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Chondroitin Sulfates Are Required for Fibroblast Growth Factor-2-Dependent Proliferation and Maintenance in Neural Stem Cells and for Epidermal Growth Factor-Dependent Migration of Their Progeny

SWETLANA SIRKO,[a] ALEXANDER VON HOLST,[a] ANIKA WEBER,[a] ANDREA WIZENMANN,[b] URSULA THEOCHARIDIS,[a] MAGDALENA GÖTZ,[b,c] ANDREAS FAISSNER[a]

[a]Cell Morphology and Molecular Neurobiology, Ruhr-University Bochum, Universitaetsstrasse, Bochum, Germany; [b]Helmholtz Center Munich, Institute for Stem Cell Research, Ingolstädter Landstraße, Neuherberg, Germany; [c]Institute of Physiology, University of Munich, Schillerstasse, Munich, Germany

Key Words. Extracellular matrix • Proteoglycans • Self-renewal • Differentiation • Neurogenesis

ABSTRACT

The neural stem cell niche of the embryonic and adult forebrain is rich in chondroitin sulfate glycosaminoglycans (CS-GAGs) that represent complex linear carbohydrate structures on the cell surface of neural stem/progenitor cells or in their intimate environment. We reported earlier that the removal of CS-GAGs with the bacterial enzyme chondroitinase ABC (ChABC) reduced neural stem/progenitor cell proliferation and self-renewal, whereas this treatment favored astroglia formation at the expense of neurogenesis. Here, we studied the consequences of CS-deglycanation further and revealed that CS-GAGs are selectively required for neurosphere formation, proliferation, and self-renewal of embryonic cortical neural stem/progenitor cells in response to fibroblast growth factor (FGF)-2. Consistently, the FGF-2-dependent activation of the MAPKinase in neural stem/progenitor cells was diminished after ChABC treatment, but unaltered after epidermal growth factor (EGF) stimulation. Upon EGF treatment, fewer radial glia were brain lipid-binding protein (BLBP)-positive, whereas more were glutamate aspartate transporter (GLAST)-positive after CS-GAG removal. Only in this latter situation, GLAST-positive radial glia cells extended processes that supported neuronal migration from differentiating neurospheres. CS-deglycanation also selectively increased astrocyte numbers and their migration in response to EGF. Thus, our approach revealed that CS-GAGs are essential for FGF-2-mediated proliferation and maintenance of neuron-generating neural stem/progenitor cells. Simultaneously, CS-GAGs act as a brake on the EGF-dependent maturation, migration, and gliogenesis of neural stem/progenitor cells. We conclude that neural stem/progenitor cell subpopulations reside in neurospheres that are distinguishable by their responsiveness to FGF-2 and EGF which is differentially regulated by CS-carbohydrate structures. STEM CELLS *2010;28:775–787*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The central nervous system develops through the controlled proliferation of neural stem cells that initially increase in number. In the forebrain, the expansion phase is followed by neurogenesis when neurons differentiate as progeny of radial glia cells (RGCs) [1, 2] and intermediate basal progenitors [3, 4] at midgestation. Toward the end of embryogenesis and during early postnatal life, astrocytes and oligodendrocytes are generated during gliogenesis when neurogenesis has largely ceased, with the marked exception of the adult neurogenic niches [5].

Two views on how the timely differentiation of neural stem cells into neurons and glia occurs have been proposed. One is based on time-lapse video microscopy studies of single cells that generated mixed clones of neuronal and glia progeny in a comparable order as observed in vivo [6]. This is interpreted as neural stem cells that undergo a switch from a neurogenic to a gliogenic mode of cell division [7, 8]. The alternative view stems from retroviral cell-lineage tracing experiments in vivo where most clones are either neuronal or glial [9, 10]. The rare observation of few multipotent clonal progeny of single infected cells also holds true for early neuroepithelial stages of forebrain development before the onset of neurogenesis [11]. These latter findings suggest that

Author contributions: S.S., A.v.H.: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; A. Weber, A. Wizenmann: Collection of data; U.T.: Collection and assembly of data; M.G.: Conception and design, financial support, administrative support; data analysis and interpretation, manuscript writing, final approval of the manuscript; A.F.: Conception and design, financial support, administrative support; manuscript writing, data interpretation, final approval of the manuscript.

Correspondence: Andreas Faissner, M.D., Prof. Cell Morphology & Molecular Neurobiology, Ruhr-University Bochum, NDEF 05/594, Universitätsstr. 150, D-44780 Bochum, Germany. Telephone: +49 234 3223851; Fax: +49 234 3214313; e-mail: andreas.faissner@ruhr-uni-bochum.de; or Alexander von Holst, Ph.D. Cell Morphology & Molecular Neurobiology, Ruhr-University Bochum, NDEF 05/594, Universitätsstr. 150, D-44780 Bochum, Germany. Telephone: +49 234 3225812; Fax: +49 234 3214313l; e-mail: Alexander.vonholst@ruhr-uni-bochum.de Received July 22, 2009; accepted for publication December 23, 2009; first published online in STEM CELLS *EXPRESS* January 19, 2010. © AlphaMed Press 1066-5099/2009/$30.00/0 doi: 10.1002/stem.309

different neural stem/progenitor cell (NSPC) populations that preferentially generate neurons or glia reside side by side in the neuroepithelium. This is in line with the observed heterogeneity of RGCs [12, 13] that can be prospectively isolated and faithfully predicted in their cellular fate [14].

In both views it remains unknown how the timing of cell fate and the later onset of gliogenesis are governed, but changes in growth factor responsiveness have been proposed [15]. Cells isolated from the developing forebrain can be clonally expanded in defined media as free-floating cellular aggregates called neurospheres [16] or, alternatively, under adherent growth conditions [17, 18]. These cells behave as neural stem cells because they self-renew in response to basic fibroblast growth factor (FGF)-2 and epidermal growth factor (EGF) and give rise to neurons, astrocytes, and oligodendrocytes upon differentiation [19, 20]. Early on, NSPCs proliferate only in response to FGF-2 and preferentially generate neuronal progeny [17, 21]. This is in accordance with the forebrain phenotype of FGF-2-deficient mice that display a smaller cortex because of reduced proliferation and neurogenesis [22–24]. At later stages, NSPCs become responsive to EGF in addition to FGF-2 [19, 25, 26]. EGF signaling supports their proliferation and has been associated with enhanced gliogenesis [27], although EGF-expanded cells generate neurons [18]. The timed acquisition of EGF-responsiveness is associated with increased expression levels of the receptor tyrosine kinase EGFR [26, 28], which allows for the isolation of late NSPCs [29]. The existence of separate FGF-2- and EGF-responsive NSPCs during forebrain development [30, 31] is in accordance with different radial glia subtypes [14, 32]. Alternatively, FGF-2-responsive NSPCs may acquire EGF-responsiveness over time and so represent different stages of the same lineage [27, 31, 33].

Telencephalic NSPCs can be immunoselected with the 473HD monoclonal antibody [34] that detects defined chondroitin sulfate glycosaminoglycan (CS-GAG) structures [35]. These complex carbohydrates are crucial for NSPCs of the embryonic forebrain because their digestion in culture or in utero suppressed neurosphere formation, self-renewal, and proliferation [36]. The degradation of CS-GAGs in vitro and in vivo also reduces the number of newborn neurons and generated glial progeny at the peak of neurogenesis [36]. Despite showing the definite importance of chondroitin sulfates for NSPCs, it remained unknown whether CS-deglycanation affected the response to FGF-2 and EGF and whether a single or separate populations of NSPCs were affected.

Here we show that digestion of CS-GAGs selectively reduced neurosphere formation, self-renewal, and proliferation of cortical NSPCs in response to FGF-2, but not in response to EGF. This effect was accompanied by reduced BLBP-positive RGC numbers and diminished activation of the MAPKinase signaling pathway. In parallel, we recorded enhanced EGF-responsiveness that resulted in maturation of GLAST-positive RGCs and increased generation and migration of astrocytes. We conclude that CS-deglycanation influences NSPCs with different growth factor requirements that represent preferentially neurogenic or gliogenic radial glia lineages. Therefore, the presence of defined CS structures accounts in part for the correct timing of FGF-2-dependent neurogenesis and EGF-mediated gliogenesis during NSPC development.

Materials and Methods

Neurosphere Cultures. All experiments were performed with embryos (E13, Theiler stage 21) derived from timed pregnancies of the NMRI or C57/Bl6 mouse strains obtained from Charles River Laboratories (Sulzfeld, Germany, http://www.criver.de). Embryonic cerebral cortices were isolated and enzymatically digested, and dissociated cortical cells were allowed to form free-floating spheres in the presence of 20 ng/ml EGF or FGF-2 with or without 50 mU/ml chondroitinase ABC (ChABC; EC 4.2.2.4; Sigma-Aldrich, Schnelldorf, Germany http://www.sigmaaldrich.com/germany.html) following the neurosphere culture protocol described previously [34, 36] and detailed in supporting information data 1. For differentiation and migration assays, individual neurospheres were washed three times in neurosphere medium before transfer onto polyornithine-coated wells and incubated for an additional 5 or 7 days.

Immunocytochemistry. The differentiated cell types were identified by immunostaining with the commercially obtained primary antibodies anti-Nestin, anti-βIII-tubulin, anti-BLBP, anti-GLAST, and anti-glial fibrillary acidic protein (GFAP), as described previously [34, 37] and detailed in supporting information online data 1. To detect the various primary antibodies, we used specific secondary antibodies (all from Dianova, Hamburg, Germany http://www.dianova.de/).

Intracerebroventricular Injections in Utero. All experimental procedures in vivo were done in accordance with the Society for Neuroscience and European Union guidelines and were approved by the institutional animal care at the Helmholtz Center (License: 55.2-1-24-2531-044-07) (Munich, Germany). Intracerebroventricular injections (ICVI) into telencephalic ventricles of E13 embryos in utero were performed as described previously [36].

BrdU Pulse Labeling. Labeling of cycling cells was performed by intraperitoneal injection of bromodeoxyuridine (BrdU) (10 mg/100 g body weight; Sigma) 1 hour before removal of the litter or by addition of 10 μM BrdU to culture medium for 12 or 24 hours. The number of BrdU-incorporating cells was determined by immunostaining according to the supplier's protocol (BrdU Labeling and Detection Kit I, Roche Applied Science, Mannheim, Germany).

Reverse Transcription Polymerase Chain Reaction. Total RNA was isolated and reverse transcribed as previously described [38]. The polymerase chain reaction (PCR) reactions were performed as reported earlier [34, 39] and detailed in supporting information online data 1.

