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Transforming Growth Factor β Promotes Neuronal Cell Fate of Mouse Cortical and Hippocampal Progenitors In Vitro and In Vivo: Identification of Nedd9 as an Essential Signaling Component

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Transforming Growth Factor β (Tgfβ) and associated signaling effectors are expressed in the forebrain, but little is known about the role of this multifunctional cytokine during forebrain development. Using hippocampal and cortical primary cell cultures of developing mouse brains, this study identified Tgfβ-regulated genes not only associated with cell cycle exit of progenitors but also with adoption of neuronal cell fate. Accordingly, we observed not only an antimitotic effect of Tgfβ on progenitors but also an increased expression of neuronal markers in Tgfβ treated cultures. This effect was dependent upon Smad4. Furthermore, in vivo loss-of-function analyses using *Tgfβ2*$^{-/-}$/*Tgfβ3*$^{-/-}$ double mutant mice showed the opposite effect of increased cell proliferation and fewer neurons in the cerebral cortex and hippocampus. *Gata2, Runx1*, and *Nedd9* were candidate genes regulated by Tgfβ and known to be involved in developmental processes of neuronal progenitors. Using siRNA-mediated knockdown, we identified Nedd9 as an essential signaling component for the Tgfβ-dependent increase in neuronal cell fate. Expression of this scaffolding protein, which is mainly described as a signaling molecule of the β1-integrin pathway, was not only induced after Tgfβ treatment but was also associated with morphological changes of the Nestin-positive progenitor pool observed upon exposure to Tgfβ.

Keywords: cerebral cortex, differentiation, Hef1, Nestin, progenitor

Introduction

Transforming Growth Factor β (Tgfβ) isoforms 1, 2, and 3 are the eponymous members of the Tgfβ superfamily that comprises more than 40 different cytokines. Although several specific functions are attributable to each of the isoforms in vivo, very likely caused by specific expression of one isoform in a specific cellular context, Tgfβ1, 2, and 3 are homologous proteins and probably replaceable in vitro. Thus, for convention, in this study, we will refer to the term "Tgfβ" whenever exogenous Tgfβ was used (normally TGFβ1, unless otherwise indicated) or when we will refer to all 3 isoforms. For in vivo studies, the specific isoform used or manipulated is indicated.

Tgfβ has versatile functions both between and within different organ systems. This also holds true for the nervous system, in which overexpression of Tgfβ1 in mice results in hydrocephalus and gliogenesis (Wyss-Coray et al. 1995), treatment of dopaminergic neurons with Tgfβ causes either neurotoxic degeneration or neurotrophic effects (Krieglstein and Unsicker 1994; Krieglstein et al. 1995; Commissiong et al. 1997; Sanchez-Capelo et al. 1999; Farkas et al. 2003), and in which Tgfβ can induce ontogenetic cell death (Krieglstein et al. 2000). Thus, to unravel Tgfβ functions in the nervous system, it is necessary to analyze its actions with respect to different cell populations and even to different developmental stages. In this study, we aimed to understand the function of Tgfβ during forebrain development because little is known about the role of this multifunctional cytokine in this biological context.

Tgfβ and corresponding receptors are expressed in the forebrain: Tgfβ2 and Tgfβ3 are found in progenitors, differentiating neurons and radial glial cells, whereas Tgfβ1 is expressed mainly in mesenchymal cells of meninges and choroid plexus during development (Flanders et al. 1991; Miller 2003; Falk et al. 2008). Tgfβ upregulates cell cycle inhibitors, mainly p21, and counteracts cell cycle progression of cortical neuroepithelial cells of E10.5 mouse brains and of E17.5 rat cortical slice cultures (Seoane et al. 2004; Siegenthaler and Miller 2005). Inactivation of *Tgfβ receptor II* in the forebrain through *Emx1*-cre does not alter neuroepithelial proliferation at E12.5 and lacks a cortical phenotype at E18.5 (Falk et al. 2008). However, *Tgfβ1* inactivation leads to a compacted and thinner neocortex accompanied by increased apoptosis in neonatal and adult mice, indicating that Tgfβ1 functions in survival of central nervous system (CNS) neurons (Brionne et al. 2003).

Further insight into Tgfβ function in the CNS comes from studies of the midbrain, which is also influenced by Tgfβ. Here, dorsal progenitors are vulnerable to the loss of *Wnt1*-cre–induced conditional loss of *Tgfβ receptor II*, leading to increased cell proliferation and self-renewal of neuroepithelial cells in vivo and in vitro (Falk et al. 2008). In these animals, ventral midbrain progenitors are not affected, although ventral dopaminergic neurons and progenitors respond to Tgfβ signaling. In chicken, Tgfβ is involved in differentiation of a dopaminergic phenotype (Farkas et al. 2003), and Tgfβ neutralization results in selective loss of ventrally located dopaminergic neurons. Mouse E12.5 ventral midbrain–derived neurospheres express dopaminergic markers TH and Nurr1 upon exposure to Tgfβ. Furthermore, dopaminergic differentiation into a TH- and Nurr1-positive phenotype is also promoted when dorsal E12.5 midbrain is used for generating neurospheres (Roussa et al. 2006). In this context of dopaminergic neuron development, Tgfβ increases differentiation but does not influence proliferation of progenitors (Roussa et al. 2006).

Several data corroborate the finding that Tgfβ exerts antimitogenic functions on early neuronal progenitors through activation of p21, which interferes with G1 progression and entry into the S phase. Thus, although the idea that Tgfβ might influence the production of neurons or glial cells from forebrain progenitors seems likely, it has actually not been proven so far. And it is conceivable that inhibition of p21 is only the start, after which further cellular events are initiated and

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influenced through Tgfβ. To unravel Tgfβ functions during forebrain development, we studied the effect of this multifunctional cytokine on cortical and hippocampal cultures and on possible target genes. This study shows, for the first time, that Tgfβ not only mediates exit from the cell cycle but also promotes adoption of a neuronal cell fate in vitro and in vivo. This process is dependent upon Smad signaling and induction of integrin-signaling protein Nedd9/Hef1.

Materials and Methods

Primary Cultures of Mouse Embryonic Hippocampal and Cortical Cells

All experiments were performed according to national and international guidelines on the ethical use of animals.

Hippocampal and cortical cells were isolated from embryonic mice at E16.5 or 14.5, dissected in Hanks' Balanced Salt Solutions (Gibco, Karlsruhe, Germany) and dissociated by trypsinization in 0.25% Trypsin–ethylenediaminetetraacetic acid (Gibco) at 37 °C for 8 min followed by gentle trituration. Cells were plated on poly-L-ornithin (0.1 mg/mL, Sigma, München, Germany) and laminin (1 μg/mL, Sigma) coated dishes at a density of 150 000 cells/cm^2 and cultured in Neurobasal-medium (Gibco) supplemented with B27 (Gibco), apo-transferrin (5 μg/mL, Sigma), glutathione (1 μg/mL, Sigma), superoxide-dismutase (0.8 μg/mL, Sigma), and glutamine (0.5 mM, Gibco). After 4 days in vitro (DIV), half of the medium was changed every 3 days.

Immunocytochemistry

Tgfβ2/Tgfβ3 mutant mice were generated as described (Roussa et al. 2006). Cryosections were processed with microwaving for 7 min twice in 0.01 M citrate buffer and subsequent blockage of endogenous peroxidases for Diaminobenzidine (DAB)-based staining. Primary antibodies NeuN (mouse, 1:150, Millipore) and Ki67 (rabbit, 1:100, Epitomics) were applied overnight and detected with DAB-based staining (Ki67) or donkey antimouse Cy3 (NeuN). In the latter, red fluorescence image was converted to black and white using Photoshop. Only the red channel in the channels palette was activated and inverted under image-adjustments-invert.

