Chondroitin Sulfate Acts in Concert with Semaphorin 3A to Guide Tangential Migration of Cortical Interneurons in the Ventricle Telencephalon

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Chondroitin sulfate (CS) carrying proteoglycans (PGs) are widely expressed in the nervous system, and there is increasing evidence that they regulate developmental mechanisms like neurite outgrowth, axonal guidance and neuronal migration. Moreover, they can also act indirectly by organizing and/or modulating growth factors and guidance molecules. We found that chondroitin-4-sulfate is coexpressed with semaphorin 3A (Sema 3A) in the striatal mantle zone (SMZ), a nontarget region of neuropilin (Nrp)-1–expressing cortical interneurons flanking their migratory route in the subpallium. Using in vitro assays, we showed that CS PGs exert a repellent effect on cortical interneurons, independently of Sema 3A, due to the CS side chains. We further showed that extracellular Sema 3A binds to CS. Disrupting Sema 3A–Nrp-1 signaling led migrating medial ganglionic eminence neurons to inappropriately invade the SMZ and even more so after removal of the CS side chains. Moreover, we found that soluble Sema 3A enhances the CS-induced repulsion in vitro. We concluded that CS acts as a repellent for cortical interneurons and that, in addition, CS restricts secreted Sema 3A within SMZ. Thus, both molecules act in concert to repel cortical interneurons from the SMZ during tangential migration toward the cerebral cortex.

Keywords: ganglionic eminences, neuronal migration, neuropilin, proteoglycans, subpallium

Introduction

Cortical function relies on the delicate equilibrium between inhibition and excitation. Imbalance affects cortical physiology and plasticity (reviewed in Hensch and Fagiolini 2005) and is thought to be associated with a number of human conditions such as epilepsy, anxiety, schizophrenia, and depression (reviewed in Sanacora et al. 2000; Lewis et al. 2005; Levitt et al. 2006). Excitatory projection neurons and inhibitory interneurons undergo different developmental strategies to become integrated into the cortical circuits. Cortical projection neurons are born in the ventricular zone (VZ) and subventricular zone (SVZ) of the dorsal telencephalon and migrate radially to establish the different cortical layers following an inside-out pattern of positioning (Rakic 1995). In contrast, most inhibitory cortical interneurons in rodents arise from subcortical germinative zones in the medial ganglionic eminence (MGE) and caudal ganglionic eminence and migrate tangentially over long distances along well-defined pathways toward the cortex (Lavdas et al. 1999; Anderson et al. 2001; Marin and Rubenstein 2001; Wichterle et al. 2001; Nery et al. 2002). Repulsive and attractive signaling molecules act together guiding the cortical interneurons from the subpallium to the cortex (Marin et al. 2001; Flames et al. 2004; Zimmer et al. 2008). An important example are the class III semaphorins, expressed in the developing striatum, which act as repellent cues for cortical interneurons bearing neuropilin (Nrp) receptors (Marin et al. 2001).

Semaphorins constitute a family of secreted or membrane-bound glycoproteins, which play essential roles during nervous system development (Culotti and Kolodkin 1996; Raper 2000; Bagri and Tessier-Lavigne 2002; He et al. 2002; Zhou et al. 2008; Roth et al. 2009). The secreted semaphorin 3A (Sema 3A) was the first vertebrate semaphorin described and was shown to act as a repellent for motor (Varela-Echavarria et al. 1997), sensory (Luo et al. 1993; Messersmith et al. 1995), hippocampal (Chedotal et al. 1998) and cortical neurons (Bagard et al. 1998). The receptors for class III semaphorins consist of dimerized Nrp-1 and/or Nrp-2 transmembrane proteins with a short intracellular domain lacking known signaling motifs, which form stable complexes withplexins (Chen et al. 1997; He and Tessier-Lavigne 1997; Kolodkin et al. 1997; Giger et al. 1998). Plexins are a large family of integral membrane proteins with a highly conserved cytoplasmic domain performing signal transduction (Fiore and Puschel 2003). Moreover, the L1 immunoglobulin (Ig) superfamily adhesion molecule was described to associate with Nrp-1 to form the Sema 3A receptor (Castellani et al. 2002).

Another group of signaling molecules involved in nervous system development are proteoglycans (PGs). PGs are a very heterogeneous group of extracellular or membrane-bound molecules with covalently bound glycosaminoglycan chains (GAGs). During nervous system development, PGs carry heparan sulfate (HS) and chondroitin sulfate (CS) side chains showing diverse patterns of sulfation, which are tightly regulated in vivo (Kitagawa et al. 1997; Bulow and Hober 2006; Bulow et al. 2008). Heparan sulfate proteoglycans (HSPGs) are essential for proper axonal pathfinding, influencing several axonal guidance cues in a sulfation-specific manner (Bulow and Hober 2004). Chondroitin sulfate proteoglycans (CSPGs) were also reported to influence neuronal growth cones following central nervous system injury and during development, causing mainly repulsive effects (Yamada et al. 1997; Schmalfeldt et al. 2000; Properzi et al. 2005; Wang et al. 2008). In addition, CSPGs were reported to bind growth factors, guidance cues, and other matrix molecules, thus influencing development by “organizing” signaling molecules to be recognized by their appropriate ligands by serving as a scaffold (Emerling and Lander 1996). Because our initial results showed that expression of CS strongly overlaps with Sema 3A in the striatal anlage and that extracellular Sema 3A binds to CS in situ, we postulated that CS might play a role in guidance of tangential migration of cortical interneurons. Using different in vitro assays, we found that CS acts as a repellent for
cortical interneurons and that this repulsion is enhanced by the presence of soluble Sema 3A, pointing to a molecular interaction that contributes to regulate the migration and positioning of interneurons into the cerebral cortex.

Materials and Methods

Animals
Enhanced green fluorescent protein (EGFP)-expressing mice (Okabe et al. 1997) and wild type (WT) (from the C57BL/6 and Swiss strains) were used. For staging of the embryos, the day of insemination was considered embryonic day (E) 1. All animal procedures were authorized by the Animal Ethics Committee of the Federal University of Rio de Janeiro (Brazil) in agreement with international regulations and with the institutional regulations of the University of Jena (Germany).

