THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 41, pp. 27927–27936, October 10, 2008

β 1 Integrin-mediated Effects of Tenascin-R Domains EGFL and FN6–8 on Neural Stem/Progenitor Cell Proliferation and Differentiation *in Vitro*[*] [S]

Received for publication, June 23, 2008 Published, JBC Papers in Press, July 24, 2008, DOI 10.1074/jbc.M804764200

Hong Liao[‡1,2], Wenhui Huang[‡§1], Melitta Schachner[§¶], Yue Guan[‡], Jingjing Guo[‡], Jun Yan[‡], Jing Qin[‡], Xianshu Bai[‡], and Luyong Zhang[‖3]

From the [‡]Neurobiology Laboratory, New Drug Screening Centre and the [‖]Jiangsu Center for Pharmacodynamic Research and Evaluation, China Pharmaceutical University, Nanjing 210038, China, the [§]Zentrum für Molekulare Neurobiologie, University of Hamburg, D-20251 Hamburg, Germany, and the [¶]Keck Centre for Collaborative Neuroscience and Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, New Jersey 08854

Neural stem/progenitor cells (NSCs) have the capacity for self-renewal and differentiation into major classes of central nervous system cell types, such as neurons, astrocytes, and oligodendrocytes. The determination of fate of NSCs appears to be regulated by both intrinsic and extrinsic factors. Mounting evidence has shown that extracellular matrix molecules contribute to NSC proliferation and differentiation as extrinsic factors. Here we explore the effects of the epidermal growth factor-like (EGFL) and fibronectin type III homologous domains 6–8 (FN6–8) of the extracellular matrix molecule tenascin-R on NSC proliferation and differentiation. Our results show that domain FN6–8 inhibited NSC proliferation and promoted NSCs differentiation into astrocytes and less into oligodendrocytes or neurons. The EGFL domain did not affect NSC proliferation, but promoted NSC differentiation into neurons and reduced NSC differentiation into astrocytes and oligodendrocytes. Treatment of NSCs with β 1 integrin function-blocking antibody resulted in attenuation of inhibition of the effect of FN6–8 on NSC proliferation. The influence of EGFL or FN6–8 on NSCs differentiation was inhibited by β 1 integrin antibody application, implicating β 1 integrin in proliferation and differentiation induced by EGFL and FN6–8 mediated triggering of NSCs.

Extracelluar matrix (ECM)[4] molecules in the central nervous system, are secreted from both neurons and glial cells and accumulate in the extracellular space (1). ECM constituents play important roles in synaptic plasticity, formation of developmental compartments, and control of cell adhesion, migration, and differentiation (2–4). Tenascin-R (TN-R) is an extracellular matrix glycoprotein, mainly expressed in the central nervous system and predominantly by differentiating oligodendrocytes as well as some interneurons in the spinal cord, retina, cerebellum, and hippocampus (5–7). TN-R contains a cysteine-rich amino-terminal region, epidermal growth factor-like repeats (EGFL), a region consisting of fibronectin type III (FN) homologous repeats, and a fibrinogen-like domain at the carboxyl terminus (Fig. 1) (8, 9). TN-R is a multifunctional molecule with multiple domains that confer different effects on neuronal cell functions, such as neuronal cell adhesion, neurite outgrowth, modulation of sodium channels, and synaptic plasticity. The EGFL domain is anti-adhesive for microglia and hippocampal neurons, whereas the FN6–8 domain promotes adhesion of microglia or hippocampal neurons (10–14). *In vivo* experiments have shown that in TN-R-deficient mice functional recovery after spinal cord injury was better than in wild type control littermates, and cortical and hippocampal neuronal excitability were enhanced (13, 15, 16). Our previous work has shown that TN-R plays a role in neuroprotection via domains EGFL and FN6–8 (14). We have further shown that TN-R is responsible for radial migration of NSCs of the rostral migratory stream by attracting NSCs to the olfactory bulb or, when TN-R secreting fibroblasts are transplanted into the striatum, NSCs are attracted to the source of TN-R expression and deviate from tangential migration by attraction to the TN-R source.

The role of TN-R in NSC proliferation and differentiation has, however, not been explored. NSCs are mainly found in the subgranular layer of the dentate gyrus of the hippocampus and in the subventricular zone (SVZ) of the lateral ventricles in the adult mammalian central nervous system, where they have the capacity for self-renewal and differentiation into major classes

* This work was supported by grants from the National Natural Science Foundation of China (30670793), Natural Science Foundation of Jiang Su Province (BK2006150), International Coorperation Projects of Jiang Su Province (BZ2006050), Pharmacodynamics Research and Evaluation Fund of Jiang Su Province, and Initial Fund of China Pharmaceutical University (to H. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

[1] Both authors contributed equally to this work.

[2] To whom correspondence may be addressed: 1 Shen Nong Rd., Nanjing 210038, China. Tel.: 86-25-85391036; Fax: 86-25-85303260; E-mail: liaohong56@hotmail.com.

[3] To whom correspondence may be addressed: 1 Shen Nong Rd., Nanjing 210038, China. Tel.: 86-25-83271500; Fax: 86-25-85303260; E-mail: lyzhang@cpu.edu.cn.

[4] The abbreviations used are: ECM, extracellular matrix; bFGF, basic fibroblast growth factor; BrdUrd, 5-bromodeoxyuridine; EGF, epidermal growth factor; EGFL, epidermal growth factor-like repeats containing the NH_2-terminal domain of TN-R; FCS, fetal calf serum; FN6–8, fibronectin-type III homologous repeats 6–8; GFAP, glial fibrillary acidic protein; GST, glutathione *S*-transferase; NSCs, neural stem/progenitor cells; PBS, phosphate-buffered saline; SVZ, subventricular zone; TN-R, tenascin-R; TN-C, tenascin-C; DMEM, Dulbecco's modified Eagle's medium.

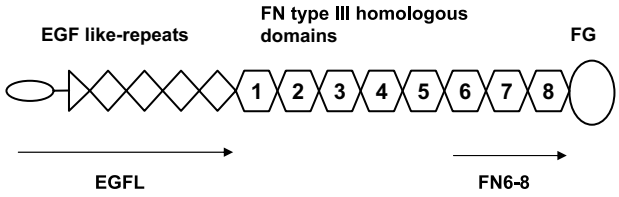

FIGURE 1. **Domain structure of TN-R.** Shown from the amino-terminal end (*small oval*). The epidermal growth factor (*EGF*)-like repeats (*rhombuses*), fibronectin (*FN*) homologous domains (*hexagons*), and the fibrinogen (*FG*)-like knob (*large oval*). *Double arrows* represent the recombinant proteins produced in *Escherichia coli* as GST fusion proteins.

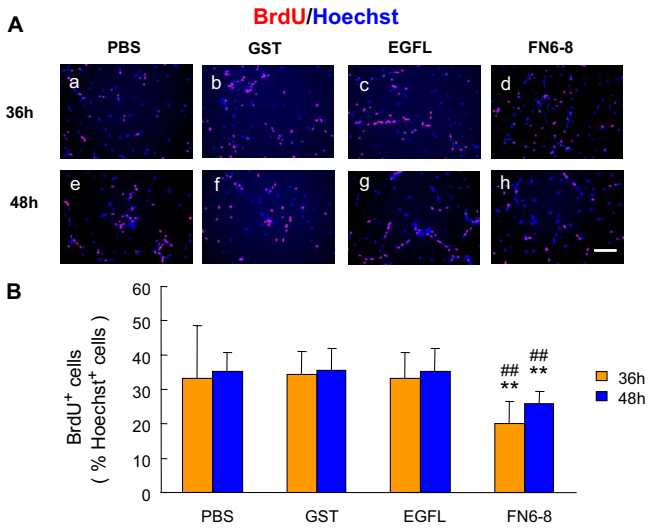

FIGURE 2. **TN-R domain FN6–8 reduces proliferation of NSCs.** Single cell suspensions of NSCs were seeded onto coverslips pre-coated with poly-L-lysine and maintained in DMEM/F-12 medium supplemented with B27, bFGF, and EGF for 36 and 48 h. BrdUrd was added to the culture medium 24 h before fixation of cells and immunostaining for BrdUrd (*red*) and nuclei (Hoechst, *blue*). *A*, the proliferation pattern of NSCs was measured in the presence of PBS as vehicle control (*a* and *e*), GST (*b* and *f*), EGFL (*c* and *g*), and FN6–8 (*d* and *h*). Fluorescence overlay micrographs are shown. *Scale bar* (in *h*) = 25 μm. *B*, percentages of BrdUrd$^+$ cells within the population of Hoechst$^+$ cells are shown as mean ± S.D. **, $p < 0.01$ *versus* PBS group; ##, $p < 0.01$ *versus* GST group.

