Nuclear Factor Kappa B Signaling Initiates Early Differentiation of Neural Stem Cells

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Abstract

Inflammatory mediators, many of which activate the signaling of nuclear factor kappa B (NFκB), have received increasing attention in the field of neurogenesis. NFκB signaling regulates neurite outgrowth and neural plasticity as well as the proliferation/apoptosis and terminal differentiation of neural stem cells (NSCs). Early neurogenesis from NSCs produces identical progeny through symmetric division and committed daughter cells through asymmetric division. Here, we show that NFκB signaling is required for NSC initial differentiation. The canonical IKKβ/IκBα/ p65 pathway is activated during the initial stages of neural differentiation induced by treatment with TNFα or with- drawal of epidermal growth factor/basic fibroblast growth factor. NSC-specific inhibition of NFκB in transgenic mice causes an accumulation of Nestin$^+$/Sox2$^+$/glial fibrillary acidic protein$^+$ NSCs. Inhibition of NFκB signaling in vitro blocks differentiation and asymmetric division and maintains NSCs in an undifferentiated state. The induction of initial differentiation and asymmetry by NFκB signaling occurs through the inhibition of C/EBPβ expression. Our data reveal a novel function of NFκB signaling in early neurogenesis and provide insight into the molecular mechanisms underlying neurodevelopmental disorders and neurodegenerative diseases.

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The authors indicate no potential conflicts of interest.

Keywords

Neural stem cells; Nuclear factor kappa B; Neurogenesis; C/EBPβ; Glial fibrillary acidic protein; Cell division

Introduction

Neurogenesis plays an important role in the pathogenesis of a number of disorders, including neurodevelopmental defects, depression, epilepsy, stroke, and neurodegenerative diseases [1]. Both embryonic and adult neurogenesis involves the proliferation, migration, and lineage differentiation of neural stem cells (NSCs). During embryonic development, NSCs are derived from neuroepithelial cells (NECs) and differentiate sequentially into neural progenitor cells (NPCs), various lineage-restricted precursor cells (RPCs), and mature neural cells [2]. During adult neurogenesis, NSCs are derived from radial glial cells and/or ependymal cells and follow a differentiation pattern similar to embryonic neurogenesis [3]. Although many extrinsic factors and intrinsic proteins have been identified that regulate both embryonic and adult neurogenesis [4], the signaling pathways and molecular mechanisms remain poorly understood. In particular, very few factors that control the initiation of NSC differentiation into NPCs have been identified [5].

Inflammatory mediators, many of which activate the nuclear factor kappa B (NFκB) signaling pathway, have received increasing attention in neurogenesis, because inflammatory and immune responses play critical roles in various injuries and diseases of the nervous system [6–8]. The members of the NFκB family and related signaling components are widely expressed in all cell types of the nervous system. Mice deficient in p50 show impairments in learning, defects in short-term memory, and a reduction in anxiety-like behavior [9]. Mice deficient in p65 display spatial learning defects [10], whereas c-Rel-deficient mice display hypomotility [11]. An enrichment of phosphorylated inhibitor of NFκB (IκB)α and IκB kinase (IKK) in the axon initial segment [12] indicates a novel role of NFκB signaling in the regulation of axo-nal polarization. NFκB activation is required for the growth and branching of dendrites and axons [13]. These findings suggest that NFκB signaling plays a critical role in the regulation of neurite outgrowth and neural plasticity. NFκB has also been shown to control the proliferation/apoptosis and terminal differentiation of NPCs and RPCs [6, 14, 15]. The disruption of NFκB signaling by an IKKα and IKKβ double knockout in mice causes dramatic apoptosis in NECs and impairs the formation of neural tissue [16]. Stress selectively activates NFκB and decreases proliferation of NSCs, but not early NPCs, in the adult hippocampus [17]. These observations reveal the important functions of NFκB signaling in the proliferation/apoptosis of NECs, NSCs, and NPCs. However, little is known about the role of NFκB signaling in regulating neural differentiation of NSCs/NPCs. Toll-like receptor 2 (TLR2) promotes neuronal differentiation of NSCs/NPCs via NFκB activation [15], although it has also been reported that this signaling has no effect [18]. The neuronal differentiation of adult hippocampal NSCs/NPCs is reduced in p50-deficient mice [19]. However, whether NFκB signaling regulates the initial differentiation of NSCs still remains unknown.

Cellular division is a critical step for NSC self-renewal and lineage differentiation through symmetric or asymmetric machinery [20]. However, the mechanisms behind the initiation and control of the asymmetric division of NSCs and their further development into intermediate NPCs are as yet unclear.

In this study, we investigated the role of NFκB signaling in regulating asymmetric division and neural differentiation of NSCs in vitro and in vivo. We demonstrate that canonical

NFκB signaling is activated during NSC differentiation. Phar-macologic, small hairpin RNA (shRNA), and transgenic inhibition of NFκB signaling attenuated neural differentiation and promoted self-renewal of NSCs. Furthermore, we identified CCAAT/enhancer binding protein (C/EBP)β as an effector of NFκB signaling for the modulation of early neurogenesis. These findings highlight the importance of NFκB signaling in regulating initial neural differentiation of NSCs and add to our understanding of the molecular mechanisms involved in neurodevelopmental or neuroregenerative deficits.

Materials and Methods

Animals

All procedures involving the use of animals were conducted in compliance with the guidelines of the National Institutes of Health and approved by the Animal Care and Use Committee of the Temple University and Sichuan University. Glial fibrillary acidic protein (GFAP) dominant-negative IκBα (dnIκBα) mice were obtained from Dr. Bethea's Laboratory at the University of Miami and maintained by breeding heterozygous GFAP-dnIκBα males with wild-type (*WT*) females [21]. *WT* littermates were used as controls. Animals were housed in a pathogen-free facility with a 12-hour light/dark cycle and ad libitum access to water and food.

Neurosphere and Monolayer Cultures

Primary neurospheres were cultured from mouse E14.5 embryos, neonatal (p5–7), and adult (2–4 months) brain. The embryonic cerebral cortices or neonatal and adult subventricular zone (SVZ) were dissected and placed in phosphate-buffered saline (PBS) containing 2% glucose on ice. For embryos, cerebral cortices were collected in a 15-ml tube containing 2 ml NeuroCult NSC proliferation medium (StemCell Technologies, Vancouver, Canada, www.stemcell.com). Using a fire-polished glass pipette, the tissues were triturated approximately 10 times until a fine single cell suspension was achieved. For neonatal or adult SVZ, the tissues were cut into pieces, digested with Accutase (Sigma, St. Louis, MO, www.sigmaaldrich.com) for 10–15 minutes, and triturated. The dissociated cells were cultured at a density of 2×10^5 cells per milliliter in NeuroCult NSC proliferation medium containing 20 ng/ml epidermal growth factor (EGF) and 10 ng/ml basic fibroblast growth factor (bFGF) (StemCell Technologies). Medium was changed every other day, and primary neurospheres were formed within 3–7 days.

For adherent monolayer culture, primary neurospheres were dissociated with Accutase and plated in plates or cover-slips coated with poly-ornithine (Sigma) and fibronectin (R&D Systems, Minneapolis, MN, www.rndsystems.com). Half of the medium was changed every other day.

Inhibitor Treatment

NFκB signal inhibitors Bay-11-7028, SC-514, IKKβ inhibitor V, and 4-methyl-N^1-(3-phenylpropyl)benzene-1,2-diamine (JSH-23) (EMD Chemicals, Philadelphia, PA, www.emdchemicals. com) dissolved in dimethyl sulfoxide (DMSO) were added in NeuroCult NSC proliferation medium or NeuroCult NSC differentiation medium, and half of the medium was changed every day.

Neural Colony-Forming Cell Assay

NeuroCult neural colony-forming cell (NCFC) assay kit (StemCell Technologies) was used with a minor modification (Fig. 3A) [22]. Briefly, monolayer NSCs/NPCs from primary neurospheres were treated for 24 or 72 hours (medium changed every day) with indicated inhibitors in NeuroCult NSC differentiation medium or tumor necrosis factor (TNF)α in

NeuroCult NSC proliferation medium. Cells were dissociated by treatment with collagenase II and seeded at a clonal density of 250 cells per milliliter (500 cells per 35 mm dish) in collagen semisolid medium containing 20 ng/ml of EGF and bFGF. After 14 days, each clone (> 50 μm in diameter) was picked and dissociated into single cells for culture of secondary neurospheres. After 10 days, the secondary neurospheres (> 30 μm in diameter) were picked individually and dissociated into single cells. Cells from each neurosphere were seeded into two wells of 48-well plate coated with poly-L-ornithine/fibronectin and cultured with differentiation medium for 10 days, and the lineage differentiations were analyzed by multilabeled immunocytochemistry.

Single Cell Division Assay

Clonal pair-cell analysis was performed as described [23, 24]. Briefly, the single cells dissociated from primary neurospheres were seeded at very low density (500 cells per well of a 24-well culture plate) on a coverslip coated with poly-ornithine/ fibronectin. When single cells attached were verified under microscope within 4–6 hours after seeding, the medium was changed to differentiation medium or proliferation medium with/without NFκB inhibitor or TNFα as indicated. After 24 or 72 hours, the cells were fixed for multilabeled immunocytochemistry.

5-bromo-2′-deoxyuridine (BrdU) Pulse Labeling

Short-term BrdU labeling was performed as previously described [17, 25]. For in vitro studies, the cultured cells were treated with 10 μm BrdU (Sigma) for 4 hours before fixation with 4% paraformaldehyde/PBS. For in vivo analysis, animals were administrated with an i.p. injection of BrdU (100 mg/kg, Sigma) 4 hours before being euthanized (see below). Before immunostaining, the cells or tissue sections were incubated in HCl (1N) for 10 minutes on ice to rupture the DNA structure of the labeled cells and then treated with HCl (2 N) for 10 minutes at room temperature and 20 minutes at 37°C. Immediately after the acid washes, borate buffer (0.1 M) was added to buffer the cells for 12 minutes at room temperature. The samples were then washed in PBS/glycine (1 M).

Immunocytochemistry of Cultured Cells

Standard immunocytochemistry was carried out. Primary antibodies included Nestin (Developmental Studies Hybridoma Bank, Iowa, IO, dshb.biology.uiowa.edu), GFAP, myelin basic protein (MBP), microtubule-associated protein 2 (MAP2) (Chemicon, Millipore-Chemicon, Billerica, MA, www.millipore.com), C/EBPβ, NFκB p65, IκBα, phosphor-histone H3(Ser10), sex determining region Y-box 2 (Sox2), Tau, tripartite motif-containing protein 32 (TRIM32) (polyclonal), Numb (Santa Cruz Biotechnologies, Santa Cruz, CA, www.scbt.com), TRIM32 (monoclonal, Abnova, Walnut, CA, www.abnova.com), phosphor-p65 (Cell Signaling Technologies, Danvers, MA, www.cellsignal.com), BrdU, and Tuj1 (Sigma). All Alexa-Fluor-labeled secondary antibodies were from Invitrogen (Grand Island, NY, www.invitrogen.com). Cells were fixed for 20 minutes in 4% paraformaldehyde/ PBS. After three rinses, the cells were treated with 0.5% Triton X-100 PBS for 20 minutes and blocked by 10% serum or 2% bovine serum albumin/PBS for 1 hour. Cells were incubated overnight at 4°C with primary antibodies. After rinsing three times, cells were incubated with secondary antibodies for 1 hour and with 4′-6-diamidino-2-phenylindole (DAPI) for 5 minutes. After three rinses with PBS, the cells were mounted with a coverslip using antifading aqueous mounting media (Biomeda, Foster City, CA, www.biomeda.com) and analyzed under fluorescence microscope.

Immunohistochemistry and Stereological Analysis of Adult Brains

Littermates of GFAP-dnIκBα *TG* and *WT* mice were euthanized with an overdose of pentobarbital solution and transcar-dially perfused with 4% paraformaldehyde. The brains were dissected, postfixed overnight in the same fixative, and cryopreserved with buffered 25% sucrose. A series of coronal sections of brain at 40 μm thickness were cut on a freezing sliding microtome and each 12th section was collected into buffered 25% sucrose and stored at −80°C. Then standard multiple-labeled immunofluorescent staining was performed. For Sox2/GFAP, Nestin/GFAP, or BrdU/GFAP double-labeling, five to six coronal sections containing SVZ (separated by 12×40 μm) per animal were analyzed for the percentage of $Sox2^+/GFAP^+$, $Nestin^+/GFAP^+$, or $BrdU^+/GFAP^+$ over total $Sox2^+$, $Nestin^+$, or $BrdU^+$ cells per field at ×40 objective, respectively. Each section covered three to six fields. The average percentage per animal was used for statistical analysis. The significant difference in the percentage of NSCs was analyzed from three to four animals each genotype.