For quantification of marker proteins, 0.8×10^5 (hippocampus) or 1×10^5 (cortex) cells were plated on poly-L-ornithin/laminin precoated 12-mm glass coverslips. For most experiments, TGFβ1 (5 ng/mL, Peprotech, London, United Kingdom) or ALK4,5,7-Inhibitor SB431542 (10 μM, Biozol, Eching, Germany) was added to the medium on DIV2 at a concentration of 5 ng/mL and replaced with each medium exchange. TGFβ2 and TGFβ3 (Peprotech) were used under the same conditions.

Before analysis, cells were fixed in 4% PFA for 10 min, blocked 30 min in 10% normal goat or donkey serum, 0.1% Triton X-100 in PBS, and incubated overnight at 4 °C with primary antibody in blocking solution, followed by the corresponding secondary antibody for 1 h at room temperature in PBS. Primary antibodies used were 5-Bromo-2-deoxyuridine (BrdU; sheep, 1:500, Abcam), Doublecortin (goat, 1:100, Santa Cruz Biotechnology), Pax6 (rabbit, 1:300, Covance), Nestin (rabbit, 1:500, Abcam), GFAP (mouse, 1:500, Sigma), HuC/D (mouse, 1:100, Invitrogen), Ki67 (rat, 1:25, Dako), HEF1 (NEDD9, mouse, 1:500, Abcam), PSA Ncam (mouse, 1:20, Developmental Studies Hybridoma Bank), and Smad 1,2,3 (mouse, 1:100, Santa Cruz Biotechnology). Secondary antibodies used were donkey antimouse, antirabbit, or antisheep Cy3 (1:800); goat antimouse, antirabbit FITC (1:100); and donkey antirat FITC (1:100) (all Jackson ImmunoResearch, West Grove, PA). Cell bodies were counterstained with DAPI (1:1000, Sigma), coverslips mounted with Fluoromount-G (Southern Biotechnology, Birmingham, AL) and visualized with an Axio Imager Z1 microscope (Zeiss, Jena, Germany). Quantification was performed by counting 10 random fields from 2 slides per experimental set. Data were collected from at least 3 independent experiments and are represented as mean ± standard error of mean (SEM). To verify whether differences between TGFβ1 treatment and untreated control reached the significance level $P < 0.05$, student's *t*-tests were used.

Smad Nuclear Translocation Assay

Hippocampal cells seeded on coverslips were preincubated with ALK4,5,7-Inhibitor SB431542 (10 μM) for 24 h to suppress endogenous TGFβ signaling. Then, cells were washed twice and stimulated with TGFβ1 (5 ng/mL) for 1 h. After fixation, cells were stained with mouse anti-Smad 1,2,3 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA).

TGFβ Secretion Assay

For detection of active and latent Tgfβ released in the culture medium by cortical and hippocampal cells, the mink lung epithelial cell (MLEC) luciferase bioassay was used as described (Abe et al. 1994; Krieglstein et al. 2000).

BrdU Labeling

Cells were plated on coverslips and stimulated with TGFβ1 until fixation at DIV8. Twenty-four hours prior to fixation, 20 nM BrdU (Sigma) was added to the medium. Fixed cells were incubated in 1 N HCl for 10 min on ice followed by an incubation for 20 min in 2 N HCl at 37 °C, neutralization in 0.1 M borate buffer and blocking in 5% normal donkey serum, 1% Triton X-100 in PBS, and incubation with sheep-anti-BrdU (1:500, Abcam, Cambridge, United Kingdom) overnight at 4 °C. The number of BrdU-positive cells and the total number of cells were counted. For determination of the quitting fraction (QF), cells were treated with TGFβ1 from DIV2 onward. BrdU was added for 24 h prior to fixation at DIV4. Cells were processed for immunocytochemistry and double labeled with anti-BrdU and anti-Ki67. All BrdU-positive cells either positive or negative for Ki67 were counted.

FACS Analysis of Apoptotic Cell Death

Hippocampal and cortical cells were treated with 5 ng/mL TGFβ1 starting at DIV2. At DIV4 or DIV8, cells were trypsinized, fixed with 70% ethanol in PBS, and stored at –20 °C for at least 2 h. Directly before analysis, DNA was stained with propidium iodide by resuspending the cells in fluorescence activated cell sorting (FACS) buffer containing 100 μg/mL propidium iodide, 10 μg/mL RNAse in PBS for 30 min at 37 °C. Data are expressed as percentage of cells present in the sub-G1 peak, representing apoptotic cells.

RNA Isolation and RT-PCR

For Tgfβ target gene expression analyses, cells were preincubated with ALK4,5,7-Inhibitor SB431542 (10 μM) for 24 h to decrease endogenous Tgfβ signaling, washed twice, and stimulated with 5 ng/mL TGFβ1 for 2 or 24 h. Expression of Tgfβ isoforms and receptors was assessed from untreated control cells.

Total RNA was isolated at DIV4 or DIV12 using the RNeasy Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA with RevertAid MMuLV reverse transcriptase (Fermentas) using oligo(dT) primers (Invitrogen). One microliter of the RT reaction mixture was subjected to PCR amplification.

Quantitative real-time RT-PCR analysis was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), using SYBR Green PCR Master Mix (Applied Biosystems) in a MicroAmp 96-well reaction plate following the manufacturer's protocols. Gapdh was used as a reference to obtain the relative fold change for target samples using the comparative CT method. Primer sequences are shown in supplemental data table S1.

Lentivirus-Delivered RNA Interference

All shRNA containing lentiviral transduction particles were purchased from Sigma (Mission shRNA). One or 2 constructs for each gene with more than 85% knockdown efficiency were used. For control infection, nontarget shRNA control was used. Cortical and hippocampal cells were infected with the viruses at DIV2 for 48 h and then subjected to selection by 0.8 μg/mL puromycin for 24 h prior to TGFβ1 treatment. Sequences used in the shRNA vectors are shown in supplemental data table S2.

Statistics

Data are presented as the mean ± SEM. Statistical analysis was performed using 2-tailed unpaired Student's *t*-test for in vitro data and 1-tailed unpaired Student's *t*-test for evaluation of in vivo results. Differences were considered statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

Primary Cortical and Hippocampal Cultures Secrete Tgfβ and Activate Tgfβ-Dependent Smad-Signaling Pathways

To determine whether Tgfβ signaling is necessary for proper development of neurons, we used cultures of primary cortical and hippocampal neurons isolated from E14.5 and E16.5 mouse embryonic brains. Under the applied cell culture conditions, primary neurons of both origins produced Tgfβ in its active or inactive, protein-bound latent form over several DIV (Fig. 1*A* and data not shown). In vivo studies show that different Tgfβ isoforms are active in specific parts of the brain during development, for example, Tgfβ1 is mainly produced by mesenchymal cells of meninges and choroid plexus, whereas Tgfβ2 and Tgfβ3 are produced in neural cells (Flanders et al. 1991; Pelton et al. 1991). In vitro, cortical and hippocampal cells produced all 3 Tgfβ isoforms, as well as their main receptors mediating signal transduction (Fig. 1*B*). Thus, primary neuronal cells in culture are endogenously exposed to Tgfβ indicating that this cytokine might play an important role in neuronal development and function. Furthermore, these data imply that loss-of-function studies focussing on Tgfβ ligands 1, 2, and 3 would require a knockdown of all 3 isoforms. To circumvent this difficulty, we decided to study Tgfβ effects primarily using exogenous supplementation.