of central nervous system cell types, such as neurons, astrocytes, and oligodendrocytes (17–19). The most active neurogenic region and the richest source of NSCs is the SVZ of the forebrain, and from the SVZ, newly generated NSCs migrate long distances to reach their final position in the olfactory bulb according to a well defined path called the rostral migratory stream. Cells then shift their migration pattern from the tangential to the radial orientation and finally differentiate into neurons (18, 20–22). Much evidence has demonstrated the existence of a "niche," a specialized microenvironment where stem cells are located *in vivo* and that contributes to NSC migration, proliferation, and differentiation as an extrinsic factor. Niches are composed of cells that affect NSC behavior by producing soluble factors (growth factors, chemokines, and neurotrophins), membrane-bound molecules, and ECM molecules. The developmental program of NSCs is regulated by these extrinsic factors (23, 24). ECM molecules in the SVZ are likely to control, at least in part, NSC behavior by instructive cues. For instance, TN-R mediates activity-dependent recruitment of neuroblasts in the adult mouse forebrain (25). In tenascin-C (TN-C)-deficient mice the rate of oligodendrocyte precursor cell migration increased and the rate of cell proliferation decreased (25, 26). TN-C contributes to the generation of a stem cell niche within the SVZ, acting to orchestrate growth factor signaling so as to accelerate neural stem cell development (27). However, the functions of distinct domains of TN-C or TN-R acting as extrinsic factors on NSC behavior have remained unclear. In particular, the molecular mechanisms of distinct TN-R domains have not been investigated with regard to their migration and differentiation into different neural cell types, such as neurons, astrocytes, and oligodendrocytes. Thus, we investigated the consequences of proliferation and differentiation of NSCs exposed to TN-R. For this study, we chose to investigate the EGFL and FN6–8 domains, because these domains have emerged as the most effective in modulation of functional properties of target cell types, such as neurons, in their capacity to generate neurites on a uniform substrate or being repelled at a boundary (11, 12, 28–30). Furthermore, the EGFL domain is anti-adhesive for microglia, whereas the FN6–8 domain promotes adhesion of microglia, whereas domains FN1–2 and FN3–5 do not affect microglial adhesion (23). Our results show that both domains modulate NSC proliferation and differentiation via the cell surface receptor β 1 integrin.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant EGFL and FN6–8 Domains—Generation and purification of the recombinant EGFL and FN6–8 domains of mouse TN-R as fusion proteins with GST were performed as described (11), and the plasmids of pGEX-EGFL and pGEX-FN6–8 were a kind gift of Dr. Zhi-Cheng Xiao (Singapore General Hospital, Singapore). The fusion proteins were analyzed for purity by SDS-PAGE (31) and shown to have a purity of at least 90%.

Neural Stem Cell Culture—Sprague-Dawley rat embryos (E13–14 days) were stripped of meninges, and coronal sections (2 mm thick) of tissue containing the SVZ of the lateral ventricles were removed under a dissection microscope and mechanically dissociated into single cells. Cells were seeded into a 6-well plate (Costar), and maintained in DMEM/F-12 medium containing B27, bFGF (20 ng/ml), EGF (20 ng/ml) (all from Invitrogen), at 37 °C in an incubator with 5% CO_2. Neurospheres formed within 3–5 days were dissociated mechanically into single cells and seeded into a new 6-well plate. Neurospheres from 3 to 5 passages were used for all experiments. Cells in the neurospheres were all positive for nestin, a marker for NSCs.

Proliferation of Neural Stem Cells—Dissociated NSCs from neurospheres were cultured in DMEM/F-12 medium supplemented with B27, bFGF (20 ng/ml), and EGF (20 ng/ml) in the presence of GST fusion proteins (GST, EGFL, and FN6–8, 100 μg/ml, respectively) or seeded onto glass coverslips precoated with 0.1% poly-L-lysine and GST fusion proteins (GST, EGFL, and FN6–8, 100 μg/ml, respectively). After culturing for 12 and 24 h, BrdUrd (5-bromodeoxyuridine, 10 μg/ml) was added into the culture medium and cells were continuously maintained at 37 °C in a CO_2 incubator up to 36 and 48 h, respectively. Then, cell proliferation on coverslips was analyzed for BrdUrd incorporation by immunocytochemistry. For the β 1 integrin blocking assay, after

