Cerebral Cortex July 2007;17:1712–1721
doi:10.1093/cercor/bhl082
Advance Access publication October 4, 2006

Functional Interaction between Matrix Metalloproteinase-3 and Semaphorin-3C during Cortical Axonal Growth and Guidance

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In the developing cortex, axons and dendrites extend progressively in response to environmental cues attracting or repelling growing processes. Recent evidence suggests the existence of a functional link between guidance molecules and metalloproteinases. Here, we analyzed the putative functional interaction of matrix metalloproteinases (MMPs) with guidance cues of the semaphorin family during growth and guidance of cortical axons. Our results demonstrate that the expression pattern and the proteolytic activity of MMP-3 are consistent with a role of this particular MMP during cortical axon outgrowth. We found that MMP-3 is required for an optimal axon extension and is involved in the Sema3C-dependent chemoattraction of cortical axons by modulating both the growth capacity and the orientation of growth. Interestingly, the inhibitory Sema3A decreased both the expression and activity of MMP-3. Taken together, our results reveal a molecular interaction between MMPs and semaphorins providing new insight into the molecular mechanism allowing axonal growth cone to respond to environmental guidance cues in the context of cortical development.

Keywords: axon guidance, axon outgrowth, cortex, development, matrix metalloproteinase, semaphorin

Introduction

The development of the cortical connectivity requires sophisticated mechanisms controlling neurite outgrowth. During embryonic development in the mouse, cortical layers are progressively established within the last week of gestation. Each wave of postmitotic neurons reaches its final laminar position before axonal and dendritic projections start to be established. Once neurons are appropriately located, they achieve their differentiation by growing an axon and several dendrites with stereotyped orientations. This process is under the control of environmental signals determining the growth and the orientation of the neuritic extensions. Recent progress identified several families of guidance molecules such as netrins, ephrins, or semaphorins, attracting or repelling growing axons by the regulation of cytoskeletal dynamics (Dickson 2002; He et al. 2002). Indeed, members of the semaphorins' family are key regulators of axon outgrowth in the cortex (Bagnard et al. 1998, 2000, 2001; Polleux et al. 1998, 2000) where semaphorins act as bifunctional chemoattractant (Sema3C) or chemorepellent (Sema3A) guidance molecules for cortical axons. The signaling cascade leading to axonal growth inhibition by semaphorins is being well understood (Castellani and Rougon 2002; Fiore and Puschel 2003). Receptor complexes including neuropilins, plexins, adhesion molecules, or vascular endothelial growth factor receptors are selectively recruited to ultimately control the cytoskeletal dynamics. On the other hand, little is known about the signaling cascade mediating neurite elongation in response to positive-growth–promoting semaphorins.

Recent findings indicate that matrix metalloproteinases (MMPs), a family of proteolytic enzymes performing different functions in developing and mature tissues (Chang and Werb 2001), might be crucial components of the response to guidance cues in the nervous system (McFarlane 2003). MMPs are important in numerous cellular behaviors ranging from cell proliferation, differentiation, migration, and extracellular matrix remodeling (Chang and Werb 2001). In the central nervous system (CNS), neuronal proteinases are important for axon elongation by modulating cell–cell and cell–matrix contacts (Seeds et al. 1997). Several studies indicate that MMPs are necessary for appropriate axon elongation both in vitro and in vivo (Fawcett and Housden 1990; Muir 1994; Sheffield et al. 1994; Fambrough et al. 1996). Nevertheless, the biological functions of metalloproteinases are more complex as suggested by compelling evidence for a role in axon guidance (Galko and Tessier-Lavigne 2000; Hattori et al. 2000; Webber et al. 2002). Moreover, MMP-9 expression and activation contribute to the remodeling of dendrites in the adult hippocampus (Szklarczyk et al. 2002). Hence, because of their well-established functions in the CNS we decided to investigate the role of MMPs in the developing E15 cortex as well as their potential interactions with guidance cues of the semaphorins' family shown to be key regulators of cortical efferent fibers development.