To prove that supply of exogenous Tgfβ would result in the activation of downstream signaling, we analyzed Smad phosphorylation and translocation into the nucleus. Endogenous Tgfβ signaling also activated Smad signaling, and this background activity hampered direct investigation of Smad phosphorylation and translocation after exongenous supply of Tgfβ. We therefore exposed neuronal cultures for 24 h to the Alk4,5,7 inhibitor SB431542 to block endogenous Tgfβ signaling and subsequently replaced the inhibitor with 5 ng/mL TGFβ1. Using these conditions, we were able to observe increased Smad phosphorylation (data not shown) and translocation (Fig. 1*C*). Taken together, these data showed that primary neuronal cultures not only produced all 3 Tgfβ isoforms but also expressed the respective Tgfβ receptors and activated Smad signaling. Expression of all these different components of Tgfβ signal transduction machinery therefore raised the question whether Tgfβ would influence cellular processes such as proliferation, apoptosis, or differentiation.

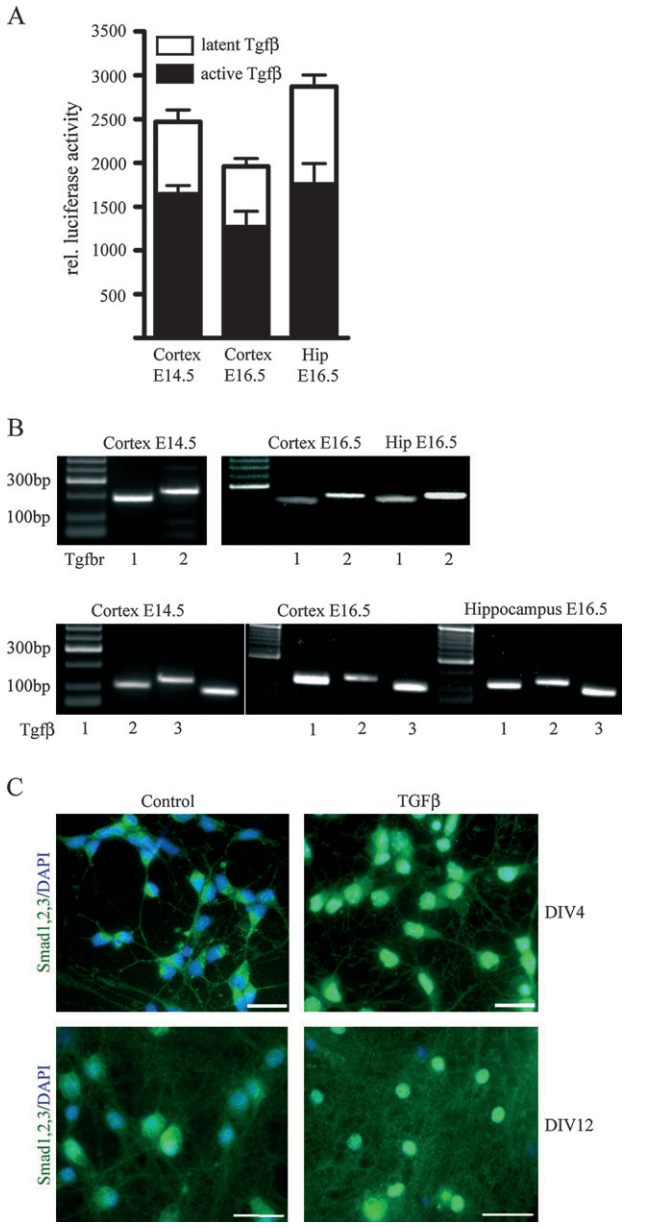


Figure 1. Primary cortical and hippocampal neurons isolated from E14.5 and E16.5 mouse embryonic brains express Tgfβs and their receptors and are responsive to TGFβ treatment. (*A*) MLEC/PAI–luciferase assay with conditioned medium from DIV4 cortical and hippocampal cultures. Data as mean ± SEM ($n = 3$). (*B*) RT-PCR reveals that cortical and hippocampal neurons express *Tgfβ receptors I* and *II*, as well as *Tgfβ* isoforms 1, 2, and 3. (*C*) Hippocampal neurons activate Smad proteins upon TGFβ stimulation. Smad1/2/3 immunoreactivity in control untreated cells was localized diffusely in the cytoplasm, whereas TGFβ treatment caused a translocation of Smad proteins in the nucleus. Upper panels: DIV4, lower panels: DIV12. Scale bars = 50 μm.

Tgfβ Reduces Progenitor Proliferation and Favors Differentiation into Neurons

Tgfβ signaling is context dependent and involved in diverse cellular processes affecting different cell populations. Such cellular processes include proliferation, apoptosis, and differentiation. We therefore analyzed the effect of Tgfβ on embryonal hippocampal and cortical cells with regard to these cellular processes.

Accordingly, quantitative analyses of the proliferative cell population in hippocampal cultures after 8 DIV and 6 days of Tgfβ treatment revealed about 12% less Ki67- or BrdU-positive progenitors compared with untreated cultures (Fig. 2*A*). About 7% less Ki67- or BrdU-positive cells were counted in cortical cultures after Tgfβ treatment compared with untreated control samples (Fig. 2*A*). This finding was corroborated by the observation that Tgfβ treatment for 48 h at DIV2 resulted in a smaller cycling fraction (CF = $(BrdU^{+};Ki67^{+})/(BrdU^{+})$) and a higher quitting fraction (QF = $(BrdU^{+};Ki67^{-})/(BrdU^{+})$) in hippocampal and cortical cells (Fig. 2*B*).

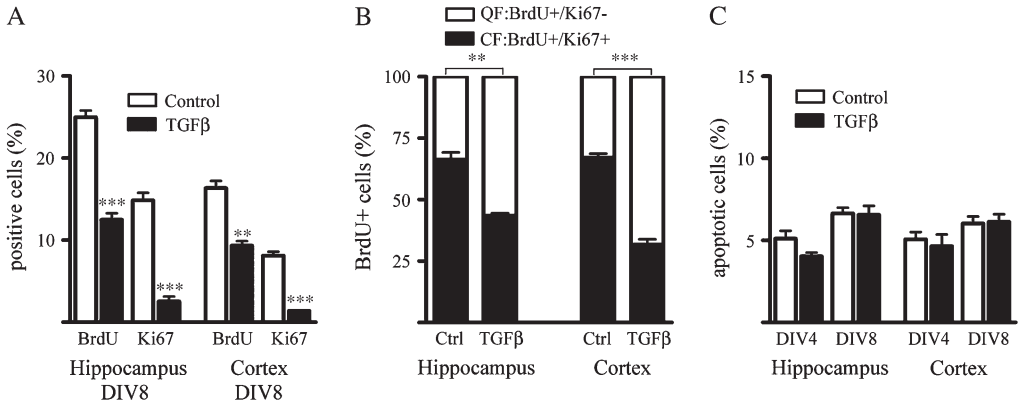


Figure 2. TGFβ promotes exit from the cell cycle. (*A*) Hippocampal and cortical cultures were treated for 6 days with TGFβ, labeled with BrdU (20 nM) in the last 24 h, and stained for BrdU and Ki67, detecting decreased numbers of proliferating progenitors ($n = 3$), *P* values: $^{***}P < 0.001$, $^{**}P < 0.01$, student's *t*-test. (*B*) Determination of cells leaving the cell cycle within the first 48 h of TGFβ exposure. Treatment started at DIV2; after 24 h, BrdU was added and after another 24 h, cells were fixed and costained for BrdU and Ki67. BrdU incorporation had not changed at this time point, but there was an increase in the number of cells quitting the cell cycle (QF) and a corresponding decrease of cells that reenter the cell cycle (CF) (all $n = 3$), *P* values: $^{***}P < 0.001$, $^{**}P < 0.01$, student's *t*-test. (*C*) PI-FACS analysis of apoptotic cells after short-time (DIV2–DIV4) and long-time (DIV2–DIV8) treatment with TGFβ ($n = 3$).

