29], and the generation of multiprotein signaling complexes [30]. We therefore asked whether Caspr4 promotes neuronal differentiation of NPCs through C4ICD. C4ICD plasmid and Caspr4 shRNA were cotransfected to NPCs. The transfected cells were immunostained for β III-tubulin and GFP at 3 days after being cultured under differentiation condition. Interestingly, the abnormally reduced neuronal differentiation of Caspr4 shRNA-transfected NPCs was rescued by

coexpression of C4ICD (Fig. 2C). These results indicate that Caspr4 promotes neuronal differentiation of NPCs through its intracellular domain.

LNX2 interacts with Caspr4

Given that Caspr4 promotes neuronal differentiation of NPCs through C4ICD, we were interested in identifying binding proteins interacting with C4ICD by yeast two-hybrid screening. Using bait that consisted of C4ICD (amino acid residues 1,265–1,310), we identified prey that coded amino acids 433–690 of LNX2, an N-terminal truncated form of LNX2 (Fig. 3A). To confirm this interaction, we transformed yeast cells with the following three groups of plasmids: (1) Caspr4ICD/pGBKT7 and pGADT7; (2) pGBKT7 and LNX2/pGADT7 (full length of LNX2 fused with the GAL4 activation domain); and (3) Caspr4ICD/pGBKT7 and LNX2/pGADT7 (Fig. 3B). On SD plates that lack amino acids Ade, His, Leu, and Trp and contain X- α -Gal, blue