Several studies have clearly demonstrated the role of MMPs during developmental processes in the cerebellum (Vaillant et al. 1999) or process outgrowth (Oh et al. 1999; Ferguson and Muir 2000; Duchossoy et al. 2001; Szklarczyk et al. 2002). Here, we focused our interest on the role of MMP-3 (stromelysin-1) during cortical development, whereas most of the studies have shown MMP-3 to be expressed in the brain in various pathological conditions including for example traumatic brain injuries or cerebral ischemia (Sole et al. 2004; Kim et al. 2005). Our investigations reveal that MMP-3 is expressed and activated in the developing cortex. Immunocytochemical experiments, enzymatic activity assays, and functional growth assays suggest a selective role of MMP-3 in axon extension. Further findings indicate that the chemoattractive effect of Sema3C on cortical axons requires the upregulation of MMP-3 expression and activity both for axon growth promotion and orientation.

Methods

Histology and Immunocytochemistry

E15 mouse brains were fixed at 4 °C during 24 h in a 4% formaldehyde solution balanced with phosphate buffered saline (PBS: NaCl 140 mM + Na_2HPO_4 $2H_2O$ 9 mM + Na_2PO_4 H_2O 1.3 mM) and 20% sucrose. Fixed brains were sliced (70 μm thickness) using a vibratome (Leica VT 1000S, Paris, France). The sections were blocked with 3% bovine serum

albumin–tris buffer saline (BSA-TBS) for 30 min before incubation with the primary anti-MMP-3 antibody (Chemicon International, Hampshire, UK, ref. AB810, dilution 1:700) overnight at 4 °C under agitation. To verify the specificity of the signal, we performed a signal extinction test in which the primary antibody anti-MMP-3 (1:700 corresponding to 2 μg of antibody) was preincubated 1 h at 4 °C with 0.5 μg of purified MMP-3 (Sigma, Saint-Quentin Fallavier, France, ref. M1677). The sections were rinsed with TBS and incubated for 1 h at room temperature with the secondary antibody F(ab')2 anti-IgG (H + L) rabbit horse radish peroxydase (H.R.P.) (P.A.R.I.S., Compiègne, France, ref. BI 4407) at a final dilution of 1:200. After washing in TBS, the peroxidase activity was revealed by incubating the sections for 20 min at room temperature in a solution containing 0.018% chloronaphthol + 0.009% H_2O_2 in TBS. Fast freezing of the brains by direct immersion in −45 °C isopentane was carried out to prepare sections for in situ zymography and double immunostaining of MMP-3 and neurofilaments (NF). These sections were prepared using a cryostat (Reichert-Jung, Leica). Slices were blocked in 3%-BSA–TBS for 30 min at 37 °C before overnight incubation at 4 °C with primary antibodies (anti-MMP3 and/or anti-NF) diluted in 3%-BSA–TBS. Mouse monoclonal antibody against phosphorylated NF SMI312 (Sternberger Monoclonals Incorporated, Berkeley, California) was used to identify axons. MMP-3 and NF signals were revealed by the use of a secondary antibody coupled with the avidin–biotin–fluorochrome Cy3 (Biosys, Compiègne, France, ref. BI 4807, and Jackson ImmunoResearch Laboratories, Suffolk, UK, ref. 016-160-084 or secondary antibody coupled with Alexa 488 (Molecular Probes, Eugene, Oregon, ref. A-11017) respectively. Images were collected using a microscope equipped with a standard fluorescent device (Axiovert 200, Zeiss, Le Pecq, France) or a confocal microscope (LSM510, Zeiss).