Because Tgfβ is also implicated in apoptotic processes, we used propidium iodide–based FACS analyses of Tgfβ-treated samples to test for increased apoptosis that might have led to a loss of progenitor cells. We did not observe enhanced apoptosis in hippocampal and cortical cultures that were exposed either short-term (DIV4) or longer-term (DIV8) to Tgfβ (Fig. 2*C*). To confirm this finding, we counted TUNEL-positive cells and confirmed that Tgfβ did not promote cell death under the applied conditions (data not shown).

Taken together, these observations showed for the first time that exposure to Tgfβ resulted in an antiproliferative response of progenitor cells in hippocampal cultures and corroborated earlier studies of cortical progenitors.

Because we also determined a higher fraction of cells that were exiting the cell cycle (Fig. 2*B*) after Tgfβ treatment, we next investigated whether exposure to Tgfβ would result in an increase of differentiated cells, for example, neurons or glia. We visualized neurons using HuC/D and NeuN, neural progenitors using Nestin and Pax6, and glial cells using GFAP (Fig. 3*A*). Quantitative analyses revealed that Tgfβ treated cultures contained 17% more neurons in cortical, and 20% more neurons in hippocampal cultures, but less neuronal progenitors, for example, 12% less Nestin- and 5% less Pax6-expressing cortical cells, and 9% less Nestin-positive cells in hippocampal cultures, respectively. The number of GFAP-positive glial cells was unaffected by Tgfβ (Fig. 3*B*,*C*).

These experiments were all performed with TGFβ1. In vivo, Tgfβ1 is mainly expressed in mesenchymal cells, and Tgfβ2 and Tgfβ 3 are active in CNS-derived cells, whereas all 3 isoforms are expressed in vitro (Fig. 1*B*). Thus, we also analyzed whether the observed effect mediated through TGFβ1 was also seen when using the other 2 isoforms. Indeed, we observed that TGFβ2 and TGFβ 3 were also capable of increasing the number of neurons (Fig. 3*D*).

To investigate whether the length of Tgfβ treatment would be of critical influence, we exposed cells for 24 h at DIV2 to Tgfβ and analyzed the number of neurons at DIV8. Distribution of HuC/D-positive neurons indicated that the 24 h Tgfβ pulse was sufficient to increase the number of neurons (Fig. 3*E*). Next, we analyzed whether the time point of starting Tgfβ treatment would be important by exposing cultures at DIV5 to Tgfβ for 3 days, and counting HuC/D-positive cells at DIV8. Under these conditions, we observed only a slight increase in neurons (7%) (Fig. 3*E*). We therefore hypothesized that Tgfβ is capable of initiating neuronal differentiation at DIV2 and DIV5 but that the differences observed were due to a shorter time period during which differentiation would have had time to progress (6 and 3 days, respectively). Indeed, Tgfβ treatment for 48 h at DIV2 and analysis at DIV4 did not result in more HuC/D-positive cells, reflecting a critical time period that is necessary for the differentiating neurons to express the respective markers (data not shown). Cultures treated at DIV5 for 24 h or 6 days, and analyzed for HuC/D at DIV11, contained again up to 20% more neurons than untreated cells (Fig. 3*E*). Thus, Tgfβ was capable of promoting a neuronal cell fate at DIV2 and DIV5 in similar ratios when cultures were analyzed after a 6-day differentiation interval. However, Tgfβ treatment produced fewer differentiated neurons in older cultures treated at DIV8 up to DIV14 (Fig. 3*E*). This might reflect that older cultures generally contained more neurons and fewer progenitors under the conditions in B27 medium, which supports differentiation.

In summary, neuronal progenitors are competent to generate neurons upon exposure to Tgfβ over the entire culture period, where upon a minimal time frame of 2–3 days is necessary to detect changes in the expression of differentiation markers.

To further investigate the association of this neurogenic effect with a functional Tgfβ signaling pathway, we treated cells with Tgfβ together with the Alk4,5,7-inhibitor SB431542, which blocks Tgfβ receptor I kinase activity, and observed a block in the increase in HuC/D positive cells (Fig. 3*F*). We next investigated whether the neurogenic effect of Tgfβ was dependent upon Smad signaling, using a lentivirus expressing a *Smad4* shRNA (validation shown in supplementary fig. 1) that was transduced in cortical and hippocampal cells at DIV2. We did not observe an increase in neurons after Tgfβ treatment when we interfered with Smad-pathway activity. Rather, we observed that neurons were generated in similar numbers as in transduced but non-Tgfβ exposed control cells (Fig. 3*G*,*H*), suggesting that Tgfβ activation of the Smad-signaling pathway is a central component of Tgfβ-mediated neuronal differentiation.