Western Blotting. The total protein content was harvested by cell lysis or directly from the conditioned medium as described previously [38, 40]. The samples were fractionated and transferred to polyvinylidene fluoride membranes, which were then incubated with anti-phosphorylated mitogen-activated protein kinase (pMAPK) and antitubulin antibodies or with mab 473HD (1:600) and pk-anti-phosphacan, batch KAF13 (1:2,000) overnight at 4°C (details given in supporting information online data 1). The band intensities were quantified using the freely available ImageJ software (NIH, http://rsb.info.nih.gov/ij/) on digital images of the scanned films.

Statistical Analysis. Statistical significance of differences observed between distinct experimental groups was assessed using Student's t test and is given as p values in the figures and legends with * $p < .05$; ** $p < .01$; and *** $p < .001$.

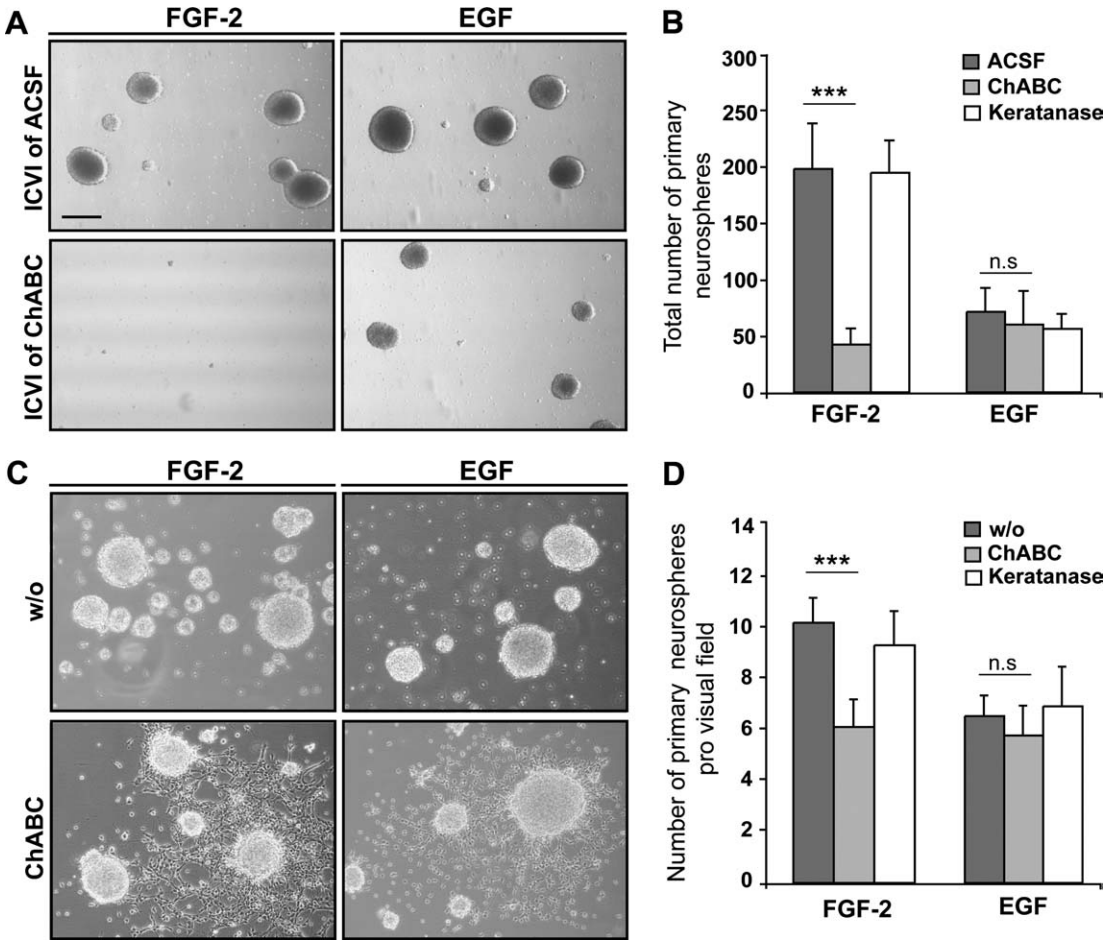

Figure 1. The removal of chondroitin sulfate glycosaminoglycans (CS-GAGs) selectively affects neurosphere formation of neural stem/progenitor cells in response to FGF-2. **(A):** Phase-contrast photomicrographs documenting the formation of neurospheres generated from cortical cells derived 1 day after intracerebroventricular injection (ICVI) of ACSF (upper panel) or ChABC (lower panel) E13.5 embryos in response to FGF-2 or EGF. Note that the total number of neurospheres obtained after 7 days in the presence if FGF-2 was markedly reduced. **(B):** Bar charts of the quantification of the total number of primary neurospheres formed in response to FGF-2 or EGF from cortical cell suspension of ChABC-injected embryos (light gray bars) in comparison with the control ACSF- (dark gray bars) or Keratanase-injected (white bars) embryos as indicated. The data are expressed as mean ± SD ($n = 5$; *** indicates p value $<.001$; Student's t test; n.s., not significant). **(C):** Phase-contrast images of cortical neurosphere cultures obtained in response to FGF-2 or EGF. Cell suspensions dissociated from normal (uninjected) E13.5 embryos were grown for 7 days in the presence or absence of ChABC as indicated. **(D):** Bar charts of the quantification of the total number of primary neurospheres formed in response to FGF-2 or EGF from cortical cell suspensions grown under control conditions (without, dark gray bars), in the presence of ChABC (light gray bars) or in the presence of Keratanase (white bars) as indicated. The data are expressed as mean ± SD ($n = 6$; *** indicates p value of $<.001$; Student's t test, n.s not significant). Scale bar: 150 μm. Abbreviations: ACSF, artificial cerebrospinal fluid; ChABC, chondroitinase ABC; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; ICVI, intracerebroventricular injection; w/o, without.

Results

The Removal of CS-GAGs Selectively Affects Neurosphere Formation of Neural Stem/progenitor Cells in Response to FGF-2

We have previously shown that the deglycanation of CS-GAGs by injection of the bacterial enzyme ChABC into the lateral ventricle of mid-gestation embryos at E13.5 followed by dissection and seeding single cells 1 day later reduced the number of cortical and striatal neurospheres obtained in the presence of EGF and FGF-2 [36]. In the light of the heterogeneity of the neural stem/progenitor cell (NSPC) population [12] and their ability to grow as neurospheres in response to FGF-2 and/or EGF [31], it remained unanswered whether deglycanation of CS-GAGs affected the responsiveness of NSPCs toward FGF-2, EGF, or both growth factors. Therefore, we monitored neurosphere formation in response to FGF-2 or EGF in cortical cell suspensions that were obtained from E 14.5 forebrains 1 day after intracerebroventricular (ICVI) of ChABC, keratanase, or artificial cerebrospinal fluid (ACSF) into the lateral ventricles (Fig. 1A). Cortical cell suspensions obtained from ChABC-injected forebrains generated significantly lower numbers of neurospheres in the presence of FGF-2 compared with parallel seeded cultures from ACSF-injected or keratanase-injected control embryos (Fig. 1B). The number of neurospheres generated in response to EGF was threefold lower compared with the FGF-2 condition. Also, similar quantities of EGF-grown neurospheres emerged under all three conditions, showing that the selective removal of CS-GAGs affected neurosphere formation of cortical NSPCs in response to FGF-2, but not in response to EGF (Fig. 1B). To confirm the requirement of CS-GAGs for FGF-2-dependent neurosphere formation, cortical cell suspensions from non-injected control embryos were grown in the presence of ChABC and

compared to keratanase-treated or control sister cultures. In line with the results from the ICVI injection experiments, removal of CS-GAGs by ChABC selectively reduced the number of primary neurospheres in the presence of FGF-2 by half compared with the control cultures, whereas the number of neurospheres produced in the presence of EGF and ChABC was not significantly altered (Fig. 1C, 1D). Thus, CS-GAGs are required for FGF-2-dependent neurosphere formation of embryonic cortical NSPCs.

FGF-2-Sensitive Neural Stem/progenitor Cells Require Intact CS-GAGs for Proliferation

The formation of neurospheres depends on the addition of mitogenic growth factors [7], and our previous study showed that ChABC treatment affected proliferation when neurospheres were grown in FGF-2 in combination with EGF [36]. To assess the treatment in the presence of singular mitogens, the relative number of actively cycling cells was measured by BrdU incorporation. Approximately half of the cells in FGF-2- and EGF-grown neurospheres were identified as actively cycling after an overnight pulse of BrdU (Fig. 2A). Quantification of BrdU-positive cells in ChABC-exposed neurospheres revealed a significant reduction to almost half the number of cells undergoing S-phase in the presence of FGF-2 compared with control cultures (Fig. 2B). Also, in EGF-grown neurospheres, fewer cells incorporated BrdU upon exposure to ChABC, but the reduction was less extensive compared with the situation with FGF-2 (Fig. 2B). This latter finding was unexpected because ChABC-treatment did not alter the number of neurospheres obtained in response to EGF. However, it may indicate that endogenously expressed FGF-2 contributes to proliferation within neurospheres growing in response to EGF. Therefore, we plated acutely dissociated cortical cells from E13 embryos on polyornithine-coated dishes, added FGF-2 or EGF, and assessed BrdU-incorporation after 12 and 24 hours in the presence or absence of ChABC (Fig. 2). The removal of CS-GAGs significantly suppressed the increase in BrdU-positive cells in response to FGF-2 observed after a 12 and 24-hour pulse, but the number of BrdU-positive cells undergoing S-phase in EGF was unaltered by ChABC treatment (Fig. 2C). The reduced number of cells in S-phase in FGF-2 cultures exposed to ChABC could be caused by an increased fraction of cells leaving the cell cycle or by an impaired cell survival. We consider alterations in cell survival an unlikely explanation for the reduced proliferation in response to FGF-2 after CS-GAG removal because the number of cells undergoing apoptosis as determined by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was low and not significantly increased by treatment with ChABC (supporting information Table 1). The documented propagation of BrdU-incorporated cells argues, however, for a delayed S-phase entry but otherwise similar cell cycle kinetics. In fact, the initial number of BrdU-positive cortical cells (after a 1-hour pulse) was 7.4 ± 1.7 ($n = 3$), and we observed a near doubling of BrdU-positive cells between 12 and 24 hours in the presence of FGF-2. In the same time window, a similar doubling of cortical cells undergoing S-phase occurred in the presence of FGF-2 and ChABC, whereas during the first 12 hours, the pool of S-phase cells remains unchanged when compared with the initial number of BrdU-positive cells. Taken together, CS-GAGs are crucial for FGF-2-mediated proliferation of cortical NSPCs.