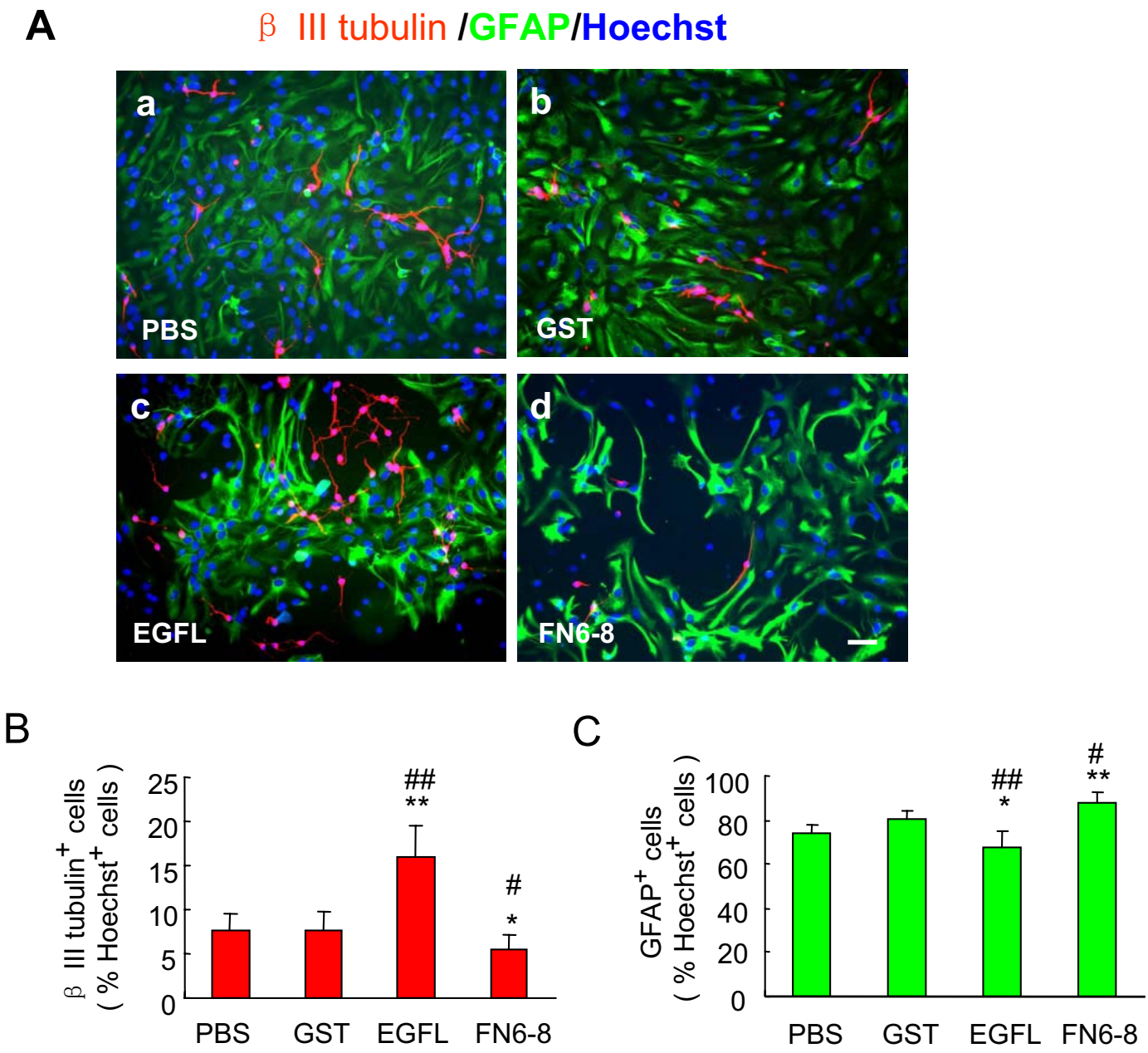

FIGURE 3. **TN-R domains EGFL and FN6–8 affect differentiation of NSCs into neurons.** Single cell suspensions of NSCs were seeded onto coverslips pre-coated by poly-L-lysine and maintained in DMEM/F-12 culture medium supplemented with B27 and 1% FCS. After 7 days cells were immunostained for the neuronal marker β-tubulin III, for the astrocytic marker GFAP, and for cell nuclei marker Hoechst. *A*, differentiation pattern of NSCs maintained in the presence of the PBS control (*a*), GST (*b*), EGFL (*c*), or FN6–8 (*d*). In the fluorescence overlay micrographs β-tubulin III$^+$ cells (*red*), GFAP$^+$ cells (*green*), and Hoechst$^+$ cells (*blue*) are shown. *Scale bar* (in *d*) = 25 μm. *B*, percentages of β-tubulin III$^+$ cells, and *C*, of GFAP$^+$ cells within all Hoechst$^+$ cells are shown. Values are shown as mean ± S.D. *, $p < 0.05$; **, $p < 0.01$ *versus* PBS group; #, $p < 0.05$; ##, $p < 0.01$ *versus* GST group.

pre-blocking of cells with 1% bovine serum albumin for 1 h, NSCs seeded onto coverslips were incubated with a function-blocking β 1 integrin antibody (50 μg/ml, Pharmingen) or rabbit IgG (50 μg/ml, Pierce) for 1 h at 37 °C before adding GST fusion proteins into the culture medium. Three independent experiments were performed.

Differentiation of Neural Stem Cells—Neurospheres were dissociated mechanically into single cells and maintained in the DMEM/F-12 medium with B27 and 1% fetal calf serum (FCS) at 37 °C in the CO_2 incubator for 7 days in the presence of GST fusion proteins (GST, EGFL, and FN6–8, 100 μg/ml, respectively), or GST fusion proteins (GST, EGFL, and FN6–8, 100 μg/ml, respectively) were precoated onto glass coverslips. Then, cells were identified for marker protein expression to identify neurons, astrocytes, and oligodendrocytes by immunocytochemistry. For the β 1 integrin blocking assay, after pre-blocking of cells with 1% bovine serum albumin for 1 h, NSCs seeded onto coverslips incubated with a function-blocking β 1 integrin antibody (50 μg/ml, Pharmingen) or rabbit IgG (50 μg/ml, Pierce) for 2 h at 37 °C before adding GST fusion proteins into the culture medium. Three independent experiments were carried out.

Immunocytochemistry—Cells on coverslips were washed with PBS, fixed with 4% paraformaldehyde, blocked with 10%

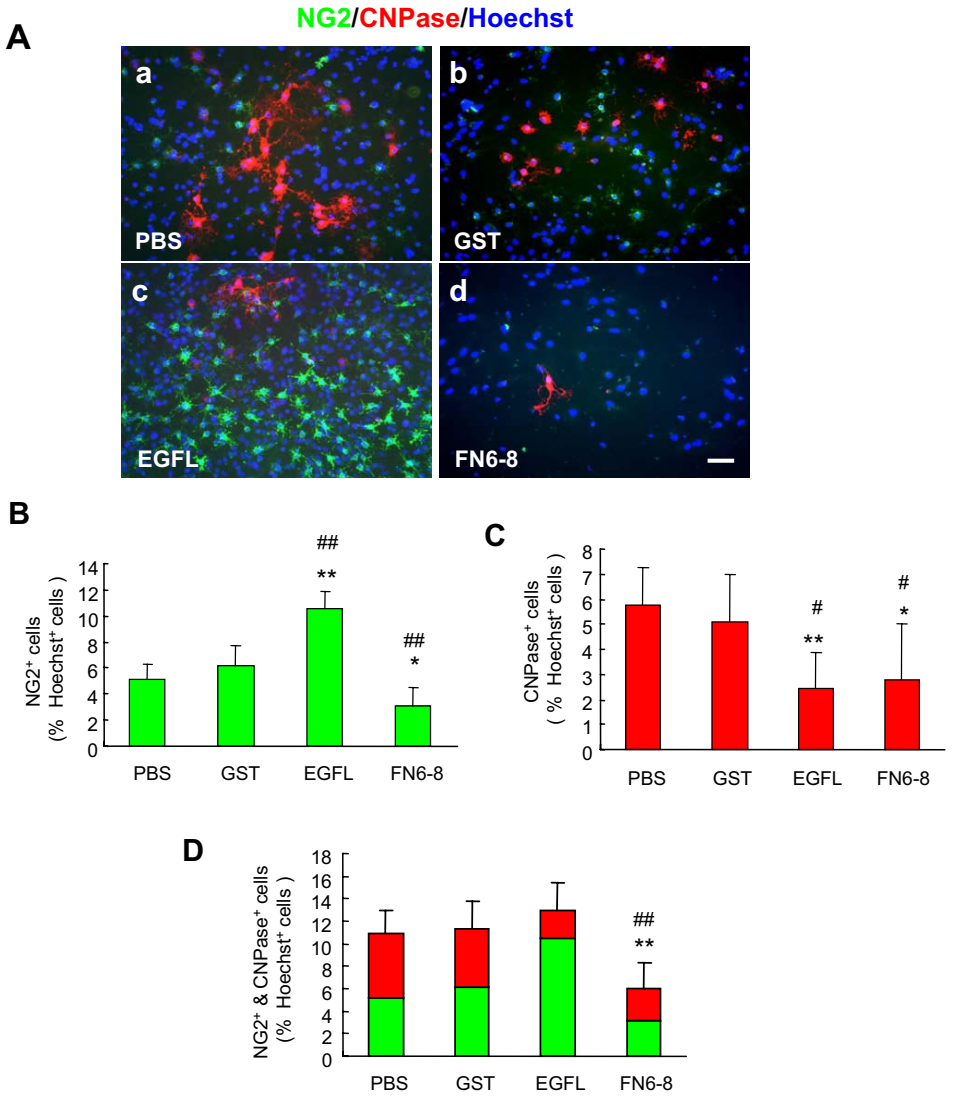

FIGURE 4. **TN-R domains EGFL and FN6–8 affect differentiation of NSCs into oligodendrocytes.** Single cell suspensions of NSCs were seeded into coverslips pre-coated with poly-L-lysine and maintained for 7 days in DMEM/F-12 medium supplemented with B27 and 1% FCS. *A*, fluorescence overlay micrographs showed differentiation of NSCs into oligodendrocytes in the presence of the PBS control (*a*), GST (*b*), EGFL (*c*), or FN6–8 (*d*). Immature oligodendrocytes are identified with antibodies to NG2 (*green*) and more mature oligodendrocytes are identified with antibodies to CNPase (*red*). Cells were counterstained with Hoechst (*blue*). Fluorescence overlay micrographs are shown. *Scale bar* (in *d*) = 25 μm. Percentages of NG2$^+$ cells (*B*), CNPase$^+$ cells (*C*), and NG2$^+$ and CNPase$^+$ cells (*D*) of Hoechst$^+$ cells are shown. Values are shown as mean ± S.D. *, $p < 0.05$; **, $p < 0.01$ *versus* PBS group; #, $p < 0.05$; ##, $p < 0.01$ *versus* GST group.

normal goat serum in PBS, and incubated with primary antibody in 10% normal goat serum at 4 °C overnight followed with Cy2- or Cy3-labeled secondary antibody (1:1000, Beyotime, China). As primary antibodies, anti-β-tubulin III (1:1000, StemCell), anti-GFAP (1:500, DAKO), anti-NG2 (1:200, Chemicon), anti-nestin (1:300, Chemicon), anti-BrdUrd (1:200, Santa Cruz), and anti-β 1 integrin (1:200, Chemicon) were used. Nuclei were labeled with Hoechst 33342 (10 μg/ml, Molecular Probes). For the BrdUrd assay, cells were incubated in 2 M HCl for 30 min at room temperature before blocking in 10% normal goat serum. After mounting in fluorescent mounting medium (Beyotime, China), cells were visualized with an Olympus fluorescence microscope. At least 1000 cells from 10 to 15 viewing fields per group were used to calculate percentages of cells.