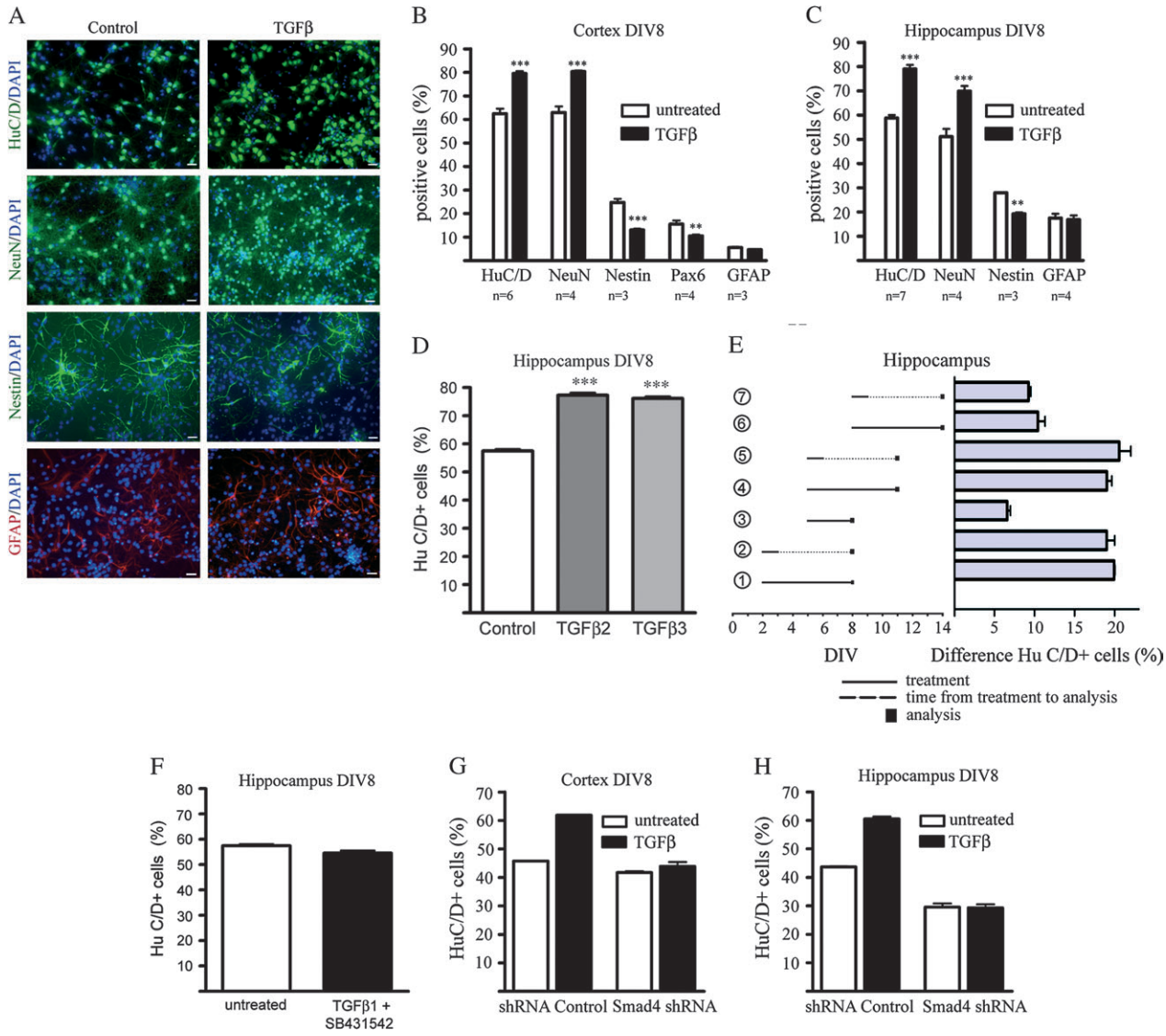


Figure 3. TGFβ promotes neuronal differentiation from hippocampal and cortical progenitors in a Smad-dependent way. (*A*) Immunohistochemical appearance of HuC/D and NeuN expressing neurons, Nestin-positive progenitors, and GFAP-positive glia cells in hippocampal cultures after 6 days of TGFβ treatment compared with untreated control cells. Scale bars = 50 μm. (*B*,*C*) Quantification of markers reveals an increase of neurons and a decrease in progenitors in TGFβ-treated cultures compared with untreated controls. The number of glia cells remains unchanged. Data are presented as a percentage of the total cell number. Statistical significance was determined by student's *t*-test with *P* values: $***P < 0.001$, $**P < 0.01$. (*D*) Quantification of HuC/D-positive neurons after 6-day stimulation of hippocampal cells with Tgfβ2 and Tgfβ3, respectively, at 8 DIV. (*E*) Influence of the duration of the TGFβ pulse and the age of the cultures on the TGFβ-induced neurogenetic effect in hippocampal cultures. A short TGFβ pulse of 24 h was sufficient to enhance neuronal differentiation (2,5,7) in the same way as a long pulse of 6 days over the entire cultivation period (1,4,6). TGFβ was also able to promote a neuronal cell fate in older cultures from DIV5 (4,5) at a similar rate as in cultures from DIV2 (1,2). In cultures where TGFβ treatment was started on DIV8 (6,7), there was less increase in the number of HuC/D neurons. Data are presented as percent difference of HuC/D-positive cells of the TGFβ-treated cultures based on the untreated controls and given as mean ± SEM ($n = 3$). (*F*) Quantification of HuC/D-positive cells under control conditions and after blocking Tgfβ signaling with Alk4,5,7 inhibitor SB431542. (*G*,*H*) TGFβ-mediated neuronal differentiation was abolished after knockdown of *Smad4* by lentiviral transduction of cortical (*G*) and hippocampal (*H*) cultures. HuC/D-positive neurons were counted after transduction with *Smad4* shRNA or nontarget shRNA control with and without TGFβ treatment ($n = 3$).

In summary, these data showed for the first time that Tgfβ not only had antiproliferative functions on hippocampal and cortical progenitors but also that Tgfβ treatment introduced a bias toward differentiation into neurons, which was dependent on a functional Tgfβ/Smad-signaling pathway.

Tgfβ2/Tgfβ3 Mutant Mice Display Fewer Neurons and Increased Progenitor Proliferation

To confirm our finding that Tgfβ signaling interferes with cell cycle progression and promotes neuronal differentiation in hippocampal and cortical primary cultures in vivo, we analyzed $Tgf\beta2^{-/-}/Tgf\beta3^{-/-}$ double mutant forebrains. Because these mutations are lethal by E15.5 (Dunker and Krieglstein 2002), we used E14.5 mutants for analyzing expression of Ki67 and NeuN. As shown in Figure 4, NeuN staining revealed that a loss of Tgfβ signaling resulted in fewer neurons in the cerebral cortex (Fig. 4*A*,*B*), as well as in the hippocampus (Fig. 4*C*,*D*). The quantification of these observations is shown in Figure 4*I*. Loss of Tgfβ2 and Tgfβ3 signaling resulted also in increased cell proliferation in the cerebral cortex (Fig. 4*F*) and in the hippocampus (Fig. 4*H*) compared with control littermates

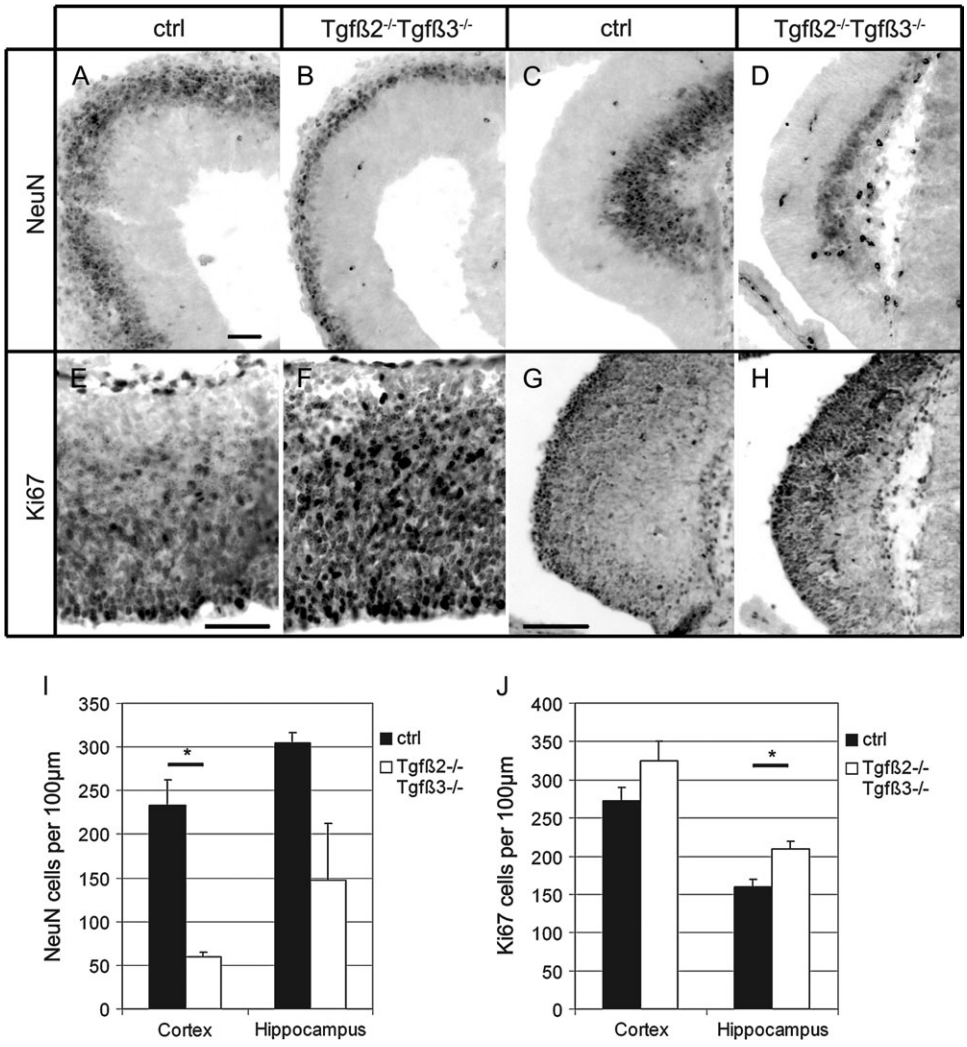


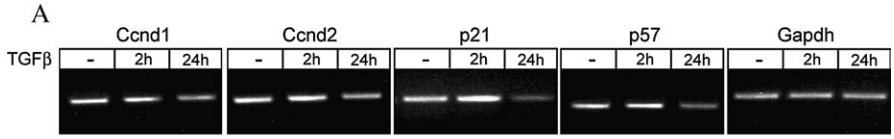
Figure 4. Increased proliferation and decreased neuronal differentiation in Tgfβ2/Tgfβ3 double mutant mice. (*A–D*) Expression of NeuN in control cortex (*A*) and hippocampus (*C*), and *Tgf*β*2/Tgf*β*3* double mutant cortex (*B*) and hippocampus (*D*) from E14.5 littermates. Scale bar = 50 µm. (*E–H*) Expression of Ki67 in control (*E*) and in *Tgf*β*2/Tgf*β*3* double mutant cerebral cortex (*F*), in control (*G*) and in *Tgf*β*2/Tgf*β*3* double mutant hippocampus (*H*). Scale bar *E,F* = 50 µm, *G,H* = 100 µm. (*I*, J) Quantification of NeuN (*I*) and Ki67 (*J*) positive cells in the cerebral cortex and hippocampus of age-matched control and *Tgf*β*2/Tgf*β*3* double mutants ($n = 2$, *P* value: * $P < 0.05$, one-tailed student's *t*-test).