CS-GAGs Are Required for FGF-2-Dependent Self-Renewal of Neural Stem/Progenitor Cells

The observed reduction in cell proliferation of NSPCs in response to FGF-2 upon ablation of CS-GAGs would have predicted smaller neurospheres in FGF-2 containing media, but not necessarily a reduced number of primary neurospheres as described above. This suggested that ChABC-treatment could compromise the self-renewal and maintenance of FGF-2-sensitive NSPCs. To probe this assumption, an equal number of cells derived from different primary neurospheres grown in FGF-2 or EGF in the presence or absence of ChABC were allowed to form secondary neurospheres in response to FGF-2 or EGF (Fig. 3A). Under all employed combinations, we observed the generation of secondary neurospheres, indicating that subpopulations of NSPCs exist in primary FGF-2- or EGF-grown neurospheres, which can be distinguished by their responsiveness to either FGF-2 or EGF in secondary neurosphere assays in the absence of ChABC. The removal of CS-GAGs in primary FGF-2-grown neurospheres severely compromised the ability of neurosphere-derived cells to generate secondary neurospheres in response to FGF-2 (Fig. 3B). This clearly indicated that FGF-2-mediated self-renewal depends on CS-GAGs as suspected. Regardless of the primary growth factor condition, when cortical neurospheres were initially exposed to ChABC, only about 10% of the cells formed secondary neurospheres in response to FGF-2 in comparison with control cultures (Fig. 3C, 3D). Indeed, ChABC-treatment of primary FGF-2-grown neurospheres also significantly reduced the number of secondary neurospheres in response to EGF (Fig. 3C), which indicates that half of the EGF-responsive NSPCs depends on FGF-2 for maintenance. Similarly, the compromised response of ChABC-exposed primary EGF-grown neurospheres to FGF-2 (Fig. 3D), which was not seen when secondary neurospheres were allowed to form in EGF (Fig. 3D), suggests the presence of an FGF-2-dependent subpopulation of NSPCs in EGF-grown neurospheres. Thus, the complex protocol of alternating the growth factor conditions after CS-GAG removal revealed that the FGF-2-responsive neurosphere-forming cells requires intact CS-GAGs for maintenance and self-renewal.

The Removal of CS-GAGs Biased Subsets of Radial Glia Cells

The diversity of NSPCs observed during normal forebrain development [12, 14, 41] is reflected by the known heterogeneity of cell types within neurospheres [34, 42, 43]. Our observation of selectively affected subpopulations of NSPCs and their differential responsiveness in the secondary neurosphere assay after CS-GAG removal led us to examine the relative number of Nestin-positive, BLBP-positive, and GLAST-positive radial glia cells (RGCs) within neurospheres. As suspected because of the above findings and from our previous work [36], ChABC-treatment reduced the fraction of Nestin-positive cells by one third and led to a two-fold reduction of BLBP-positive cells, independent of the growth factor condition (Fig. 4B). This was confirmed by reverse transcription polymerase chain reaction (RT-PCR) analysis of neurospheres grown for 7 days with or without ChABC in that BLBP mRNA levels were significantly reduced in response to CS-GAG degradation in both FGF-2- and EGF-expanded neurospheres (Fig. 4C). Interestingly, BLBP is present in both the cytoplasm and nucleus of RGCs and its expression level is dynamically modulated by environmental cues during corticogenesis [44]. We further noticed a significant doubling of GLAST-positive cells after ChABC-exposure that occurred selectively in EGF-grown neurospheres, but not in response to FGF-2 (Fig. 4B).

To confirm these observations, we also performed an analysis of acutely dissociated cortical cells plated on polyornithine (Fig. 5). The adherent cortical cells were grown for 24

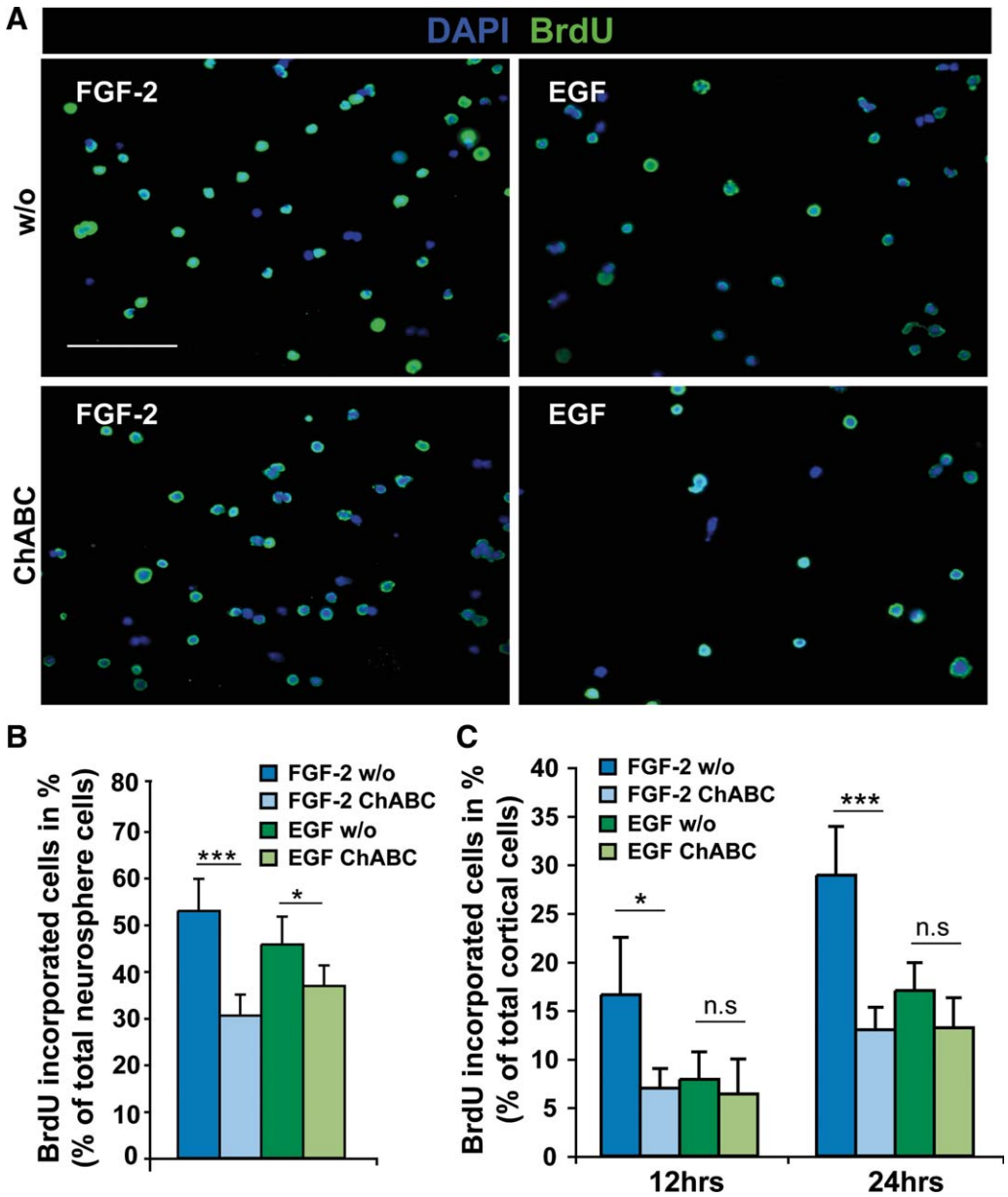

Figure 2. Cultivated FGF-2-sensitive neural stem/progenitor cells require intact chondroitin sulfate glycosaminoglycans (CS-GAGs) for proliferation **(A):** Photomicrographs of BrdU-labeled neurosphere-derived cells originating from neurospheres grown in response to FGF-2 or EGF under control (upper panel) or ChABC-treated conditions (lower panel) after an overnight BrdU-pulse are shown. Cell nuclei were counterstained with bisbenzimide and are shown in blue (4',6-diamidino-2-phenylindole [DAPI]). **(B):** Bar chart of the quantification of the total number of BrdU-positive cells obtained in primary neurospheres that formed in response to FGF-2 (blue bars) or EGF (green bars) from cortical cell suspension grown under control conditions (without, dark bars) or in the presence of ChABC (light bars) as indicated. The data are expressed as means ± SD ($n = 6$; * indicates p value $<.05$; *** indicates p value of $<.001$; Student's t test). **(C):** Bar charts depicting the relative number of freshly dissociated E13.5 cortical cells plated on a polyornithine substrate that incorporated BrdU in response to FGF-2 (blue bars) or EGF (green bars). The cells were pulsed with BrdU for 12 or 24 hours after plating in the presence (light bars) or absence of ChABC (without, dark bars). Note that the removal of CS-GAGs selectively reduced the number of BrdU-incorporating cells in response to FGF-2. All data are expressed as means ± SD ($n = 3$; * indicates p value of $<.05$; *** indicates p value of $<.001$; n.s denotes not significant; Student's t test). Scale bar: 50 μm. Abbreviations: BrdU, bromodeoxyuridine; ChABC, chondroitinase ABC; DAPI, 4',6-diamidino-2-phenylindole; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; hrs, hours; w/o, without.

hours in neurosphere medium with growth factors and immunostained with antibodies against Nestin, BLBP, and GLAST (Fig. 5A). Nearly a third of FGF-2-grown and EGF-grown cells were Nestin-positive and coexpressed the radial glia markers GLAST and/or BLBP. Only a small fraction of FGF-2-stimulated cells expressed GLAST, but were negative for Nestin and BLBP (Fig. 5B). This cell fraction was selectively expanded in the EGF-containing cultures. This trend in phenotypic differences between the FGF-2- and EGF-grown precursor cell pools was most extensive in cultures supplemented with ChABC (Fig. 5B). Moreover, stimulation with EGF also supported morphologic differentiation of GLAST-positive cells (Fig. 5C). When EGF was applied in conjunction with ChABC, the outgrowth of GLAST-labeled processes was