Oligodendrocyte Differentiation Assay—The oligodendroglioma cell line OLN-93 (32) was used to measure the effect of EGFL on oligodendrocyte differentiation (provided kindly by Dr. Zhi-Cheng Xiao, Singapore General Hospital, Singapore). Cells were cultured in DMEM with high glucose and supplemented with 10% FCS for 6 h at 37 °C in an incubator with 5% CO_2. The culture medium was then replaced with fresh DMEM containing 0.5% FCS, and cells were maintained in the presence of EGFL and GST proteins (100 μg/ml). After 5 days, cells were lysed using 1% Nonidet P-40 and analyzed for CNPase, a marker of oligodendrocytes, by immunoblotting.

Co-immunoprecipitation, GST Pull-down, and Immunoblot Analysis—Rat brain Nonidet P-40 lysates (~200 μg) and rabbit IgG (10 μg) were first incubated with 50 μl of protein A-agarose at 4 °C for 1 h to reduce nonspecific binding to the beads. Cleared lysates were then incubated with the specific anti-β 1 integrin antibody (Chemicon) for 1 h at 4 °C and then with 50 μl of protein A-agarose beads overnight at 4 °C. Beads were washed, bound proteins were eluted in 50 μl of gel loading buffer, separated by 8% SDS-PAGE, blotted onto nitrocellulose membranes, and immunoblotted with anti-TN-R antibody (1:1000, Santa Cruz). The immunoprecipitated β 1 integrin was checked as loading control by anti-β 1 integrin antibody (1:1000, Chemicon).

For pull-down analysis, GST fusion proteins (15 μg/ml) immobilized on glutathione-agarose beads (Sigma) were incubated with NSC lysates (200 μg of protein) at 4 °C for 16 h. Bound proteins were eluted, resolved by 8% SDS-PAGE, and processed for immunoblotting with anti-β 1 integrin antibody. GST antibody (1:200, Dragonfly Science) was used to investigate the GST fusion proteins as loading control.

Statistical Analysis—Data were analyzed by one-way analysis of variance and are expressed as mean ± S.D. Significance was set as $p < 0.05$.

RESULTS

FN6–8 Domain of TN-R Inhibits the Proliferation of NSCs—Because NSCs are capable of self-renewal, we first assessed the

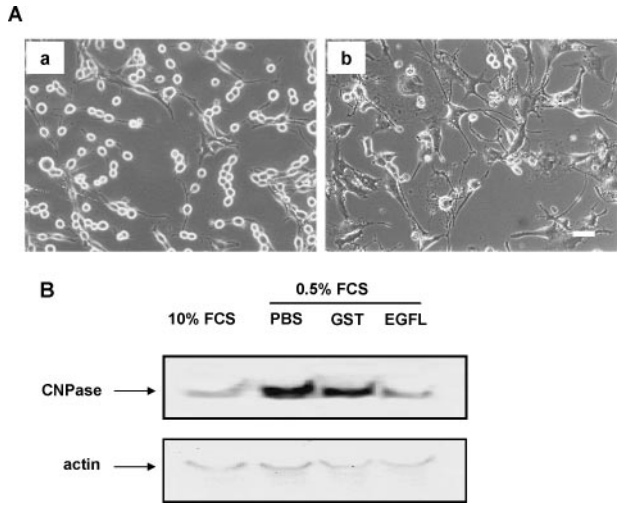

FIGURE 5. **TN-R domain EGFL inhibits differentiation of OLN-93 cells.** *A*, single cell suspensions of OLN-93 cells were seeded into plastic dishes and maintained for 5 days in DMEM supplemented with 10% FCS (*a*) or DMEM supplemented with 0.5% FCS (*b*). *Scale bar* (in *b*) = 25 μm. *B*, single cell suspensions of OLN-93 cells were maintained in DMEM supplemented with 10% FCS or 0.5% FCS and PBS vehicle as control, GST, or EGFL. Cells were then harvested and the levels of CNPase in detergent lysates were determined by Western blot analysis using antibody to CNPase and actin as loading control.

influence of TN-R EGFL and FN6–8 domains on NSC proliferation by measuring the extent of BrdUrd incorporation into NSCs obtained by dissociation of neurospheres. After maintenance of NSCs in culture for 36 or 48 h the percentage of proliferating cells as measured by incorporation of BrdUrd was decreased by FN6–8, when compared with cells treated with culture medium on GST or PBS (Fig. 2, *A* and *B*). EGFL did not show a significant difference in the percentage of the BrdUrd positive cells when compared with the culture medium and GST controls (Fig. 2, *A* and *B*). The effects of GST fusion proteins on NSCs proliferation in solution were the same as precoated on coverslips (data not shown, see supplemental Fig. S1). These results indicate that the FN6–8 domain of TN-R inhibits the proliferation of NSCs.

TN-R Domains EGFL and FN6–8 Regulate NSC Differentiation—We next tested whether EGFL and FN6–8 influence the differentiation of NSCs. Single cell suspensions of NSCs were plated onto coverslips precoated with poly-L-lysine and maintained in DMEM/F-12 culture medium supplemented with B27 and 1% fetal calf serum for 7 days in the presence of soluble proteins, with PBS as a control (Fig. 3*A*). Cells were then fixed and subjected to immunocytochemistry using antibodies to β-tubulin III and GFAP, to label neurons and astrocytes, respectively. In the presence of EGFL the percentage of β-tubulin III-positive cells was increased by a factor of 2, from ~8 to ~16%, when compared with controls (PBS and GST) (Fig. 3*B*). FN6–8 decreased the percentage of β-tubulin III-positive cells from ~8 to ~6%, when compared with the controls (Fig. 3*B*). Differentiation of NSCs into $GFAP^+$ cells was slightly decreased from ~80 to 70% of all cells (Fig. 3*C*). In the presence of FN6–8 differentiation into GFAP positive cells was slightly increased (from ~80 to 90% of all cells) (Fig. 3*C*). These observations indicate that EGFL enhances NSCs differentiation into neurons at the expense of astrocytes, whereas FN6–8 enhances NSC differentiation into astrocytes at the expense of neurons.