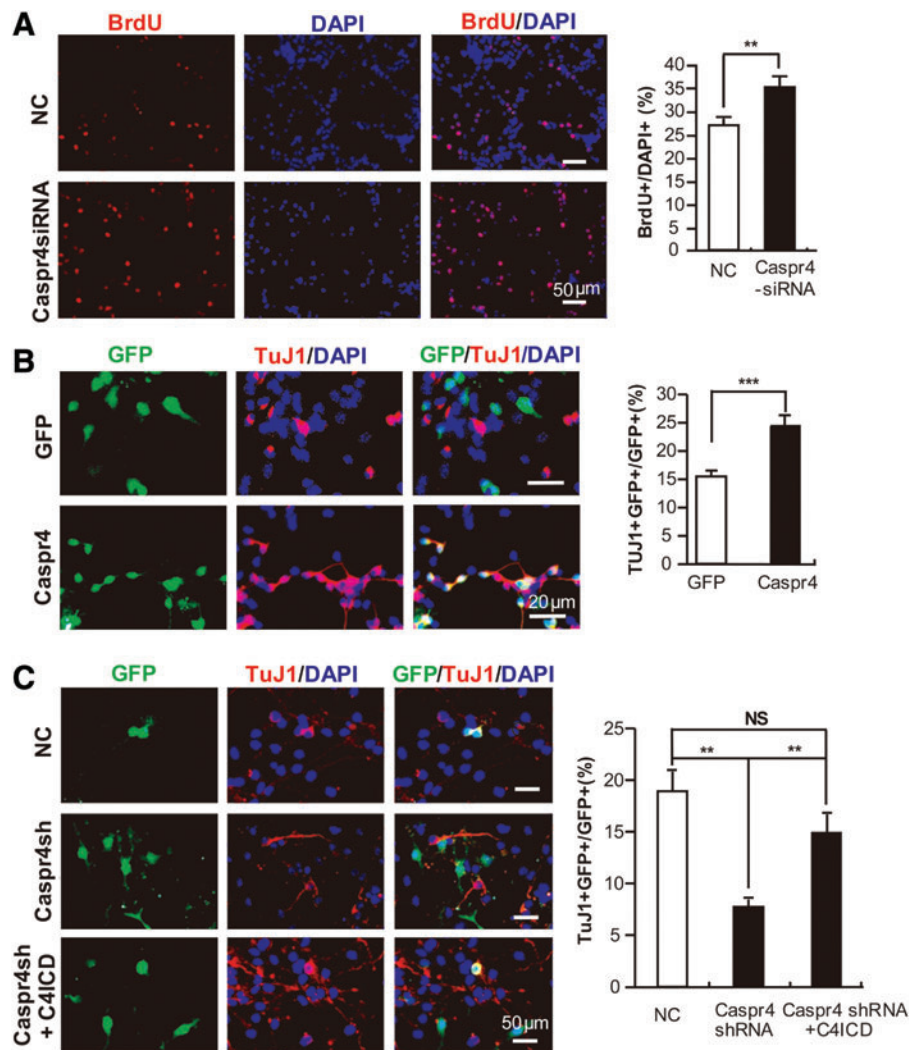


FIG. 2. Caspr4 inhibits the proliferation while promoting neuronal differentiation of NPCs. (A) NPCs were transfected with Caspr4 small interference RNA (siRNA) and control siRNA (NC), respectively. Transfected cells were stained for 5-bromo-2-deoxyuridine (BrdU) and 4',6-diamidino-2-phenylindole (DAPI) after being cultured in the medium containing BrdU for 3 h. The numbers of BrdU⁺ cells were counted and expressed as the percentage of the number of DAPI⁺ cells (NC: 26.89 ± 1.26; Caspr4-siRNA: 35.11 ± 2.06). (B) NPCs were transfected with Caspr4 plasmid in a pCDF1-MCS1-EF1-copGFP vector that expresses coGFP (Caspr4) or in an empty vector containing only copGFP (GFP). After 3–4 days of differentiation in vitro, cells were immunostained for β III tubulin (TUJ1), GFP, and DAPI. The numbers of GFP⁺TUJ1⁺ cells were counted and expressed as the percentage of the number of GFP⁺ cells (GFP: 15.33 ± 1.01; Caspr4: 24.33 ± 1.70). (C) NPCs were transfected with Caspr4 small hairpin RNA (shRNA) or Caspr4 shRNA plus intracellular domain of Caspr4 (C4ICD) as well as a scrambled shRNA (NC) as the control. After 3–4 days of differentiation in vitro, cells were immunostained for β III tubulin (TUJ1), GFP, and DAPI. The numbers of GFP⁺TUJ1⁺ cells were counted and expressed as the percentage of the number of GFP⁺ cells (NC: 18.59 ± 1.97; Caspr4-shRNA: 7.69 ± 0.80; Caspr4-shRNA plus C4ICD: 14.89 ± 1.82). Scale bars: 50 μ m (A–C); 20 μ m (B). Values are represented as mean \pm standard error of the mean (SEM), Student's *t*-test (A, B); one-way analysis of variance (C). ***P* < 0.01. ****P* < 0.001. NS, not significant. Color images available online at www.liebertpub.com/scd

colonies were only apparent when the bait plasmid, C4ICD/pGBKT7, and the prey plasmid, LNX2/pGADT7, were co-transformed into the yeast cells (Fig. 3B). The interaction between Caspr4 and LNX2 was further confirmed by the GST pull-down assay and IP. C4ICD-GST, but not GST, could pull-down LNX2-HA protein from LNX2-HA-transfected-COS7 cells (Fig. 3C). IP analysis showed that anti-myc antibody could only precipitate Caspr4 from Caspr4- and LNX2-myc-cotransfected HEK293T cells (Fig. 3D). Furthermore, immunostaining using antibodies against Caspr4 and LNX2 showed that Caspr4 and LNX2 were

colocalized along cell membranes of Caspr4- and LNX2-myc-cotransfected HEK293T cells (Fig. 3E). These results indicate that Caspr4 interacts with LNX2 in both yeast and mammalian cells.

LNX2 binding to Caspr4 requires the second PDZ domain

LNX2 consists of an N-terminal ring-finger domain and four PDZ domains. The ring-finger domain functions as an E2-dependent, E3 ubiquitin ligase [23,24]. The PDZ