Preparation of Dissociated Neurons from the MGE
Time pregnant EGFP and WT mice were deeply anesthetized with ether and killed by opening the thorax and interrupting breathing. E13 mouse embryos were removed from the uterus, and the brains were prepared. The MGEs of E13 mouse embryos were dissected and collected in ice-cold phosphate-buffered saline (PBS) supplemented with 0.65% glucose. The tissue was incubated with 0.025% trypsin in PBS for 25 min at 37°C and dissociated by mechanical homogenization followed by filtration through a nylon gauze to remove cell aggregates. Neurons were cultured for 1 day in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin, 0.065% glucose, and 0.4 mM L-glutamine (Sigma, São Paulo, Brazil) at 37°C and 5% CO2 in a humid atmosphere.

Stripe assay
Stripe assays were performed as previously described (Vielmetter et al. 1990) using silicone matrices obtained from the Max Planck Institute for Developmental Biology (Tübingen, Germany). Coverslips were apposed to the silicone matrices, and a mixture containing 10 µg/ml extracellular CSPG (Chemicon, Temecula, Canada) and 40 µg/ml Alexa-488 labeled anti-human IgG (Invitrogen, Karlsruhe, Germany) in PBS was injected into the matrix channels. Coverslips were incubated for 2 h at 37°C. After removal of the matrix and washing with PBS, the coverslips were coated with 20 µg/ml laminin and 5 µg/ml poly-l-lysine in PBS (Invitrogen) for 1 h at 37°C. The cleavage of the CS side chains of the CSPG stripes was performed with 0.2 U/ml chondroitinase ABC (ChABC) from Proteus vulgaris (Sigma) at 37°C for 1 h prior to coating with 20 µg/ml laminin and 5 µg/ml poly-l-lysine in PBS. For the formation of alternating stripes of Sema 3A-Fc and control protein, 50 µg/ml of recombinant Sema 3A-Fc (R&D Systems, Minneapolis, MN) were injected without the Alexa-488 anti-human IgG. Dissociated E15 neurons were added at a density of 300 cells/mm² and incubated during 24 h at 37°C and 5% CO2 for migration on the stripe carpet. After that period, cells were fixed for 15 min with 4% paraformaldehyde (PFA) and washed in PBS/0.2% Triton X-100. After blocking with 10% normal goat serum (NGS) in PBS/0.2% Triton X-100 for 2 h, primary antibody (mouse anti-Calbindin; 1:200, Swant, Bellinzona, Switzerland) was applied overnight at 4°C. After washing with PBS/0.2% Triton X-100, a goat anti-mouse Cyanine3 (Cy3) (Jackson Immuno Research Laboratory, West Grove, PA) secondary antibody and an anti-human IgG-Alexa-594 secondary antibody were used in order to visualize the Sema 3A-Fc stripes. When 5 µg/ml recombinant Sema 3A-Fc were added to the medium of neurons growing on CSPG stripes, the protein was preclustered with 10 µg/ml anti-human IgG-Alexa-488 for 30 min at room temperature.

Boyden Chamber assay
Boyden chamber assays were performed using transfilter chemotaxis assay (Chemicon, Schwalbach, Germany) with inserts containing a collagen membrane with 8-µm pore size. Dissociated MGE neurons from E13 WT embryos were prepared and plated in the upper compartment of the transfilter inserts on the collagen membrane at a density of 900 000 cells in 1-ml serum-free neurobasal medium (Invitrogen) supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 0.065% glucose, and 0.4 mM L-glutamine. The medium of the lower compartment was either supplied with 10 µg/ml Fc protein (Rockland Immunochemicals Inc., Hamburg, Germany) as a control or 10 µg/ml CSPG mixture (Chemicon). After 6 h at 37°C and 5% CO2 in a humid atmosphere, cells were fixed with 4% PFA in PBS and stained with 4’-diamidino-2-phenylindole (DAPI). Cells that migrated through the membrane were counted using a Zeiss Axiovert S100 inverted microscope (Zeiss, Jena, Germany; 50 pictures per coverslip) with a ×20 phase contrast objective (Zeiss Plan Neofluar, numerical aperture 0.5) in combination with fluorescence excitation to visualize the DAPI-stained nuclei. Cells per frame with Fc or CSPG mixture in the lower compartment were counted (60 frames of 3 independent experiments were analyzed). The number of cells that migrated through the membrane in control conditions was set to “1,” and the cells that migrated into the CSPG-containing compartment were calculated relatively.

Preparation of the Slice Cultures
Time pregnant EGFP heterozygote mice were deeply anesthetized, and E13 embryos were removed from the uterus and decapitated. In the same litter, EGFP+/- and EGFP+/- embryos were discerned by UV illumination under fluorescence light at a Zeiss Axiovert microscope (excitation 365 nm, dicroic filter 395 nm, emission 420 nm long pass). Their brains were removed, sectioned coronally into 225-µm slices using a tissue chopper, and transferred to Gey’s balanced salt solution. WT slices, containing the MGE, lateral ganglionic eminence (LGE), and the entopeduncular area (AEP) were placed on glass coverslips coated with poly-l-lysine (10 µg/ml). Explants of the VZ of the MGE of corresponding green fluorescent slices were transplanted homotopically into the WT slices. Slice cultures were incubated in culture medium composed of 60% DMEM/F12 (Sigma) and 30% Hanks’ balanced salt solution supplemented with 10% PBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.65% glucose for 2 days at 37°C, 5% CO2 in a humid atmosphere. Depending on the culture conditions, 0.1 U/ml ChABC from P. vulgaris (Sigma) and/or 1 µg/ml of a functional blocking goat anti-rat Nef-1 antibody (R&D Systems) were added to the medium 1 h after transplantation. After 1 day in vitro (d.i.v.), 50% of the culture medium was replaced with medium containing the respective substances. Slices were fixed after 2 d.i.v. with 4% PFA in PBS for 2 h and prepared for double immunostaining for chondroitin-sulfate (CS-4) and EGFP. After washing with PBS/0.3% Triton, slices were blocked with 20% NGS for 2 h at room temperature. For the CS-4 immunostaining, we used an antibody binding to mouse ChABC-digested cartilage PGs (monoclonal mouse anti-CS-4; Chemicon, Temecula, CA). In addition, we incubated the control slices that were not treated with 0.1 U/ml ChABC during the time of incubation for 2 h at 37°C with the enzyme. To assure effective digestion of the CS side chains in the slice assay, we performed the anti-CS-4 immunostaining without additional ChABC cleavage on these samples. Primary antibodies (mouse anti-CS-4, 1:2500; rabbit anti-EGFP, 1:2500, Invitrogen, Paisley, UK) were applied for 48 h at 4°C. Fluorescein isothiocyanate–coupled goat anti-rabbit (1:100; Jackson Immuno Research Laboratory) and a goat anti-mouse Cy3 secondary antibody (1:400; Jackson Immuno Research Laboratory Inc.) were applied for 2 h at room temperature after washing with PBS/0.5% Triton. Negative controls were performed by omitting either the primary antibody or ChABC digestion were performed and resulted only in background staining.