In situ Zymography

To determine the enzymatic activity of MMPs in situ, we applied the method described by Oh et al. (1999). We used the fluorescein-conjugated casein (Molecular Probes) as a substrate of MMP-3. In situ zymography was carried out on E15 brain mouse sections (30 μm thickness) prepared with a cryostat (Reichert-Jung, Leica). The sections were isolated using Dako Pen (DAKO) and incubated for 5 min with reaction buffer (Tris-HCl 50 mM + NaCl 0.15 M + $CaCl_2$ 5 mM + 0.2 mM sodium azide: pH 7.6). Reaction mix consisting of 50 μl of reaction buffer + 50 μg/ml of substrate (fluorescein-5-isothiocyanate-casein) was added to the section for 24 h in a wet darkroom at 37 °C. Negative control was obtained by the addition of 50 μM 1,10-phenantrolin to the reaction solution. We also used a specific inhibitor of MMP-3 (Calbiochem, MMP-3 inhibitor I, from VWR INTERNATIONAL S.A.S, Fontenay sous Bois, France) (Fotouhi et al. 1994) at the final concentration of 5 μM. Sections were mounted in Aqua Polymount (Poly Sciences, Eppelheim, Germany) without washes. The observations were performed with an inverted optical microscope (Axiovert 200M, Zeiss). The quantification of the fluorescence was determined using Metaview (Universal Imaging Corporation, Evry, France). In some experiments, in situ zymography was combined to immunohistochemistry. In this case, the zymographic reaction was performed during primary antibody incubation (using anti-SMI312 antibody, see details above) by addition of appropriate buffers.

Gel Zymography

MMP-3 activity was also analyzed on gelatin-containing gels (12.5% polyacrylamide gel containing 0.675 mg/ml gelatin). Following electrophoresis, gels were washed with 2.5% (v/v) Triton-X 100 for 15 min under smooth agitation at 37 °C to remove SDS. Finally the gels were placed in a MMP activation buffer (Tris-base 100 mM, $CaCl_2$ 15 mM, pH = 7.4) and incubated 20 h at 37 °C under smooth agitation to allow MMP substrate proteolysis. The gels were stained with 0.1% (w/v) Coomassie Brilliant Blue-R250 (30% [v/v] methanol, 10% [v/v] acetic acid in water) before discoloration in 30% (v/v) methanol, 10% (v/v) acetic acid in water without Coomassie blue to obtain a uniform background coloration except in regions where MMPs had migrated and cleaved the gelatin. Numerical pictures of the gels were taken with a Nikon Coolpix 990 and the images were contrasted using Gimp 2.2 software.

Western Blot Analysis of MMP Expression

Western blots were performed on fresh conditioned culture media obtained from coculture experiments or dissociated cultured E15 neurons. The different fractions were probed with the same antibodies used for immunohistochemistry (Chemicon International, ref. AB810). Samples migrated in 10% polyacrylamide gels for 2 h at 25 mA before transfer on a nitrocellulose membrane (Protan, Perkin Elmer Life Sciences, Courtaboeuf, France, ref. NBA085C) for 1 h at 250 mA. Membranes were blocked in 5%-milk-TBS for 30 min and incubated with the primary antibody (dilution 1:5000) overnight at 4 °C under agitation. After several washes with TBS, the membranes were incubated with the secondary antibody F(ab')2 anti-IgG (H + L) rabbit H.R.P. (P.A.R.I.S., ref. BI 4407) at a dilution of 1:2000 in TBS for 1 h at room temperature. After washing in TBS, the blots were revealed with Super-Signal West Dura Extended Duration Substrate (Pierce, Rockford, Illinois, ref. 34076). Protein concentration was determined in the different samples by the Bradford method. Equal loading was verified by red Ponceau staining of the proteins in the absence of known referenced protein secreted at a constant concentration in culture medium.