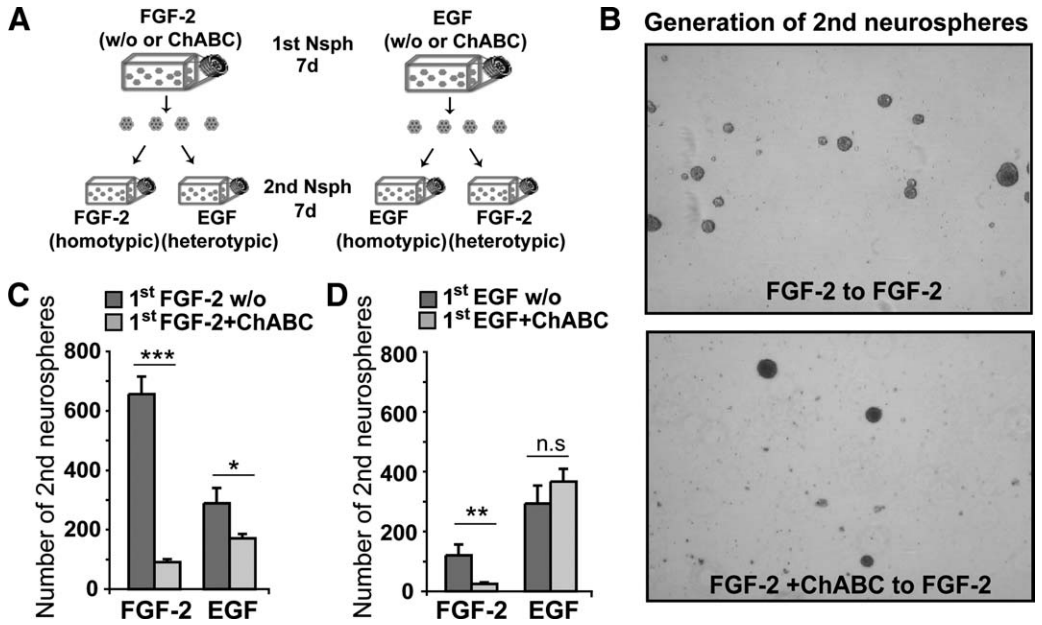

Figure 3. Chondroitin sulfate glycosaminoglycans (CS-GAGs) are required for FGF-2-dependent self-renewal of neural stem/progenitor cells. **(A):** Primary neurospheres were grown in EGF- or FGF-2-containing medium in the presence or absence of ChABC. After 7div the generated neurospheres were dissociated to single cell suspensions. An equal number of the dissociated cells were then plated in homotypic (the first and second culture phases in the same growth factor conditions) or in heterotypic (first and second culture period in distinct growth factor conditions) neurosphere cultures without further addition of ChABC and assessed for secondary neurosphere formation (2°) as indicated in the scheme. **(B):** Phase-contrast photomicrographs of secondary neurosphere cultures obtained in response to FGF-2 after 1 week. Note that previous treatment of primary neurospheres with ChABC clearly compromised the capacity for self-renewal of neurosphere-derived neural stem/progenitor cells in response to FGF-2. **(C):** Bar chart of the quantification of the total number of secondary (2°) neurospheres obtained in response to FGF-2. Equal cell numbers of primary neurosphere-derived cell suspension grown under control conditions (without, dark blue bars) or in the presence of ChABC (light blue bars) were plated under clonal conditions. **(D):** Bar chart of the quantification of the total number of secondary (2°) neurospheres obtained in response to EGF. Equal cell numbers of primary neurosphere-derived cell suspension grown under control conditions (without, dark green bars) or in the presence of ChABC (light green bars) were plated under clonal conditions. All data are expressed as means ± SD ($n = 5$; * indicates p value of $<.05$; *** indicates p value of $<.001$; n.s denotes not significant; Student's t test). Scale bar: 200 μm. Abbreviations: ChABC, chondroitinase ABC; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; w/o, without, div, days in vitro.

even more accentuated. In this regard, the morphologic maturation of GLAST-positive cells, sometimes referred to as astroglial precursors [45], was most pronounced in response to EGF after deglycanation of CS-GAGs.

Removal of CS-GAGs Selectively Reduces the Activation of the MAPK by FGF-2

ChABC-treatment reduced FGF-2-dependent proliferation, neurosphere formation, and self-renewal, and we wondered which signaling pathway could be perturbed. Since both growth factors, FGF-2 and EGF, act as canonical activators of the mitogen-activated protein kinase (MAPK) cascade [46] and since the MAPK pathway has been implicated in glial differentiation of NSPCs [47, 48], we hypothesized that ChABC treatment could interfere with the activation of the MAPK signaling pathway in response to FGF-2 and/or EGF. To test this idea, neurospheres obtained in the presence of EGF plus FGF-2 were dissociated. The neurosphere-derived cells were plated on a laminin-1 substrate and starved from the two growth factors in the absence or presence of ChABC. The cultures were next stimulated with low amounts of FGF-2 or EGF for 15 minutes, and the level of phosphorylated MAPKinase (pMAPK) was determined by Western blotting. As expected, the levels of phosphorylated mitogen-activated protein kinase (pMAPK) were low in control cultures and increased in response to FGF-2 or EGF (Fig. 4D). The stimulation of ChABC-treated cultures with FGF-2 resulted in a significantly decreased level of pMAPK (values, $n = 3$; $p < .05$), whereas CS-deglycanation did not alter pMAPK activation in response to EGF (Fig. 4D). The differential stimulation of the MAPK pathway by EGF and FGF-2 after ChABC treatment in this short-term assay correlates well with the distinguished long-term response of NSPCs to EGF and FGF-2 in the neurosphere assay. Taken together, CS-GAGs are crucial for activation of the MAPKinase pathway in response to FGF-2 but not to EGF.

Removal of CS-GAGs Supports EGF-Mediated Differentiation of Radial Glia Cell Processes

Because MAPK-signaling in response to FGF-2 was reduced and MAPK-signaling was proposed to be important for functional maturation of RGC in vitro [49], we explored this approach further by seeding primary EGF- or FGF-2-initiated neurospheres that were expanded for 5 days in the presence or absence of ChABC on polyornithine-coated dishes. The individual primary neurospheres continued to grow in serum-free medium in either the same (homotypic) or switched growth factor (heterotypic) conditions without further addition of ChABC (Fig. 6A). Regardless of the initial growth conditions, almost no neurospheres gave rise to elongated cell processes in the presence of FGF-2 (Fig. 6B). The extension of elongated RGC processes from neurosphere cells was exclusively observed in EGF-containing cultures (Fig. 6C). To distinguish radial glia cell processes from axons, the expression of the neuronal marker βIII-tubulin and the RGC marker GLAST was examined. Indeed, adherent neurospheres

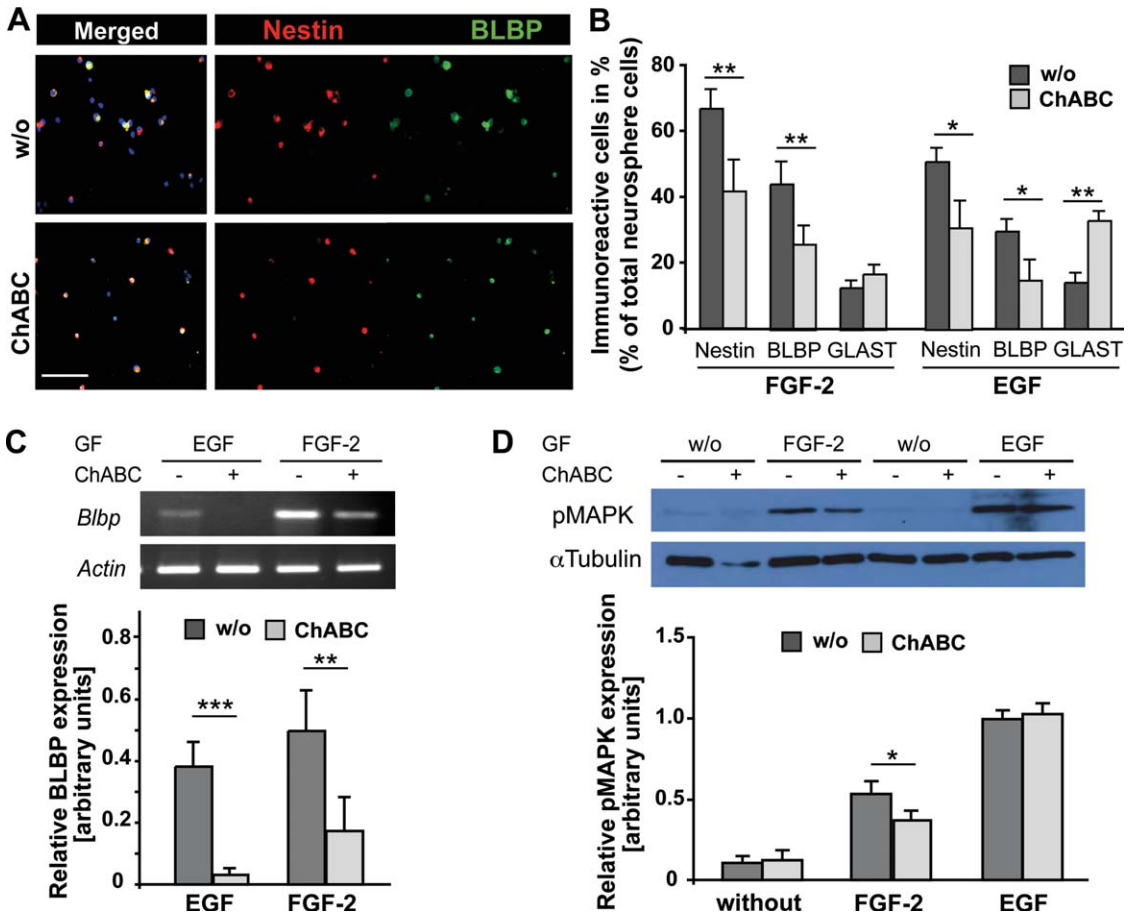

Figure 4. The selective elimination of chondroitin sulfate glycosaminoglycans (CS-GAGs) biases subsets of radial glia cells and reduces the activation of the mitogen-activated protein kinase (MAPK) by FGF-2. **(A):** Double-immunofluorescence images of dissociated neurosphere-derived cells stained for Nestin (red) and BLBP (green) are shown together with the merged pictures that include the DAPI-channel to visualize the Hoechst-stained cell nuclei (blue). **(B):** These observations were quantified using acutely dissociated suspensions of control (w/o, dark gray) or ChABC-treated neurospheres (light gray) that had been expanded in FGF-2 or in EGF and plotted as %-fractions. ChABC-treatment of neurospheres led to two-fold reduction of the frequency of BLBP-positive cells in both FGF-2 and EGF stimulated neurospheres. This was paralleled by a twofold increase of GLAST-positive cells in the presence of EGF that was more attenuated when the cells has been precultivated in presence of FGF-2. **(C):** The BLBP expression level in response to different treatments was determined by RT-PCR and quantified by densitometry. In comparison with EGF-generated neurospheres, the level of BLBP mRNA was more prominent in the FGF-2-generated neurospheres. Note that the degradation of CS-GAGs upon ChABC-treatment significantly reduced the BLBP mRNA levels in both FGF-2- and EGF-expanded neurospheres. Data are expressed as mean ± SD ($n = 3$). **(D):** The level of phosphorylated MAPK levels upon stimulation with growth factors and/or treatment with ChABC was measured. Growth factor treatment induced the upregulation of pMAPK to a comparable degree in control and in ChABC treated cultures. In the control condition without ChABC an increased phosphorylated MAPK (pMAPK) level in the EGF condition relative to other conditions was detected. Relative to the control situation, addition of ChABC caused decrease of phosphorylation levels of MAPK after stimulation with FGF-2. Scale bars: 100 μm. Abbreviations: BLBP, brain lipid-binding protein; ChABC, chondroitinase ABC; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; w/o, without.

growing in FGF-2-containing media did not display GLAST-positive cell processes, whereas the thin, elongated processes that extended in response to EGF stained positive for GLAST and were negative for βIII-tubulin (Fig. 6B, 6C). The digestion of CS-GAGs during the first culture period caused a substantial increase in the number of RGC fibers in response to EGF. These GLAST-expressing fibers were decorated with immature neurons, suggesting that they supported neuronal migration. In contrast, the addition of FGF-2 did not evoke a comparable morphologic evolution accompanied by neuron-glia interactions, although many GLAST-positive cells appeared and migrated out of neurospheres after CS-GAG removal. Thus, consistent with the selective effect of EGF for differentiation of functional RGCs reported previously [49], the removal of CS-GAGs supports EGF-mediated maturation of RGCs.