Immunohistochemistry on Cryo sections
WT time pregnant mice were deeply anesthetized and embryos removed from the uterus. Embryonic brains (E13-E16) were dissected and immersion fixed with 4% PFA in PBS for 2 h at room temperature. Fixed brains were then cryoprotected overnight with 15% 30% sucrose at 4°C and frozen in liquid nitrogen for coronal sectioning at 20-µm thickness.

Two rows of alternating sections were placed on one slide and washed with PBS. For the immunostaining against Sema 3A, one row of sections was pretreated with 0.2 U/ml ChABC, whereas the adjacent sections were incubated in PBS as a control for 2 h at 37°C. After washing in PBS without Triton, probes were blocked with 20% NGS.
Sections were incubated in rabbit anti-Sema 3A polyclonal antibody (1:100, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) for 48 h at 4 °C, washed in PBS, and incubated for 2 h at room temperature with Cy3 goat anti-secondary antibody (1:600; Jackson Immuno Research Laboratory Inc.). Sections were processed similarly for CS staining, except that all were treated with ChABC, and 0.2% Triton was added to the PBS and blocking reagent (20% NGS). The primary antibody (mouse anti-CS-4; Invitrogen) was used at 1:2500 dilution and the secondary antibody used at 1:600 dilution (Cy3 goat anti-mouse; Jackson Immuno Research Laboratory). Negative controls omitting either the primary antibody or ChABC were performed and resulted only in background signals. For the Sema 3A/CS double labeling, the rabbit anti-Sema 3A (1:100, Santa Cruz) was applied in combination with a monoclonal mouse anti-CS-56 antibody (1:200; Sigma, München, Germany). As secondary antibodies, we used a Texas red goat anti-mouse IgM (1:2000; Jackson Immuno Research Laboratory Inc., West Grove, PA) and an Alexa®880 goat anti-rabbit IgG antibody (1:2000, Invitrogen). Pictures were taken as described in the Confocal imaging.

**In Situ Hybridization**

In situ hybridizations were carried out on 20-μm coronal cryosections of the heads of E14 WT embryos using digoxigenin-labeled antisense RNA probes. For this purpose, embryos were decapitated and the heads were freshly frozen in liquid nitrogen. Sections were thaw mounted on SuperFrost Plus slides (Thermofisher, Schwerte, Germany). In situ hybridization was performed as described previously (Weth et al. 1996) with slight modifications. In brief, sections were fixed for 10 min in 4% PFA in PBS at room temperature, permeabilized with 0.2 M HCl, and acetylated with 5 μM acetic anhydride. Hybridization and washing were carried out at high stringency using a probe concentration of 3 ng/μl. After blocking with 2% blocking reagent (Roche, Mannheim, Germany), hybrids were detected using an anti-Digoxigenin Fab fragment conjugated with alkaline phosphatase (1:750, Roche) and nitroblue tetrazolium chloride/5-bromo-4-chloro-indolyl-phosphate as colorigenic substrates. Probes were derived by in vitro transcription in the presence of Digoxigenin-11-UTP (Roche) from polymerase chain reaction fragments of respective cDNAs cloned into pBluescript KS (Stratagene, Amsterdam, The Netherlands). For Sema 3A, the template corresponded to nucleotides 1002-1639 and for Nrp-1 to nucleotides 897-1622 of the coding sequence with position +1 being the adenine of the start codon.

**Immunoprecipitation and Western Blot**

Mechanically dissociated ganglionic eminences were preincubated with 0.5 U/ml ChABC (Sigma) or PBS for 2 h at 37 °C. Cells were shorty centrifuged and miened in lysis buffer containing the following: 50 mM Tris, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM Na_2VO_4, 1 mM NaF, 2.5 mM Na-pyrophosphate, protease inhibitor mixture (1 mM phenylmethylsulfonl fluoride, 5 μg/ml pepstatin, 25 μg/ml aprotinin, 15 μg/ml 2,3-dihydro-neuramic acid, and 25 μg/ml leupeptin), and 1% Triton X-100 under sonication at 4 °C for 10 min. After incubation of the lysed cells on ice for 30 min with occasional shaking, the probes were centrifuged. Supernatant was preincubated with protein A-sepharose (Amersham Bioscience, Freiburg, Germany) for 1 h at 4 °C. Precipitation with 30 μg/ml rabbit anti-Sema 3A antibody (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) was performed overnight at 4 °C. Precipitates were recovered with protein A-agarose (Amersham Bioscience), washed 3 times with lysis buffer without protease inhibitors in MicroSpin columns (Amersham Bioscience), and resuspended in sample buffer. After separation of proteins with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, they were blotted to Hybond nitrocellulose membranes (Amersham Bioscience). The following primary antibodies were used: mouse anti-CS-56 IgM monoclonal (1:1000 Sigma), goat anti-human Sema 3A (1:1000 R&D Systems, Wiesbaden, Germany), and goat anti-Akt (1:1000, Santa Cruz). Horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Europe Ltd, Newmarket, Suffolk, UK) and ECL chemiluminescent and chemiluorescent detection (Amersham Bioscience) were used for detection. To digest the CS band on the nitrocellulose membrane, they were washed overnight and incubated with 0.5 U/ml ChABC at 37 °C.