MMP-3 Enzyme-Linked Immunosorbent Activity Assays

MMP-3 enzymatic activity was determined in conditioned media using the MMP-3 Biotrak Activity Assay System (Amersham Biosciences, Buckinghamshire, UK, ref. RPN2639). The whole procedure to measure the total activity of MMP-3 was performed according to manufacturer's instructions. Briefly, enzyme-linked immunosorbent activity (ELISA) plates were incubated with culture media overnight at 4 °C. The plates were then washed and incubated with a reaction solution containing a specific substrate of MMP-3 at 37 °C and *p*-aminophenylmercuric acetate to activate all the pro-MMPs. To determine the specificity of MMP-3 inhibitor, we used 2 ELISA activity assay kit (Amersham Biosciences, ref. RPN2639 for MMP-3 activity and ref. RPN2631 for MMP-2 activity). Purified MMP-2 or MMP-3 standards were incubated with increasing concentrations of MMP-3 inhibitor. The plates were read with an optical plate reader (Labsystems Multiskan MS, Elsinki, Finland) (see supplemental data). Function blocking experiments were performed by using neuropilin-2 (NRP2) rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) raised against a recombinant peptide corresponding to amino acids 560–858 mapping within the extracellular domain of NRP2 of human origin. This so called meprin/A5/μ-phosphatase domain is considered to be important for biological function of NRP2 (Giger et al. 1998). Nasarre et al. (2003) have previously shown the efficacy of this antibody to block NRP2 and thereby inhibit semaphorin signaling.

Cell Culture and Neocortical Explants

Cortical neurons were prepared from E15 mouse embryos as previously described (Hanbali et al. 2004). Cerebral hemispheres were dissected in cold Gey's Balanced Salt Solution-glucose in order to obtain blocks of neocortex. The blocks of neocortex were placed in 5 ml of a solution of Hank's Balanced Salt Solution/glucose + 0.25% of trypsin for 20 min under agitation at 37 °C. A volume of 5 ml of culture medium with serum (2 mM L-glutamine, 1 mg/ml glucose, 0.5 unit/ml penicillin, 0.5 μg/ml streptomycin, 10% fetal calf serum [FCS] in DMEM [Dulbecco' s Modified Eagle Medium], Gibco, Cergy Pontoise, France) was added in order to stop trypsin. The cells were centrifuged 5 min at 1000 × *g* to obtain a pellet recovered in 1 ml of culture medium with serum. The cells were dissociated mechanically using a Gilson pipette, filtered through 48-μm-diameter pores and again centrifuged. A cellular counting was carried out to allow a homogeneous distribution of 2×10^5 cells per well. Cortical neurons were grown on glass coverslips covered with a neutral substrate of poly-L-lysine (100 μg/ml). After 12 h in culture, the culture medium was changed with fresh serum-free medium consisting of 2 mM L-glutamine, 1 mg/ml glucose, 0.5 unit/ml penicillin, 0.5 μg/ml streptomycin, 16 μg/ml putrescein, 52 ng/ml selenium sodium, 10 μg/ml transferrin, 5 μg/ml insulin, and 3 ng/ml progesterone in DMEM. At this stage, neurons were treated with pharmacological inhibitors of MMPs or with Sema3A ± inhibitors. An immunostaining of phosphorylated NF (see immunocytochemistry section) was performed on the cells to identify axonal processes. Axon length was determined using Image Tool software.

HEK-C293 cells were cultured in MEM with 5000 U/ml penicillin, 5 mg/ml streptomycin, 2 mM L-glutamine, and 10% FCS (Gibco). Aggregates of cells stably expressing recombinant semaphorins (Sema3A or Sema3C) were formed as described previously (Bagnard et al. 1998). Cell aggregates were cocultured with cortical explants prepared from blocks of neocortex cut into cubes of 200 μm using a tissue chopper (McIlwain,