We next tested whether EGFL and FN6–8 would influence the differentiation of NSCs into oligodendrocytes. Single cell suspensions of NSCs were plated onto coverslips precoated with poly-L-lysine and cultured in DMEM/F-12 medium supplemented with B27 and 1% fetal calf serum in the presence of PBS vehicle control and GST, EGFL, and FN6–8 for 7 days, when cells were fixed and sub-

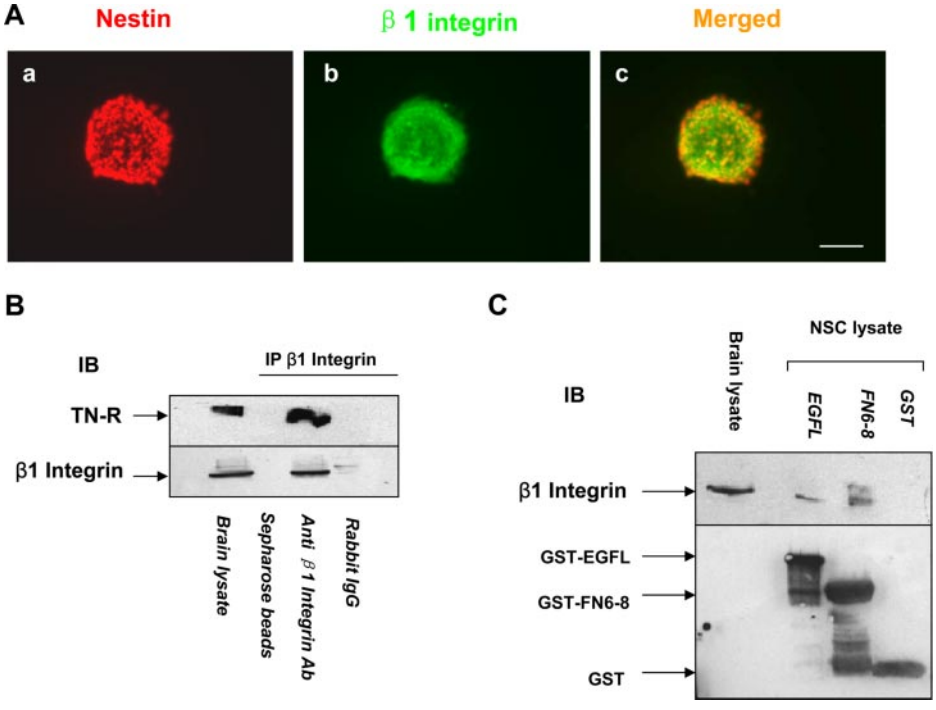

FIGURE 6. **TN-R domains EGFL and FN6–8 co-immunoprecipitate with β1 integrin.** *A*, expression of β 1 integrin in neurospheres derived from NSCs is shown by double-labeling for nestin (*a*) and β 1 integrin (*b*). The fluorescence overlay is shown in *panel c*. *Scale bar* (in *c*) = 25 μm. *B*, antibodies to β 1 integrin co-immunoprecipitate TN-R from detergent lysates of adult rat brain. Sepharose-protein A beads and beads coated with rabbit IgG were used as negative controls. Immunoprecipitates (*IP*) were resolved by 8% SDS-PAGE and immunoblotted with rabbit anti-TN-R antibody. For control, rat brain lysate was loaded in the same gel for a comparison. *C*, pull-down of β 1 integrin from detergent lysates of NSCs seeded into plastic dishes and maintained in culture medium for 7 days. Lysates were incubated with glutathione-agarose beads coated with GST-EGFL, GST-FN6–8, or GST. Bound proteins were eluted from the beads, resolved by 8% SDS-PAGE, and immunoblotted (*IB*) with antibodies to β 1 integrin. Rat brain lysate was loaded in the same gel as positive control. Anti-GST antibody was used to clarify that GST fusion proteins bind to the beads, as loading control.

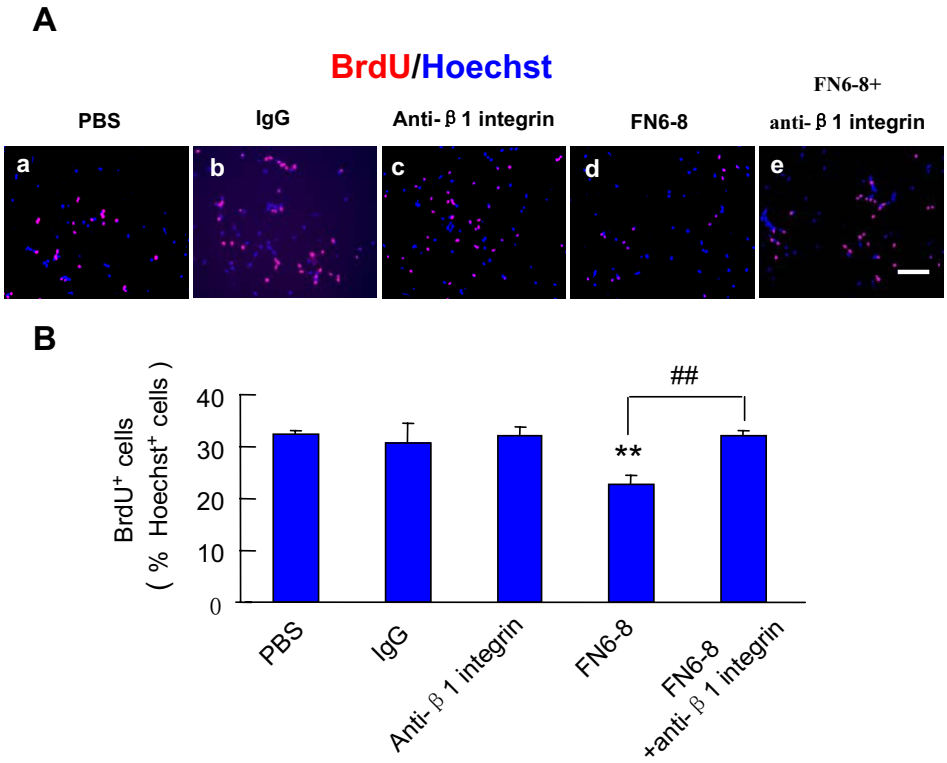

FIGURE 7. **TN-R domain FN6–8 reduces the proliferation of NSCs via β1 integrin.** Single cell suspensions of NSCs were seeded onto coverslips pre-coated by poly-L-lysine and maintained in DMEM/F-12 medium supplemented with B27, bFGF, and EGF for 48 h. Cultures were exposed to BrdUrd 24 h before cells were fixed and immunostained for BrdUrd (*red*) and nuclei (Hoechst, *blue*). *A*, fluorescence overlay micrographs show BrdUrd$^+$ cells (*red*) and nuclei (*blue*), maintained in culture medium with PBS as vehicle control (*a*), IgG (*b*), antibodies to β 1 integrin (*c*), FN6–8 (*d*), and antibodies to β 1 integrin (*e*) for 48 h. *Scale bar* (in *e*) = 25 μm. *B*, percentages of BrdUrd$^+$ cells of all cells counterstained with Hoechst. Values are shown as mean ± S.D. **, $p < 0.01$ *versus* PBS group; ##, $p < 0.01$ *versus* β 1 integrin antibody group.

jected to immunocytochemistry using antibodies to chondroitin sulfate proteoglycan NG2 and CNPase to mark oligodendrocyte progenitor cells and more mature oligodendrocytes, respectively (Fig. 4). EGFL increased the percentage of NG2 positive cells from 5 to 6% in the control to ~11% (Fig. 4*B*), whereas it decreased the percentage of CNPase positive cells from 5 to 6% to about 2% (Fig. 4*C*). FN6–8 slightly decreased the percentage of NG positive cells *versus* the controls (from 5–6% to ~3%) and reduced the percentage of CNPase-positive cells to approximately the same level as achieved by EGFL (Fig. 4*C*). These observations suggest that EGFL favors the existence of NSCs in the oligodendrocyte progenitor stage rather than the more differentiated state. The results also show a tendency for FN6–8 to reduce a percentage of cells in the oligodendrocyte progenitor state, but less so at the expense of the more mature state than seen with EGFL. We suggest that FN6–8 reduces the differentiation of NSCs into the oligodendrocyte lineage (Fig. 4*D*). The effects of GST fusion proteins on NSCs differentiation were the same with that of GST fusion proteins precoated on coverslips (data not shown, see supplemental Figs. S2 and S3).