**Confocal Imaging**

Pictures were taken using the LSM 510 multiphoton laser scanning microscope (Zeiss) using a ×10 (NA 0.60) and ×20 objective (NA 0.75), and ×40 water immersion objective (NA 1.2). A helium-neon laser (wavelength 543 nm) in combination with an emission long pass filter set (560 nm) was used for scanning Cy3/Texas red signals. Alexa®488/EYFP were excited using an Argon laser (488 nm), and the emission passed a 500- to 550-nm band-pass filter set. The DAPI-stained nuclei were visualized using a tunable 2-Photon Titanium-Sapphire laser (Mai-Thai, Newport Spectra-Physics, Darmstadt, Germany) with a wavelength of 790 nm and a ±50 to 850-nm band-pass filter set. Images were taken using the LSM 510 software and exported in TIFF file formats for offline analysis. The extraction of a single optical plane along the X-Y-axis was performed with the LSM 510 software.

**Results**

To determine the distribution of neurons in the stripe assay, pictures were taken with a Zeiss Axiovert S100 inverted microscope (Zeiss, 50 pictures per coverslip) using the ×20 phase contrast objective (Zeiss Plan Neofluar, NA 0.5) in combination with fluorescence excitation to visualize the stripes (long pass filter ±20 nm). At least 10 random frames were taken per coverslip. The percentage of cells on the respective stripes were calculated for each frame and statistically compared with a paired t test using Microsoft Origin 6.0 software. Results (mean ± standard error of the mean [SEM]) are presented in percentage. The number of analyzed frames is presented as “n.”

For the analysis of the migration pattern in the grafting experiments, we chose a region of interest in the LGE (Fig. 5B) and measured the distribution of green fluorescence intensity across an area from the VZ to the ventral border of the slice using the open domain ImageJ analysis software (W. S. Rasband, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/). To ensure that the analyzed region is representative of the same region in each slice, we measured the distance from the corticostriatal angle to the AEP (marked as A in Fig. 5B, left drawing). The exact location of the midline of the area analyzed was at a distance of A/2.5 from the lateral side of the LGE, with a width of A/6, which was then divided along its vertical axis into 20 equal segments, with segments 1-9 representing the VZ/SVZ and the segments 10-18 the striatal anlage, according to the CS-4 staining in the slices (Fig. 5A.C). The segment with the highest absolute fluorescence intensity in each slice was set to 100%, whereas the segment with the lowest fluorescence intensity was defined as 0%. The relative fluorescence intensities of the remaining segments were determined in relation to these values. Results (mean ± SEM) are presented in percentage. Student’s t test was used for statistical comparison of the fluorescence signals of the single segments in the different conditions using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

For quantification of the Sema 3A fluorescence signal, immunostaining of the brain sections with and without ChABC digestion was performed analogously, except that the absolute fluorescence intensities were measured in each of the 20 segments. Sections pretreated with ChABC and with PBS were processed with the same solutions and on the same slides, and images were taken with the same parameters (amplifier offset and detector gain). Background correction was performed by subtracting the lowest value in each section from all the measured values. Results (mean ± SEM) are presented as absolute values, n = number of analyzed sections from 2 brains for each embryonic age.

**CSPGs Are CoExpressed with Sema 3A in the Striatal Anlage**

Previous work demonstrated that migrating cortical interneurons avoid entering the striatal mantle zone (SMZ) due to chemorepulsive signals of class III semaphorins present in the striatal anlage, which is mediated by Nrp receptors expressed by cortical interneurons (Marin et al. 2001). The complementary expression patterns of Sema 3A in the SMZ and Nrp-1 in

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the SVZ at E14 are shown in Figure 1B,C using in situ hybridization. In addition to the mRNA, we could also detect Sema 3A in the striatal anlage using immunohistochemistry during the period of tangential migration (Fig. 1D and Supplementary Fig. 1A,B). We further found that CS-4 is also expressed in the striatal anlage from E13–E16 (Fig. 1E, Supplementary Fig. 1C). To illustrate the overlapping expression, we performed immunostaining for CS-4 and Sema 3A on adjacent coronal brain sections at E14 and imaged the slices using a Zeiss LSM 510 confocal microscope. The images were color coded and overlayed using ImageJ software (Fig. 1D–F). Nissl and DAPI staining allowed the identification of the SMZ in the coronal brain sections (Fig. 1A,G, respectively), as the cell density in the mantle zone is clearly lower than in the VZ and SVZ. The combination of DAPI staining and CS-4 immunohistochemistry illustrates the CS-4 expression within the mantle zone (Fig. 1F). We conclude that, in addition to the chemorepulsive signal of semaphorins, the developing SMZ is further characterized by another group of signaling molecules, the CSPGs, reportedly conveying repulsive effects in other regions of the nervous system (Snow et al. 1990; Oakley and Tosney 1991; Yamada et al. 1997; Wang et al. 2008).

**CS Side Chains Act as a Repulsive Cue for Cortical Interneurons**

CSPGs not only modulate or present signaling molecules but they also can act as guidance cues themselves (Snow et al. 1990; Oakley and Tosney 1991; Wang et al. 2008). Both the core protein and the CS chains can be involved in mediating these effects (Milev et al. 1994; Yamada et al. 1997; Schmalfeldt et al. 2000; Wang et al. 2008). To clarify this issue, we first addressed the possible role of CSPGs as guidance cues for cortical interneurons using the stripe assay according to Vielmetter et al. (1990). As a control experiment, we prepared glass coverslips with alternating stripes of 50 μg/ml recombinant Sema 3A-Fc and a growth permissive substrate (laminin/poly-lysine). The Sema 3A-Fc stripes were visualized with an Alexa488-labeled anti-IgG antibody. We prepared dissociated cells from MGE tissue of E13 embryos, plated them on the stripes, and fixed them after 1 d.i.v. When we analyzed the distribution of the MGE cells, about 60% were seen off the Sema 3A-Fc lanes (Supplementary Fig. 2A,B; paired Student’s t-test, P < 0.001; n = 50; 3 independent experiments). To identify cortical interneurons, we performed immunostaining with an anti-calbindin antibody, as calbindin was shown to be expressed by a subpopulation of γ-aminobutyric acidergic (GABAergic) interneurons (Andrews et al. 2006, 2008). The distribution of the calbindin-positive cells was very similar to the distribution of all the MGE cells (Supplementary Fig. 2C); about 59 ± 1.2% were located on the control stripes and 41 ± 1.2% on the Sema 3A-Fc stripes (paired Student’s t-test, P < 0.001; number of analyzed frames n = 56, 2 independent experiments). This demonstrates that Sema 3A acts as a repulsive cue for cortical interneurons, confirming previous results (Marin et al. 2001). As the distribution of the MGE cells is nearly identical to the distribution of the calbindin-positive neurons, we analyzed total numbers of MGE cells in subsequent experiments.