Removal of CS-GAGs Enhances Astrogliogenesis and Cell Migration in Response to EGF

We immediately noted the increased cell migration from adherent neurospheres after CS-deglycanation and therefore determined the maximal distance that cells migrated from the neurosphere core (Fig. 6A). Neurosphere-derived cells from both initially FGF-2- as well as EGF-grown neurospheres migrated over significantly longer distances in response to EGF compared with FGF-2-treated sister cultures (Fig. 6D). However, independent of the applied growth factors, a massive increase in cell migration was visible after removal of CS-GAGs (Fig. 6D). Remarkably, after removal of CS-GAGs, the maximal distance of cell migration in response to FGF-2 or EGF was comparable for all culture conditions, which suggests that general inhibitory activities are relieved rather than the migration rate of individual cells.

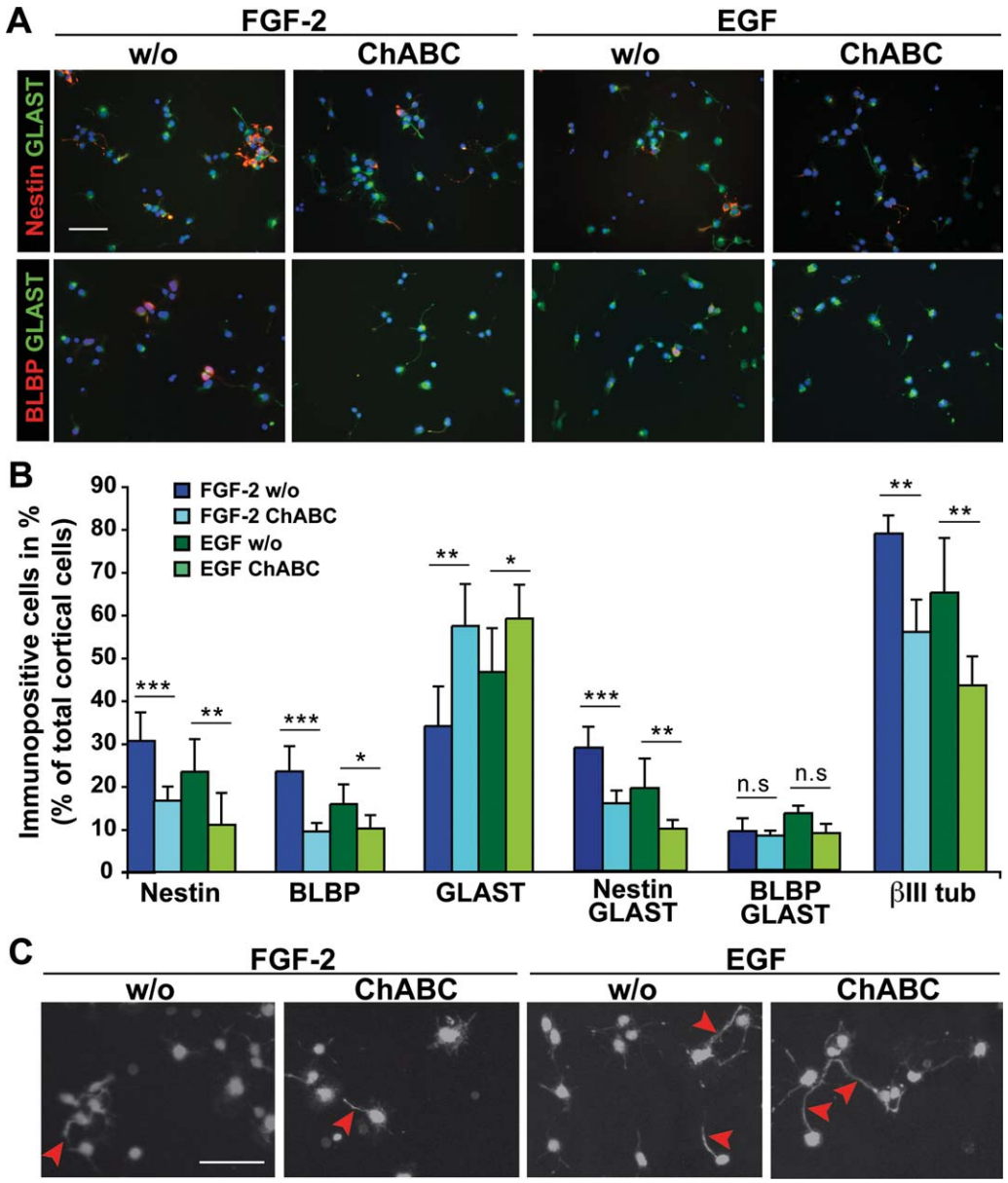

Figure 5. The effect of selective chondroitin sulfate glycosaminoglycans (CS-GAGs) elimination on the differentiation of acutely dissociated cortical cells after 24 hours. **(A):** Double-immunofluorescence images of adherent cortical cells stained for Nestin/GLAST and BLBP/GLAST after 24 hours in neurosphere medium with addition of growth factors in the presence or absence of ChABC are shown. The 4',6-diamidino-2-phenylindole (DAPI) channel visualizes the Hoechst-stained cell nuclei (blue). **(B):** The addition of ChABC to cultures of the dissociated E13 cortical cells led to reduction of the BLBP-positive cell number in each growth factor condition. This was paralleled by a significant increase of GLAST-positive cells. When the cells were plated in FGF-2-containing medium the fraction of βIII-tubulin positive neurons was more prominent in comparison with EGF-grown cells. Note that the degradation of CS-GAGs significantly reduced the pool of immature neurons in each culture condition. These observations were quantified and plotted as %-fractions. Data are expressed as means ± SD ($n = 6$; * indicates p value $<.05$; *** indicates p value $<.001$; n.s. denotes not significant; Student's t test). **(C):** Morphologic differences between GLAST-positive cells grown in the presence of FGF-2 or EGF. Most of the FGF-2-grown GLAST-positive cells gave rise to short cell fibers (5.3 ± 1.4 μm) (red arrowhead). Stimulation with EGF supported morphologic differentiation of GLAST-positive cells, which developed elongated cell processes in response to EGF (18.7 ± 3.2 μm). The addition of ChABC to each of the growth factor conditions supported the morphologic extension of GLAST-positive cell fibers. When EGF was applied in conjunction with ChABC, the outgrowth of GLAST-labeled processes was even more accentuated, reaching an average length of 37 ± 6.4 μm. Scale bars: 50 μm in **(A)** and 25 μm in **(C)**. Abbreviations: BLBP, brain lipid-binding protein; ChABC, chondroitinase ABC; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; GLAST, glutamate aspartate transporter; w/o, without.

Because neurospheres also contain a minority of neurons and astrocytes [36, 43], it remained open whether their migration would also be affected by CS-deglycanation. As expected from the phase contrast images under control conditions, most of the βIII-tubulin-positive neurons and GFAP-positive astrocytes remained confined to neurospheres in the presence of FGF-2, and only a limited number of differentiated cells had migrated in the presence of EGF (Fig. 7, upper panels). In contrast, ChABC treatment during the initial culture period resulted in a dramatic increase in the migration of neurons