Deconvolution and Confocal Image Analysis

Fluorescence was captured using sequential acquisition under a fluorescent inverted microscope (Nikon Instruments Inc., Melville, NY, www.nikoninstruments.com) equipped with a cooled CCD camera using Slidebook 5.0 digital imaging software. In some cases, the three-dimensional (3D) reconstructions were made by nearest-neighbor deconvolution of fluorescent images taken in a Z-stack of focal planes (0.25 μm).

For confocal imaging analysis, samples were viewed on a Leica SP5 confocal microscope system. Differential visualization of four fluorophores Alexa-488, Alexa-594, Alexa-647, and DAPI was obtained via specific filter combinations. Samples were scanned sequentially to avoid any potential for fluorophore bleedthrough. The Z-stack images (1,024×1,024 pixels) through 0.5 μm optical sections were obtained under identical exposure conditions and processed through processing tools. Tiff images and 3D movies were explored for analysis.

Western Blots

Cells were resuspended in ice-cold cell lysis buffer with protease and phosphatase inhibitor cocktail (Sigma) and incubated for 30 minutes on ice. Sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) was performed in 12% Tris-glycine gels and transferred to nitrocellulose membranes (BioRad, Hercules, CA, www.bio-rad.com). The membranes were blocked in Tris-buffered saline with 0.05% Tween-20 (TBST) and 5% milk for 1 hour, incubated with primary antibodies at 4°C overnight, washed three times in TBST, and then incubated with horseradish peroxidase-conju-gated corresponding secondary antibodies for 1 hour. After washing, immunoreactive proteins were visualized using SuperSignal Femto maximum sensitivity substrate kit (Pierce, Rockford, IL, www.piercenet.com).

NFκB-Luciferase Reporter Assay

Cells were cultured in 96-well plate and infected with adenovirus carrying NFκB-*firefly*-luciferase vector (Vector Biolabs, Philadelphia, PA, www.vectorbiolabs.com) at 100 multiplicity of infection. After incubation for the indicated time periods in the absence or presence of stimulators, the cell lysate was used for measurement of *firefly* luciferase activity with ONE-Glo luciferase assay system (Promega, Madison, WI, www.promega.com/). The luminescence was measured on the 2104 EnVision Multilabel Reader (PerkinElmer, Waltham, MA, www.perkinelmer.com). Four separate experiments were conducted and, in each experiment, data were calculated as the average of four to six samples.

Enzyme-linked Immunosorbent Assay (ELISA)

The PathScan Phospho-NFjB p65 (Ser536) Sandwich ELISA Kit (Cell Signaling Technology) was used to quantitatively determine the level of phosphor-p65 in whole cell lysates as indicated.

Cell Cycle and Apoptosis Analysis

Cells dissociated from primary neurospheres were cultured on the dishes and treated as indicated. Cells were dissociated and fixed with ice-cold 70% ethanol. After staining with DAPI, the cell cycles were analyzed by fluorescence-activated cell sorting (FACS; BD, FACS Aria, San Jose, CA, www.bdbiosciences.com). For apoptosis assay, the cells were incubated with AnexinV and propidium iodide (Sigma) at room temperature for 20 minutes and analyzed by FACS.

CellTiter-Glo Luminescent Cell Viability Assay

Dissociated cells were cultured at 1×10^4 cells per well in a 96-well plate for 3 days, and the cell viability was determined by adding 50 μl of CellTiter-Glo luminescent reagent (Promega) and then recording the luminescence for each well on the 2104 EnVision Multilabel Reader (PerkinElmer). Four separate experiments were conducted and, in each experiment, data were calculated as the average of four to six samples.

Lentiviral Vector and Infection

IKKβ shRNA and control lentiviral vectors were constructed as described [26]. Liver-enriched inhibitory protein (LIP) and liver-enriched activator protein (LAP)* were subcloned into the pCDF1-MSC2-EF1-copGFP vector (SBI, Mountain View, CA, www.systembio.com) by polymerase chain reaction (PCR). Lentiviral vectors were packaged into HEK293T cells, and the lentiviruses were concentrated by centrifugation. Dissociated NSCs/NPCs were cultured in NeuroCult NSC proliferation medium. The next day, lentiviruses were added into the medium. After 6 hours of incubation, the media were changed back into NeuroCult NSC proliferation medium. The transduction efficiency was validated by the expression of internal fluorescent reporters.

Conventional and Real-Time Reverse Transcription (RT)-PCR

Total RNA was isolated from NSCs/NPCs with the Trizol reagent (Invitrogen) and treated with TURBO DNase (Invitro-gen). RNA (1 μg) was used to synthesize cDNA using Super-Script III reverse transcriptase (Invitrogen) with a random hexanucleotide primer. Conventional PCR was performed on the cDNA using HotMaster Taq DNA polymerase kit (5 Prime, Gaithersburg, MD, www.5prime.com). PCR products were purified and cloned into T-A vector for confirmation by sequencing.

Real-time PCR analysis was carried out on the LightCy-cler480 (Roche, Indianapolis, IN, www.roche-applied-science.com) using SYBR Green I kit (Roche). Each sample was tested in triplicate. Cycle threshold (Ct) values were obtained graphically. The difference in Ct values between the target gene and house-keeping gene were represented as ΔCt values. The ΔΔCt values were obtained by subtracting the ΔCt values of the control samples from that of the experimental samples. Relative fold change in gene expression was calculated as $2^{-\Delta\Delta Ct}$.

Expression Microarray

Monolayer NSCs/NPCs dissociated from primary neuro-spheres of mouse E14.5 embryonic cortex were cultured in 10-cm dishes and treated with DMSO or IKKβ inhibitor V for 24 hours. Total RNA was isolated with the Trizol reagent (Invitrogen) and treated with TURBO

DNase (Invitrogen). The quality of isolated RNA was determined by ultraviolet-spectrophotometry and denaturing agarose gel electrophoresis. Microarray analysis was performed by using mouse WG-6 v2.0 Expression BeadChip (Illumina, Inc., San Diego, CA, www.illumina.com). The samples were then processed for array hybridization and data accumulation at Illumina China (Shanghai). In brief, biotinylated cRNAs were prepared from 0.55 μg quantities of total RNA using the Illumina TotalPrep RNA Amplification Kit (Invitrogen). Following fragmentation, cRNA was hybridized to the Illumina mouse WG-6 v2.0 Expression BeadChip in 0.75 μg quantities using protocols provided by the manufacturer. Arrays were scanned using the Illumina Bead Array Reader Confocal Scanner. Array data export processing and analysis were performed using Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8).

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay was performed using SimpleChIP Enzymatic Chromatin Immunoprecipitation kit with magnetic beads (Cell Signaling Technology). Briefly, dissociated NSCs/NPCs from primary neurospheres of neonatal mouse SVZ were cultured in 10-cm dishes for 3 days (>90% confluence) and treated with or without TNFa (10 ng/ml) for 1 hour. The DNA-chromatin of cells were crosslinked with 1% formaldehyde for 10 minutes at room temperature and stopped with 0.125 M glycine. Cells were washed twice with PBS, followed by chromatin digestion and immunoprecipitation according to the manufacturer's protocol. Rabbit monoclonal antibodies anti-p65 and anti-p50 (Cell Signal Technology) were used. The supernatant of an immunoprecipitation reaction carried out in the absence of antibody was purified and diluted 1:100 as total input DNA control. Conventional and real-time PCR were carried out as described above. The TRANSFAC 8.3 Version of PROMO [27] program identified six potential NFκB binding sites within 2 κb of the mouse C/EBPβ promoter (−300/−309, −487/499, −634/−643, −1,175/−1,185, −1,369/−1,378, and − 1,847/−1,858). Thus, three pairs of PCR primers were designed: C/EBPβ-a (−330 to −203 covering the first site, 5′-tcgtgtagctggaggaacgatctg-3′ and 5′-ctcgggaacacggaggagcgcgg-3′), -b (−680 to −400 covering the second and third sites, 5′-tgacaccactactaacaataccaac-3′ and 5′-atgtcctcccacccaggtgctgcc-3′) and -c (−1,420 to −1,142 covering the fourth and fifth sites, 5′-cagatagtgtgctcactctctgcc-3′ and 5′-taagacgctagccctgc ctgcctg-3′). The primers for the NFκB binding site within the mouse interleukin (IL)-6 promoter (−376 to −144, 5′-gtgtatgtgtgtgtcgtctgt cat-3′ and 5′-catcgaaagaatcacaactaggaa-3′) was used as a positive control for the ChIP assay.

Statistical Analysis

Results were expressed as means ± SEM. Data were evaluated by Student's t test or ANOVA and Newman-Keuls multiple comparison test. A value of $p < .05$ or .01 was considered as statistically significant.

Results

Activation of NFκB Signaling Is Required for Neural Differentiation of Nestin[+] and Sox2^{+} NSCs/NPCs

The members of the NFκB family are highly expressed in cells within the zones of active neurogenesis in neonatal and adult brain [15, 28, 29]. Extensive transgenic studies have demonstrated the critical role of NFκB signaling in regulating embryonic neural development [9–11, 14, 16]. The complete disruption of NFκB signaling in IKKa and IKKβ double knockout mice induces embryonic lethality at E12 before NECs undergo full neurogenesis [16], which makes it difficult to analyze the detailed functions of NFκB signaling at the early stages of neurogenesis in animal embryos. Therefore, we cultured primary neurospheres from embryonic, neonatal, and adult SVZ, where NSCs/NPCs harbor

extensive similarities to their endogenous counterparts in vivo [30]. In neurosphere or monolayer cultures, the p65 protein was located in the cytoplasm of Nestin/Sox2$^+$ NSCs/ NPCs (Fig. 1A, 1B), indicating that NFκB signaling is inactive during NSC/NPC proliferation, which is consistent with previous reports [14, 15]. When NSCs/NPCs were subjected to differentiation by withdrawing EGF/bFGF, the activation of NFκB signaling was evident by the degradation of IκBα, the phosphorylation and nuclear translocation of p65, and the increase of NFκB reporter activity (Fig. 1, Supporting Information Fig. S1A). The loss of p65 phosphorylation at 24–48 hours may reflect a transient process of NFκB signaling activation during neural differentiation. Nestin$^+$ NSCs/ NPCs in the SVZ of neonatal and adult mouse brain also contained inactive p65 in their cytoplasm (Supporting Information Fig. S1B) [15, 29]. These data indicate that NFκB signaling is inactive in Nestin$^+$ NSCs/NPCs during nondifferentiation cellular states and becomes active during neural differentiation, suggesting that NFκB signaling plays a role in the neural differentiation of NSCs/NPCs.

The data above identified the constitutive activation of NFκB signaling during neural differentiation. Various inflammatory mediators have been shown to regulate proliferation/ apoptosis of NSCs/NPCs [6–8, 14, 15]. To address whether these mediators affect the NFκB signaling and neural differentiation of NSCs/NPCs, we used the adenovirus-mediated NFκB reporter assay in cultured primary NSCs/NPCs and an immortalized enteric NPC line [31]. Both TNFα and lympho-toxin α1β2 dramatically induced activation of the NFκB reporter (Supporting Information Fig. S2A), which was corroborated by Western blot analysis (Supporting Information Fig. S2B). Consistent with previous reports [32, 33], the addition of TNFa to the proliferation or differentiation media induced or promoted neural differentiation, as determined by immunocyto-chemistry and Western blot analysis (Fig. 2A, 2B, Supporting Information S2C, S2D). Treatment with other cytokines failed to activate the canonical NFκB pathway (Supporting Information Fig. S2) and were unable to induce neural differentiation. TNFα also activates other signaling pathways, such as c-Jun N-terminal kinase (JNK) (Supporting Information Fig. S2E, S2F), which may regulate neural differentiation of NSCs/NPCs. However, the JNK-specific inhibitor SP600125 promoted [34] TNFα-induced upregulation of Tuj1 expression, while the NFκB specific inhibitor IKKβ-V prevented this effect (Fig. 2C). These data indicate that TNFα induces activation of the canonical NFκB pathway and the subsequent differentiation in NSCs/NPCs.

To clarify the role of NFκB signaling in neural differentiation, we treated primary NSCs/ NPCs from embryonic and neonatal brain with specific IKKβ shRNA (Supporting Information Fig. S3A) or with inhibitors at various levels of the NFκB pathway (Fig. 2D, 2E, Supporting Information Fig. S3B). When NSCs/NPCs were subjected to differentiation for 24 hours, the number of Nestin$^+$ cells dramatically decreased (Fig. 2D, 2E) and Tuj1$^+$ cells (Tuj1 expression) increased (Fig. 2F). After 72 hours, very few Nestin$^+$ cells remained (Fig. 2E). Inhibition of NFκB signaling blocked the differentiation of NSCs/NPCs (Fig. 2E, 2F, Supporting Information Fig. S3A). These results indicate that NFκB activation plays a critical role in neural differentiation of NSCs/NPCs.