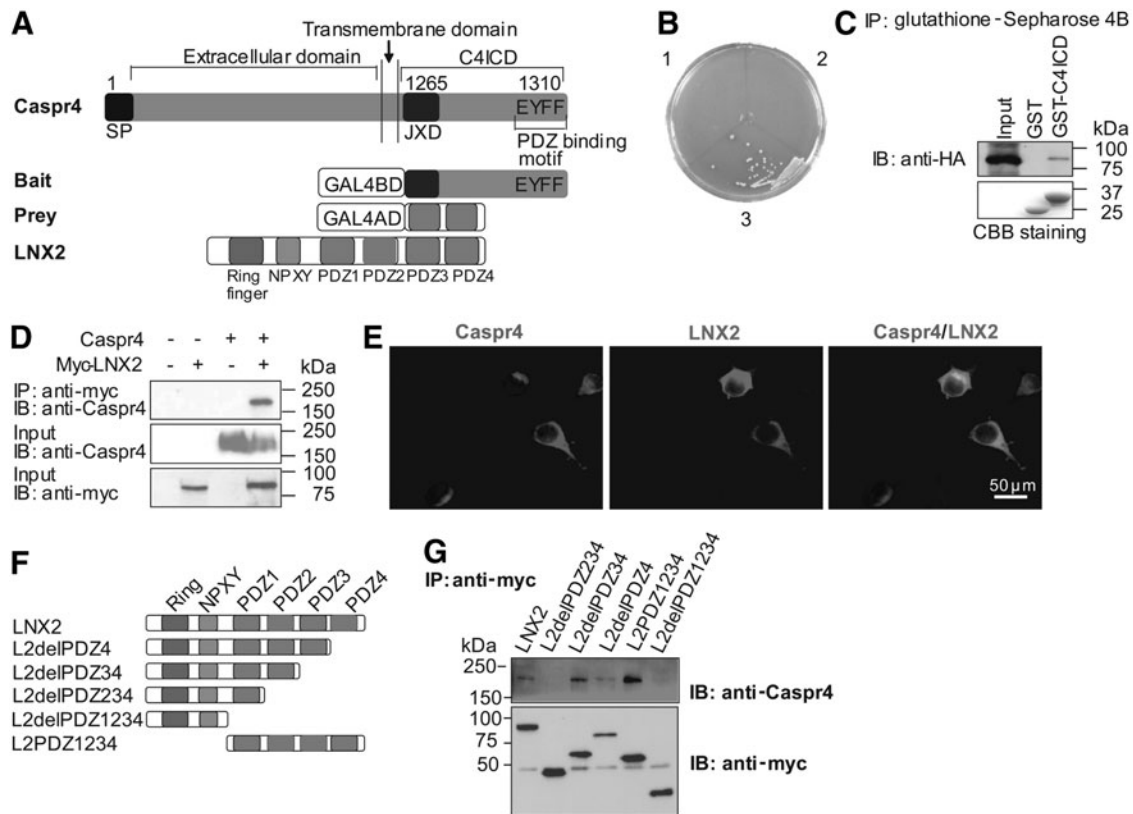


FIG. 3. Ligand of Numb protein X2 (LNX2) interacts with Caspr4. **(A)** Schematic representations of full-length Caspr4, the bait protein that has the last 47 amino acids of the Caspr4 cytoplasmic domain (C4ICD, amino acids 1,265–1,310), the prey clone that codes the amino acids 433–690 of LNX2, and full-length LNX2 that contains one Ring finger, one NPXY, and four PDZ domains. **(B)** Yeast cells were transformed with the following plasmids: Caspr4ICD/pGBKT7 and pGADT7 (1), pGBKT7 and LNX2/pGADT7 (2), and Caspr4ICD/pGBKT7 and LNX2/pGADT7 (3). Transformants were spread onto SD/–Ade/–His/–Leu/–Trp medium plate containing X- α -Gal. Colonies were only observed in yeast cells coexpressing C4ICD and LNX2. **(C)** Protein extracts from LNX2-HA-transfected COS7 cells were incubated and pulled-down with GST and GST-C4ICD proteins. Bound proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted with anti-HA antibody (*upper panel*) or stained with Coomassie brilliant blue (CBB staining; *lower panel*). **(D)** Caspr4 was cotransfected with LNX2-myc into HEK293T cells. Immunoprecipitation was performed with an anti-myc antibody and probed with an anti-Caspr4 antibody. The inputs were immunoblotted with both antibodies against Caspr4 and myc as indicated. **(E)** HEK293T cells were cotransfected with Caspr4 and LNX2-myc. After 24 h post-transfection, double immunofluorescence staining was performed on these cells by using antibodies against Caspr4 and myc. Scale bar: 50 μ m. **(F, G)** Schematic representation of truncated LNX2 constructs, which were tagged with a myc at their N-terminus **(F)**. Caspr4 was cotransfected with a myc-tagged full-length (FL-LNX2) and different truncated LNX2 (L2delIPDZ234, L2delIPDZ34, L2delIPDZ4, L2PDZ1234, and L2delIPDZ1234) into HEK293T cells. Coimmunoprecipitation was performed using the anti-myc antibody and probed with antibodies against Caspr4 (*upper panel*) and myc (*lower panel*) **(G)**.

domains of LNX2 contain the binding sites for one of the proteins own PDZ domains and so LNX proteins can form PDZ-dependent oligomeric as well as heteromeric complexes with each other [23]. To identify the binding sites of LNX2 to Caspr4, we constructed various truncated LNX2, which were tagged with myc (Fig. 3F), and cotransfected them respectively with Caspr4 cDNA into HEK293T cells. IP analysis showed that Caspr4 could be precipitated by full-length LNX2, truncated LNX2 deleted PDZ3 and PDZ4 domains (L2delIPDZ34), truncated LNX2 deleted PDZ4 domain (L2delIPDZ4), and the PDZ (1–4) domains of LNX2 (L2PDZ1234) (Fig. 3G). In contrast, truncated LNX2 deleted PDZ2, PDZ3, and PDZ4 (L2delIPDZ234) and truncated LNX2 deleted all four PDZ domains (L2delIPDZ1234) failed to precipitate Caspr4 from the cotransfected cells (Fig. 3G). These results suggest that the binding of LNX2 to Caspr4

requires its second PDZ domain. Thus, LNX2 binds to Caspr4 in a PDZ domain-dependent manner.

LNX2 coexpression with Caspr4 in the SVZ and NPCs

Caspr4 is expressed in NPCs in the SVZ and modulates the proliferation and neuronal differentiation of NPCs. We then asked whether LNX2, as a novel binding partner of Caspr4, might play a role in NPCs as well. The expression of LNX2 in the cortex was detected from E10, increased at E12, E14, E16, E18, and peaked at P0 (Supplementary Fig. 6A), which showed a similar expression profile to Caspr4. Similar to Caspr4, LNX2 was detected in the SVZ during the embryonic stage (Supplementary Fig. 6B). Immunostaining by using antibodies against LNX2 and Nestin in E14 (Fig. 4A), P0 (Supplementary Fig. S2B), and adult