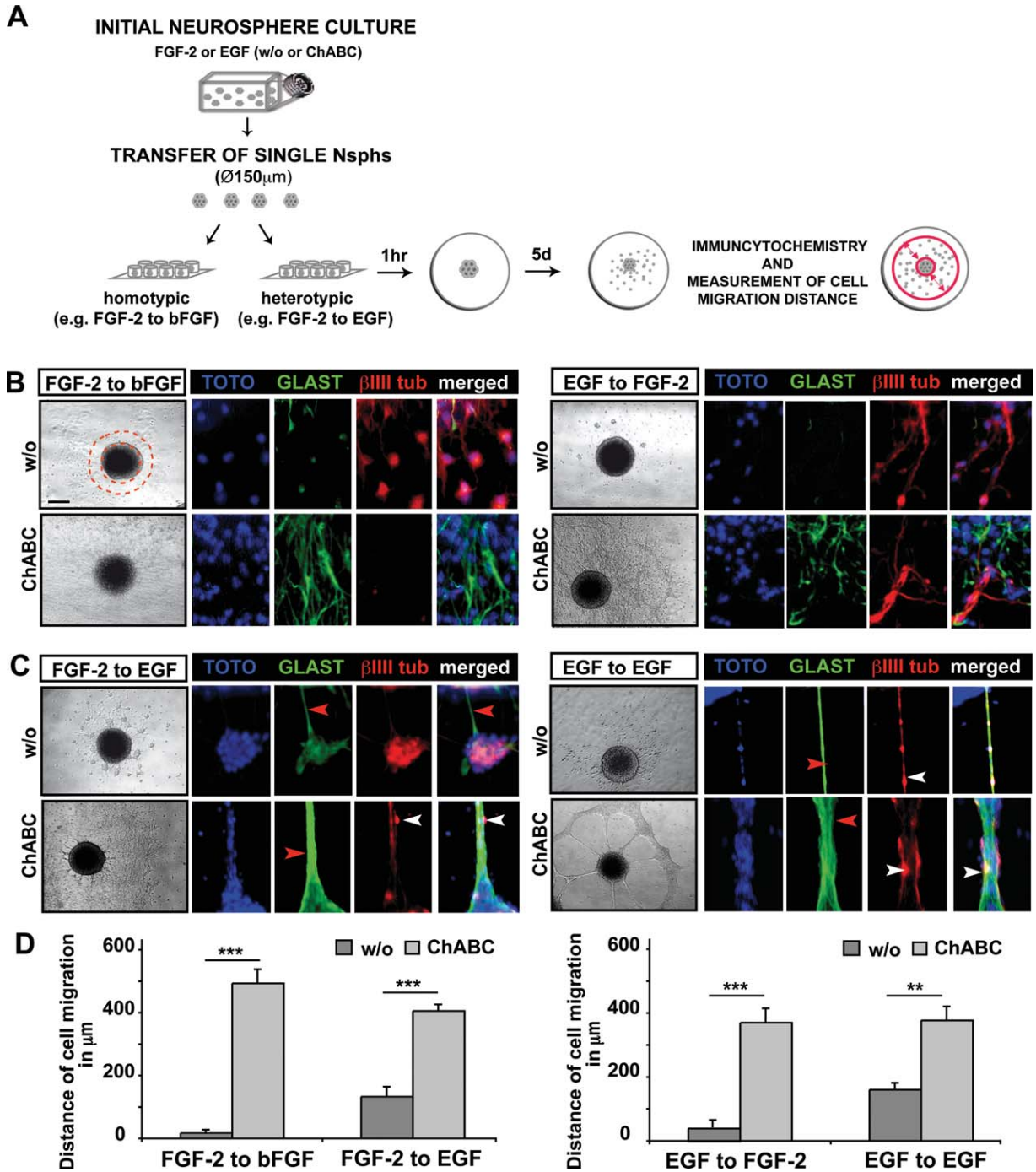

Figure 6. Digestion of chondroitin sulfate glycosaminoglycans (CS-GAGs) in EGF generates GLAST-positive radial glia cells that support neuron migration. **(A):** Schematic representation of the experimental layout. Cortical cell suspensions of E13.5 embryos were grown in EGF- and FGF-2-containing medium with or without ChABC. After 5 days, individual primary neurospheres (⌀; 150 μm) were transferred to polyornithine-coated substrates and allowed to continue growth under homotypic (first and second culture phases in the same growth factor) or heterotypic (first and second culture period in distinct growth factors) conditions for a further 5 days as indicated. Cell migration was quantified by measuring the distance between the border of the neurosphere and the front of migrated cells. **(B):** Cell migration and differentiation in response to FGF-2. Phase-contrast images show examples of individual neurospheres. Note that initial treatment with ChABC (lower panels) caused massive cell migration that was not seen in control cultures (without, upper panels). Higher power confocal photomicrographs of TOTO-positive cell nuclei (blue), GLAST-positive radial glia cells (green), βIII-tubulin-positive neurons (red) and the merged images obtained under the four indicated conditions are shown. Note that initial ChABC-treatment increased the number of GLAST-expressing radial glia cells and impaired neuronal differentiation. **(C):** Cell migration and differentiation in response to EGF. Phase-contrast images show examples of individual neurospheres. Note that initial treatment with ChABC (lower panels) strongly increases structured cell migration that is less apparent in control cultures (w/o, upper panels). Higher power confocal photomicrographs stained as in B are depicted. Note that initial ChABC-treatment increased the number of extending GLAST-expressing radial glia cell processes (red arrowheads), which support neuronal migration (white arrowheads). **(D):** Bar graphs that plotted the maximal distance of cell migration determined as schematically indicated in **(A)**. Cell migration in response to FGF-2 or EGF in control cultures (without, dark gray bars) is compared with cultures that were initially treated with ChABC (light gray bars). Note that ChABC-exposed cells migrated 10 times the distance in the presence of FGF-2 and still three times the distance in the presence of EGF. All data are expressed as mean $\pm$ SD ($n = 3$; ** indicates p value of $<.01$; *** indicates p value of $<.001$; Student's t test). Scale bar: 100 μm for the phase-contrast and 50 μm for the confocal images. Abbreviations: bFGF, basic fibroblast growth factor; BLBP, brain lipid-binding protein; ChABC, chondroitinase ABC; d, days; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; GLAST, glutamate aspartate transporter; hr, hours; w/o, without. TOTO is a dimeric cyanine nucleic acid dye and used as nuclear stain. It is a trademark of molecular probes and stands for toto®-3 iodide.

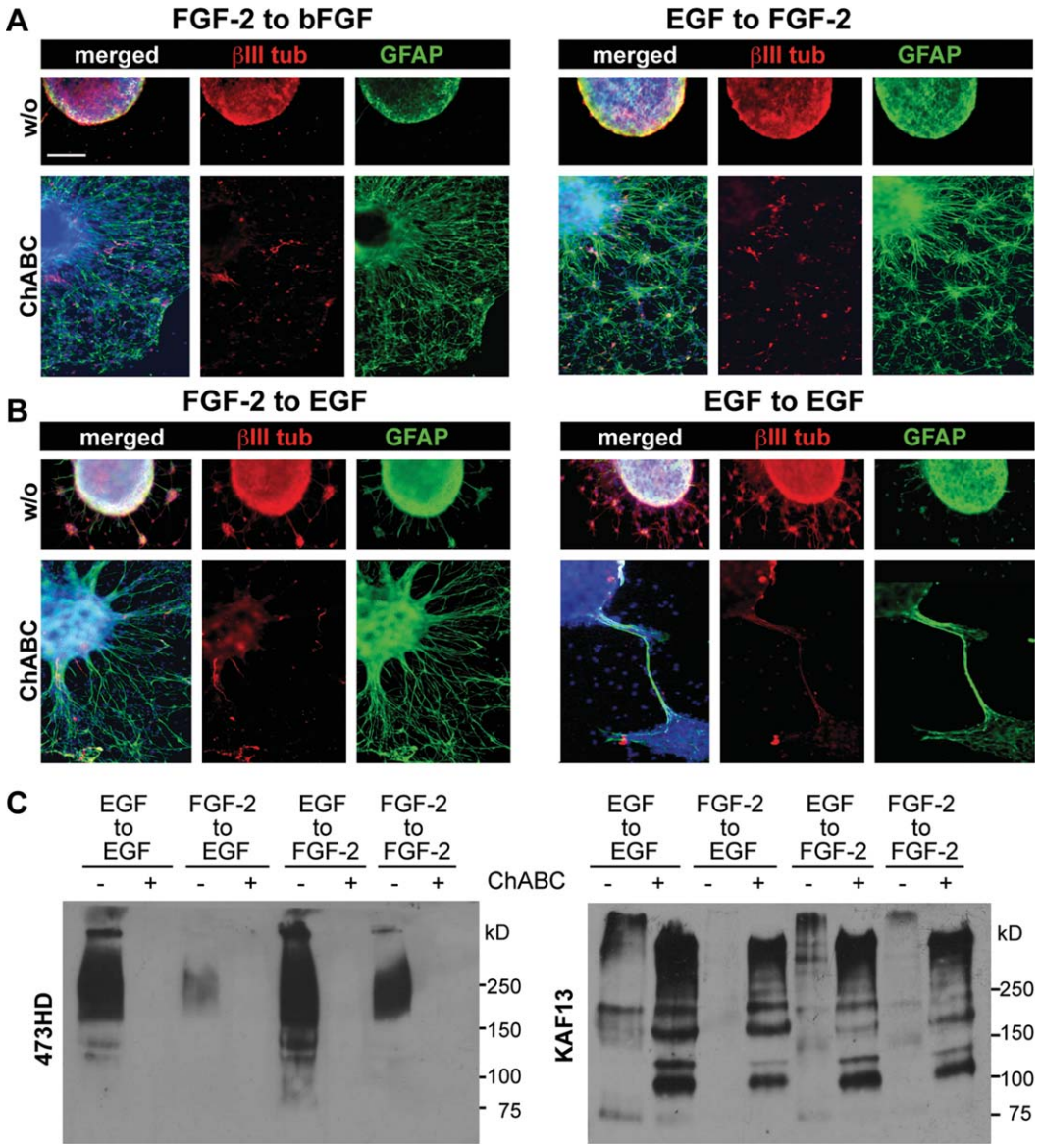

Figure 7. Removal of chondroitin sulfate glycosaminoglycans (CS-GAGs) enhances astrogliogenesis and cell migration in response to EGF. **(A):** Cell migration and differentiation in response to FGF-2. Photomicrographs of individual cortical neurospheres generated as indicated in Figure 5A after double-immunocytochemical staining for βIII-tubulin-positive neurons (red) and GFAP-positive astrocytes are presented. Merged confocal images that include TOTO-positive cell nuclei (blue) are shown left to the individual channels under the four indicated conditions. Note that initial treatment with ChABC (lower panels) caused massive migration of many astrocytes and few neurons that was not observed in control cultures (without, upper panels). **(B):** Cell migration and differentiation in response to EGF. Photomicrographs of individual cortical neurospheres are shown that were generated and documented as **(A)**. Note that under control conditions, some βIII-tubulin- and GFAP-positive cells migrated from neurospheres (upper panels). Initial ChABC-treatment enhanced the number of migrating astrocytes while very few βIII-tubulin-positive neurons developed (lower panels). **(C):** Western blot analysis with mab 473HD revealed the presence of CS-GAGs in the conditioned medium of control cultures after 5 days of migration assay. When neurospheres were pre-treated with ChABC, the 473HD-epitope was removed. On the other hand, ChABC treatment did not affect the core glycoproteins of the RPTPβ gene products, which were visualized with the pab anti-phosphacan. The CSPG DSD-1-PG/phosphacan is a released soluble variant of the RPTPβ/ζ gene and its highly glycosylated form migrates in the high Mr range in undigested samples. Persistence of the core proteins in the ChABC-pretreated cultures may reflect the release of a variant of phosphacan barely derivatized by CS-GAGs from the expanding immature glial cells. Scale bar: 100 μm. Abbreviations: bFGF, basic fibroblast growth factor; ChABC, chondroitinase ABC; CSPG, chondroitinsulfate proteoglycan; CS-GAGs, chondroitinsulfate glycosaminoglycans; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; GFAP, glial fibrillary acidic protein. pab, polyclonal antibodies; mab, monoclonal antibody; Mr, Mass relative.

and astrocytes in response to FGF-2 (Fig. 7A). Similarly, the migration of astrocytes was larger in response to EGF after CS-GAG removal (Fig. 7B). In line with earlier reports that FGF-2 promotes neurogenesis [21] and EGF favors an astroglial fate [26, 27] and our previous observations that ChABC treatment reduced neurogenesis [36], not many neurons were expected to differentiate under the chosen culture conditions, and, indeed, very few neurons migrated from ChABC-exposed neurospheres in response to EGF.