Next, we plated dissociated MGE cells on alternating stripes containing either 10 μg/ml CSPG mixture or control protein. To visualize the CSPG stripes, we employed 40 μg/ml Alexa488-labeled anti-IgG, coinjected with the CSPG mixture. As illustrated in Figure 2A,B, most MGE cells avoided the CSPG-containing stripes and positioned preferentially on the control lanes (64 ± 1.3%; paired Student’s t-test, P < 0.001; 4 independent experiments). When we performed the same assay but pretreated the CSPG stripes with 0.2 U/ml ChABC, CS repulsion was clearly abolished and MGE cells were homogeneously distributed (Fig. 2C,D). CSPG stripes: 48 ± 1.4%; control lanes: 52 ± 1.4%; 3 independent experiments).

To confirm this repulsive effect with another approach, we further performed a Boyden chamber assay using an insert containing a collagen membrane with 8-μm pore size. Dissociated MGE neurons (900 000 cells/ml) were plated in the upper compartment of the insert, whereas the lower compartment contained either 10 μg/ml Fc protein or 10 μg/ml...
CSPGs have a repulsive effect on cortical interneurons in vitro. Dissociated MGE cells were plated on alternating stripes containing either CSPG or control protein (A) or CSPG and control previously treated with ChABC (C). Quantification of the distribution of MGE cells on the stripes after 24 h in vitro is shown in (B) and (D). (F) Schematic drawing of the setup of the transwell chemotactic assay (Boyden chamber assay). (F) Quantification of the number of cells migrated through the membrane in the lower compartment after application of CSPGs relative to Fc. Scale bars: 100 μm; n = number of analyzed frames (including 4 independent experiments in B, 3 independent experiments in D, and 3 independent experiments in F; *** P < 0.001; mean ± SEM).

Figure 2. CSPGs have a repulsive effect on cortical interneurons in vitro. Dissociated MGE cells were plated on alternating stripes containing either CSPG or control protein (A) or CSPG and control previously treated with ChABC (C). Quantification of the distribution of MGE cells on the stripes after 24 h in vitro is shown in (B) and (D). (F) Schematic drawing of the setup of the transwell chemotactic assay (Boyden chamber assay). (F) Quantification of the number of cells migrated through the membrane in the lower compartment after application of CSPGs relative to Fc. Scale bars: 100 μm; n = number of analyzed frames (including 4 independent experiments in B, 3 independent experiments in D, and 3 independent experiments in F; *** P < 0.001; mean ± SEM).

CSPGs mixture (Fig. 2E). After 6 h in vitro, the number of cells that migrated through the membrane was determined. As illustrated in Figure 2F, the relative number of migrating cells was clearly reduced when the lower compartment contained CSPGs compared with control conditions. These results confirm the repulsive effect of CSPGs observed in the stripe assay. Together, these findings indicate that CSPGs act as repellent cues for cortical interneurons and that repellus is due to the CS side chains.

Secreted Sema 3A Binds to CS Side Chains in the SMZ

The data obtained from the in vitro assays described above indicate that CS acts as a chemorepellent for cortical interneurons in vitro. As Sema 3A is coexpressed with CS in the striatum and was already described to repel cortical interneurons during tangential migration (Marin et al. 2001), we next asked whether there is a possible interaction between CS and Sema 3A or whether Sema 3A and CS act as independent repellents for cortical interneurons.

De Wit et al. (2005) already showed that cortical neurons transfected with Sema 3A-green fluorescent protein (GFP) displayed a punctate extracellular Sema 3A pattern on dendrites and axons, and this cell surface Sema 3A could be removed with ChABC. To test if Sema 3A interacts with CS, we performed double immunostaining against Sema 3A and CS combined with confocal imaging. As illustrated in Figure 3A (arrowhead and asterisk, see also Fig. 4F), in addition to the cellular staining, the immunostaining for Sema 3A resulted in a punctate labeling in the striatum, similar to the secreted cell surface Sema 3A observed by De Wit et al. (2005). The corresponding CS immunostaining in the same section is shown in Figure 3B. The Sema 3A and CS labeling depicted in Figure 3A,B (arrowhead and asterisks) considerably overlap as illustrated in Figure 3C,D. Direct association of the Sema 3A and CS signals is evident in X and Y line scans through a single optical plane as shown in the top and the right side panels in Figure 3E,F and Supplementary Movie 1. Another example is presented in Supplementary Figure 3 and in the corresponding Supplementary Movie 2. These data suggest that Sema 3A can bind to CS in the SMZ.

The colocalization of Sema 3A and CS observed by immunohistochemistry suggests a structural interaction between these molecules. To ascertain a binding of Sema 3A to CS, we next used a biochemical approach. We performed immunoprecipitation against Sema 3A in neuronal tissue extracts of the ganglionic eminences of E14 embryos and tested for coprecipitated CS. Immunoblotting with the CS-56 antibody resulted in a band of about 250 kDa. When the tissue was pretreated with 0.5 U/ml ChABC, this band of ~250 kDa could not be detected (Supplementary Fig. 4A). In contrast, the immunoblotting of the total lysate with CS-56 resulted in a broad smear, indicating that only a fraction of the total CS binds to Sema 3A. When the tissue was digested with ChABC, the CS smear could not be selected in the total lysate, confirming the ChABC activity (Supplementary Fig. 4C). We further treated the presumptive CS band after Sema 3A immunoprecipitation on the nitrocellulose membrane with 0.5 U/ml ChABC for 4 h at 37 °C. Subsequent staining with the CS-56 antibody showed that the CS band disappeared (data not shown), confirming that Sema 3A binds to CS side chains in neuronal tissue of the ganglionic eminences.