(Fig. 4*E,G,J*). Taken together, these in vivo data clearly corroborated our findings in primary cell cultures of the hippocampus and cerebral cortex.

*Tgf*β*-Mediated Neuronal Differentiation is Associated with Cell Cycle Exit and Induction of Integrin-Associated Protein Nedd9*

Tgfβ controls cell cycle progression at the G1/S checkpoint (Urano et al. 1999; Hu and Zuckerman 2001) and it has been recognized that Tgfβ exerts antiproliferative effects during cortical development (Siegenthaler and Miller 2005). As expected, genes associated with cell cycle progression were also regulated in hippocampal cultures upon exposure to Tgfβ. Specifically, RT-PCR from cells that were exposed to Tgfβ for 2 h revealed that p21 and p57 expression was induced, whereas a 24-h Tgfβ treatment resulted in downregulation of p21, p57, Ccnd1, and Ccnd2 in E16.5 hippocampal cells cultured for 4DIV (Fig. 5*A*). The Cdk inhibitors p21 and p57, and the Cdk activators Ccnd1 and Ccnd2 converge in the regulation of cell progression through G1 phase and the ratio of p21 to CyclinD–Cdk complexes determines Cdk activity where high ratios of Cdk inhibitors to CyclinD–Cdk complexes suppress Cdk activation and interfere with G1 progression (Sherr 1995). Thus, induction of p21 and downregulation of Ccnd1 and Ccnd2 through Tgfβ likely prevented neuronal progenitors from progressing through G1 and entering the S phase.

However, induction of cell cycle inhibitors might be a prerequisite for entering differentiation but may not be sufficient to generate neurons. Because it is unknown whether inhibition of cell cycle progression is the only function of this multifunctional cytokine in this context, we examined whether the neurogenic capacity of Tgfβ is associated with the regulation of further target genes. Several published microarray data (Verrecchia et al. 2001; Zavadil et al. 2001; Ota et al. 2002; Kang et al. 2003; Yang et al. 2003; Zhao et al. 2004) identified Tgfβ-regulated genes in non-CNS-derived cells. We therefore based our analyses by screening these published data for genes that might also be relevant for developmental processes of the forebrain. Altogether, we selected 114 genes (supplementary



B

Gen	Fold change 2h TGFβ	24h TGFβ
Ctgf	3,58	1,93
Gata2	3,19	-7,16
Hes1	2,23	3,61
Id3	-2,03	-3,25
Itga3	1,15	-2,07
Itga5	2,49	-1,50
Nedd9	3,79	3,34
Smad7	3,77	2,62
Tle3	2,14	-1,22

C

Gen	Fold change Control-shRNA 2h TGFβ	24h TGFβ	Smad4 shRNA 2h TGFβ	24h TGFβ
Ctgf	9,82	12,36	1,40	2,10
Dvl1	1,10	-4,20	-1,40	1,18
Dvl3	-1,14	-2,55	1,05	1,31
Fstl3	3,72	1,42	1,31	1,53
Gata2	2,59	4,61	-1,06	-1,40
Itga3	-1,31	-2,07	1,46	1,60
Itga5	4,78	1,10	4,08	1,20
Nedd9	3,45	2,51	1,80	1,28
Notch2	3,34	2,31	-1,23	1,52
Runx1	10,26	8,45	17,38	9,80
Smad7	3,16	3,97	2,86	2,92
Timp3	1,57	2,72	1,09	1,50
TnC	1,17	2,27	1,11	1,27

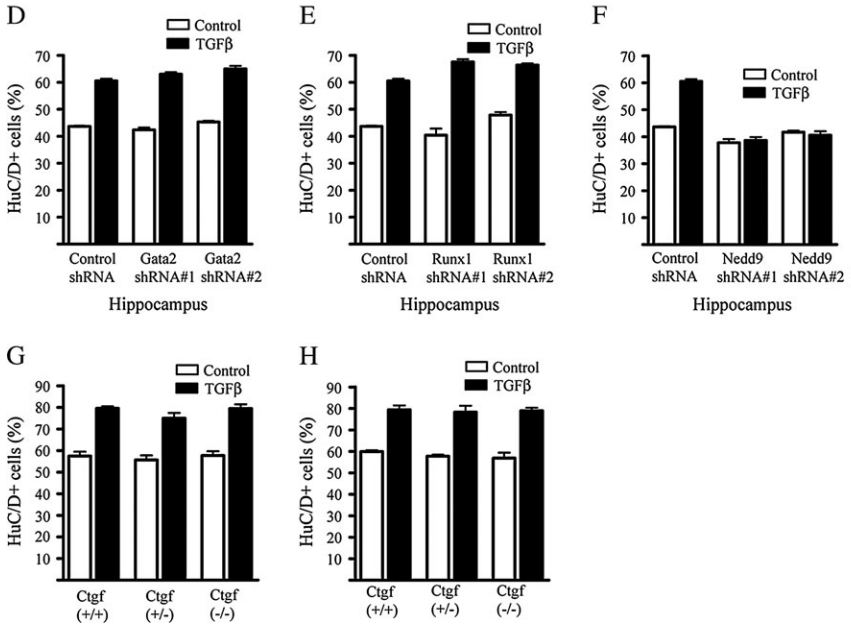


Figure 5. TGFβ-promoted neuronal differentiation depends on the expression of *Nedd9*. (*A*) Semiquantitative RT-PCR of cell cycle regulating genes after TGFβ stimulation of hippocampal cultures at 4DIV for 2 and 24 h. (*B*) Real-time RT-PCR of different Tgfβ-regulated genes using cDNA from hippocampal cultures at 4DIV. Given are fold changes after 2 and 24 h of Tgfβ treatment, respectively. (*C*) Real-time RT-PCR of different Tgfβ-regulated genes using cDNA from hippocampal cultures at 12DIV. Given are fold changes after 2 and 24 h of Tgfβ treatment, respectively. Cells were transduced with a nontarget shRNA control lentivirus or with a *Smad4* shRNA lentivirus to assess *Smad4*-dependent gene regulation. (*D–H*) shRNA-mediated knockdown of target genes showed that TGFβ-promoted neuronal differentiation was independent of Gata2 (*D*) and Runx1 (*E*). No increased neuronal differentiation was observed in cultures transduced with *Nedd9* shRNA lentivirus (*F*). Cultures of hippocampal or cortical cells from *Ctgf* mutant mice ($^{-/-}$) showed increased neuronal differentiation after Tgfβ treatment in similar ranges as control mice ($^{+/+}$ and $^{+/-}$) (*G*,*H*).