The recovery of CS-GAGs on cell surfaces was examined by immunochytochemistry. Mab 473HD specifically recognizes a CS-dependent GAG epitope on the large form of DSD-1-PG/receptor protein tyrosine phosphatase-β/ζ, and decorates the surface of both embryonic and adult neural stem/

progenitor cells [34, 36]. After 5 days in the migration assay under control condition, 10.6 ± 2.2% of cells showed a strong expression of CS-GAGs on their cell membrane. Although the fraction of 473HD-positive cells which originated from initially ChABC-treated neurospheres was diminished by half compared with control cultures (5.8 ± 1.4%, $n = 3$), there were still twice as many as in the case of neurospheres that had been continuously exposed to ChABC (2.8 ± 0.9%, $n = 3$) (data not shown). The propagation of the 473HD-positive cell population and, hence, the presence of the CS-specific GAG structure on the cells obtained from ChABC-pretreated neurospheres argue against the persistence of enzyme activity in the migration culture system. The presence of CS-GAGs in the cell culture supernatant in the different culture conditions was examined by Western blotting (Fig. 7C). Taken together, CS-GAGs differentially fine tune the response to FGF-2 and EGF in subpopulations of NSPCs and appear to balance their maturation and differentiation along the neuronal and astroglial lineages.

Discussion

In the present study we revealed that chondroitin sulfates are essential for the proliferation, self-renewal, and maintenance of FGF-2, rather than of EGF-responsive NSPCs. The removal of CS-GAGs by ChABC treatment reduced FGF-2-mediated MAPKinase phosphorylation and resulted in fewer BLBP-positive RGCs. In parallel, CS-GAG removal allowed maturation of GLAST-positive RGC processes that supported neuronal migration in the presence of EGF. CS-deglycanation selectively increased EGF-dependent generation and migration of GLAST-expressing RGCs and GFAP-positive astrocytes. Our data imply that CS-GAGs constitute an important part of the environment that orchestrates growth factor responsiveness of neurogenic and gliogenic neural stem/progenitor cell populations.

These observations are in line with our previous work in which we reported a shift from neurogenesis to gliogenesis for FGF-2 plus EGF grown neurospheres upon CS-deglycanation [36]. However, with the results obtained here, we interpret our data in the following way: treatment with ChABC affects two separate NSPC populations. CS-removal suppressed the expansion of FGF-2-responsive, BLBP-positive, and preferentially neurogenic NSPCs, which is not entirely surprising when one considers that FGF-2 in conjunction with Notch signaling regulates BLBP expression [50, 51]. These NSPCs may correspond to those RGCs that directly generate neurons [4, 13, 14, 52]. In line with the forebrain phenotype of FGF-2-deficient mice that have a smaller cortex caused by reduced proliferation and neurogenesis [22–24], we propose that intact CS-GAG structures are required for normal proliferation and neurogenesis of BLBP-expression in RGCs in response to FGF-2. Indeed, CS-GAG preparations purified from the embryonic rat telencephalon changed in their composition during development and supported proliferation of neurosphere-derived cells in response to FGF-2 [53]. At the same time, CS-deglycanation favored the increase of GLAST-positive, EGF-responsive progenitors that preferentially generated astroglia. This preferential expansion of GLAST-positive RGCs could reflect an enhanced propensity of dividing cortical progenitors to generate two GLAST-positive daughters in response to EGF. Notably, the treatment with ChABC seems to favor this type of cell division and, consequently, the generation of glial-restricted progenitors, whereas the generation of BLBP-expressing progenitors simultaneously diminished (supporting information Fig. 1). This switch in phenotype of neural progenitor cells mostly likely corresponds to RGC subtypes that appear at later developmental stages [12, 14, 27]. In this way, a selective expression of CSPGs in subsets of cells may account for the generation of NSPC diversity. The digestion of CS-GAGs from the cell surface of progenitors limited not only their cell proliferation, but also the process of cell division itself (supporting information Fig. 1). CS-GAGs are required for the interaction between the receptor protein tyrosine phosphatase-β/ζ (RPTPβ/ζ) long [54] and its ligand pleiotrophin [55]. Therefore, it is conceivable that the digestion of CS-GAGs impairs the activity of RTPTβ/ζ to dephosphorylate p190RhoGAP, a negative regulator of Rho which also interacts with GRB2, a molecule involved in the MAPKinase pathway. Interestingly, other GRB2-binding proteins, such as FRS2 or sprouty1, differentially influence the FGF-2 and the EGF signaling pathways [56].

Moreover, the GLAST-positive cells displayed enhanced EGF-dependent migration after ChABC treatment. Remarkably, EGFR-expressing neural stem cells fluorescence-activated cell sorted with Alexa-EGF migrate on cortical tissue sections in an EGF-dependent fashion [29]. However, not only the migration of GLAST-positive RGCs themselves, but also their ability to support neuronal migration along elongated RGC processes, was marked in response to EGF, but not FGF-2, after the digestion of CS-GAGs. Indeed, EGF-mediated MAPKinase signaling has been implicated in this particular differentiation of RGCs [49]. With the later onset of GLAST-expression in RGC subtypes [12, 45, 57, 58], we suggest that a gradually declining or changing composition of CS-GAGs during forebrain development [53] would contribute to an environment permissive for the differentiation of NSPCs into the astroglial lineage and emigration of astroglial progeny in response to EGF. Taken together, defined CS structures could control the growth factor responsiveness of separate NSPCs and so balance neurogenesis and gliogenesis—at least in vitro.

Chondroitin sulfates and their carrier proteoglycans display an enormous structural diversity [59], and this diversity is the basis for their wide variety of functions during development [60]. With regard to proliferation, the interactions of CS chains with some FGF-family members have been described [61] and can potentiate the mitogenic activity of FGF-2 in NIH/3T3 [62] or neurosphere-derived cells [53]. In fact, the biologic activity of FGF-2 depends on the ability to bind cell surface or extracellular matrix heparan sulfate proteoglycans (HSPGs), and cells that express FGF-receptors but lack these proteoglycans neither bind nor respond to their ligands [63, 64]. Based on our data, CSPGs serve as essential cofactors for FGF-2-mediated MAPKinase signaling and proliferation of primary NSPCs. This role of CSPGs in regulating the MAPK signaling pathway by FGF-2 is novel, since previous studies have solely focused on HSPGs in mammary fibroblasts [65, 66]. This implies a selective activity of CSPGs because the presence of heparin did not rescue the loss of FGF-2-responsiveness after removal of CS-GAGs. Notably, defined glycosaminglycan (GAG)-dependent responses have been reported for semaphorin5A-mediated axon guidance, which is attractive in the presence of HS-GAGs and repulsive in the presence of CS-GAGs [67].

The molecular interactions that lead to the increased EGF-responsiveness after CS-deglycanation are less obvious because EGF has not been reported to bind to or to require CS-GAGs or HS-GAGs for signaling. However, CS-GAGs interact with heparin binding epidermal growth factor (HB-EGF) [61], which is expressed at lower levels during early stages of corticogenesis and acts as bona fide ligand for the EGFR, causing proliferation [68] or cell migration [69]. HB-

EGF could be a shared ligand between CS-GAGs and the EGFR, which activates the EGFR directly and not competitively when CS-GAGs are removed. It is noteworthy that cell migration is orchestrated by multiple molecular events that involve ECM molecules and cell surface receptors, and EGFR-dependent migration of late telencephalic NSPCs has been described [29]. Of particular interest is the localization of the two alternatively spliced proteoglycan gene products of the *Ptprz1* gene, DSD-1-PG/phosphacan and RPTPβ long [54] and their ligand pleiotrophin [55] along forebrain RGC fibers during neurogenesis [34]. Pleiotrophin/HB-GAM not only interacts with RPTPβ, but was shown to promote NSPC differentiation [70] and EGFR-mediated neuronal migration [71]. The latter depended on the HSPG N-syndecan and thus renders a direct competition between growth factors bound to the different proteoglycan families for receptor tyrosine kinases plausible. In this scenario, a competitive ligand-receptor mechanism between RPTPβ, pleiotrophin/HB-GAM, and HB-EGF would play a role in the EGF-mediated cell migration upon CS-GAGs digestion. Alternatively, the selective expression of CS-GAGs and their interaction partners within cortical germinal regions during neurogenesis could act as a barrier for the EGF-mediated long-distance migration [69], thereby preventing the emigration of neural stem/progenitor cells from the stem cell niche.

Perspectives

CSPGs become upregulated in the glial scar of CNS lesions, interact with a variety of ECM constituents and cell adhesion molecules, such as F3/contactin, N-CAM, L1-CAM, TAG1, or tenascin-C, and represent the major inhibitory entity with regard to axon regeneration [72]. Regarding the ability of isolated core proteins of CSPGs and CS-GAGs by themselves to inhibit cell migration [73–75], the digestion of GS-GAGs entirely coincided with a supportive effect on EGF-mediated radial glia cell migration. However, the importance of CS structures for NSPCs to undergo neurogenesis in response to FGF-2 may be a good reason to caution when inhibitory activities are removed to enhance regeneration of the lesioned CNS.