NFκB Signaling Initiates the Differentiation of NSCs

To determine which cellular stage(s) of neural differentiation are initially regulated by NFκB signaling, we performed the NCFC assay (Fig. 3A), which is an established approach to discriminate NSCs from more committed NPCs [22]. Under proliferation conditions, 9%– 16% of seeded cells formed tripotential clones (Fig. 3B). Approximately 2%–3% of the cells formed tripotential clones when cultured under differentiation conditions or treated with TNFa under proliferation conditions for 24 hours. After pharmacological inhibition of various levels of the NFκB pathway in cells under differentiation conditions, the percentage of tripotential clones was restored to or even exceeded the levels in the untreated group

under proliferation conditions (Fig. 3B). Over 72 hours of treatment, tripotential clones were undetectable, whereas NFκB inhibition led to more clones with tripotential capacity than that of the 24-hour treatment group (Fig. 3B). These results indicate that inhibition of NFκB signaling induces an accumulation of NSCs/NPCs with tripotential differentiation abilities.

To discriminate the tripotential NSCs with self-renewal capacity from the tripotential NPCs without self-renewal, we assessed the frequency of stemness by determining the self-renewal and tripotential capacity of the secondary neurospheres (Fig. 3A). Upon neural differentiation or TNFα treatment for 24 or 72 hours, the number of NSCs was dramatically reduced or completely lost (Fig. 3C). Treatment with various NFκB signaling inhibitors under differentiation conditions maintained or increased the frequency of NSCs (Fig. 3C). These results are supported by a previous report that treatment with SN50 (a cell-permeable direct inhibitor for NFκB nuclear translocation) in E14.5 neurospheres increased the number of secondary neurospheres [35]. Similar pheno-types were obtained in NSCs with shRNA knockdown of IKKβ (Fig. 3D–3F, Supporting Information S3C), which showed an increase in the proliferation of tripotential and self-renewal NSCs. These results indicate that the blockage of NFκB signaling maintains NSCs in a nondifferentiation state and suggest that NFκB signaling initiates NSC differentiation.

NFκB Signaling Controls the Asymmetric Division of Neural Cells at Early Stages

Neural differentiation involves sequential rounds of asymmetric divisions of NSCs/NPCs [36, 37]. To address whether NFκB signaling regulates asymmetric division, we examined the distribution of Nestin, Sox2, and Tuj1 proteins as well as the asymmetric determinants TRIM32 [38] and Numb [39] in NSCs/NPCs at early stages of differentiation. Using a single cell division assay, we found that one Nestin$^+$ neural cell underwent asymmetric division to produce one Nestin$^+$ cell and one Nestin $^{low/-}$ cell (Fig. 4A, Supporting Information Fig. S4A, S4B). The Sox2$^+$ and TRIM32$^+$ cells underwent a similar distribution under asymmetric division (Fig. 4A–4D, Supporting Information S4A, S4B). During asymmetry, TRIM32 also translocated into the nucleus (Fig. 4C, 4D, Supporting Information Fig. S4A), which was consistent with previous report [40]. The asymmetric segregation of TRIM32 did not entirely match that of Nestin or Sox2. Of TRIM32 asymmetry, 85% and 42% displayed asymmetry for Nestin and Sox2, respectively (Supporting Information Fig. S4B). However, 89% of Nestin and 28% of Sox2 at asymmetry were asymmetric for TRIM32. In two-thirds of cases, TRIM32 went to Nestin $^{low/-}$ or Sox2 $^{low/-}$ cells (Fig. 4C), implying the neural induction by TRIM32 [38, 40]. In the two daughter cells, Tuj1 protein was observed in Nestin$^{low/-}$ or Sox2$^{low/-}$ neural cells but was absent in Nestin$^+$ or Sox2$^+$ cells (Fig. 4C). In contrast, higher expression and nuclear translocation of TRIM32 were observed in Tuj1$^+$ daughter cells of asymmetric dividing and in both Tuj1$^+$ daughter cells of symmetric dividing at the initial stage (Fig. 4D, Supporting Information Fig. S4c1), consistent with previous reports [40]. Of TRIM32 asymmetry, 83% showed Tuj1 asymmetry and 13% existed in Tuj1$^-$ paired cells (Fig. 4B). Interestingly, TRIM32 expression was reduced or lost in the well-differentiated Tuj1$^+$ neurons with long processes (Supporting Information Fig. S4c2, Fig. S4c3). Under TRIM32 symmetry, 34% showed Tuj1 symmetry and 46% were in Tuj1$^-$ paired cells (Fig. 4B), implying that TRIM32 symmetric distribution occurred not only in the dividing Tuj1 symmetric cells but also in undifferentiated NSCs/ NPCs as shown previously [38]. The expression of Numb was found to be equally distributed in two daughter cells at the point of Nestin-related asymmetric division (Fig. 4E). The asymmetric distribution of Numb was found when Nestin expression was absent (Fig. 4E). Upon inhibition of NFκB signaling, the Nestin$^+$, Sox2$^+$, or TRIM32$^+$ neural asymmetric divisions were significantly blocked and the corresponding symmetric divisions were increased (Fig. 4A, 4B), while the generation of Tuj1$^+$ neural cells including asymmetric and symmetric pairs was impeded (Figs. 2E, 4B, 4D). The nuclear translocation of TRIM32 was also

blocked by NFκB inhibition (Fig. 4D). The increased proportion of TRIM32 symmetric distribution after NFκB inhibition derived largely from Tup cells (mainly NSCs/NPCs) [38], whereas the increase in the TRIM32 asymmetric Tuj1⁻ cells may reflect the differentiation of other lineages (Fig. 4B). These results suggest that NFκB signaling is required for neural asymmetric division at the very early stages of neural differentiation.

Nestin⁺/GFAP⁺/Tuj1⁻ NSCs Accumulate in Mouse Brains with Partially Disrupted NFκB Signaling in NSCs

It is generally recognized that neonatal and adult NSCs are GFAP positive, while NPCs are GFAP negative [5, 17, 41–44]. Therefore, the transgenic inhibition of NFκB signaling in GFAP⁺ NSCs would provide direct evidence for the role of NFκB signaling in early neurogenesis. To address this, we used a transgenic (*TG*) mouse line expressing a dnIκBα driven by the GFAP promoter [21]. This mouse line displays a deficit in learning and memory [45]. The expression of transgene dnIκBα in NSCs was validated by RT-PCR (Fig. 5A), and the marked inhibition of TNFa-induced or differentiation-initiated activation of NFκB signaling was corroborated with an adenovirus-NFκB reporter assay (Fig. 5B) and Western blot analysis (Fig. 5C). To directly examine the effect of NSC NFκB inhibition on the accumulation of Nestin⁺ NSCs/NPCs in vitro, we obtained primary neurospheres from the SVZ of neonatal (P6) and adult (10 weeks old) *TG* and *WT* littermates. At passage 2–3, the *TG* neurospheres grew faster and were larger in proliferation media than *WT* neurospheres (Fig. 5D, Supporting Information Fig. S5A). The *TG* NSCs/NPCs contained more Nestin⁺ cells (Fig. 5E). The higher proliferating capacity of *TG* NSCs/NPCs was further confirmed by single cell division, a cell-titer viability assay, and BrdU incorporation (Fig. 5F, Supporting Information Fig. S5B–S5D). To distinguish the effect of the transgene on NSCs or NPCs, a clonogenic assay was performed (Fig. 3A). Consistent with the effects of NFκB inhibitors, the frequency of NSCs with self-renewal and tripotential potency from the secondary neurospheres was significantly higher in *TG* than in *WT* mice (Fig. 5G, Supporting Information Fig. S5E). Upon differentiation, the expression of Sox2 started to decrease at 12 hours after differentiation in *WT* mice but not in *TG* mice (Fig. 5H, Supporting Information Fig. S5F). At 24–72 hours, the TG NSCs/NPCs had higher levels of Sox2 protein expression (Fig. 5H) and *Nestin* mRNA expression (Fig. 5I). Taken together, these data suggest that transgenic inhibition of NFκB signaling in GFAP⁺ NSCs promotes the self-renewal and accumulation of NSCs and delays early neural differentiation.

These in vitro data were further validated by the in vivo studies using adult (10 weeks old) *WT* and *TG* littermates. The Nestin⁺/Tuj1⁻ NSCs/NPCs were enriched in the SVZ of *TG* mice (Fig. 6A, Supporting Information Fig. S6). The percentage of Nestin⁺/GFAP⁺ NSCs within total Nestin⁺ cell population was approximately 31% in the SVZ of *WT* mice and significantly increased to 43% in *TG* mice (Fig. 6B, 6D). This was further supported by the significant increase (from 35% to 55%) in the percentage of Sox2⁺/GFAP⁺ NSCs in TG SVZ compared with *WT* SVZ (Fig. 6D). Short-term BrdU labeling confirmed that the number of proliferating BrdU⁺/ GFAP⁺ NSCs was significantly increased in TG SVZ (Fig. 6C, 6D). These data demonstrate that partial inhibition of NFκB signaling in GFAP⁺ NSCs enriches Nestin⁺/Sox2⁺/ GFAP⁺ NSCs in vivo.

NFκB Signaling Controls Neural Differentiation by Inhibiting C/EBPβ

To identify the downstream molecular events by which NFκB signaling modulates the differentiation of NSCs/NPCs, we performed a gene expression profile assay in primary embryonic NSCs/NPCs treated for 24 hours with or without an IKKβ inhibitor (Supporting Information Table 1). Most genes with significant changes (more than threefold) were related to neurogenesis. We further evaluated the dynamic correlation of selected neurogenetic genes by RT-PCR (Supporting Information Table 2). The expression profile of

C/EBPβ, one of the NFκB target genes and a regulator of neurogenesis [46, 47], was in line with that of *Nestin* and *Sox2*, and was downregulated by NFκB signaling during neural differentiation. The ChIP assay validated the binding activity of p65, but not p50, with the C/EBPβ promoter, which was significantly dependent on TNFα (Fig. 7A, 7B). The clono-genic assay revealed that the number of C/EBPβ+ clones decreased dramatically during neural differentiation (Fig. 7C). Moreover, the C/EBPβ+ neural cells underwent asymmetric division (Fig. 7D). C/EBPβ was expressed in Nestin+, Sox2+, and Tuj1− daughter cells but was remarkably decreased in Tuj1+ and Numb+ daughter cells (Fig. 7E, Supporting Information Fig. S4C). Inhibition of NFκB signaling maintained the expression of C/EBPβ in NSCs and blocked the asymmetric divisions of C/EBPβ NSCs when they were subjected to differentiation (Fig. 7C, 7D). These results suggest that C/EBPβ is downregulated by NFκB signaling and retains NSC self-renewal.

To further characterize the function of C/EBPβ in neural differentiation regulated by NFκB signaling, we expressed full-length C/EBPβ (LAP*) as well as the natural inhibitor of C/EBPβ (LIP) [48]. Ectopic expression of active LAP* inhibited the neural differentiation under differentiation conditions and disrupted TNFα-induced neural differentiation under non-differentiation conditions (Fig. 7F, 7G). Inhibition of C/EBPβ function by LIP induced neural differentiation under the non-differentiation conditions (Fig. 7F) and rescued the defects in neural differentiation induced by the NFκB signaling inhibitors (Fig. 7G). These data indicate that C/EBPβ is a major effector of NFκB signaling in the control of neural differentiation in NSCs/NPCs.

Discussion

The salient finding of this study was that NFκB signaling has a critical role in the regulation of the very early stages of neurogenesis (Supporting Information Fig. S7). In this model, NFκB activation downregulates C/EBPβ expression and initiates NSC asymmetric division and further differentiation. The following evidence supports our findings: (a) NFκB signaling was activated during the initial stages of neural differentiation; (b) inhibition of NFκB signaling blocked asymmetric division and neural differentiation at a very early stage, leading to accumulation of NSCs; and (c) C/EBPβ downregulation by NFκB signaling controlled the initial differentiation of NSCs/NPCs.