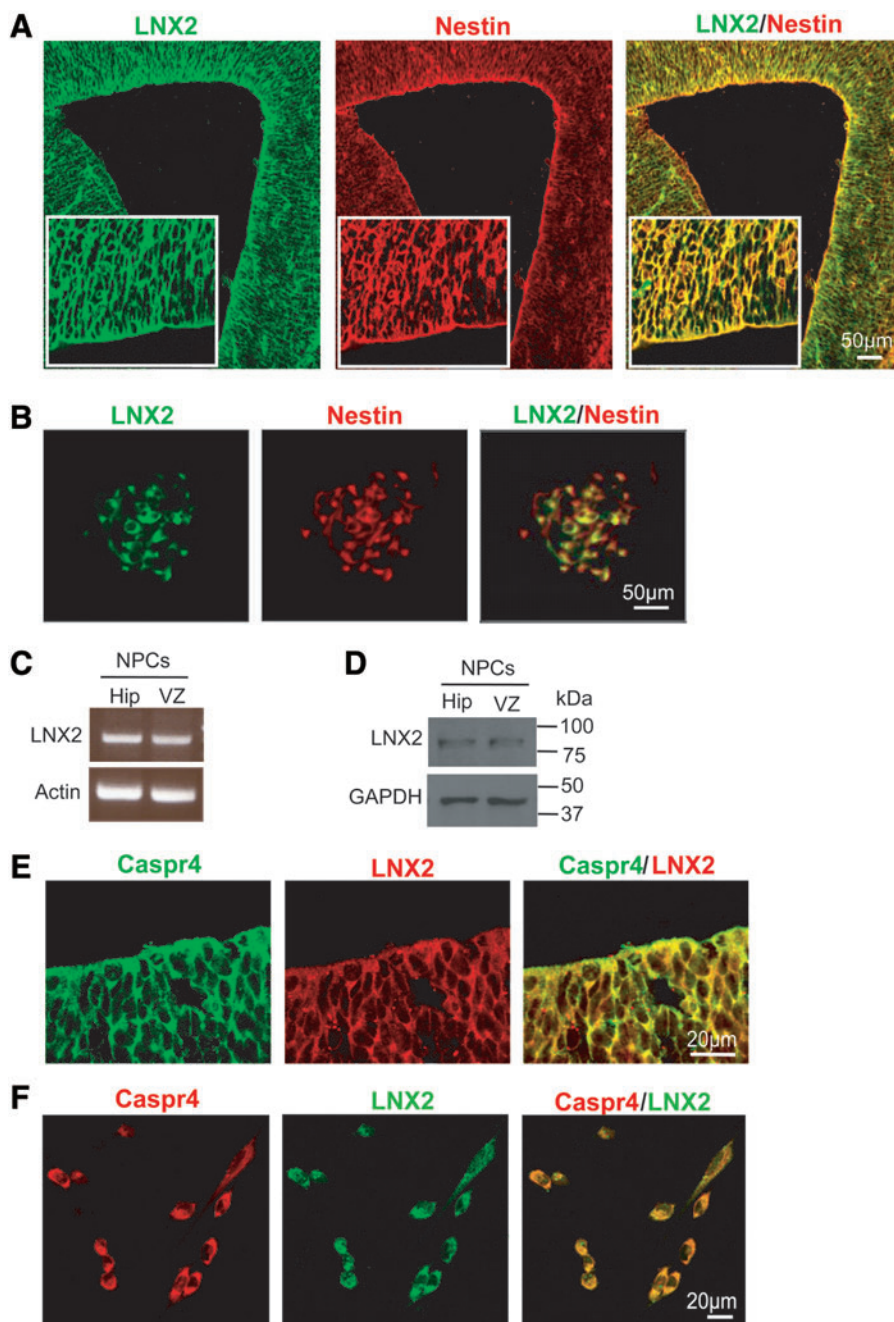


FIG. 4. Expression of LNX2 in the SVZ and NPCs. **(A)** Double immunostaining for Nestin and LNX2 in the SVZ of E14 mouse brain. Scale bar: 50 μ m. **(B)** Cultured NPCs isolated from the SVZ of E14 mouse brain were immunostained for LNX2 and Nestin. Scale bar: 50 μ m. **(C)** RT-PCR analysis of the expression of LNX2 mRNA in cultured NPCs derived from E14 SVZ and hippocampus (Hip). Actin was detected as loading control. **(D)** Western blot analysis of the expression of LNX2 in cultured NPCs derived from E14 SVZ and hippocampus (Hip). GAPDH was detected as loading control. **(E)** Double immunostaining for LNX2 and Caspr4 in the SVZ of E14 mouse brain. Scale bar: 20 μ m. **(F)** NPCs isolated from the SVZ of E14 mouse brain were immunostained for LNX2 and Caspr4. Scale bar: 50 μ m. Color images available online at www.liebertpub.com/scd