Harvard Apparatus, Les Ulis, France). HEK-C293 cell aggregates and cortical explants were placed in 20 µl chicken plasma on a glass coverslips. During coagulation of the clot with 20 µl thrombin, explants were arranged around aggregates at a distance of 100–500 µm. After 30–45 min, coverslips were placed in 30-mm dishes containing 2 ml of culture medium consisting of 50% Eagle's basal medium, 25% Hank's balanced salt solution, and 25% horse serum supplemented with 0.1 mM glutamine and 6.5 mg/ml glucose (all from Gibco) and transferred to the incubator (37 °C, 5% CO_2). To determine the involvement of the MMPs we used 1,10-phenantroline or the large spectrum inhibitor of MMPs GM6001 (Galardin, Calbiochem) and its inactive control (here called GM1006, *N-t*-butoxycarbonyl-L-leucyl-L-triptophan methylamide) at 1–10 µM, and selective inhibitors of MMP-2 and -9 (MMP-2, -9 inhibitor I: (2*R*)-2-[(4-biphenylylsulfonyl)amino]-3-phenylpropionic acid; Calbiochem see also Tamura et al. 1998) at the concentration of 620 nM or MMP-3 (MMP-3 inhibitor I: Ac-RCGVPD-NH_2; Calbiochem see also Fotouhi et al. 1994) at 1 µM. These inhibitors were added directly to the culture medium at the beginning of the culture in serum-free medium. After 48 h in vitro, outgrowth was sufficiently progressed and cultures were fixed in 4% formaldehyde and then analyzed. Guidance effects of semaphorins were quantified in the cocultures by attributing −1 to explants experiencing repulsion, 0 for explant with a symmetrical outgrowth, and +1 to explants showing a preferential outgrowth toward the cell aggregates secreting semaphorins (Castellani et al. 2000). Camera lucida drawing of axons emerging perpendicular to the semaphorin gradients was performed to determine the turning angles of growing axons as previously described (Bagnard et al. 1998). Statistical analysis was performed using a Student's *t*-test for neuritic length and χ^2 analysis for cocultures.

Results

Cortical Neurons Express MMP-3 at E15

To address the role of MMP-3 in the development of cortical projections, we performed immunohistochemical experiments with a specific antibody recognizing MMP-3. As seen in Figure 1(*b*), a strong signal was detected in the entire thickness of the neocortex. The strongest expression was observed in the ventricular zone and the cortical plate together with an intense staining of growing axons in the intermediate zone. Specificity control of the staining was performed by preincubating the anti-MMP3 antibody with a purified version of MMP-3 before application onto the slices. As expected, signal intensity was drastically reduced thereby demonstrating the specificity of the observed staining (Fig. 1*c*). Double immunostaining with the axonal marker NF revealed that all axons detected in the intermediate zone expressed MMP-3. Colocalization profiles (Fig. 1*g*) between MMP-3 (Fig. 1*d*) and NF (Fig. 1*e*) obtained from confocal microscopy (overlay, Fig. 1*f*) confirmed the axonal localization of MMP-3.

Enzymatic Activity of MMP-3 in the Developing Cortex

Although the immunohistochemical localization revealed that cortical neurons express MMPs, it is necessary to show that MMP-3 is activated and exerts a proteolytic activity along cortical axon pathway. To address this issue we performed in situ zymography combined to immunocytochemical labeling of cortical axons. As depicted in Figure 2, NF-positive axons were surrounded by a fluorescent signal corresponding to the degradation of casein by secreted MMPs. The specificity of the signal was verified by experiments in which a general pharmacological inhibitor of MMPs (1,10-phenantroline, Fig. 2*e*) or a selective MMP-3 inhibitor (Fig. 2*f*) was added to the slices. Quantitative analysis of the fluorescent signals showed a significant decrease of fluorescence intensity in the presence of 1,10-phenantroline thereby confirming the implication of MMPs in this proteolytic activity in the developing cortex. Despite the