Both in vitro and in vivo studies have demonstrated that inhibition of NFκB signaling induces an accumulation of Nestin+/Sox2+ NSCs/NPCs. This accumulation may have resulted from a decrease in apoptosis, increase in proliferation, and/or blockage of neural differentiation. The data from our studies and previous reports [14, 32, 35, 49–51] support our hypothesis that the blockage of early neural differentiation by the inhibition of NFκB signaling is a predominant cause for the accumulation of Nestin+/Sox2+ NSCs/NPCs. Under proliferation conditions, pharmacologic inhibition of endogenous NFκB neither enhances apoptosis nor changes the proliferation of Nestin+ NSCs/NPCs (Supporting Information Fig. S8A, S8B), which are findings supported by previous studies [14, 35]. Under differentiation conditions, inhibition of endogenous NFκB decreased the proliferation of NSCs/NPCs at 24 hours and increased apoptosis at 72 hours (Supporting Information Fig. S8C, S8D). Conversely, activation of NFκB signaling by TNFα treatment increased the proliferation but decreased apoptosis of NSCs/NPCs under proliferation conditions (Supporting Information Fig. S8E, S8F), whereas inhibition of NFκB sensitized TNFα-induced apoptosis of cultured NSCs/NPCs. These results were consistent with previous reports [32, 49, 50] and suggested that NFκB signaling plays a role in regulating neural survival/apoptosis during neurogenesis [51]. Accordingly, the accumulation of Nestin+ cells by NFκB inhibition for 72 hours during differentiation may result from the selective apoptosis of Nestin-negative cells. It is also possible that NSCs are more resistant to cell death versus NPCs and RPCs [52]. However,

TNFα treatment under proliferation conditions did not increase the percentage of Nestin$^+$ cells (Fig. 2D) but led to the loss of NSCs with tripotential and self-renewal potency (Fig. 3B, 3C). These data suggest that NFκB signaling is required for the proliferation and survival of Nestin$^+$ NPCs, which is against the hypothesis that decreased apoptosis or increased proliferation contributed to the accumulation of Nestin$^+$ NSCs/NPCs by NFκB inhibition. In contrast, NFκB signaling-stimulated proliferation of the rapid amplifying NPCs could promote the differentiation of its upstream slow-dividing NSCs.

Distinguishing the quiescent or slow-dividing NSCs from the rapidly amplifying early NPCs remains a challenge [15]. Both NSCs and NPCs are Sox2$^+$/Nestin$^+$. Several studies have used GFAP expression as a cellular marker and the self-renewal with tripotential capacity as a functional marker to distinguish NSCs from NPCs [5, 17, 41–44, 53, 54], although this remains controversial [55]. Using these markers, we demonstrated that selective inhibition of canonical NFκB pathway with pharmacologic inhibitors, NSC-specific transgene dnIκBα expression, and shRNA-mediated silencing retained the tripotential ability of differentiation and restored self-renewal capacity of NSCs. This is the first evidence to show that NFκB signaling predominantly mediates the differentiation of NSCs into NPCs at the very early stages of neurogenesis. This finding may explain the interesting observation that the proliferation of NSCs (GFAP$^+$/Nestin$^+$), but not NPCs (GFAP$^-$/Nestin$^+$), is significantly reduced by stress-induced NFκB activation in the adult brain [17], which is more likely to reflect the differentiation of NSCs. Our finding is also supported by a recent finding that pigment epithelium-derived factor enhances NSC self-renewal through the promotion of p65 nucleo-cytoplasmic export [56], which is a process that counteracts the nuclear translocation of p65. The critical role of NFκB in the initial differentiation step of NSCs will provide a new molecular mechanism for embryonic and adult neurogenesis. The enrichment of NSCs by NFκB inhibition may provide a valuable tool to expand neurospheres. Our finding is also in agreement with previous reports that TLR4 deficiency or small interfering RNA (siRNA) promotes self-renewal of NSCs/NPCs [15], and that Nanog represses the prodifferentiation activities of NFκB signaling to maintain pluripotency of mouse embryonic stem cells [57]. In IKKα/ IKKβ knockout mice, the apoptosis of NEC leads to abortive neurogenesis at an early developmental stage (E9.5) [16]. Therefore, time-controllable manipulation of NFκB signaling at various stages of neurogenesis would be a provocative method for addressing the mechanisms involved in the development of different neural cell lineages during in vivo neurogenesis.

Several proinflammatory cytokines have been shown to regulate neural differentiation, such as TNFα, IL-6, IL-1β, TWEAK, and interferon (IFN)γ [7, 8, 58]. The effects of TNFα on the neural differentiation of NSCs/NPCs have been extensively studied, but the reports remain controversial [49]. The enhancing effect of TNFα on neuronal differentiation was first reported in rat neonatal mesencephalic NSCs/NPCs [33]. However, TNFα has no effect on neuronal differentiation in mouse and rat adult SVZ NSCs/NPCs [6]. Recent studies suggest that TNFα promotes glial differentiation instead of neuronal differentiation in NSCs/NPCs [49, 59]. TNFα-induced neuronal differentiation is dose-dependent [32]. Moreover, TNFα treatment of nondissociated neurospheres has no effect on neural differentiation under proliferation conditions [49]. The studies with opposite results regarding the effects of TNFα on neural differentiation may have resulted from the use of different protocols (culture media, coating matrix, and exposure time), passage numbers, brain regions, species, or doses [49]. In addition, these reports have targeted the late stages (from progenitor to precursor and terminal cells) of neural differentiation. In our study, we examined the effects of TNFα on the dissociated neural cells cultured on a matrix-coated plate under differentiation and nondifferentiation conditions. Consistent with previous reports [32, 49], TNFα controlled the survival and neuronal differentiation of neural cells at some late stages during neural differentiation. Under proliferation conditions, TNFα

induced neural differentiation through NFκB activation at a very early stage of neurogenesis. The neurogenic effects of inflammatory cytokines highlight the important role of inflammatory and immune responses in early neurogenesis after injury or diseases of the nervous system [15].

During neurogenesis, cells undergo asymmetric division due to the segregation and function of multiple asymmetric proteins [38, 39]. Numb mediates neurogenesis by recycling E-cadherin in adherens junctions [60]. Asymmetric Numb distribution has been observed in the NSCs/NPCs of mice, rats, and chickens [39]. Asymmetric segregation of TRIM32, the mouse homolog of Brat/Mei-P26, is required for suppressing NSC/NPC self-renewal and inducing neuronal differentiation [38]. In this study, we observed that the early asymmetric division was TRIM32/C/EBPβ/Nestin/Sox2-related. The inhibition of NFκB signaling significantly blocked the asymmetric distribution of TRIM32, C/EBPβ, Nestin, and Sox2. These data suggest that NFκB signaling initiates asymmetric division of NSCs/NPCs at the very early stages of neural differentiation. The correlations among Nestin/Sox2, TRIM32, C/EBPβ, and Numb during asymmetric division as well as how NFκB signaling modulates these asymmetric factors will require further investigation.

Here, we show that the canonical pathway of NFκB activation suppresses the expression of C/EBPβ, which is associated with early neural differentiation of NSCs. The function of C/EBPβ in neurogenesis remains unclear. Previous studies have shown that C/EBPβ promotes neuronal linage differentiation of NPCs but inhibits gliogenesis at the late stage [61]. This effect could be due to the binding of C/EBPβ to the promoter cis-elements of the neuronal markers Tuj1 [47] and NeuroD [62]. However, in PC12 cells, the C/EBP protein level is reduced during nerve growth factor-induced neuronal differentiation [63]. A recent report showed that the increased expression of C/EBPβ and signal transducer and activator of transcription 3C in NSCs prevents neuronal differentiation [64]. Our studies provide new evidence that C/EBPβ inhibits NSCs' differentiation into NPCs, which suggests that C/EBPβ plays an important role in early neurogenesis. The mechanism by which NFκB signaling suppresses C/EBPβ remains under investigation.

GFAP has been widely used to mark mature astrocytes. Increasing evidences have validated the expression of GFAP in radial glia-like NSCs [42, 65, 66]. Transgenic models using the GFAP promoter have been established to investigate the role of GFAP+ NSCs in both embryonic and adult neurogenesis [41, 42, 44, 65, 67–69]. In this study, transgenic inhibition of NFκB signaling in GFAP+ NSCs promoted the self-renewal and accumulation of NSCs both in vitro and in vivo. For the in vitro culture system, we focused on the early stage of neural differentiation (within 24 hours) to exclude the possible effect of GFAP+ mature astrocytes, which occurred generally at a later stage during differentiation. For the in vivo studies in the adult animals, a specific population of proliferating NSCs that are positive for GFAP and Sox2/Nestin were analyzed. Although these approaches could exclude the potential influence from mature astrocytes, further validation of the direct effect of GFAP-dnIκBα on embryonic neurogenesis could be performed in embryonic developmental brain, in which GFAP expression is more selective for radial glial NSCs [41, 42, 70].

Conclusion

In conclusion, our data have established NFκB signaling as an initiator for the neural differentiation of NSCs at the early stages of neurogenesis. Our results provide important insight into the molecular mechanisms underlying neural development and adult neurogenesis and open a potential avenue for the development of therapeutics for the treatment of neurode-velopmental disorders and neurodegenerative diseases. Furthermore in

vivo loss- or gain-of-function assays using the conditional TG or knockout mice, such as GFAP-dnIκBα-enhanced green fluorescent protein (EGFP) or GFAP-IKK2^{CA}-EGFP mice are needed to confirm the essential role of NFκB signaling in initiating early differentiation of both embryonic and adult neurogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Ma DK, Bonaguidi MA, Ming GL, et al. Adult neural stem cells in the mammalian central nervous system. Cell Res. 2009; 19:672–682. [PubMed: 19436263]

2. Alexandre P, Reugels AM, Barker D, et al. Neurons derive from the more apical daughter in asymmetric divisions in the zebrafish neural tube. Nat Neurosci. 2010; 13:673–679. [PubMed: 20453852]

3. Kriegstein A, Alvarez-Buylla A. The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci. 2009; 32:149–184. [PubMed: 19555289]

4. Conti L, Cattaneo E. Neural stem cell systems: Physiological players or in vitro entities? Nat Rev Neurosci. 2010; 11:176–187. [PubMed: 20107441]

5. Aguirre A, Rubio ME, Gallo V. Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal. Nature. 2010; 467:323–327. [PubMed: 20844536]

6. Widera D, Kaus A, Kaltschmidt C, et al. Neural stem cells, inflammation and NFκB: Basic principle of maintenance and repair or origin of brain tumours? J Cell Mol Med. 2008; 12:459–470. [PubMed: 18182066]

7. Das S, Basu A. Inflammation: A new candidate in modulating adult neurogenesis. J Neurosci Res. 2008; 86:1199–1208. [PubMed: 18058947]

8. Whitney NP, Eidem TM, Peng H, et al. Inflammation mediates varying effects in neurogenesis: Relevance to the pathogenesis of brain injury and neurodegenerative disorders. J Neurochem. 2009; 108:1343–1359. [PubMed: 19154336]

9. Kassed CA, Herkenham M. NFκB p50-deficient mice show reduced anxiety-like behaviors in tests of exploratory drive and anxiety. Behav Brain Res. 2004; 154:577–584. [PubMed: 15313047]

10. Meffert MK, Chang JM, Wiltgen BJ, et al. NFκB functions in synaptic signaling and behavior. Nat Neurosci. 2003; 6:1072–1078. [PubMed: 12947408]

11. Ahn HJ, Hernandez CM, Levenson JM, et al. c-Rel, an NFκB family transcription factor, is required for hippocampal long-term synaptic plasticity and memory formation. Learn Mem. 2008; 15:539–549. [PubMed: 18626097]

12. Sanchez-Ponce D, Tapia M, Munoz A, et al. New role of IKK alpha/ beta phosphorylated IκBα in axon outgrowth and axon initial segment development. Mol Cell Neurosci. 2008; 37:832–844. [PubMed: 18321726]

13. Gutierrez H, Hale VA, Dolcet X, et al. NFκB signalling regulates the growth of neural processes in the developing PNS and CNS. Development. 2005; 132:1713–1726. [PubMed: 15743881]

14. Young KM, Bartlett PF, Coulson EJ. Neural progenitor number is regulated by NFκB p65 and p50 subunit-dependent proliferation rather than cell survival. J Neurosci Res. 2006; 83:39–49. [PubMed: 16307445]

15. Rolls A, Shechter R, London A, et al. Toll-like receptors modulate adult hippocampal neurogenesis. Nat Cell Biol. 2007; 9:1081–1088. [PubMed: 17704767]

16. Li Q, Estepa G, Memet S, et al. Complete lack of NFκB activity in IKK1 and IKK2 double-deficient mice: Additional defect in neurula-tion. Genes Dev. 2000; 14:1729–1733. [PubMed: 10898787]

17. Koo JW, Russo SJ, Ferguson D, et al. NFκB is a critical mediator of stress-impaired neurogenesis and depressive behavior. Proc Natl Acad Sci USA. 2010; 107:2669–2674. [PubMed: 20133768]