To further elucidate the effect of EGFL on the differentiation of oligodendrocytes, we took advantage of the oligodendroglioma cell line OLN-93, which proliferates when cultured in DMEM with 10% fetal calf serum (Fig. 5*A*, *panel a*), but when maintained in DMEM culture medium with 0.5% fetal calf serum, cells are induced to differentiate morphologically (Fig. 5*A*, *panel b*) as previously observed (33). To test whether EGFL inhibits differentiation of these cells into mature oligodendrocytes, EGFL was added to the culture medium when it was changed from the 10% fetal calf serum supplement to the 0.5% fetal serum supplement. After 5 days in the presence of soluble EGFL the expression level of CNPase was tested by immunoblotting (Fig. 5*B*). In the presence of EGFL, CNPase expression was indeed reduced when compared with the PBS vehicle control and GST. The level CNPase expression in the presence of EGFL in 0.5% fetal calf serum was similar to the level of CNPase expression in 10% fetal calf serum (Fig. 5*B*). These observations support the interpretation that EGFL inhibits differentiation of progenitor cells into oligodendrocytes.

β 1 Integrin Associates with EGFL and FN6–8—To investigate whether the effects seen with EGFL and FN6–8 on proliferation and differentiation were mediated by β 1 integrin, the cognate receptor for TN-R (34, 35), we first studied the expression of β 1 integrin by NSCs using immunocytochemistry. Neurospheres labeled with nestin and β 1 integrin antibody showed expression of β 1 integrin (Fig. 6*A*). Co-immunoprecipitation experiments using β 1 integrin antibodies on detergent lysates of adult rat brain showed association of β 1 integrin with TN-R (Fig. 6*B*). Pull-down experiments using Sepharose beads coated with GST-EGFL, GST-FN6–8, or GST showed that both EGFL and FN6–8 interact with β 1 integrin (Fig. 6*C*).

FN6–8 Inhibits Proliferation of NSCs via β 1 Integrin—We then studied whether β 1 integrin mediates the inhibition of NSC proliferation by FN6–8. Single cell suspensions of NSCs were seeded onto glass coverslips precoated with poly-L-lysine and maintained in DMEM/F-12 culture medium supplemented with B27, FGF2, and EGF in the presence of FN6–8 (Fig. 7*A*) under the same conditions as shown for Fig. 2. In the absence of FN6–8 neither antibodies to β 1 integrin nor irrelevant rabbit IgG affected NSC proliferation when compared with the PBS vehicle control (Fig. 7*B*). In the presence of antibodies to β 1 integrin, the inhibition of proliferation of NSCs induced by FN6–8 was neutralized to control levels (Fig. 7*B*). These observations suggest that FN6–8 inhibits the proliferation of NSCs via β 1 integrin.

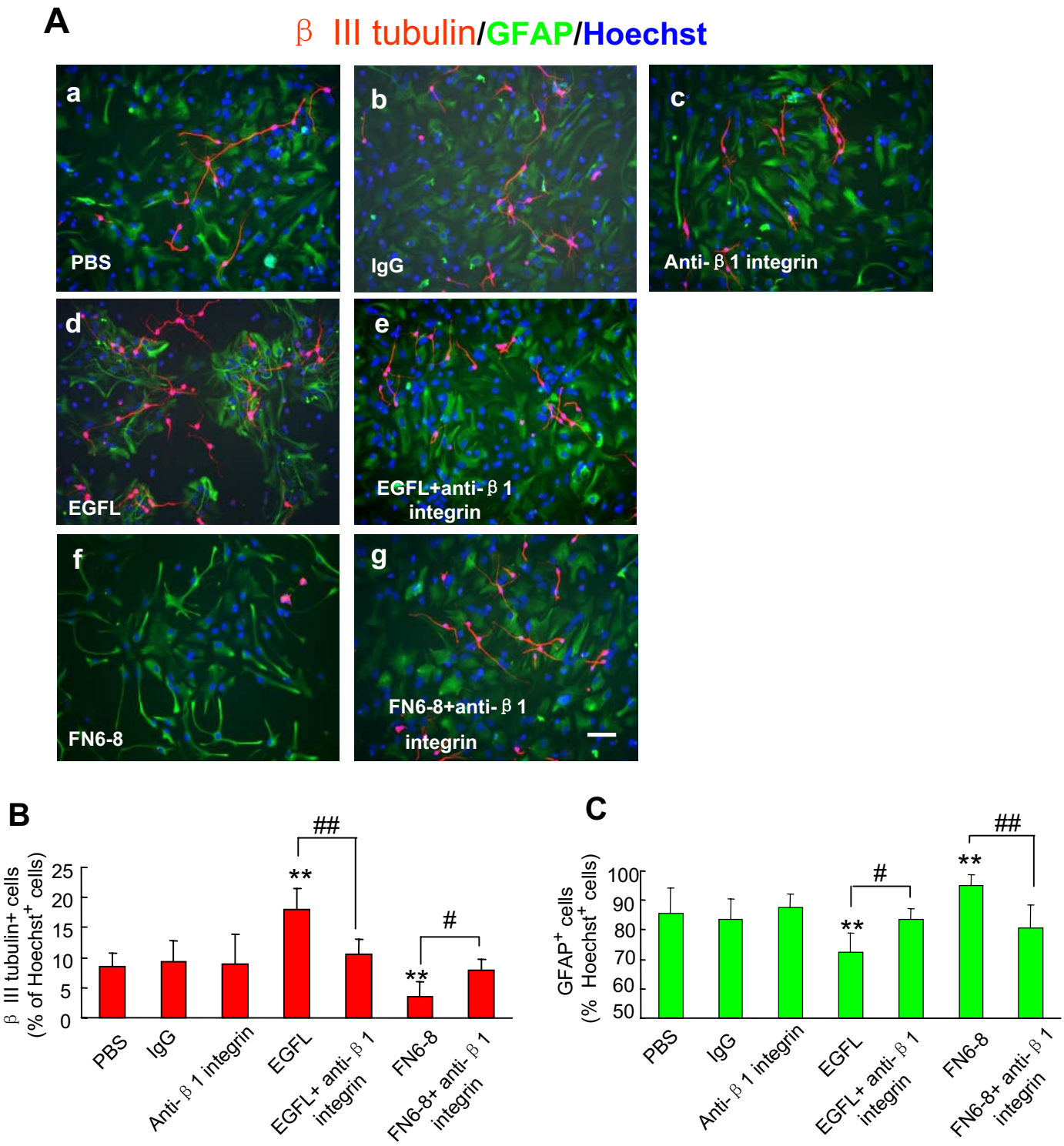

FIGURE 8. **NSC differentiation into neurons is stimulated by TN-R domains EGFL and FN6–8 via β1 integrin.** A, Single cell suspensions of NSCs were seeded onto coverslips pre-coated by poly-L-lysine and maintained in DMEM/F-12 culture medium supplemented with B27 and 1% FCS. After 7 days of exposure to the PBS vehicle control (*a*), IgG (*b*), antibodies to β 1 integrin (*c*), EGFL (*d*), EGFL + antibodies to β 1 integrin (*e*), FN6–8 (*f*), FN6–8 + antibodies to β 1 integrin (*g*), cells were then immunostained for neurons (β III tubulin$^+$, *red*), astrocytes (GFAP$^+$, *green*), and cellular nuclei (Hoechst$^+$, *blue*). *Scale bar* (in *g*) = 25 μm. *B*, percentages of β-tubulin III$^+$ cells; and *C*, percentages of GFAP$^+$ cells of all Hoechst$^+$ cells. Values are shown as mean ± S.D. **, $p < 0.01$ *versus* PBS group; #, $p < 0.05$; ##, $p < 0.01$ *versus* β 1 integrin antibody group.