If secreted Sema 3A binds to CS side chains, then ChABC treatment should reduce extracellular Sema 3A in the SMZ. To address this issue, we performed immunohistochemistry for Sema 3A on alternating coronal brain sections of E13 and E14 embryos (2 brains were used for each embryonic age) mounted on the same slide, either pretreated with 0.2 U/ml ChABC (Fig. 4D) or with PBS (Fig. 4C). For the rest of the procedure, the sections were treated equally and pictures were taken with the same parameters. ChABC treatment significantly decreased the Sema 3A signal in the striatum (Fig. 4D,E). Higher magnification revealed that the punctate extracellular Sema 3A labeling was strongly reduced (Fig. 4F,G), whereas the cellular signal was much less affected. For quantification, we measured the absolute fluorescence intensities of the Sema 3A signal in the indicated area of the LGE, whose size and location was determined relative to the dimensions of each slice (zoomed up in Fig. 4E; described in Materials and methods). Segments 1–9 represent the VZ/SVZ, and the SMZ is represented by segments 10–18. The graphs
confirm the decreased fluorescence intensities for Sema 3A in the mantle zone after CS degradation at E13 and E14 (Fig. 4H, J). Based on the mean fluorescence intensities calculated from segments 10 to 18, the overall decrease of the Sema 3A signal in the SMZ after CS cleavage is about 20% as compared with the control tissue (Supplementary Fig. 5). This relatively low difference could be explained by considering that we measured the signal intensities of both, cellular and punctate Sema 3A, but only the punctate Sema 3A was reduced by CS removal. Taken together, these results indicate that secreted Sema 3A binds to CS side chains in the SMZ.

CS--Sema 3A Interactions Are Involved in Guidance of the Tangentially Migrating Cortical Interneurons

To directly investigate a possible role of CS in guidance of tangential migration of GABAergic interneurons toward the cortex, we performed grafting experiments, in which E13 EGFP cells were homotopically transplanted into the MGE of coronal WT slices. Previously, these grafting experiments have been widely used to investigate tangential migration of cortical interneurons. As discussed in our previous publication (Zimmer et al. 2008), the migration of cortical interneurons in these brain slices exhibits many features of the migration in vivo: they largely avoid the SMZ and migrate mostly within the SVZ and VZ to the cortex (Fig. 5A).

We first checked for the expression of CS-4 in coronal slices at E13 + 2 d.i.v. when the first neurons reached the cortex (Fig. 5A,C). Immunostaining with an anti-CS-4 antibody resulted in a strong signal within the striatum and the AEP (Fig. 5A), very similar to the in situ situation (Fig. 1E,H). For a quantitative analysis of the migration pattern of the EGFP-MGE cells and the signal strength of the CS-4 immunostaining, we measured the relative fluorescence intensities in defined segments of the LGE (Fig. 5B, described in Materials and methods). The segment with the highest fluorescence intensity was set to 100%, whereas that with the lowest intensity was defined as 0%. The relative fluorescence intensities of the remaining segments were then calculated in reference to these set values. According to the CS-4 and Sema 3A signal (Fig. 5C), the SMZ corresponds to segments 10-18, whereas the VZ/SVZ consists of segments 1-9. The relative fluorescence intensities of the migrating neurons against the CS-4 signal are presented in Figure 5C. Most neurons migrated in the VZ and SVZ, and only few cells invaded the SMZ, which showed a high CS-4 signal intensity. Thus, this in vitro model can be used to investigate a possible role of CS on the tangential migration of cortical interneurons.

In the experiments described above, we demonstrated that CS represents a repulsive cue for cortical interneurons and that it binds Sema 3A in the SMZ. This suggests that in addition to exerting a repulsive effect itself, CS might present and localize secreted Sema 3A in the SMZ. To address this issue, we first used a blocking antibody for Nrp-1 (1 lg/ml) in the grafting experiments to determine and quantify the impact of the Sema 3A-induced repulsion without interfering with CS function. As expected, blocking Nrp-1 function leads to an increased number of EGFP-MGE cells in the SMZ as compared with control conditions (Fig. 6A,C). The fluorescent intensities of segments 13-15 are significantly increased, as compared with the control condition (Fig. 6C; Student’s t-test; P < 0.05).

Next, we wanted to interfere with the function of the CS in the SMZ. For this, we enzymatically removed the CS side chains by treating slices with ChABC. Addition of 0.1 U/ml ChABC to the medium of the brain slices for 2 d.i.v. resulted in a robust migration (Fig. 6B). The removal of the CS side chains was controlled by immunostaining for CS-4 (data not shown).
Analysis of the migration pattern after 2 d.i.v. revealed an increased number of neurons invading the mantle zone of the striatum, as compared with the control (Fig. 6B, D). Comparison of the relative mean fluorescence intensities of segments representing the mantle zone revealed significantly higher signals in segments 9–18 after CS degradation than under control conditions, which was even more prominent than after blocking the Nrp-1 receptors (Fig. 6C, D; Student’s t-test; \(* P < 0.05; ***, P < 0.01\)). Comparison of the fluorescence intensities

in the SMZ after blocking Nrp-1 function with the fluorescence signals obtained after CS cleavage demonstrated that ChABC treatment caused a stronger invasion of the SMZ by EGFP-MGE cells (Fig. 6E; Student’s t-test; \(P < 0.05\)). As Sema 3A binds to the CS side chains in situ (Figs 3 and 4), we presumed that CS cleavage also decreases extracellular Sema 3A in the grafting experiments. Thus, the increased number of cells after ChABC treatment seems to be due to the summed effect of the removal of both repellents rather than to the effect of CS alone, suggesting a functional interaction between CS and Sema 3A. To rule out that the enhanced invasion of the SMZ after ChABC treatment is attributed only to a CS-induced repulsion, we combined both conditions: blocking Nrp-1 signaling and removing CS side chains. If Sema 3A binds to CS and therefore is also removed by CS cleavage, the migration pattern of the EGFP-MGE cells after applying ChABC and the anti-Nrp-1 antibody should be very similar compared with the CS cleavage only. Indeed, the fluorescence intensities of the EGFP-MGE cells in the distinct segments after ChABC treatment did not differ from the data obtained after combining CS cleavage and Nrp-1 blocking (Supplementary Fig. 6; no statistical differences with Student’s t-test). These results indicate that the removal of both Sema 3A and CS by ChABC is responsible for the inappropriate invasion of the MGE cells in the SMZ.