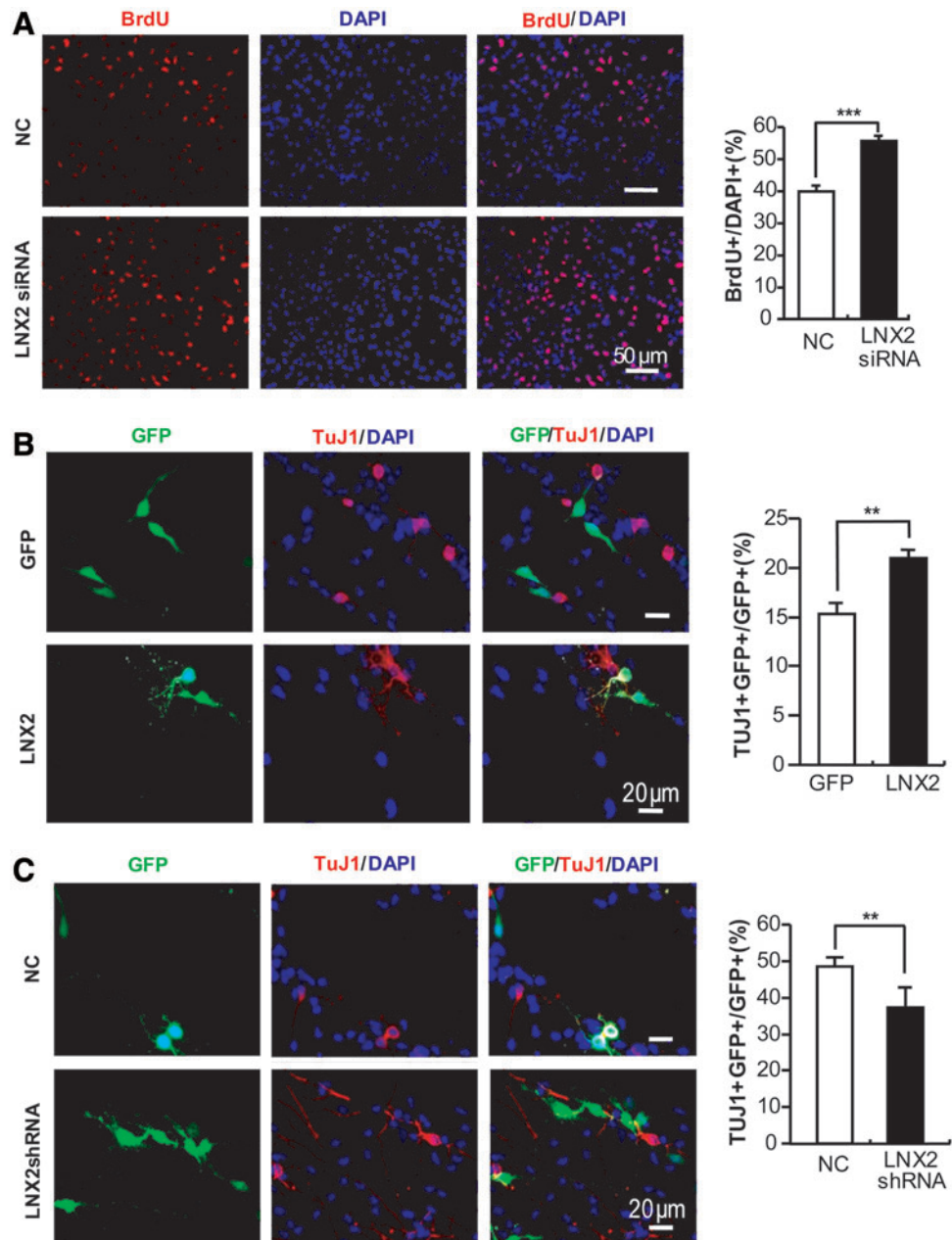
(Supplementary Fig. 2D) mouse SVZ or in the cultured NPCs derived from E14 mouse SVZ (Fig. 4B) showed that LNX2 was expressed in Nestin⁺ cells. Similar to Caspr4, LNX2 mRNA (Fig. 4C) and protein (Fig. 4D) were detected in cultured NPCs derived from E14 mouse SVZ and hippocampus by RT-PCR and western blot, respectively. In addition, similar to Caspr4, LNX2 immunoreactivity was detected in β III-tubulin⁺ cortical neurons in the cerebral cortex (Supplementary Fig. S3C) and GFAP⁺ astrocytes in the corpus callosum (Supplementary Fig. S3F). Moreover, immunostaining by using antibodies against LNX2 and Caspr4 in the E14 SVZ or in the cultured NPCs showed that LNX2 colocalized with Caspr4 in the cells along the SVZ of E14 mice (Fig. 4E) and the cultured NPCs derived from the SVZ (Fig. 4F). These results indicate that LNX2 is coexpressed with Caspr4 by NPCs in the SVZ of embryonic mouse.

LNX2 inhibits the proliferation while promoting neuronal differentiation of NPCs

We then investigated whether LNX2 might play a same role as Caspr4 in the proliferation of NPCs. NPCs transfected with LNX2 siRNA, which could downregulate the expression of LNX2 in LNX2-transfected HEK293T cells (694, Supplementary Fig. S5C) or the control scrambled siRNA (NC, Supplementary Fig. S5C), were subjected to BrdU incorporation analysis. Quantification showed that the number of BrdU⁺ cells was increased in LNX2 siRNA-transfected NPCs (Fig. 5A). Thus, similar to Caspr4, LNX2 inhibits the proliferation of NPCs in vitro.