EGFL and FN6–8 Influence NSC Differentiation via β 1 Integrin—We investigated whether β 1 integrin also mediates the effects of EGFL and FN6–8 on NSC differentiation using β 1 integrin blocking antibodies (Fig. 8). β 1 Integrin antibodies were added to the cultures of NSC monolayers as described in the legend to Fig. 3. Neurons were immunolabeled with antibodies to β-tubulin III and astrocytes with antibodies to GFAP (Fig. 8*A*). The percentage of neurons that was increased by EGFL in the

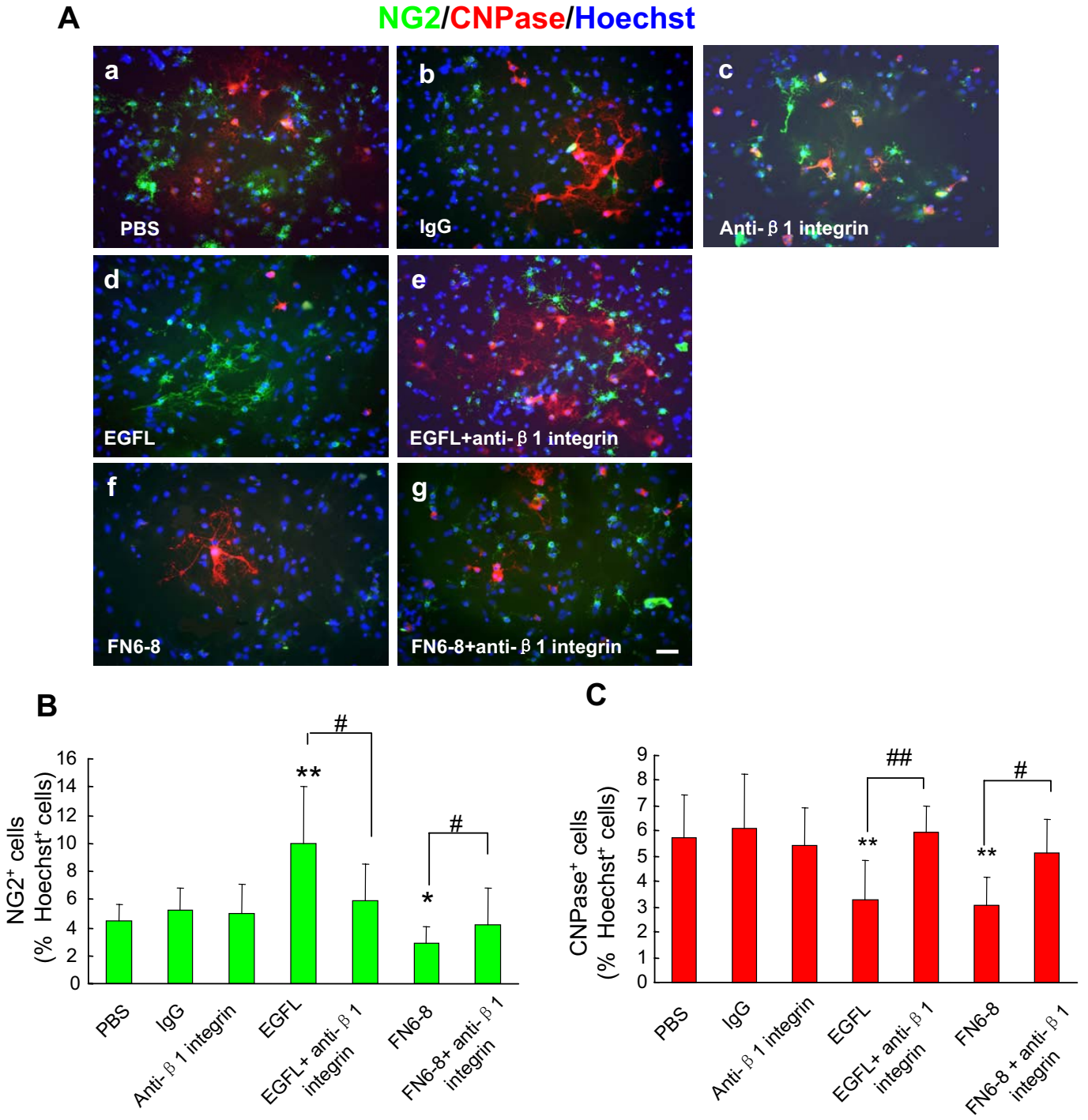

FIGURE 9. **TN-R domains EGFL and FN6–8 affect differentiation of NSCs into oligodendrocytes via β1 integrin.** *A*, fluorescence overlay micrographs show the differentiation pattern of NSCs as detailed in the legend to Fig. 8. Immature oligodendrocytes are identified with antibodies to NG2 (*green*), more mature oligodendrocytes are identified with antibodies to CNPase (*red*), and cellular nuclei are identified with Hoechst (*blue*). *Scale bar* (in *g*) = 25 μm. *B*, percentages of $NG2^+$ cells; and *C*, $CNPase^+$ cells of all $Hoechst^+$ cells are shown. Values are shown as mean ± S.D. *, $p < 0.05$; **, $p < 0.01$ *versus* PBS group; #, $p < 0.05$; ##, $p < 0.01$ *versus* β 1 integrin antibody group.

absence of antibodies to β 1 integrin was reduced when β 1 integrin antibodies were applied in the presence of EGFL (Fig. 8*B*). Similarly, the reduction of differentiation of NSCs into β-tubulin III-positive cells in the presence of FN6–8 was neutralized to control levels in the presence of antibodies to β 1 integrin (Fig. 8*B*). Also, the reduction of differentiation into GFAP-positive cells by EGFL and enhancement of differentiation into astrocytes by FN6–8 was neutralized in the presence of antibodies to β 1 integrin (Fig. 8*C*). The effects of EGFL and FN6–8 on the differentiation of NSCs into oligodendrocyte progenitor cells and oligodendrocytes were also neutralized by antibodies to β 1 integrin (Fig. 9, *A–C*). These observations suggest that all effects induced by EGFL and FN6–8 with regard to differentiation into neurons, astrocytes, and oligodendrocytes are mediated by β 1 integrin.

DISCUSSION

In the present study we have taken the first steps toward dissecting the functional influence of TN-R on neural stem cell differentiation into different cell lineages with a focus on the two major domains of TN-R, the EGFL and the FN6–8. TN-R has been implicated in multiple cellular processes in the central nervous system. It already acts early in neural development in guidance of neural precursor cells of the subventricular zone and rostral migratory stream to engage in radial migration to the olfactory bulb, by its capacity as chemoattractant (7, 25, 27). TN-R is present in perineuronal nets, a feature that may be related to its capacity to bind to voltage-dependent Na^+ channels and, by homology, also to voltage-dependent Ca^{2+} channels (36, 37). TN-R affects neurite outgrowth in a positive manner *in vitro*, when coated as a uniform substrate (10, 11), but repels growth cones in boundary with a conductive substrate, such as laminin (29, 38). TN-R also influences oligodendrocyte and microglial adhesion and repulsion (14, 39). TN-R acts as an intrinsic autocrine factor for oligodendrocyte differentiation and promotes cell adhesion by a sulfatide-mediated mechanism (40), an observation that appears relevant with regard to the localization of TN-R at nodes of Ranvier where it accumulates after termination of the myelination process (6, 41). The immunoglobulin superfamily and L1 family member contactin/F3, mainly expressed by neurons, has been described as a receptor for TN-R on neurons (11, 12, 42). TN-R also interacts with chondroitin sulfate proteoglycans that are enriched in perineuronal nets surrounding subpopulations of inhibitory interneurons (43). The involvement of these different receptors and binding partners for TN-R in mediating its functional properties has, however, remained largely unexplored. Furthermore, the question as to which domains of this multifunctional molecule are involved in distinct functions and which are the receptors for these domains are not known.