Finally, we used another approach to examine if the interaction between CS and Sema 3A leads to an increase of the repellent activity for cortical interneurons. In an attempt to mimic the in vivo condition, we performed a stripe assay with CSPGs and control protein and added 5 μg/ml soluble Sema 3A-Fc to the medium. As previously described, about 64 ± 1.3% of MGE neurons avoided the CSPG stripes with control medium (Fig. 2B). In the presence of soluble Sema 3A-Fc in the medium, the percentage of E13 MGE cells that avoided the CSPG lanes significantly increased.
by about 10% (Fig. 7A–B), compared with the control condition (Fig. 7C; unpaired Student’s t-test; \( P < 0.001 \); 3 independent experiments). Therefore, the repellent potential of the CSPG stripes is increased by the presence of soluble Sema 3A. Taken together, these data support the idea that CS–Sema 3A interactions offer an important regulating mechanism during tangential migration by repelling cortical interneurons from the SMZ.

**Discussion**

Our study suggests a novel role for CSPGs in the regulation of tangential migration of cortical GABAergic interneurons. CS is expressed at embryonic stages in the SMZ, a nonpermissive region for cortical interneurons during tangential migration. Using different assays, we demonstrated that CS acts as a repulsive cue for cortical interneurons, directing their trajectories away from the SMZ and toward the cortex. Based on the coexpression of CS with Sema 3A in the striatal anlage, we investigated a possible interaction between these molecules. Using a number of different in vitro and biochemical approaches, we showed that Sema 3A binds to CS and that secreted Sema 3A in the striatal anlage was reduced after removal of CS by ChABC treatment, indicating binding of secreted Sema 3A to CS side chains. Functional assays further revealed that CS and Sema 3A act in concert repelling cortical interneurons from the SMZ during their route to the neocortex. We therefore suggest 2 functions of CS in the regulation of tangential migration of cortical interneurons. 1) CS acts as a repellent cue for cortical interneurons by itself and therefore provides a novel signaling molecule that prevents cortical interneurons from entering the striatum. 2) CS binds Sema 3A in the SMZ and localizes the secreted protein in the SMZ. Binding of Sema 3A by CS increases the repulsive strength of the mantle zone to migrating cortical interneurons.

**Regulation of Tangential Migration of GABAergic Cortical Interneurons**

The tangential migration of cortical interneurons is a tightly regulated process by which migrating neurons follow well-defined
routes from the AEP and MGE to the cortex. The integration of attractive signals and repulsive guidance molecules is required for the regulation of this process. In the basal telencephalon, cortical interneurons migrate along a corridor that expresses a membrane-bound form of neuregulin-1, which is suggested to be a permissive cue for cortical interneurons (Hames et al. 2004). Ephrin-A5 and Slit proteins are expressed in the VZ flanking the corridor and exert repellent effects on cortical interneurons in vitro (Zhu et al. 1999; Zimmer et al. 2008). The semaphorin family of molecules, notably Sema 3A and Sema 3F, are reportedly expressed in the striatal anlage and mediate the exclusion of Nrp-1 and Nrp-2-expressing cortical interneurons from the SMZ (Marin et al. 2001; Tamamaki et al. 2003). Nrp-1 is known to mediate the repellent effect of Sema 3A, whereas Nrp-2-transduces the Sema 3F signal (Raper 2000; Tamagnone and Comoglio 2000). In situ hybridization revealed that Nrp-1 is expressed in the channel between VZ and the SMZ, whereas Nrp-2 is found ventrally (data not shown; Le et al. 2007). In addition, most cells of the MGE prepared from the VZ/upper SVZ were Nrp-1 positive (data not shown). There is very little overlap between Nrp-2 and the homeoproteins Dlx1 and Dlx2 (Tamamaki et al. 2003; Le et al. 2007), whose function is necessary for the migration of 75% cortical interneurons (Anderson et al. 1997). Nrp-2 is actually known to be repressed by Dlx1 and Dlx2 (Le et al. 2007). Therefore, we focused only on Sema 3A-CS interactions in our study.

**CSPGs as Repulsive Guidance Molecules during Development of the Nervous System**

We found that CS side chains repel cortical interneurons, indicating that they act as guidance molecules for migrating cells. CSPGs have been reported to function as axonal guidance molecules during development, as growing axons generally avoid CS-rich regions (Snow et al. 1990; Oakley and Tosney 1991; Pires-Neto et al. 1998; Braga-de-Souza and Lent 2004; Wang et al. 2008). Enzymatic removal of CS side chains using ChABC or treatment with exogenous CS causes targeting defects of motor, retinal, and forebrain axons (Brittis et al. 1992; Anderson et al. 1998; Bernhardt and Schachner 2000; Chung et al. 2000; Walz et al. 2002). Furthermore, various studies showed that CSPGs inhibit neuronal outgrowth in vitro, mediated by the core protein (Milev et al. 1994; Schmalfeldt et al. 2000) and their CS chains (Dou and Levine 1994; Yamada et al. 1997; Wang et al. 2008). This supports our finding that CS acts as a repellent signal for cortical interneurons. However, how CS induces repulsion in these interneurons and which receptors might be involved remains to be elucidated. As it was already described that sulfated polysaccharides interact with Nrp-1 (Narazaki et al. 2008) and that CS can modify Nrp-1 in nonneuronal cells (Shintani et al. 2006), Nrp-1 might be a receptor candidate which mediates the effects of CSPG. However, blocking Nrp-1 with an antibody in the stripe assay did not abolish the CS-induced repulsion (data not shown).