18. Covacu R, Arvidsson L, Andersson A, et al. TLR activation induces TNF-alpha production from adult neural stem/progenitor cells. J Immunol. 2009; 182:6889–6895. [PubMed: 19454685]

19. Denis-Donini S, Dellarole A, Crociara P, et al. Impaired adult neuro-genesis associated with short-term memory defects in NFκB p50-defi-cient mice. J Neurosci. 2008; 28:3911–3919. [PubMed: 18400889]

20. Noctor SC, Martinez-Cerdeno V, Ivic L, et al. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci. 2004; 7:136–144. [PubMed: 14703572]

21. Brambilla R, Bracchi-Ricard V, Hu WH, et al. Inhibition of astroglial nuclear factor kappaB reduces inflammation and improves functional recovery after spinal cord injury. J Exp Med. 2005; 202:145–156. [PubMed: 15998793]

22. Louis SA, Rietze RL, Deleyrolle L, et al. Enumeration of neural stem and progenitor cells in the neural colony-forming cell assay. Stem Cells. 2008; 26:988–996. [PubMed: 18218818]

23. Shen Q, Zhong W, Jan YN, et al. Asymmetric Numb distribution is critical for asymmetric cell division of mouse cerebral cortical stem cells and neuroblasts. Development. 2002; 129:4843–4853. [PubMed: 12361975]

24. Sanada K, Tsai LH. G protein betagamma subunits and AGS3 control spindle orientation and asymmetric cell fate of cerebral cortical progenitors. Cell. 2005; 122:119–131. [PubMed: 16009138]

25. Hahn CG, Han LY, Rawson NE, et al. In vivo and in vitro neuro-genesis in human olfactory epithelium. J Comp Neurol. 2005; 483:154–163. [PubMed: 15678478]

26. Hu W, Li F, Mahavadi S, et al. Interleukin-1beta up-regulates RGS4 through the canonical IKK2/IkappaBalpha/NFκB pathway in rabbit colonic smooth muscle. Biochem J. 2008; 412:35–43. [PubMed: 18260825]

27. Messeguer X, Escudero R, Farre D, et al. PROMO: Detection of known transcription regulatory elements using species-tailored searches. Bioinformatics. 2002; 18:333–334. [PubMed: 11847087]

28. Meneghini V, Francese MT, Carraro L, et al. A novel role for the receptor for advanced glycation end-products in neural progenitor cells derived from adult subventricular zone. Mol Cell Neurosci. 2010; 45:139–150. [PubMed: 20600932]

29. Denis-Donini S, Caprini A, Frassoni C, et al. Members of the NFκB family expressed in zones of active neurogenesis in the postnatal and adult mouse brain. Brain Res Dev Brain Res. 2005; 154:81–89.

30. Pluchino S, Zanotti L, Rossi B, et al. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. Nature. 2005; 436:266–271. [PubMed: 16015332]

31. Anitha M, Joseph I, Ding X, et al. Characterization of fetal and postnatal enteric neuronal cell lines with improvement in intestinal neural function. Gastroenterology. 2008; 134:1424–1435. [PubMed: 18471518]

32. Bernardino L, Agasse F, Silva B, et al. Tumor necrosis factor-alpha modulates survival, proliferation, and neuronal differentiation in neonatal subventricular zone cell cultures. Stem Cells. 2008; 26:2361–2371. [PubMed: 18583543]

33. Lou SJ, Gu P, Xu H, et al. [Effect of tumor necrosis factor-alpha on differentiation of mesencephalic neural stem cells and proliferation of oligodendrocytes in the rat]. Sheng Li Xue Bao. 2003; 55:183–186. [PubMed: 12715108]

34. Kanzawa T, Iwado E, Aoki H, et al. Ionizing radiation induces apopto-sis and inhibits neuronal differentiation in rat neural stem cells via the c-Jun NH2-terminal kinase (JNK) pathway. Oncogene. 2006; 25:3638–3648. [PubMed: 16491125]

35. Shingo T, Sorokan ST, Shimazaki T, et al. Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. J Neurosci. 2001; 21:9733–9743. [PubMed: 11739582]

36. Hirata J, Nakagoshi H, Nabeshima Y, et al. Asymmetric segregation of the homeodomain protein Prospero during *Drosophila* development. Nature. 1995; 377:627–630. [PubMed: 7566173]

37. Chenn A, McConnell SK. Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. Cell. 1995; 82:631–641. [PubMed: 7664342]

38. Schwamborn JC, Berezikov E, Knoblich JA. The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. Cell. 2009; 136:913–925. [PubMed: 19269368]

39. Cayouette M, Raff M. Asymmetric segregation of Numb: A mechanism for neural specification from *Drosophila* to mammals. Nat Neu-rosci. 2002; 5:1265–1269.

40. Hillje AL, Worlitzer MM, Palm T, et al. Neural stem cells maintain their stemness through protein kinase C zeta mediated inhibition of TRIM32. Stem Cells. 2011; 29:1437–1447. [PubMed: 21732497]

41. Bonaguidi MA, McGuire T, Hu M, et al. LIF and BMP signaling generate separate and discrete types of GFAP-expressing cells. Development. 2005; 132:5503–5514. [PubMed: 16314487]

42. Garcia AD, Doan NB, Imura T, et al. GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nat Neurosci. 2004; 7:1233–1241. [PubMed: 15494728]

43. Encinas JM, Vaahtokari A, Enikolopov G. Fluoxetine targets early progenitor cells in the adult brain. Proc Natl Acad Sci USA. 2006; 103:8233–8238. [PubMed: 16702546]

44. Ganat YM, Silbereis J, Cave C, et al. Early postnatal astroglial cells produce multilineage precursors and neural stem cells in vivo. J Neu-rosci. 2006; 26:8609–8621.

45. Bracchi-Ricard V, Brambilla R, Levenson J, et al. Astroglial NFκB regulates learning and memory and synaptic plasticity in female mice. J Neurochem. 2008; 104:611–623. [PubMed: 17953667]

46. Legraverend C, Antonson P, Flodby P, et al. High level activity of the mouse CCAAT/enhancer binding protein (C/EBP alpha) gene promoter involves autoregulation and several ubiquitous transcription factors. Nucleic Acids Res. 1993; 21:1735–1742. [PubMed: 8493090]

47. Liu XS, Chopp M, Zhang RL, et al. Angiopoietin 2 mediates the differentiation and migration of neural progenitor cells in the subventricular zone after stroke. J Biol Chem. 2009; 284:22680–22689. [PubMed: 19553662]

48. Kowenz-Leutz E, Twamley G, Ansieau S, et al. Novel mechanism of C/EBP beta (NF-M) transcriptional control: Activation through dere-pression. Genes Dev. 1994; 8:2781–2791. [PubMed: 7958933]

49. Keohane A, Ryan S, Maloney E, et al. Tumour necrosis factor-alpha impairs neuronal differentiation but not proliferation of hippocampal neural precursor cells: Role of Hes1. Mol Cell Neurosci. 2010; 43:127–135. [PubMed: 19840854]

50. Widera D, Mikenberg I, Elvers M, et al. Tumor necrosis factor alpha triggers proliferation of adult neural stem cells via IKK/NFκB signaling. BMC Neurosci. 2006; 7:64. [PubMed: 16987412]

51. Sierra A, Encinas JM, Deudero JJ, et al. Microglia shape adult hippo-campal neurogenesis through apoptosis-coupled phagocytosis. Cell Stem Cell. 2010; 7:483–495. [PubMed: 20887954]

52. Brazel CY, Rosti RT 3rd, Boyce S, et al. Perinatal hypoxia/ischemia damages and depletes progenitors from the mouse subventricular zone. Dev Neurosci. 2004; 26:266–274. [PubMed: 15711066]

53. Encinas JM, Michurina TV, Peunova N, et al. Division-coupled astro-cytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. Cell Stem Cell. 2011; 8:566–579. [PubMed: 21549330]

54. Ming GL, Song H. Adult neurogenesis in the mammalian brain: Significant answers and significant questions. Neuron. 2011; 70:687–702. [PubMed: 21609825]

55. Chojnacki AK, Mak GK, Weiss S. Identity crisis for adult periventric-ular neural stem cells: Subventricular zone astrocytes, ependymal cells or both? Nat Rev Neurosci. 2009; 10:153–163. [PubMed: 19153578]

56. Andreu-Agullo C, Morante-Redolat JM, Delgado AC, et al. Vascular niche factor PEDF modulates Notch-dependent stemness in the adult subependymal zone. Nat Neurosci. 2009; 12:1514–1523. [PubMed: 19898467]

57. Torres J, Watt FM. Nanog maintains pluripotency of mouse embryonic stem cells by inhibiting NFkappaB and cooperating with Stat3. Nat Cell Biol. 2008; 10:194–201. [PubMed: 18223644]

58. Scholzke MN, Rottinger A, Murikinati S, et al. TWEAK regulates proliferation and differentiation of adult neural progenitor cells. Mol Cell Neurosci. 2011; 46:325–332. [PubMed: 21040786]

59. Johansson S, Price J, Modo M. Effect of inflammatory cytokines on major histocompatibility complex expression and differentiation of human neural stem/progenitor cells. Stem Cells. 2008; 26:2444–2454. [PubMed: 18635871]

60. Rasin MR, Gazula VR, Breunig JJ, et al. Numb and Numbl are required for maintenance of cadherin-based adhesion and polarity of neural progenitors. Nat Neurosci. 2007; 10:819–827. [PubMed: 17589506]

61. Paquin A, Barnabe-Heider F, Kageyama R, et al. CCAAT/enhancer-binding protein phosphorylation biases cortical precursors to generate neurons rather than astrocytes in vivo. J Neurosci. 2005; 25:10747–10758. [PubMed: 16291948]

62. Uittenbogaard M, Martinka DL, Johnson PF, et al. 5′UTR of the neu-rogenic bHLH Nex1/MATH-2/NeuroD6 gene is regulated by two distinct promoters through CRE and C/EBP binding sites. J Neurosci Res. 2007; 85:1–18. [PubMed: 17075921]

63. Sterneck E, Johnson PF. CCAAT/enhancer binding protein beta is a neuronal transcriptional regulator activated by nerve growth factor receptor signaling. J Neurochem. 1998; 70:2424–2433. [PubMed: 9603207]

64. Carro MS, Lim WK, Alvarez MJ, et al. The transcriptional network for mesenchymal transformation of brain tumours. Nature. 2010; 463:318–325. [PubMed: 20032975]

65. Ihrie RA, Alvarez-Buylla A. Cells in the astroglial lineage are neural stem cells. Cell Tissue Res. 2008; 331:179–191. [PubMed: 17786483]

66. Song H, Stevens CF, Gage FH. Astroglia induce neurogenesis from adult neural stem cells. Nature. 2002; 417:39–44. [PubMed: 11986659]

67. Liu Y, Namba T, Liu J, et al. Glial fibrillary acidic protein-expressing neural progenitors give rise to immature neurons via early intermediate progenitors expressing both glial fibrillary acidic protein and neu-ronal markers in the adult hippocampus. Neuroscience. 2010; 166:241–251. [PubMed: 20026190]

68. Beckervordersandforth R, Tripathi P, Ninkovic J, et al. In vivo fate mapping and expression analysis reveals molecular hallmarks of pro-spectively isolated adult neural stem cells. Cell Stem Cell. 2010; 7:744–758. [PubMed: 21112568]

69. Weber T, Baier V, Pauly R, et al. Inducible gene expression in GFAP+ progenitor cells of the SGZ and the dorsal wall of the SVZ-A novel tool to manipulate and trace adult neurogenesis. Glia. 2011; 59:615–626. [PubMed: 21294160]