table 1) for investigating Tgfβ-dependent regulation in hippocampal and cortical cells. Our data showed that hippocampal and cortical cultures produce Tgfβ on their own and thus exhibit autocrine Tgfβ signaling. To circumvent this problem, we pretreated the cultures with the Alk4,5,7-inhibitor for 24 h to block endogenous Tgfβ signaling and then assessed changes in expression levels of putative target genes. This was done by releasing cells from this block and treating hippocampal cultures for 2 or 24 h with Tgfβ at DIV4 (Fig. 5*B*) and DIV12 (Fig. 5*C*). Using semiquantitative and quantitative RT PCR, we identified Tgfβ-regulated target genes associated with neuronal differentiation of CNS derived progenitors, including *Gata2, Runx1*, *Nedd9*, and *Ctgf*. These 4 genes were upregulated at least 3-fold in real-time PCR assays and/or were regulated in a Smad4-dependent manner (Fig. 5*C*). Therefore, we investigated whether interference with their expression would also interfere with Tgfβ-associated neuronal cell fate adoption. We transduced hippocampal cultures with lentiviruses containing shRNAs for *Gata2*, *Runx1*, and *Nedd9* (validation shown in supplementary fig. 2) and investigated hippocampal or cortical cells that were generated from *Ctgf* knockout mice (Ivkovic et al. 2003). As shown in Figure 5*D* and *E*, downregulation of *Gata2* and *Runx1* did not interfere with Tgfβ-mediated neuronal differentiation. In contrast, downregulation of *Nedd9*

prevented increased neuronal differentiation after exposure to Tgfβ (Fig. 5*F*), indicating that this focal adhesion protein, known to be involved in integrin signaling, is part of the molecular machinery that promotes the adoption of a neuronal cell fate. Loss of *Ctgf* did not affect the capacity of Tgfβ to generate more neurons in hippocampal or cortical cultures (Fig. 5*G*,*H*).

Tgfβ Treatment Alters the Morphology of Nestin-Positive Progenitors

In the course of our experiments, we observed that Tgfβ not only depleted the pool of neuronal progenitors, but immunocytochemical analyses also revealed that the morphology of this proliferative pool of cells changed as well (Fig. 6). Nestin is a marker of multipotential precursors, and Nestin-positive cells were found in clusters in our cultures. These clusters contained cells with a large round nucleus and a large cell body without processes, as well as several cells with a smaller nucleus and cell body with short processes. Treatment with Tgfβ not only reduced the overall number of Nestin clusters (data not shown), but it also increased the number of cells with small cell bodies and processes at the expense of the larger, rounder cells. In contrast, treatment with the Tgfβ-inhibitor SB431542 favored the larger, rounder phenotype, and small cells with processes were rarely found (Fig. 6). Costaining with Ki67 revealed that although Tgfβ induced striking morphological changes of Nestin-positive progenitors, this subpopulation of cells still proliferated (Fig. 6*A*). This finding suggested that

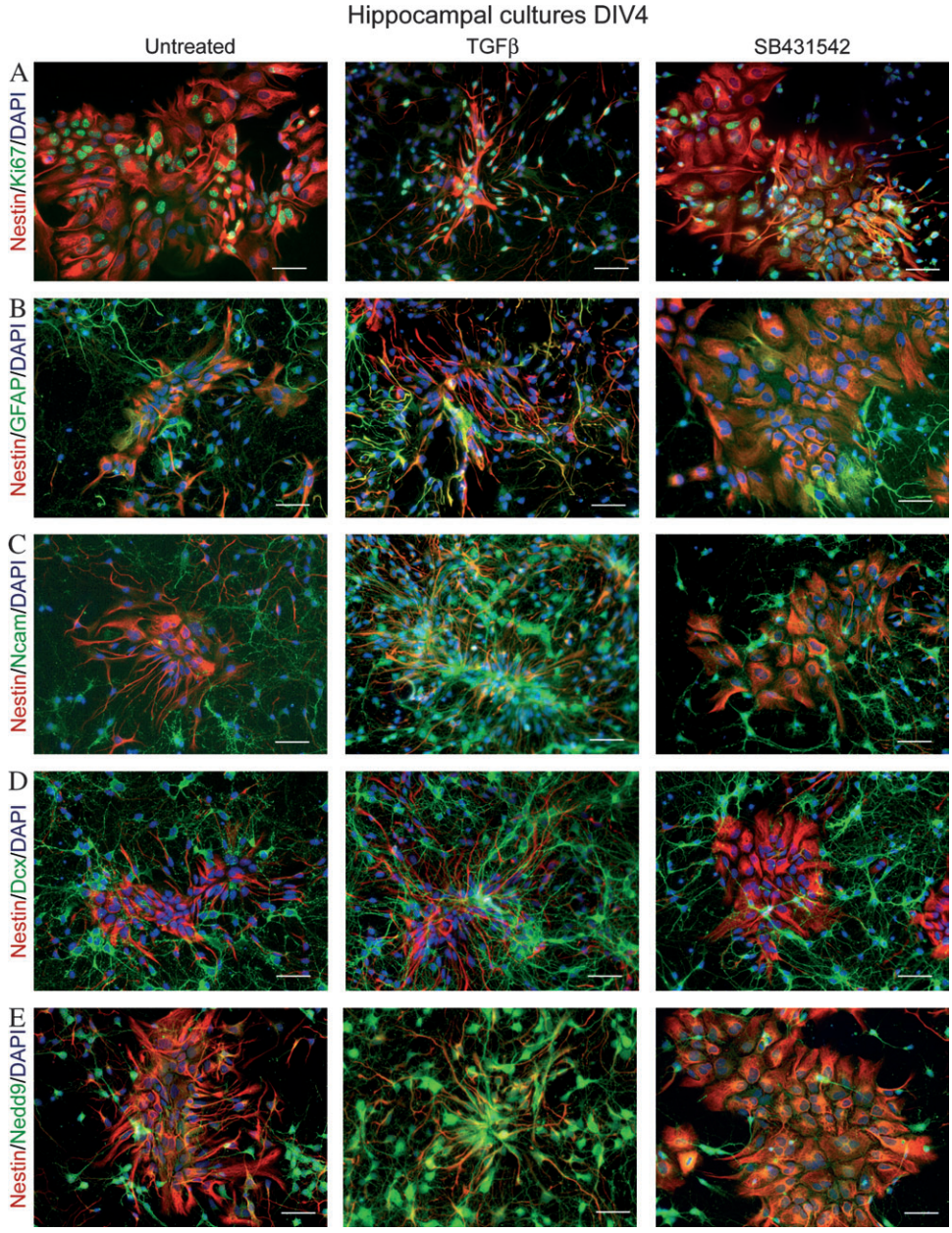


Figure 6. Morphological changes in Nestin-positive progenitors upon TGFβ treatment. (*A*–*E*) Preparations of hippocampal cells contain clusters of Nestin-positive progenitors that have a different morphology in untreated control cultures and also in cultures treated with Alk4,5,7-inhibitor SB431542 compared with TGFβ-treated samples. Immunohistochemical characterization with Nestin and (*A*) Ki67, (*B*) GFAP, (*C*) PSA-NCam, (*D*) Dcx, and (*E*) Nedd9. Scale bars = 50 μm.

Tgfβ induced a different progenitor status of Nestin-positive cells. The transition might reflect lineage commitment of naive progenitors that downregulate Nestin and start to express markers of either the glial or neuronal cell lineages. To characterize this morphologically distinct cell population, we used the marker GFAP for glial cells, PSA-NCam for immature neural precursor cells, and Dcx for precursors of the neuronal lineage (Fig. 6*B*,*C*). A minor fraction of cells displayed colocalized expression of GFAP and Nestin (Fig. 6*B*), observable in Tgfβ-treated and control cells, encompassing untreated as well as Tgfβ-inhibitor (SB431542) treated cells. PSA-NCam did not colocalize with Nestin in control cells, but Tgfβ-treated cells showed partial coexpression (Fig. 6*C*). No colocalization with Nestin was found in Dcx-expressing cells under control and Tgfβ-induced conditions (Fig. 6*D*). These findings suggested that the Tgfβ-induced morphological change did not correlate with a lineage commitment, but that these cells retain their uncommitted precursor status.