We next investigated whether LNX2 might play a role in promoting neuronal differentiation of NPCs. Using a similar

FIG. 5. LNX2 inhibits the proliferation but promotes neuronal differentiation of NPCs. **(A)** NPCs were transfected with LNX2 siRNA and its scrambled siRNA (NC), respectively. Transfected cells were stained for BrdU and DAPI after being cultured in the medium containing BrdU for 3 h. The numbers of BrdU⁺ cells were counted and expressed as the percentage of the number of DAPI⁺ cells (NC: 39.68 ± 1.39 ; LNX2 siRNA: 55.23 ± 1.75). **(B, C)** NPCs were transfected with LNX2 cDNA in a pCDF1-MCS1-EF1-copGFP vector expressing coGFP [LNX2, **(B)**] or in an empty vector containing only copGFP [GFP, **(B)**] and a LNX2 shRNA [LNX2 shRNA, **(C)**] or a scrambled shRNA as a control [NC, **(C)**]. After 3–4 days of differentiation in vitro, cells were double-stained for TUJ1, GFP, and DAPI. The numbers of GFP⁺TUJ1⁺ cells were counted and expressed as the percentage of the number of GFP⁺ cells. **(B)** Control: 15.33 ± 1.01 ; LNX2: 20.76 ± 0.77 ; **(C)** Control: 48.50 ± 2.17 ; LNX2-shRNA: 37.24 ± 5.56 . Scale bars: 50 μ m (**A**), 20 μ m (**B, C**). Values are represented as mean \pm SEM, Student's *t*-test. ***P* < 0.01. ****P* < 0.001. Color images available online at www.liebertpub.com/scd



approach as in Fig. 2B and C, LNX2-transfected NPCs showed an increased number of β III-tubulin⁺ cells (Fig. 5B), whereas LNX2-shRNA-transfected NPCs (Supplementary Fig. S5D) showed a decreased number of β III-tubulin⁺ cells (Fig. 5C), compared to the control cells (Fig. 5B, C) after differentiation. These results indicate that LNX2, similar to Caspr4, inhibits the proliferation while promoting neuronal differentiation of NPCs.

LNX2 is an essential downstream element of Caspr4 in promoting neuronal differentiation of NPCs

LNX2 functions as a RING-type E3 ubiquitin ligase and promotes degradation of Numb protein [24]. Considering the interaction between LNX2 and Caspr4, we investigated

whether LNX2 might regulate the degradation of Caspr4. Consistent with this idea, the levels of Caspr4 were reduced by overexpression of LNX2 in HEK293 cells (Fig. 6A) and were increased by knocking down of LNX2 in cultured NPCs (Fig. 6B). These results indicate that LNX2 play a role in regulation of Caspr4 protein expression. In contrast, the levels of LNX2 remain unchanged by overexpression of Caspr4 (Fig. 6C). Given that LNX2 plays a similar role to Caspr4 in neuronal differentiation of NPCs, it is unlikely that LNX2 functions on NPCs through degradation of Caspr4. Therefore, to further clarify the relationship of LNX2 and Caspr4 in modulating neuronal differentiation of NPCs, we cotransfected LNX2 plasmid and Caspr4 shRNA into cultured NPCs and compared the capability of the transfected cells to differentiate neurons. As previous result, Caspr4 shRNA-transfected NPCs differentiated less β III-tubulin⁺

neurons than the control cells transfected with the scrambled shRNA (Fig. 6D). In contrast, cotransfection of LNX2 with Caspr4 shRNA rescued the decreased neuronal differentiation of Caspr4 shRNA-transfected NPCs (Fig. 6D). However, when we cotransfected C4ICD and LNX2 shRNA into cultured NPCs, the result showed that C4ICD failed to rescue the decreased neuronal differentiation of LNX2 shRNA-transfected NPCs (Fig. 6E), although it rescued the decreased β III-tubulin⁺ neuron numbers in Caspr4 shRNA-transfected cells (Fig. 2C). These results suggest that C4ICD promotes neuronal differentiation in a LNX2-dependent manner, and LNX2 acts as the downstream molecule of Caspr4 in promoting neuronal differentiation of NPCs.