70. Sauvageot CM, Stiles CD. Molecular mechanisms controlling cortical gliogenesis. Curr Opin Neurobiol. 2002; 12:244–249. [PubMed: 12049929]
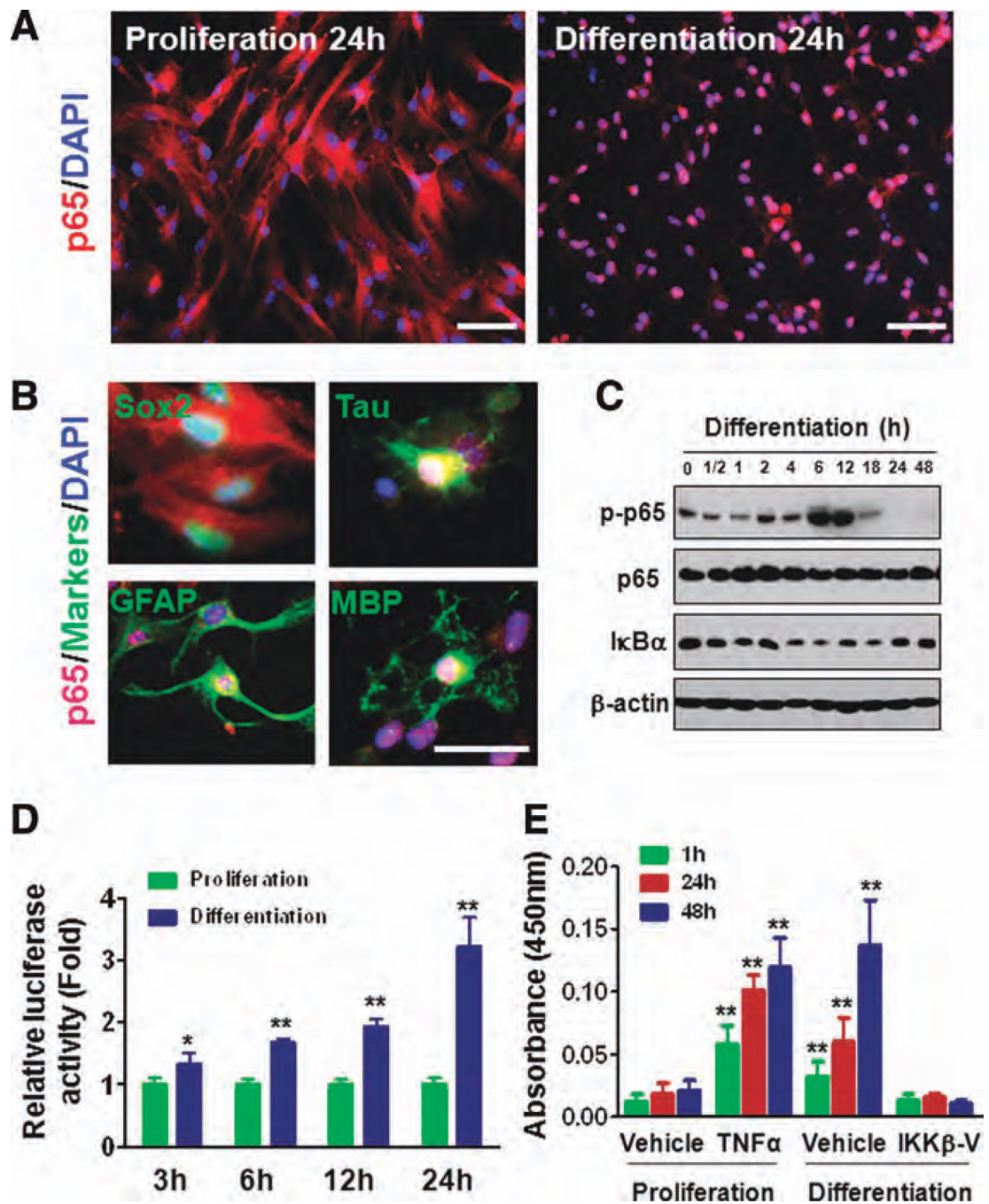
Figure 1.
Nuclear factor kappa B (NFκB) signaling is activated during neural differentiation of neural stem cells (NSCs)/neural progenitor cells (NPCs). (**A**): The NFκB subunit p65 is present in the cytoplasm during proliferation and translocated into nucleus after 24 hours of differentiation by withdrawal of epidermal growth factor (EGF)/basic fibroblast growth factor (bFGF) in cultured adult (10 weeks old) subventricular zone (SVZ) NSCs/NPCs. (**B**): p65 protein was translocated into the nucleus of differentiated neurons (Tau), astrocytes (GFAP), and oligodendrocytes (MBP). (**C, D**): Time course of NFκB activation after withdrawal of EGF/bFGF in cultured neonatal (P6) SVZ NSCs/NPCs as determined by

Western blot analysis using the indicated antibodies (C) and adenovirus-NFκB-luciferase reporter assay (D). *, $p < .05$ and **, $p < .01$ indicate a significant increase from the corresponding proliferation group by a two-tailed Student's t test ($n = 4$). (**E**): Time-dependent increase in the phosphorylation of p65 (Ser536) in cultured embryonic (E14.5) NSCs/NPCs detected by PathScan ELISA, which was blocked by the IKKβ inhibitor V. TNFα-induced p65 phosphorylation was used as a positive control. **, $p < .01$ indicates a significant increase from the corresponding vehicle control under proliferation conditions by a two-tailed Student's t test ($n = 3$). Scale bar = 20 μm. See also Supporting Information Figure S1. Abbreviations: DAPI, 4′-6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; IKK, IκB kinase; MBP, myelin basic protein; Sox2, sex determining region Y-box 2; TNFα, tumor necrosis factor alpha.
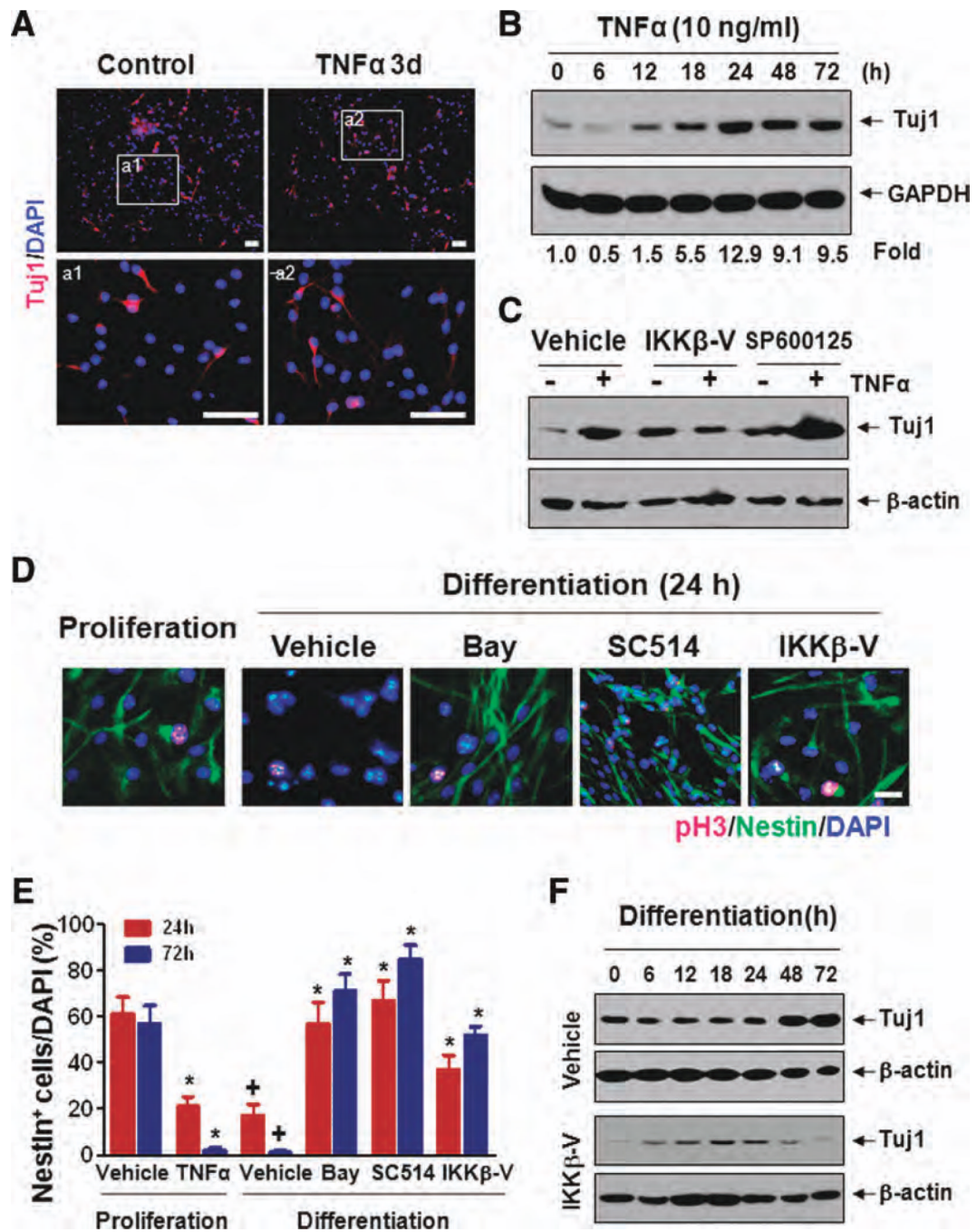
Figure 2.
Activation of nuclear factor kaapa B (NFκB) signaling controls neural differentiation of neural stem cells (NSCs)/neural progenitor cells (NPCs). (**A**): TNFα treatment for 72 hours in proliferation media in cultured neonatal (P6) subventricular zone (SVZ) NSCs/NPCs promoted neuronal differentiation as shown by immunocytochemistry with antibody against neuronal marker Tuj1. (**B**): TNFα treatment in proliferation media induced a time-dependent increase in the protein expression of Tuj1. The fold number indicates the relative optical density compared with the corresponding control after GAPDH normalization. (**C**): TNFα-induced upregulation of Tuj1 expression was blocked by the IKKβ inhibitor V but

not the JNK inhibitor SP600125. (**D, E**): Inhibition of canonical NFκB signaling maintained the Nestin^{+} cells during neural differentiation. TNFa was applied to cultured embryonic (E14.5) NSCs/NPCs under proliferation conditions. The inhibitors of NFκB pathway were added under differentiation conditions. Bay: The IκBα phosphorylation inhibitor Bay-11-7028. IKKβ-V and SC-514: The IKKβ inhibitors. *, $p < .05$ indicates a significant difference when compared with corresponding vehicle control by Student's t test, and $+p < .05$ indicates a significant decrease compared with corresponding vehicle of proliferation. (**F**): Time course changes in Tuj1 expression during neural differentiation of neonatal (P6) SVZ NSCs/NPCs after treatment with vehicle or IKK-V. Scale bar = 50 μm (A) or 10 μm (D). See also Supporting Information Figures S2 and S3. Abbreviations: DAPI, 4′-6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; TNFα, tumor necrosis factor alpha.
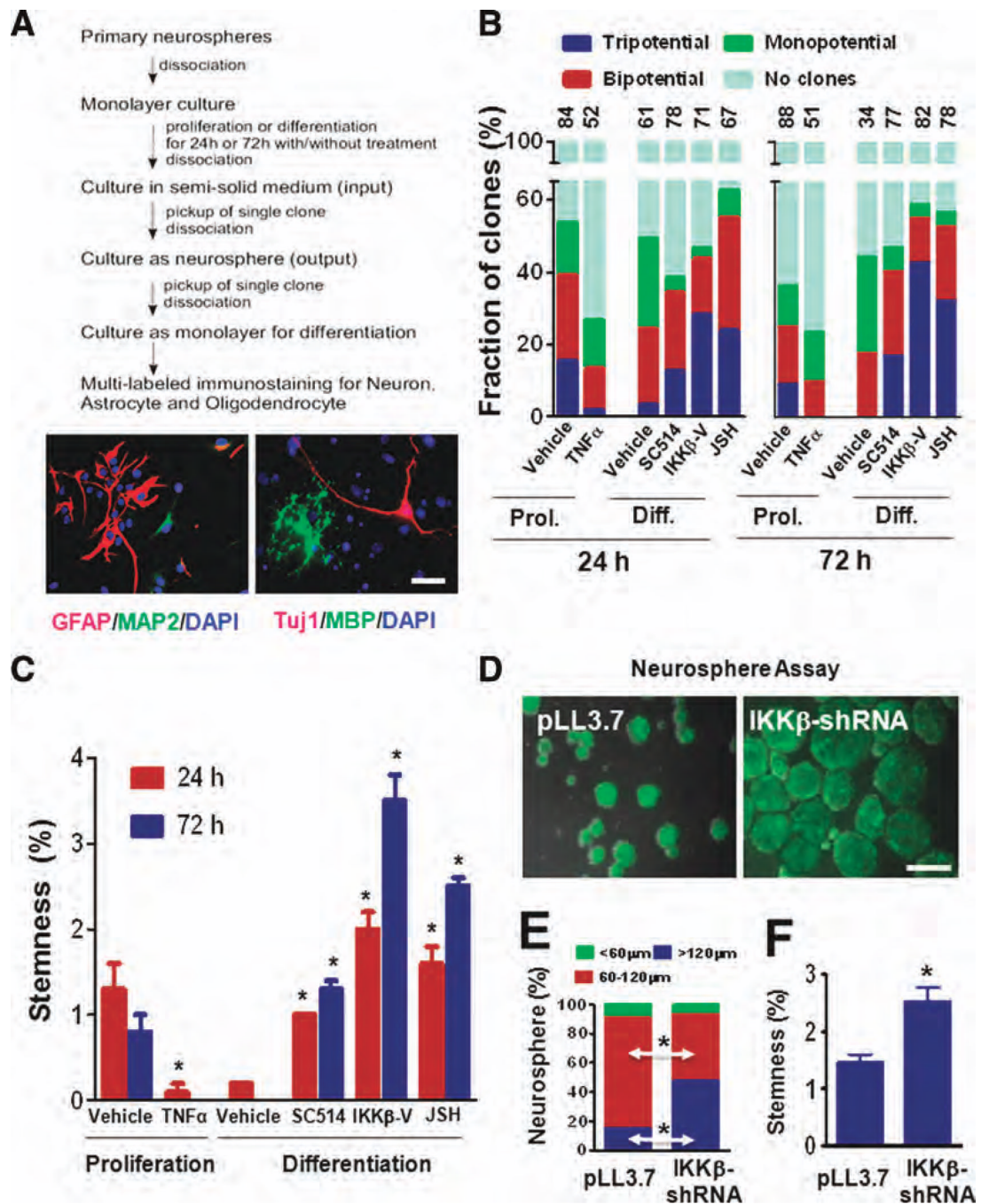
Figure 3.
Nuclear factor kappa B (NFκ) activation initiates neural differentiation of neural stem cells (NSCs). (**A**): Protocol of modified neural colony-forming cell assay and representative micrographs of multilabeled neurons (Tuji1 and MAP2), astrocytes (GFAP), and oligodendro-cytes (MBP). (**B**): Inhibition of NFκB signaling retained tripotential capacities of embryonic NSCs. The clones cultured in semisolid medium were picked for lineage differentiation. Tripotential clone: differentiating into three types of neural cells; bipotential clone: differentiating into either two cell types; monopotential clone: differentiating into one cell type. The number (*n*) shows the summary of clones forming secondary neu-rospheres