**Interactions of PGs with Other Signaling Molecules**

The data obtained from the stripe assay and the chemotaxis migration assay (Fig. 2) suggested that CS acts as a repellent for cortical interneurons by itself. We further showed that a fraction of CS binds Sema 3A (Fig. 3 and Supplementary Figs 3 and 4). Using a carbohydrate microarray-based approach, Shipp and Hsieh-Wilson (2007) recently reported that HS and CS interact with guidance proteins such as Slit-2, netrin-1, ephrin-A1 and A5, and Sema 5B in a sulfation-dependent manner. Other groups also showed that HS binds to Slit-2 (Liang et al. 1999; Ronca et al. 2001). De Wit et al. (2005) further revealed an interaction between Sema 3A with HS and CS. Sema 3A-GFP-transfected neuro-2a cells secreted this protein, which became visible as a punctate staining at the surface and bound to HS and CS. This interaction between CS and Sema 3A-GFP is at least partially specific, as soluble CS-A and CS-B could compete to bind Sema 3A, whereas CS-C could not (Smith-Thomas et al. 1994; Lander et al. 1998; Laabs et al. 2007). We also observed a punctate staining using a Sema 3A antibody, suggesting that Sema 3A protein is secreted in the striatal anlage. This punctate staining colocalizes with CS and was decreased after ChABC digestion, indicating that extracellular Sema 3A interacts with CS in the striatum. Moreover, coimmunoprecipitation experiments confirmed a binding of Sema 3A to CS. Whether the binding occurs after secretion of Sema 3A or whether they are secreted together and which cell type is producing CS remains to be elucidated, as both glial cells and neurons were reported to secrete CS (Smith-Thomas et al. 1994; Lander et al. 1998; Laabs et al. 2007).

What could be the functional implications for the interaction of guidance molecules with PGs? HS and CS have been shown to interact with secreted guidance cues such as netrin-1, Slit-2, and Sema 3A (De Wit et al. 2005; Shipp and Hsieh-Wilson 2007). Binding of these molecules to distinctly expressed PGs could lead to regionally restricted localization of these otherwise diffusible proteins, increasing their local concentration or shaping their gradients in the developing brain. Thus, binding of secreted Sema 3A to CS could restrain Sema 3A to the SMZ, where it would then repel cortical interneurons bearing the Nrp-1 receptor (Marin et al. 2001). When CS is enzymatically removed from living slices of the mouse forebrain, both the neurite repellent effects of the cortical plate and neurite-promoting effects of the intermediate zone for thalamic axons are abolished (Emerling and Lander 1996). These authors argued that these opposing activities reflect the presence of differentially localized CS-binding molecules rather than the direct activity of different CS. They suggested that CSPGs would be involved in the organization of extracellular matrix-bound cues in the cortex. Therefore, binding of PGs to distinct growth factors and guidance cues seems to be specific. Gama et al. (2006) demonstrated that certain sulfation motifs function as molecular recognition sites for growth factors. Furthermore, Bulow and Hobert (2004) showed that distinct HS modifications regulate different signaling pathways. Slit-Robo signaling requires HS modifications different from the HS modifications that are involved in integrin and ephrin signaling. As changes in the sugar residues create an enormous diversity, GAGs have been suggested to be the most informationally dense of biological molecules (Turnbull et al. 2001; Bulow and Hobert 2006). Thus, the interaction of different PGs containing specific sulfation motifs with distinct morphogens, growth and guidance molecules, would lead to a very diverse combinatorial code. Whether the interaction of Sema 3A with CS in the SMZ depends on different subclasses and sulfation motifs could not be solved in our assays, as ChABC cleaves nondistinctively CSA, CS-B, and CS-C side chains.

In addition to the organization of secreted guidance cues, PGs were reported to modulate the function of signaling molecules. Kantor et al. (2004) showed that the interaction of Sema 5A with HS via its thrombospondin repeats exerts an attractive effect, whereas Sema 5A acts as a repellent after binding to CS. Thus, the interaction with distinct PGs mediates...
a functional switch of Sema 5A from repulsive to attractive effects. Interactions between signaling molecules and PGs were also described to lead to a potentiation of activity. HS binds Slit-2, thereby increasing its repellent activity on migrating olfactory interneuron precursors (Liang et al. 1999; Hu 2001; Ronca et al. 2001). Moreover, binding of Sema 3A to Nrp-1 was promoted by heparin, and the collapsing activity of Sema 3A was increased (De Wit et al. 2005). One possible mechanism for these potentiating effects is the protection from degradation of signaling molecules by PGs, as reported for HS, leading to a potentiation of fibroblast growth factor signaling (Spivak-Kroizman et al. 1994; Schlessinger et al. 2000).

We found that the repellent potential of the SMZ is more affected by removal of both signaling molecules, Sema 3A and CS, than by blocking Sema 3A-Nrp-1 signaling alone. In addition, we observed that the repulsive activity of CS was enhanced with soluble Sema 3A-Fc in the stripe assay. Therefore, the repellent function of both groups of signaling molecules seems to be important for the proper guidance of cortical interneurons in the ventral telencephalon. Whether CS and Sema 3A activate 2 different signifying pathways via distinct receptors, resulting in a stronger repellent activity, or whether CS stabilizes the Nrp-1 binding to Sema 3A, as described for heparin (De Wit et al. 2005), remains to be clarified. But as CSPGs were reported to interact with neural cell adhesion molecules (Friedlander et al. 1994; Grumet et al. 1994) and the cell adhesion molecule L1 has already been proven necessary for Sema 3A-induced repulsion in cortical axons (Castellani et al. 2002), CS could represent an additional component stabilizing the Sema 3A receptor complex.

In conclusion, our experiments reveal that CS anchors Sema 3A in the SMZ and that both molecules act together in preventing interneurons from invading the SMZ and therefore play a critical role in regulating the tangential migration of these neurons in the ventral telencephalon.

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Supplementary Material

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

Notes

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