Discussion

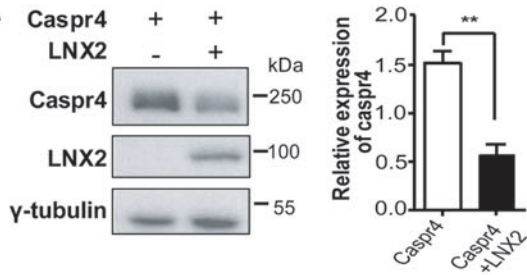
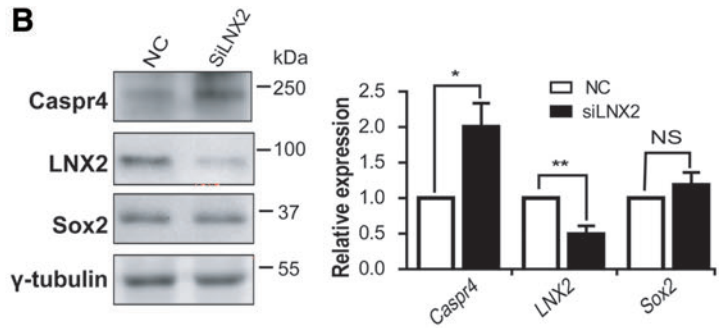
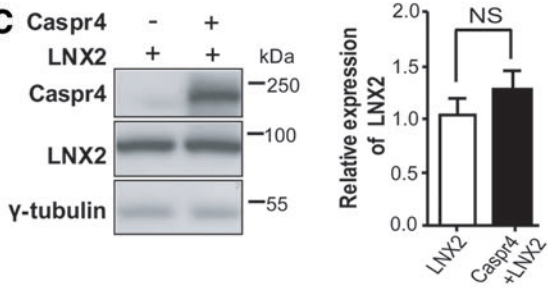
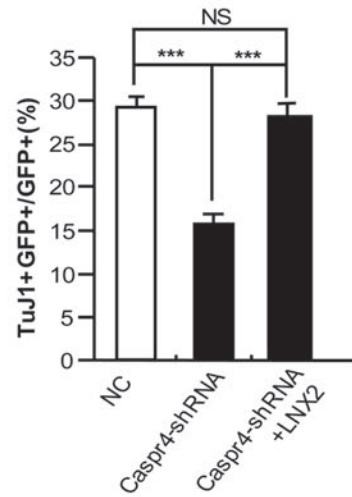
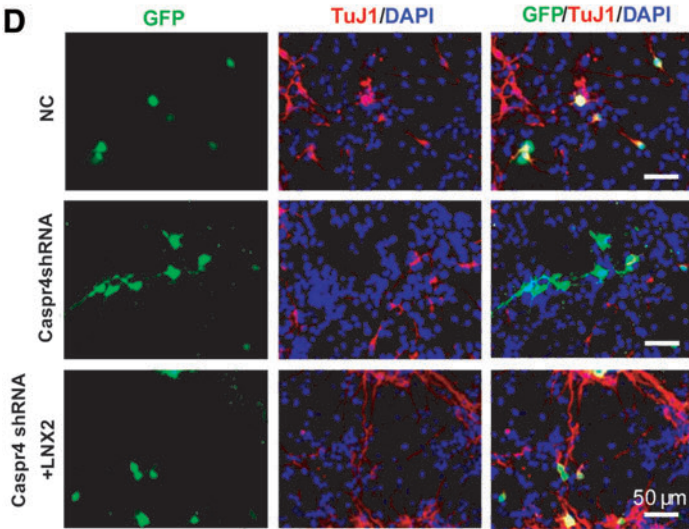
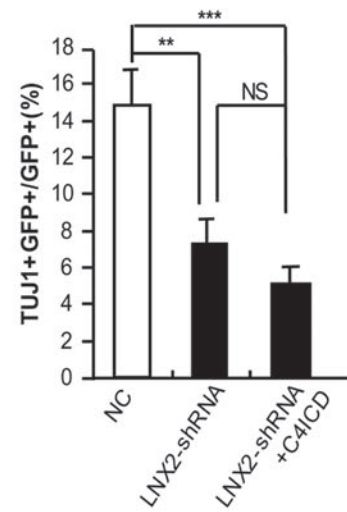
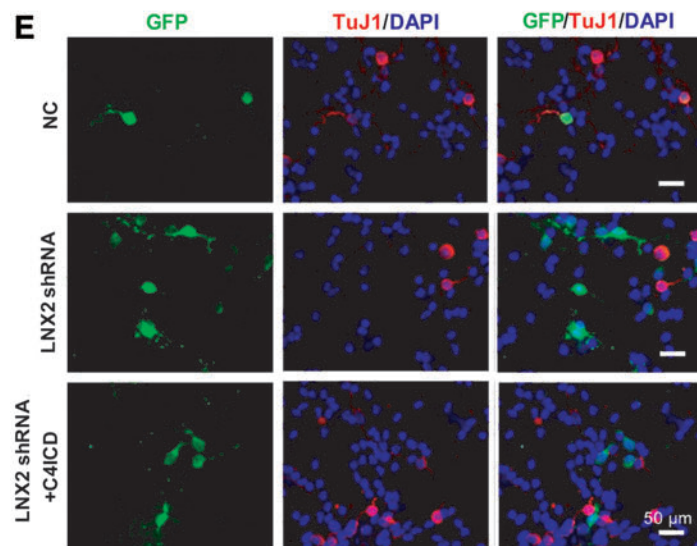
The Caspr family contains five members. Caspr1 and Caspr2 play essential roles in the establishment of the polarized domains in myelinated axons [13–17]. Caspr3 is expressed by basket cells in the cerebellum, by oligodendrocytes, and is distributed along axons in the corpus callosum, spinal cord, and peripheral nerves [26]. Caspr5 expression is detected in both the fetal development and adult brain [22]. Caspr4 is expressed in specific neuronal subpopulations in the olfactory bulb, hippocampus, deep cerebellar nuclei, and the substantia nigra [22]. However, the functions of Caspr3, Caspr4, and Caspr5 in the brain remain unknown. Both Caspr2 and Caspr4 have recently been identified as risk genes of ASDs [10,18]. Caspr2-deficient mice show deficits in social interaction behavior and abnormality in neuronal migration during the cortex development [10], suggesting a role of Caspr2 in the cortex development. In this study, we show that Caspr4 is expressed in NPCs. We further have confirmed that Caspr4 promotes neuronal differentiation of NPCs. Our study indicates Caspr4 may play important roles in the cortex development. This point is consistent with the recent genetic studies, which have shown that deletion of Caspr4 exhibits strong association with children developmental delay and ASDs [18–20].