from three experiments with duplicate wells each containing 500 cells plated after treatment. (**C**): The percentage of those cells that formed secondary neurospheres and gave rise to tripotential clones was significantly maintained by inhibition of NFκB signaling under differentiation conditions, whereas TNFα treatment significantly reduced stemness under proliferation conditions. *, $p < .05$ indicates a significant difference when compared with corresponding vehicle control by Student's *t* test. (**D–F**): Lentivirus-mediated IKKβ shRNA knockdown in neonatal NSCs/neural progenitor cells enriched neurosphere formation and tripotential clones. Equal number of dissociated single cells was cultured in an uncoated culture dish for 72 hours. Digital micrographs were taken (D), and the neurospheres were measured and grouped (E). The stemness frequency (F) was determined as above. *, $p < .05$ indicates a significant increase when compared with empty vector control by Student's *t* test. Scale bars = 10 μm (A) or 100 μm (D). See also Supporting Information Figure S3. Abbreviations: DAPI, 4′-6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; MBP, myelin basic protein; IKK, IκB kinase; JSH, NFκB activation inhibitor II; SC-514, IKKβ inhibitor; shRNA, small hairpin RNA; TNFα, tumor necrosis factor alpha.
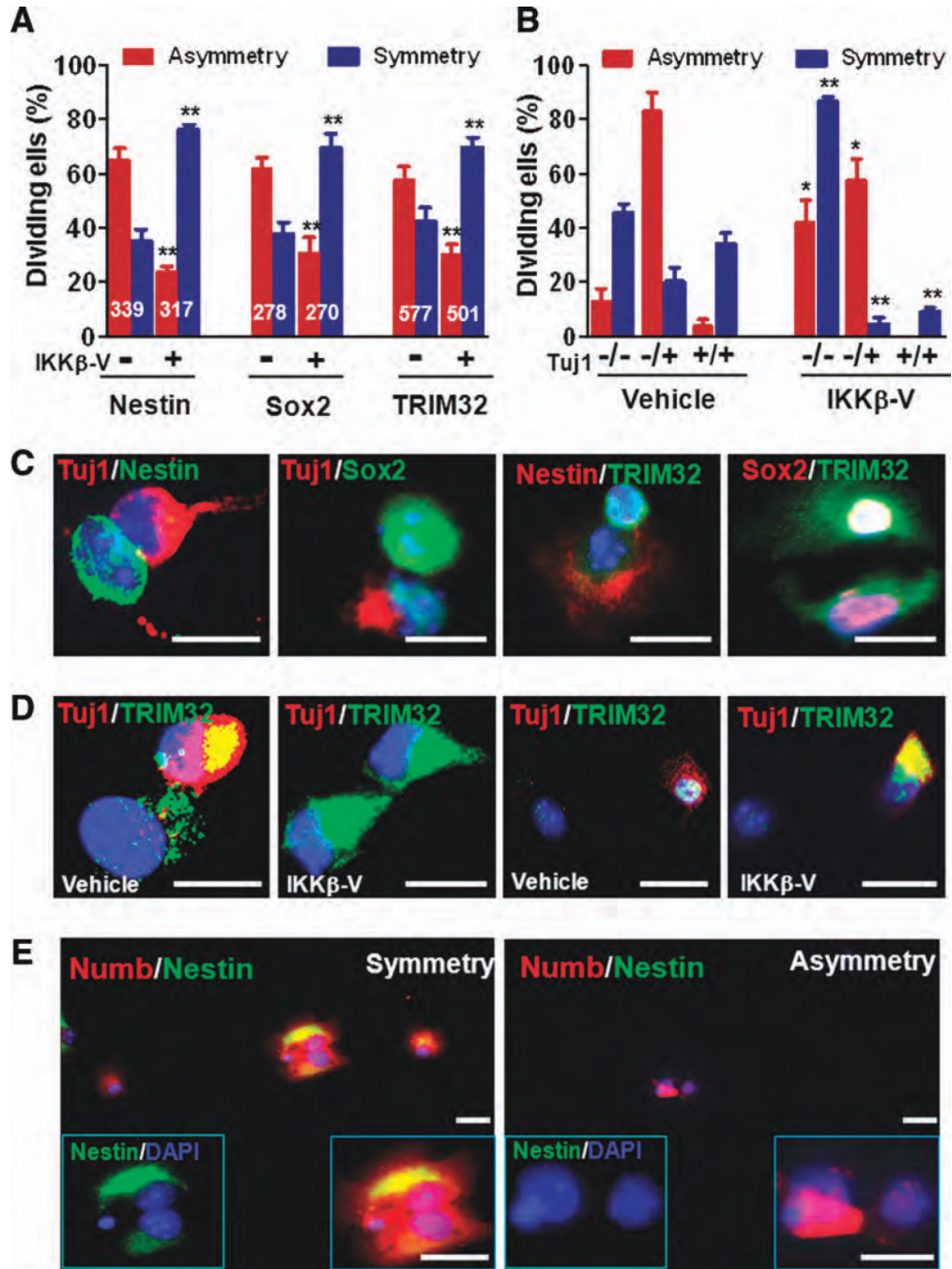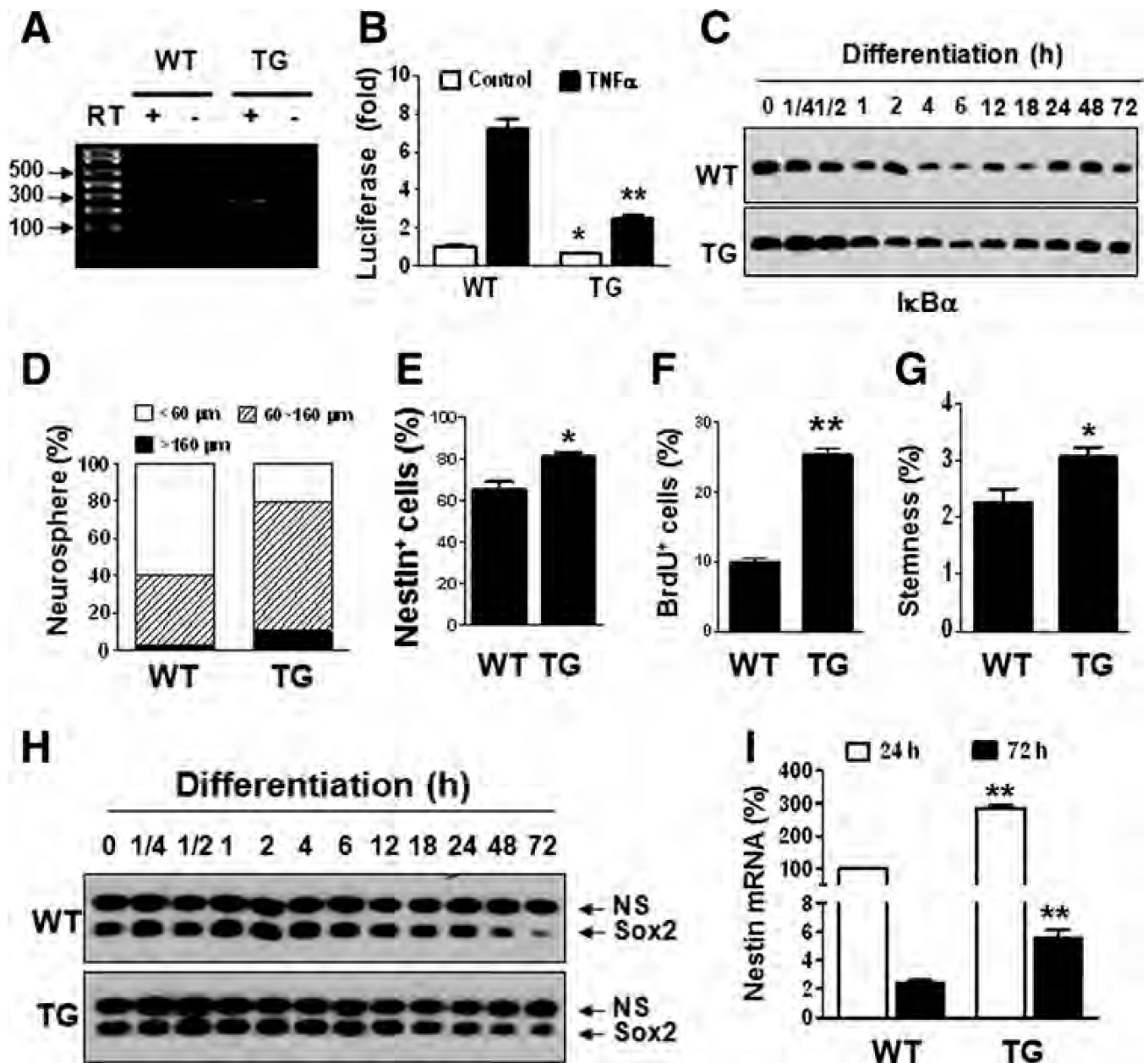
Figure 4.
Nuclear factor kappa B (NFκB) activation is required for asymmetric division of Nestin[+]/Sox2^{+}/TRIM32^{+} neural stem cells/neural progenitor cells. (**A, B**): Inhibition of NFκB signaling blocked the asymmetric division and increased symmetric division of Nestin[+], Sox2^{+}, or TRIM32^{+} cells. The effect on TRIM32 symmetry was reflected in Tuj1^{−} paired cells (B). The number (*n*) represents the summary of paired cells from at least five independent experiments. *, *p* < .05; **, *p* < .01 indicate a significant change from the corresponding vehicle control by a two-tailed Student's *t* test. (C): The Tuj1 and TRIM32 were expressed in Nestin^{low/−} Sox2^{low/−} cells during Nestin- or Sox2-related asymmetric

division. (**D**): Asymmetric distribution and nuclear translocation of TRIM32 in Tuj1^{+} cells were blocked by NFκB inhibition. (**E**): Nestin asymmetric distribution occurred prior to Numb asymmetry. Scale bar = 10 μm. See also Supporting Information Figure S4. Abbreviations: DAPI, 4′-6-diamidino-2-phenylindole; IKK, IκB kinase; Sox2, sex determining region Y-box 2;TRIM, tripartite motif-containing protein 32.

Figure 5.