We have investigated one aspect of TN-R function with regard to the differentiation of NSCs *in vitro* by focusing on the differential functions of the two major domains of this molecule, namely EGFL and FN6–8, which have previously been shown to be functionally predominant and diversely acting domains (11, 12, 14, 28). The two domains affect the functional status of NSCs in distinct ways: proliferation is reduced by FN6–8, whereas differentiation into neurons is enhanced and reduced by FN6–8 at the expense of differentiation into astrocytes in a complementary manner; and the enhancement of differentiation into neurons leads to a reduction in differentiation into astrocytes. Vice versa, FN6–8 reduces differentiation into neurons, but enhances differentiation into astrocytes. Similar effects of EGFL and FN6–8 were observed in maintenance of progenitor cells at the expense of differentiated cells in the presence of EGFL, being counteracted by FN6–8. Interestingly, embryonic stem cells transfected to overexpress TN-R as a full-length molecule enhance differentiation of precursor cells into neurons as measured in a quinolinic acid excitotoxic lesion paradigm in the adult mouse (44). All these functions appear to be mediated by β1 integrin, which is well expressed by NSCs. Both EGFL and FN6–8 interact with β1 integrin on all three cell types, namely neurons, astrocytes, and oligodendrocytes. Furthermore, proliferation of NSCs influenced by FN6–8 is mediated by β1 integrin. The question now arises how β1 integrin can mediate such diverse effects on NSCs. We can speculate that different α-subunits associating with β1 integrins could be one possibility. Another possibility is that the same α/β integrin pair interacts with distinct cell surface receptors in the plasma membrane of one cell. Candidates for such interactions are the immunoglobulin superfamily adhesion molecule L1, the close homolog of L1 (CHL1), CD9, sulfatide, and others. The combinational variations in such receptor agglomerations may thus determine signal transduction mechanisms, alternately influencing the proliferative state and differentiation of NSCs. The question remains how such differences arise in a progenitor population that is homogeneous in its expression pattern. Whether this homogeneity is indeed high is questionable, and we thus assume that small, yet decisive heterogeneity in cell populations exist in the progenitor pool that influences the susceptibility to different ligands, such as EGFL and FN6–8 on the basis of the receptor combination at the cell surface of the progenitor populations. The investigation of this question and a better understanding of the signal transduction mechanisms of a possibly heterogeneous progenitor population should prove useful in controlling stem cell behavior in specific areas of the central nervous system and should offer further hopes for manipulating progenitor cells for transplantation in the treatment of central nervous system disorders.

Acknowledgment—We thank Dr. Zhi-Cheng Xiao for providing plasmids and reagents.

REFERENCES

1. Dityatey, A., and Schachner, M. (2006) *Cell Tissue Res.* **326,** 647–654
2. Jankovski, A., and Sotelo, C. (1996) *J. Comp. Neurol.* **371,** 376–396
3. Steindler, D. A., Kadrie, T., Fillmore, H., and Thomas, L. B. (1996) *Prog. Brain Res.* **108,** 349–363
4. Scheffler, B., Horn, M., Blumcke, I., Laywell, E. D., Coomes, D., Kukekov, V. G., and Steindler, D. A. (1999) *Trends Neurosci.* **22,** 348–357
5. Pesheva, P., Spiess, E., and Schachner, M. (1989) *J. Cell Biol.* **109,** 1765–1778
6. Bartsch, U., Pesheva, P., Raff, M., and Schachner, M. (1993) *Glia* **9,** 957–969
7. Jones, F. S., and Jones, P. L. (2000) *Dev. Dyn.* **218,** 235–259
8. Fuss, B., Wintergerst, E. S., Bartsch, U., and Schachner, M. (1993) *J. Cell Biol.* **120,** 1237–1249
9. Norenberg, U., Hubert, M., and Rathjen, F. G. (1996) *Int. J. Dev. Neurosci.* **14,** 217–231
10. Lochter, A., and Schachner, M. (1993) *J. Neurosci.* **13,** 3986–4000
11. Xiao, Z. C., Taylor, J., Montag, D., Rougon, G., and Schachner, M. (1996) *Eur. J. Neurosci.* **8,** 766–782
12. Xiao, Z. C., Hillenbrand, R., Schachenr, M., Thermes, S., Rougon, G., and Gomez, S. (1997) *J. Neurosci. Res.* **49,** 698–709
13. Saghatelyan, A. K., Dityatev, A., Schmidt, S., Schuster, T., Bartsch, U., and Schachner, M. (2001) *Mol. Cell. Neurosci.* **17,** 226–240
14. Liao, H., Bu, W. Y., Wang, T. H., Ahmed, S., and Xiao, Z. C. (2005) *J. Biol. Chem.* **280,** 8316–8323
15. Gurevicius, K., Gureviciene, I., Valjakka, A., Schachner, M., and Tanila, H.

(2004) *Mol. Cell. Neurosci.* **25,** 515–523
16. Apostolova, I., Irintchev, A., and Schachner, M. (2006) *J. Neurosci.* **26,** 7849–7859
17. Gage, F. H. (2000) *Science* **287,** 1433–1438
18. Luskin, M. B. (1993) *Neuron* **11,** 173–189
19. Lois, C., and Alvarez-Buylla, A. (1994) *Science* **264,** 1145–1148
20. Alvarez-Buylla, A., and Garcia-Verdugo, J. M. (2002) *J. Neurosci.* **22,** 629–634
21. Pencea, V., Bingaman, K. D., Freedman, L. J., and Luskin, M. B. (2001) *Exp. Neurol.* **172,** 1–16
22. Magavi, S. S., Leavitt, B. R., and Macklis, J. D. (2000) *Nature* **405,** 951–955
23. Li, L., and Xie, T. (2005) *Annu. Rev. Cell Dev. Biol.* **21,** 605–631
24. Moore, K. A., and Lemischka, I. R. (2006) *Science* **311,** 1880–1885
25. Saghatelyan, A., Chevigny, A., Schachner, M., and Lledo, P. M. (2004) *Nat. Neurosci.* **7,** 347–356
26. Garcion, E., Faissner, A., and Ffrench-Constant, C. (2001) *Development* **128,** 2485–2496
27. Garcion, E., Halilagic, A., Faissner, A., and Ffrench-Constant, C. (2004) *Development* **131,** 3423–3432
28. Lochter, A., Vaughan, L., Kaplony, A., Prochiantz, A., Schachner, M., and Faissner, A. (1991) *J. Cell Biol.* **113,** 1159–1171
29. Schachner, M., Taylor, J., Bartsch, U., and Pesheva, P. (1994) *Perspect. Dev. Neurobiol.* **2,** 33–41
30. Husmann, K., Faissner, A., and Schachner, M. (1992) *J. Cell Biol.* **116,** 1475–1486
31. Laemmli, U. K. (1970) *Nature* **277,** 680–685
32. Richter-Landsberg, C., and Heinrich, M. (1996) *J. Neurosci. Res.* **45,** 161–173
33. van Meeteren, M. E., Koetsier, M. A., Dijkstra, C. D., and van Tol, E. A. (2005) *Brain Res. Dev. Brain Res.* **21,** 78–86
34. Probstmeier, R., Michels, M., Franz, T., Chan, B. M., and Pesheva, P. (1999) *Eur. J. Neurosci.* **11,** 2474–2488
35. Pesheva, P., Probstmeier, R., Skubitz, A. P., McCarthy, J. B., Furcht, L. T., and Schachner, M. (1994) *J. Cell Sci.* **107,** 2323–2333
36. Srinivasan, J., Schachner, M., and Catterall, W. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15753–15757
37. Dityatev, A., Brückner, G., Dityateva, G., Grosche, J., Kleene, R., and Schachner, M. (2007) *Dev. Neurobiol.* **67,** 570–588
38. Taylor, J., Pesheva, P., and Schachner, M. (1993) *J. Neurosci. Res.* **35,** 347–362
39. Morganti, M. C., Taylor, J., Pesheva, P., and Schachner, M. (1990) *Exp. Neurol.* **109,** 98–110
40. Pesheva, P., Gloor, S., Schachner, M., and Probstmeier, R. (1997) *J. Neurosci.* **17,** 4642–4651
41. Wintergerst, E. S., Fuss, B., and Bartsch, U. (1993) *Eur. J. Neurosci.* **5,** 299–310
42. Pesheva, P., Gennarini, G., Goridis, C., and Schachner, M. (1993) *Neuron* **10,** 69–82
43. Brückner, G., Grosche, J., Schmidt, S., Härtig, W., Margolis, R. U., Delpech, B., Seidenbecher, C. I., Czaniera, R., and Schachner, M. (2000) *J. Comp. Neurol.* **428,** 616–629
44. Hargus, G., Cui, Y., Schmid, J. S., Xu, J., Glatzel, M., Schachner, M., and Bernreuther, C. (2008) *Stem Cells* **26,** 1973–1984