Similar to other members of Caspr family, Caspr4 consists of a large extracellular domain, a single membrane-spanning domain, and a short intracellular domain [26]. The intracellular domain of Caspr4 (C4ICD) has a PDZ binding

motif, which may interact with the PDZ domain-containing proteins involved in multiple cellular and biological processes [26]. It has reported that C4ICD associates with CASK and Mint1 [26], two PDZ domain-containing proteins. In this study, we report that C4ICD interacts with LNX2. LNX2 is an E3 ubiquitin ligase, which contains a RING finger and four PDZ domains [23,24]. LNX2 interacts with the cell fate determinant Numb through the first PDZ domain and a Numb PTB domain binding site [23,24]. However, the binding of LNX2 to Caspr4 requires the second PDZ domain, suggesting that LNX2 may recognize different substrates through distinct PDZ domains. Moreover, we show that C4ICD rescues the decreased neuronal differentiation of NPCs by knocking down of Caspr4, indicating C4ICD is required for the function of Caspr4 in NPCs. This is distinct from Caspr, which inhibits neurite outgrowth through its extracellular domain [31].

Consistent with that LNX2 functions as E3 ubiquitin ligase [24], a decrease in the levels of Caspr4 by overexpression of LNX2 has been observed. However, the fact that LNX2 plays a similar role to Caspr4 in modulating the proliferation and differentiation of NPCs excludes the possibility that LNX2 promotes neuronal differentiation through the degradation of Caspr4. If LNX2 functions in NPCs through decreased Caspr4 expression, knocking down of Caspr4 should have the same effect as overexpression of LNX2 in NPCs. We further demonstrate that C4ICD rescues the decreased neuronal differentiation in Caspr4-knocking down NPCs, but it failed to do so in LNX2-knocking down NPCs. In contrast, LNX2 rescues the decreased neuronal differentiation in Caspr4-knocking down NPCs. These observations suggest that LNX2 may function as the downstream of Caspr4 in NPC differentiation. LNX2 interacts and degrades Numb and thus enhances Notch signaling [23,24], which represses neuronal differentiation during the cortical development [32]. LNX2 is recently found to enhance WNT signaling as well [33], which promotes neuronal differentiation during the development [34]. Moreover, as a PDZ domain containing protein, LNX2 interacts with various proteins such as junctional adhesion molecule 4, Coxsackievirus and adenovirus receptor, and CD8 α [35–37]. Thus, identification of the downstream targets of LNX2 may help to explain how Caspr4 modulates NPCs through LNX2.

FIG. 6. LNX2 is a downstream element of Caspr4 in promoting neuronal differentiation of NPCs. (A) Caspr4 were cotransfected with LNX2 or the empty vector into HEK293T cells. The cells were harvested at 48 h after transfection and subjected to western blot analysis using antibodies against Caspr4 and LNX2. γ -Tubulin (tubulin) was detected as a loading control. Quantification analysis of the blots was shown. (B) NPCs were transfected with LNX2 siRNA or a scrambled siRNA (NC) as a control. The cells were harvested at 48 h after transfection and subjected to western blot analysis using antibodies against Caspr4, LNX2, and Sox2. γ -Tubulin (tubulin) was detected as a loading control. Quantification analysis of the blots was shown. (C) LNX2 were cotransfected with Caspr4 or the empty vector into HEK293T cells. The cells were harvested at 48 h after transfection and subjected to western blot analysis using antibodies against Caspr4 and LNX2. γ -Tubulin (tubulin) was detected as a loading control. Quantification analysis of the blots was shown. (D) NPCs were transfected with Caspr4 shRNA, Caspr4 shRNA plus LNX2, or a scrambled shRNA (NC) as a control. Cells were immunostained for β III tubulin (TUJ1), GFP, and DAPI after 3–4 days of differentiation in vitro. The numbers of GFP⁺TUJ1⁺ neurons were counted and expressed as the percentage of the number of total GFP⁺ cells (NC: 29.23 \pm 1.37; Caspr4-shRNA: 15.46 \pm 0.78; Caspr4-shRNA plus LNX2: 28.20 \pm 1.48). (E) NPCs transfected with LNX2 shRNA or LNX2 shRNA plus C4ICD or a scrambled shRNA (NC) were immunostained for β III tubulin (TUJ1), GFP, and DAPI after 3–4 days of differentiation in vitro. The numbers of GFP⁺TUJ1⁺ neurons were counted and expressed as the percentage of the number of total GFP⁺ cells (NC: 14.84 \pm 1.79; LNX2 shRNA: 7.24 \pm 1.31; LNX2 shRNA plus C4ICD: 5.04 \pm 0.85). Scale bars: 50 μ m. Values are represented as mean \pm SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; NS, non-significant. Color images available online at www.liebertpub.com/scd

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Author Disclosure Statement

No competing financial interests exist.

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