Transgenic inhibition of nuclear factor kappa B (NFκB) in glial fibrillary acidic protein positive neural stem cells (NSCs) promotes self-renewal and multipotency. (**A**): Reverse transcription polymerase chain reaction (RT-PCR) analysis verified the mRNA expression of trans-gene dnIκBα in cultured neonatal subventricular zone (SVZ) NSCs/neural progenitor cells (NPCs). (**B, C**): Adenovirus-NFκB reporter assay and Western blot analysis confirmed the significant inhibition of NFκB activation by the transgene in NSCs/NPCs. (**D**): Transgenic inhibition of NFκB increased the size of neurospheres. Equal number of dissociated NSCs/NPCs was cultured in an uncoated flask under proliferation for 72 hours. Digital micrographs were taken, and the neurospheres were measured and grouped. (**E**): Multilabeled immunocytochemistry showed a higher percentage of Nestin+ cells in *TG* NSCs/NPCs in a monolayer culture after 72 hours of incubation in proliferation media. (**F**):

BrdU pulse labeling (4 hours) in cultured NSCs/NPCs showed a significant increase in cell proliferation by the transgene. (**G**): The clonogenic assay showed a significantly higher frequency of NSCs with tripotential and self-renewal potency in *TG* mice than that in *WT* mice. (**H**): Western blot analysis showed the delay and attenuation of Sox2 loss after differentiation in *TG* mice. (**I**): RT-quantitative PCR analysis confirmed a higher level of *Nestin* mRNA at both 24 and 72 hours after differentiation in *TG* NSCs/NPCs. *, $p < .05$; **, $p < .01$ indicate a significant change from the corresponding *WT* by a two-tailed Student's *t* test. See also Supporting Information Figure S5. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; NS: nonspecific band; Sox2, sex determining region Y-box 2; TG, transgenic; TNFα, tumor necrosis factor alpha; WT, wild type.
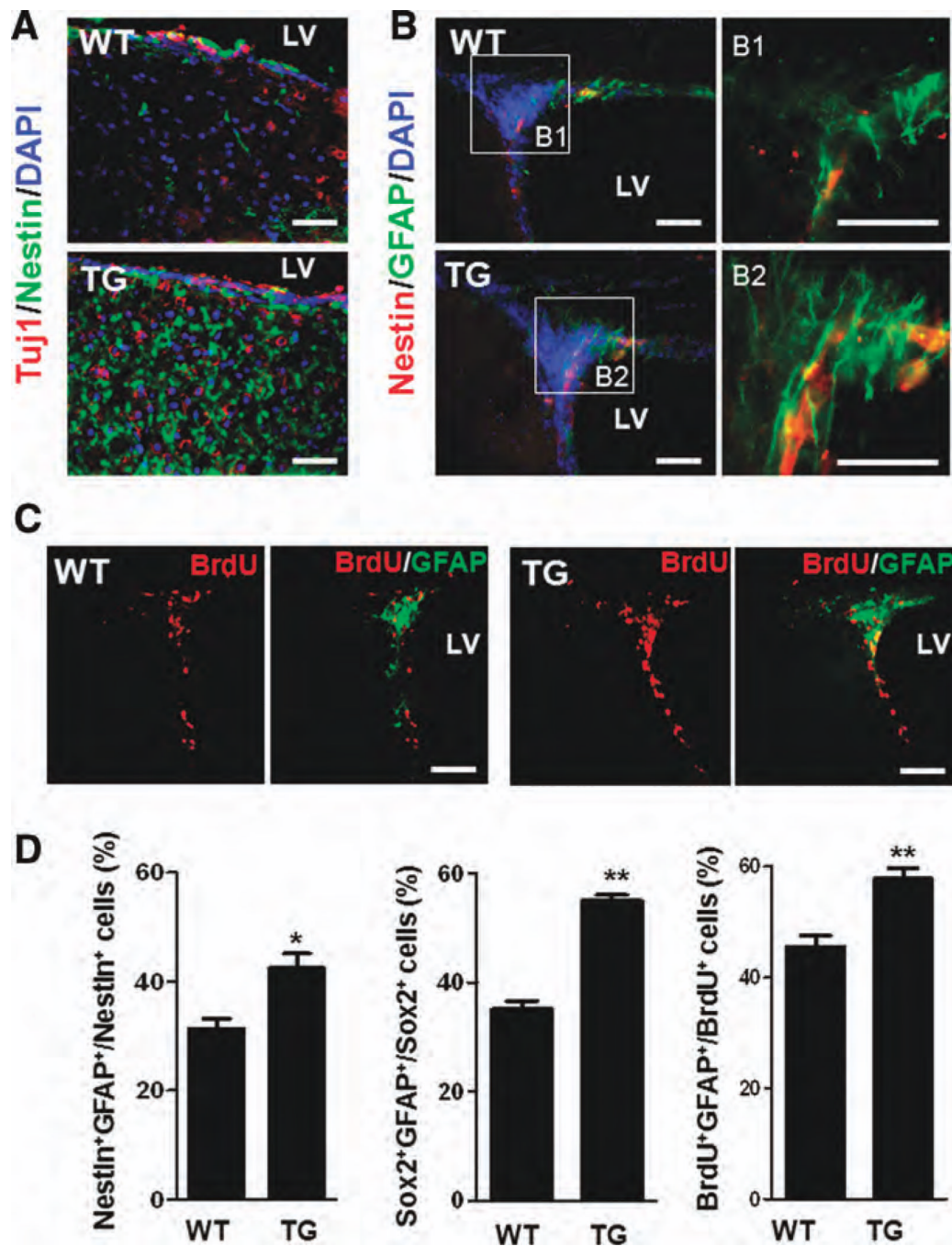
Figure 6.
Nestin$^+$/Sox2$^+$/GFAP$^+$ neural stem cells (NSCs) are accumulated by partial inhibition of nuclear factor kappa B signaling in GFAP$^+$ NSCs in vivo. (**A**): Immunohistochemical staining of transverse brain sections showed dramatic accumulation of Nestin$^+$ NSCs/neural progenitor cells (NPCs) in *TG* mouse subventricular zone (SVZ) around the LV. (**B**): Representative micrographs showed the colocalization of Nestin and GFAP in SVZ NSCs and its increase in *TG* mouse. (**C**): BrdU pulse labeling (4 hours) and immunohistochemical staining showed an increase in BrdU$^+$/GFAP$^+$ cells in *TG* mouse SVZ. (**D**): Quantitative analysis of a series of brain sections showed a significant increase of Nestin$^+$/GFAP$^+$,

Sox2$^+$/GFAP$^+$, or BrdU$^+$/GFAP$^+$ NSCs in the *TG* mouse SVZ. *, $p < .05$; **, $p < .01$ indicate a significant difference from the corresponding *WT* by a two-tailed Student's *t* test ($n = 3$). Scale bar = 40 μm. See also Supporting Information Figure S6. Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; DAPI, 4′-6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; LV, lateral ventricle; Sox2, sex determining region Y-box 2; TG, transgenic; WT, wild type.
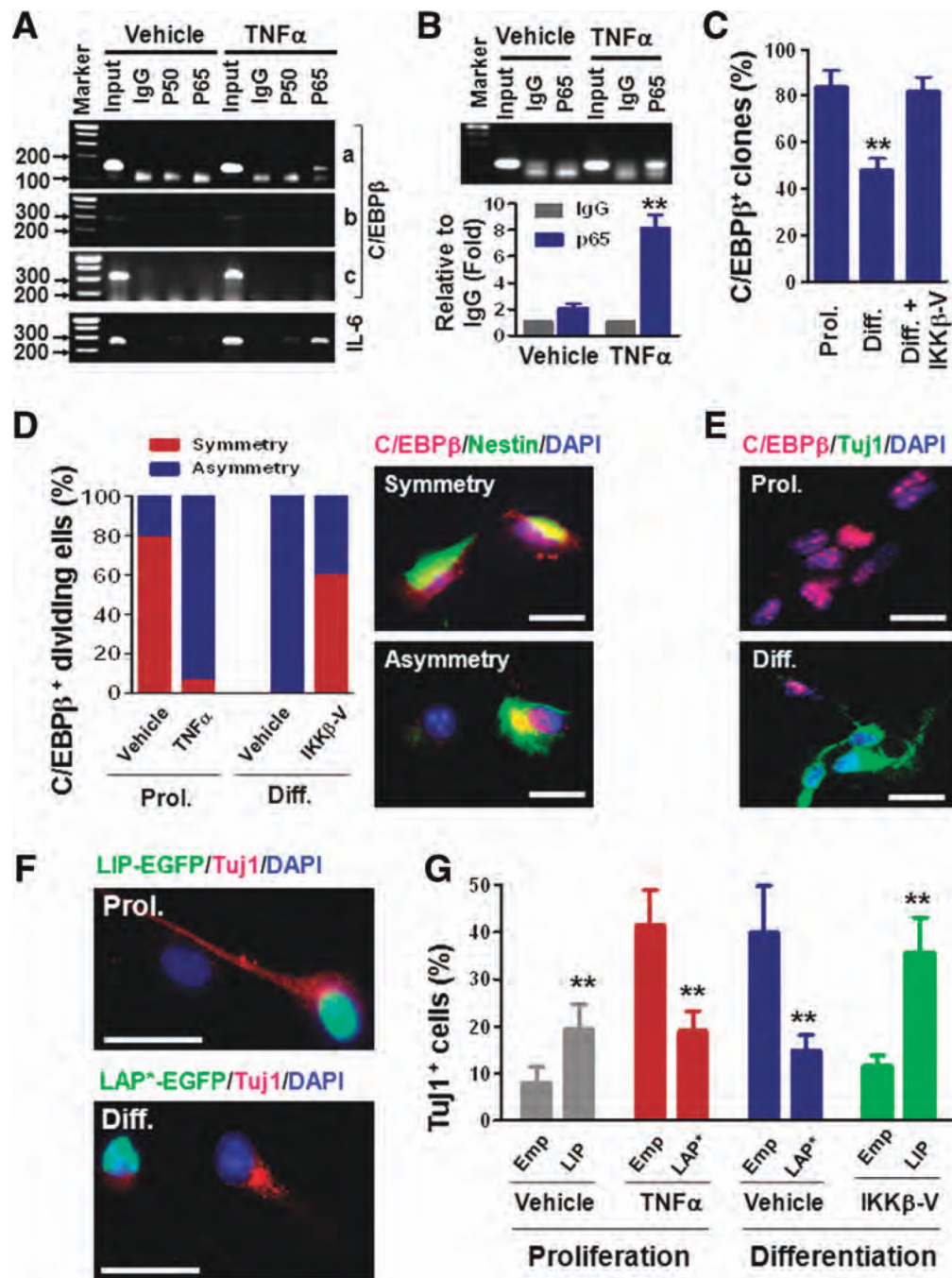
Figure 7.
Nuclear factor kappa B (NFκB) inhibits the expression of C/EBPβ for neural differentiation.
(**A**): Chromatin immunoprecipitation assay identified TNFα-dependent binding of p65, but
not p50, to the promoter regions of C/EBPβ as determined by conventional polymerase
chain reaction (PCR). IL-6 promoter binding was used as positive control. (**B**): Real-time
PCR analysis showed the quantitative change in p65 binding of the first NFκB site within C/
EBPβ. The top panel shows the endpoint PCR product. **, $p < .01$ indicates a significant
increase compared with corresponding vehicle control. (**C**): Inhibition of NFκB prevented
differentiation (Diff.)-induced decrease in C/EBPβ$^+$ clones. The data were derived from six

experiments. **, $p < .01$ indicates statistical significance compared with the proliferation (Prol.) group as determined by a Student's *t* test. (**D**): C/EBPβ protein underwent asymmetric division after 24 hours of differentiation by TNFα treatment or growth factor withdrawal, which was attenuated by NFκB inhibition. Representative micrographs showed a similar asymmetric distribution of C/EBPβ and Nes-tin. (**E**): Representative double-labeled immunostaining showed the loss of C/EBPβ cells and the gain of Tuj1 cells after differentiation for 72 hours. (**F**): Inhibition of C/EBPβ by LIP induced neural differentiation under the proliferation conditions, whereas activation of C/EBPβ by LAP* inhibited neural differentiation. Neural stem cells/neural progenitor cells were infected with the LIP-EGFP or LAP*-EGFP lentivirus for 48 hours, and immunocytochemistry was performed with an anti-Tuj1 antibody (red). (**G**): The expression of C/EBPβ inhibits the formation of Tuj1$^+$ cells. TNFα was applied under proliferation conditions, and IKKβ inhibitor V was applied under differentiation conditions. LAP* and LIP were cloned into Emp. The percentage of Tuj1$^+$ cells was determined by immunostaining 72 hours after the lentivirus infection under proliferation or differentiation conditions with or without TNFα or IKKβ inhibitor V as indicated. The data were derived from three experiments. Scale bar = 10 μm. See also Supporting Information Figure S4C. Abbreviations: C/EBP, CCAAT/enhancer binding protein; DAPI, 4′-6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein; Emp, empty lentiviral vectors; IKK, IκB kinase; IL, interleukin; LAP*, the full length of C/EBPβ; LIP, the C-terminal version of C/EBPβ; TNFα, tumor necrosis factor alpha.