We identified Nedd9 as an essential downstream component of Tgfβ-mediated neuronal differentiation. Additionally, Nedd9 has been described as a molecule involved in changes of cell morphology (Singh et al. 2007), and that is expressed in multipotent Nestin-positive cells in vivo (Aquino et al. 2008). We therefore investigated whether the observed Tgfβ-dependent morphological changes correlated with the expression of Nedd9 in Nestin-positive progenitors. As shown in Figure 6*E*, most of the Nestin-positive cells did not express Nedd9 or expressed Nedd9 only at a low level under both control conditions. In Tgfβ-treated samples, Nestin-positive cells displayed a higher degree of colocalization with Nedd9, and nearly all cell bodies of progenitors with Nestin-positive processes stained positive for Nedd9. Thus, Nedd9 is expressed in a subset of Nestin-positive cells albeit at a low level, and exposure to Tgfβ induced a higher proportion of Nedd9–Nestin double-positive progenitors. However, although these findings render Nedd9 as a candidate target that might be involved in the morphological changes of progenitor cells induced by Tgfβ, additional studies with *Nedd9* mutant mice will be necessary to prove this hypothesis.

Discussion

In this study, we have shown that forebrain-derived embryonic progenitor cells are responsive to Tgfβ, which introduces a shift in differentiation into the neuronal but not into the glial cell lineage. Thus, Tgfβ not only promotes cell cycle exit but also activates pathways leading to neuronal differentiation, a process that depends upon the activation of Smad signaling and the induction of the focal adhesion protein Nedd9.

Our study was focussed on cortical and hippocampal cells that were cultured in vitro and our findings extend earlier reports that Tgfβ influences the developing cerebral cortex. Using slice cultures of E17.5 rat cerebral cortices, Siegenthaler and Miller (2005) showed that Tgfβ1 promotes cell cycle exit through induction of p21. We now provide evidence that Tgfβ also influences hippocampal cells and not only induces exit from the cell cycle, but that Tgfβ also leads to a decrease of progenitors and increasing numbers of neurons in the developing hippocampus as well as in the cerebral cortex.

The observed neurogenic potential of Tgfβ signaling was confirmed in vivo using $Tgf\beta2^{-/-}/Tgf\beta3^{-/-}$ double mutants. This is the first Tgfβ-related mouse model in which enhanced cell proliferation and impaired neuronal differentiation has been shown in the forebrain.

Tgfβ1 knockout mice, which survive to early adulthood, have a thinner cerebral cortex. This mutant displayed higher rates of apoptosis in cortical $Tgf\beta1^{-/-}$ neurons, which was considered to be the main cause for loss of neurons (Brionne et al. 2003). Our data now provide evidence that defects in neuronal differentiation during development might also have contributed to the observed loss of neurons. Interestingly, survival of neurons in our cultures also depended upon Tgfβ signal transduction, because we observed higher rates of apoptosis when we blocked the TgfβRI with the inhibitor SB431542 over several days (data not shown).

Other published mouse mutants in which Tgfβ signaling is hampered do not develop an observable phenotype in the forebrain, as is the case for many TgfβRII conditional mutants (our own unpublished data, and Falk et al. 2008). In the latter, one reason for a lack of an observable phenotype might be that restricting the knockout to a subset of cells using cre-expressing mice may not have the same effect as a complete knockout of the 2 major Tgfβ ligands that are expressed in neurons and progenitors (Flanders et al. 1991). Another possibility might be that loss of Tgfβ signaling is accompanied with an increase of progenitor proliferation. Because neuronal differentiation is not solely dependent on Tgfβ signaling, it might be sufficient if more precursors are generated, which subsequently differentiate. This higher number of differentiating precursors might compensate for the loss of the Tgfβ-mediated differentiation signal during the entire course of development.

Further support for our finding that Tgfβ signaling is involved in neuronal differentiation comes from studies on the development of dopaminergic neurons. Here, midbrain-derived E12.5 progenitors differentiated into dopaminergic neurons upon exposure to Tgfβ in vitro, and this developmental process was as significantly impaired in E14.5 *Tgfβ2/Tgfβ3* double mutants (Roussa et al. 2006).

That Tgfβ influences progenitors not only through cell cycle exit is corroborated by our observation of morphological changes of Nestin-positive progenitors. These multipotent cells nevertheless continue proliferating and do not show any signs of early lineage restriction after Tgfβ treatment. Furthermore, apart from cell cycle control genes, we identified other components that are regulated through Tgfβ signaling in hippocampal and cortical cultures, 4 of which were tested for their implication in Tgfβ-mediated neuronal differentiation. Although Ctgf, Runx1, and Gata2 are not involved in this process, siRNA-mediated downregulation of Nedd9 abolished the neurogenic potential of the Tgfβ signaling pathway.

Nedd9 (Neural precursor cell expressed, developmentally downregulated 9), which was initially identified in embryonic brains, is downregulated during developmental progression (Kumar et al. 1992) and enriched in neural progenitor cells (Abramova et al. 2005). Further studies identified Nedd9 in Nestin- and Sox2-positive progenitors in the neuroepithelium, and its downregulation is linked to neuronal lineage commitment (Aquino et al. 2008). Furthermore, Nedd9 variation is associated with susceptibility of late-onset Alzheimer's disease and Parkinson's disease (Chapuis et al. 2008; Li et al. 2008).

Nedd9, also known as Hef1 or Cas-L, is a scaffolding protein that is implicated in β1-integrin-signal transduction because it is localized at focal adhesions and associates with focal adhesion kinase upon β1-integrin ligation. Like Tgfβ, it is

implicated in diverse biological processes including cell attachment, migration, and invasion, as well as apoptosis and cell cycle regulation (reviewed in Singh et al. 2007). Little is known about Nedd9 function in the nervous system. Our study shows for the first time that Nedd9 is implicated in Tgfβ-mediated differentiation into the neuronal lineage. Taken together with its expression pattern, our data suggest that Nedd9 might promote a progenitor status that renders the cells competent to differentiation into neurons.

Several studies describe an association of Tgfβ-signaling and Nedd9 activity, although not in the nervous system. In human dermal fibroblasts, TGFβ1 upregulates Nedd9 mRNA and protein levels but does not change posttranslational modifications, for example, phosphorylation of Nedd9 (Zheng and McKeown-Longo 2002). Furthermore, Nedd9 potentiates Tgfβ signaling by relieving negative feedback by the inhibitory Smads 6 and 7 (Inamoto et al. 2007). Smad3 also interacts with Nedd9, which can lead to proteasomal degradation of the latter after Tgfβ exposure and therefore facilitates Smad3-mediated nuclear responses. However, sustained Tgfβ signaling leads to increased levels of Nedd9, which binds Smad3 and negatively regulates Smad3-dependent Tgfβ signaling (Liu et al. 2000; Nourry et al. 2004). Such pathways might promote differentiation of specific progenitors as has been shown during spinal cord development (Garcia-Campmany and Marti 2007) and might also take place during forebrain development.

Taken together, our data provide new findings on the role of Tgfβ on hippocampal as well as cortical cells and highlight a role for Nedd9 in neuronal differentiation. These data will be the basis for extended studies to enlighten the function of Nedd9 in different CNS-derived progenitor populations and with respect to different developmental time points.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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