Acknowledgments

We are very grateful to Anke Baar, Andrea Steiner-Mezzadri, Marie-Therese Schmid, and Sören Moritz for excellent technical support and helpful assistance. We thank Michael Karus for comments on earlier versions of the manuscript. This work was supported by the Federal Ministry of Research and Technology (Bundesministerium für Bildung und Forschung (BMBF), Stem Cells for Therapies of the Central Nervous System, 01GN0504), the German Research Council (D.F.G.) and the Ministerium für Innovation, Wissenschaft, Forschung und Technologie (MIWFT) of Nordrhein-Westfalen (NRW) (to A.F.). M.G. was supported by grants from the DFG, European union (EU), BMBF, and the Bavarian Ministry of Science and Arts.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Malatesta P, Hack MA, Hartfuss E et al. Neuronal or glial progeny: Regional differences in radial glia fate. Neuron 2003;37:751–764.
2 Anthony TE, Klein C, Fishell G et al. Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 2004; 41:881–890.
3 Haubensak W, Attardo A, Denk W et al. Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: A major site of neurogenesis. Proc Natl Acad Sci U S A 2004;101:3196–3201.
4 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.
5 Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. Cell 2008;132:645–660.
6 Qian X, Shen Q, Goderie SK et al. Timing of CNS cell generation: A programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron 2000;28:69–80.
7 Temple S. The development of neural stem cells. Nature 2001;414: 112–117.
8 Miller FD, Gauthier AS. Timing is everything: Making neurons versus glia in the developing cortex. Neuron 2007;54:357–369.
9 Groves AK, Barnett SC, Franklin RJ et al. Repair of demyelinated lesions by transplantation of purified O-2A progenitor cells. Nature 1993;362:453–455.
10 Luskin MB, Parnavelas JG, Barfield JA. Neurons, astrocytes, and oligodendrocytes of the rat cerebral cortex originate from separate progenitor cells: An ultrastructural analysis of clonally related cells. J Neurosci 1993;13:1730–1750.
11 McCarthy M, Turnbull DH, Walsh CA et al. Telencephalic neural progenitors appear to be restricted to regional and glial fates before the onset of neurogenesis. J Neurosci 2001;21:6772–6781.
12 Hartfuss E, Galli R, Heins N et al. Characterization of CNS precursor subtypes and radial glia. Dev Biol 2001;229:15–30.
13 Malatesta P, Hartfuss E, Gotz M. Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development 2000;127:5253–5263.
14 Pinto L, Mader MT, Irmler M et al. Prospective isolation of functionally distinct radial glial subtypes–lineage and transcriptome analysis. Mol Cell Neurosci 2008;38:15–42.
15 Barnabé-Heider F, Wasylnka JA, Fernandes KJ et al. Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. Neuron 2005;48:253–265.
16 Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 1992;255:1707–1710.
17 Davis AA, Temple S. A self-renewing multipotential stem cell in embryonic rat cerebral cortex. Nature 1994;372:263–266.
18 Conti L, Pollard SM, Gorba T et al. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol 2005;3:e283.
19 Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 1996;175:1–13.
20 Pollard SM, Conti L, Sun Y et al. Adherent neural stem (NS) cells from fetal and adult forebrain. Cereb Cortex 2006;16(suppl 1):i112–120.
21 Qian X, Davis AA, Goderie SK et al. FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. Neuron 1997;18:81–93.
22 Vaccarino FM, Schwartz ML, Raballo R et al. Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. Nat Neurosci 1999;2:246–253.
23 Raballo R, Rhee J, Lyn-Cook R et al. Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. J Neurosci 2000;20:5012–5023.
24 Korada S, Zheng W, Basilico C et al. Fibroblast growth factor 2 is necessary for the growth of glutamate projection neurons in the anterior neocortex. J Neurosci 2002;22:863–875.
25 Kilpatrick TJ, Bartlett PF. Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF. J Neurosci 1995;15:3653–3661.
26 Burrows RC, Wancio D, Levitt P et al. Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. Neuron 1997;19:251–267.
27 Lillien L, Raphael H. BMP and FGF regulate the development of EGF-responsive neural progenitor cells. Development 2000;127:4993–5005.

28 Kornblum HI, Hussain RJ, Bronstein JM et al. Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. J Comp Neurol 1997;380:243–261.
29 Ciccolini F, Mandl C, Holzl-Wenig G et al. Prospective isolation of late development multipotent precursors whose migration is promoted by EGFR. Dev Biol 2005;284:112–125.
30 Tropepe V, Sibilia M, Ciruna BG et al. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. Dev Biol 1999;208:166–188.
31 Ciccolini F. Identification of two distinct types of multipotent neural precursors that appear sequentially during CNS development. Mol Cell Neurosci 2001;17:895–907.
32 Pinto L, Gotz M. Radial glial cell heterogeneity–the source of diverse progeny in the CNS. Prog Neurobiol 2007;83:2–23.
33 Garcion E, Halilagic A, Faissner A et al. Generation of an environmental niche for neural stem cell development by the extracellular matrix molecule tenascin C. Development 2004;131:3423–3432.
34 von Holst A, Sirko S, Faissner A. The unique 473HD-Chondroitinsulfate epitope is expressed by radial glia and involved in neural precursor cell proliferation. J Neurosci 2006;26:4082–4094.
35 Ito Y, Hikino M, Yajima Y et al. Structural characterization of the epitopes of the monoclonal antibodies 473HD, CS-56, and MO-225 specific for chondroitin sulfate D-type using the oligosaccharide library. Glycobiology 2005;15:593–603.
36 Sirko S, von Holst A, Wizenmann A et al. Chondroitin sulfate glycosaminoglycans control proliferation, radial glia cell differentiation and neurogenesis in neural stem/progenitor cells. Development 2007;134: 2727–2738.
37 von Holst A, Rohrer H. Culture of avian sympathetic neurons. Curr Top Dev Biol 1998;36:161–182.
38 von Holst A, Egbers U, Prochiantz A et al. Neural stem/progenitor cells express 20 tenascin C isoforms that are differentially regulated by pax6. J Biol Chem 2007;282:9172–9181.
39 Moritz S, Lehmann S, Faissner A et al. An induction gene trap screen in neural stem cells reveals an instructive function of the niche and identifies the splicing regulator sam68 as a tenascin-C regulated target gene. Stem Cells 2008;26:2321–2331.
40 von Holst A, Heller S, Junghans D et al. Onset of CNTFRalpha expression and signal transduction during neurogenesis in chick sensory dorsal root ganglia. Dev Biol 1997;191:1–13.
41 Kriegstein AR, Gotz M. Radial glia diversity: A matter of cell fate. Glia 2003;43:37–43.
42 D'Amour KA, Gage FH. Genetic and functional differences between multipotent neural and pluripotent embryonic stem cells. Proc Natl Acad Sci U S A 2003;100(suppl 1):11866–11872.
43 Campos LS, Leone DP, Relvas JB et al. Beta1 integrins activate a MAPK signalling pathway in neural stem cells that contributes to their maintenance. Development 2004;131:3433–3444.
44 Feng L, Hatten ME, Heintz N. Brain lipid-binding protein (BLBP): A novel signaling system in the developing mammalian CNS. Neuron 1994;12:895–908.
45 Shibata T, Yamada K, Watanabe M et al. Glutamate transporter GLAST is expressed in the radial glia-astrocyte lineage of developing mouse spinal cord. J Neurosci 1997;17:9212–9219.
46 Marshall CJ. Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179–185.
47 Bonni A, Sun Y, Nadal-Vicens M et al. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 1997;278:477–483.
48 Rajan P, McKay RD. Multiple routes to astrocytic differentiation in the CNS. J Neurosci 1998;18:3620–3629.
49 Gregg C, Weiss S. Generation of functional radial glial cells by embryonic and adult forebrain neural stem cells. J Neurosci 2003;23: 11587–11601.
50 Yoon K, Nery S, Rutlin ML et al. Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. J Neurosci 2004;24:9497–9506.
51 Anthony TE, Mason HA, Gridley T et al. Brain lipid-binding protein is a direct target of Notch signaling in radial glial cells. Genes Dev 2005;19:1028–1033.
52 Noctor SC, Flint AC, Weissman TA et al. Neurons derived from radial glial cells establish radial units in neocortex. Nature 2001;409: 714–720.
53 Ida M, Shuo T, Hirano K et al. Identification and functions of chondroitin sulfate in the milieu of neural stem cells. J Biol Chem 2006; 281:5982–5991.
54 Garwood J, Schnadelbach O, Clement A et al. DSD-1-proteoglycan is the mouse homolog of phosphacan and displays opposing effects on neurite outgrowth dependent on neuronal lineage. J Neurosci 1999;19: 3888–3899.
55 Meng K, Rodriguez-Pena A, Dimitrov T et al. Pleiotrophin signals increased tyrosine phosphorylation of beta beta-catenin through inactivation of the intrinsic catalytic activity of the receptor-type protein tyrosine phosphatase beta/zeta. Proc Natl Acad Sci U S A 2000;97: 2603–2608.
56 Guy GR, Wong ESM, Yusoff P et al. Sprouty: How does the branch manager work? J Cell Sci 2003;116:3061–3068.
57 Misson JP, Edwards MA, Yamamoto M et al. Identification of radial glial cells within the developing murine central nervous system: Studies based upon a new immunohistochemical marker. Brain Res Dev Brain Res 1988;44:95–108.
58 Mori T, Tanaka K, Buffo A et al. Inducible gene deletion in astroglia and radial glia–a valuable tool for functional and lineage analysis. Glia 2006;54:21–34.
59 Sugahara K, Mikami T. Chondroitin/dermatan sulfate in the central nervous system. Curr Opin Struct Biol 2007;17:536–545.
60 Bandtlow CE, Zimmermann DR. Proteoglycans in the developing brain: New conceptual insights for old proteins. Physiol Rev 2000;80: 1267–1290.
61 Deepa SS, Umehara Y, Higashiyama S et al. Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues. J Biol Chem 2002;277:43707–43716.
62 Milev P, Monnerie H, Popp S et al. The core protein of the chondroitin sulfate proteoglycan phosphacan is a high-affinity ligand of fibroblast growth factor-2 and potentiates its mitogenic activity. J Biol Chem 1998;273:21439–21442.
63 Rapraeger AC, Krufka A, Olwin BB. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. Science 1991;252:1705–1708.
64 Yayon A, Klagsbrun M, Esko JD et al. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 1991;64:841–848.
65 Delehedde M, Seve M, Sergeant N et al. Fibroblast growth factor-2 stimulation of p42/44MAPK phosphorylation and IkappaB degradation is regulated by heparan sulfate/heparin in rat mammary fibroblasts. J Biol Chem 2000;275:33905–33910.
66 Delehedde M, Lyon M, Gallagher JT et al. Fibroblast growth factor-2 binds to small heparin-derived oligosaccharides and stimulates a sustained phosphorylation of p42/44 mitogen-activated protein kinase and proliferation of rat mammary fibroblasts. Biochem J 2002;366: 235–244.
67 Kantor DB, Chivatakarn O, Peer KL et al. Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. Neuron 2004;44:961–975.
68 Kornblum HI, Zurcher SD, Werb Z et al. Multiple trophic actions of heparin-binding epidermal growth factor (HB-EGF) in the central nervous system. Eur J Neurosci 1999;11:3236–3246.
69 Caric D, Raphael H, Viti J et al. EGFRs mediate chemotactic migration in the developing telencephalon. Development 2001;128:4203–4216.
70 Hienola A, Pekkanen M, Raulo E et al. HB-GAM inhibits proliferation and enhances differentiation of neural stem cells. Mol Cell Neurosci 2004;26:75–88.
71 Hienola A, Tumova S, Kulesskiy E et al. N-syndecan deficiency impairs neural migration in brain. J Cell Biol 2006;174:569–580.
72 Carulli D, Laabs T, Geller HM et al. Chondroitin sulfate proteoglycans in neural development and regeneration. Curr Opin Neurobiol 2005;15:116–120.
73 Snow DM, Lemmon V, Carrino DA et al. Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth in vitro. Exp Neurol 1990; 109:111–130.
74 Dou CL, Levine JM. Inhibition of neurite growth by the NG2 chondroitin sulfate proteoglycan. J Neurosci 1994;14:7616–7628.
75 Smith-Thomas LC, Fok-Seang J, Stevens J et al. An inhibitor of neurite outgrowth produced by astrocytes. J Cell Sci 1994;107:1687–1695.

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