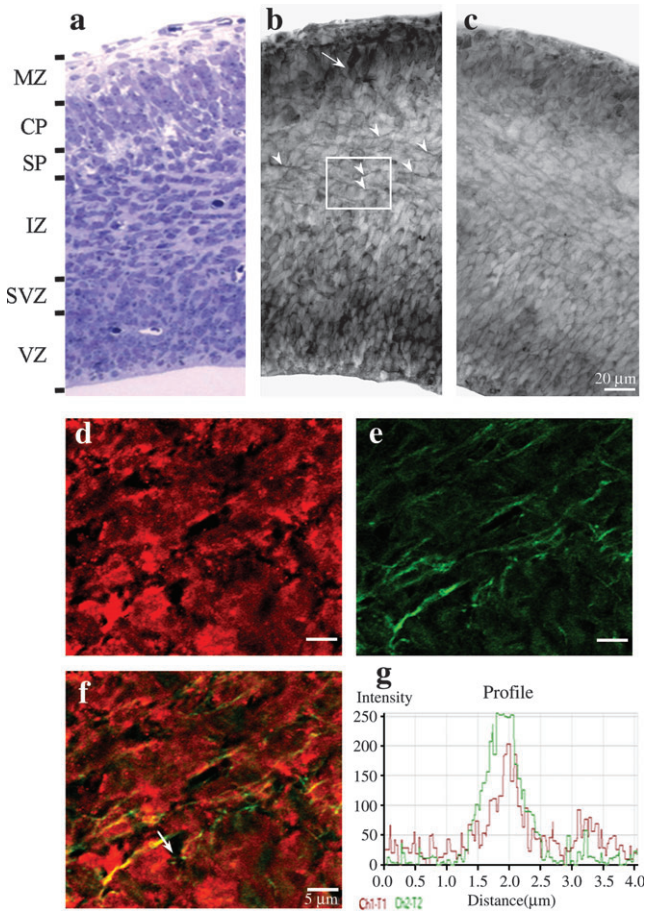

Figure 1. Expression pattern of MMP-3 in the mouse neocortex at E15. (*a*) Cytoarchitectural organization of the E15 neocortex visualized by semithin section of E15 neocortex stained with toluidin blue. (*b*) Immunostaining of MMP-3 in the E15 neocortex. MMP-3 expression was detected in the entire thickness of the cortical plate but is particularly strong in the layer 6 (white arrow) and in axons of the intermediate zone (white arrow head). (*c*) Control of the signal specificity was performed in signal extinction test in which anti-MMP-3 antibody (2 µg) was preincubated with purified MMP-3 (0.5 µg). The decrease of the signal between pictures *b* and *c* demonstrates the specificity of the staining. (*d–f*) Microphotographs were obtained from confocal microscope analysis of the double staining for MMP-3 (*d*), and NF (as an axonal marker) in axons growing in the intermediate zone (*e*). (*g*) Colocalization profiles of MMP-3 and NF signals demonstrating the presence of MMP-3 in the axons. MZ: marginal zone; CP: cortical plate; SP: subplate zone; IZ: intermediate zone; SVZ: subventricular zone; VZ: ventricular zone.

widespread and intense MMP activity observed in all of the 3 regions of the cortex analyzed (Fig. 2*b–j*), we were able to extract the fraction of the fluorescent signal due to the specific activity of MMP-3 in the cortical plate by the use of the selective pharmacological inhibitor of MMP-3 that induced a significant 15% ($P < 0.01$, Student's *t*-test, $n = 32$) reduction of the fluorescent intensity (Fig. 2*f,g*). This significant decrease was measured in the cortical plate (−23%, $P < 0.01$, Student's *t*-test, $n = 211$), the ventricular zone (−11%, $P < 0.01$, Student's *t*-test, $n = 184$), and, interestingly in the intermediate zone (−20%, $P < 0.01$, Student's *t*-test, $n = 221$) (Fig. 2*b–j*). Thus, MMP-3 is expressed in vivo by cortical neurons and is activated during their growth process.

MMP-3 is Required for Cortical Axon Outgrowth

To determine the function of MMP-3 in growing cortical axons, we performed in vitro experiments with cultured embryonic

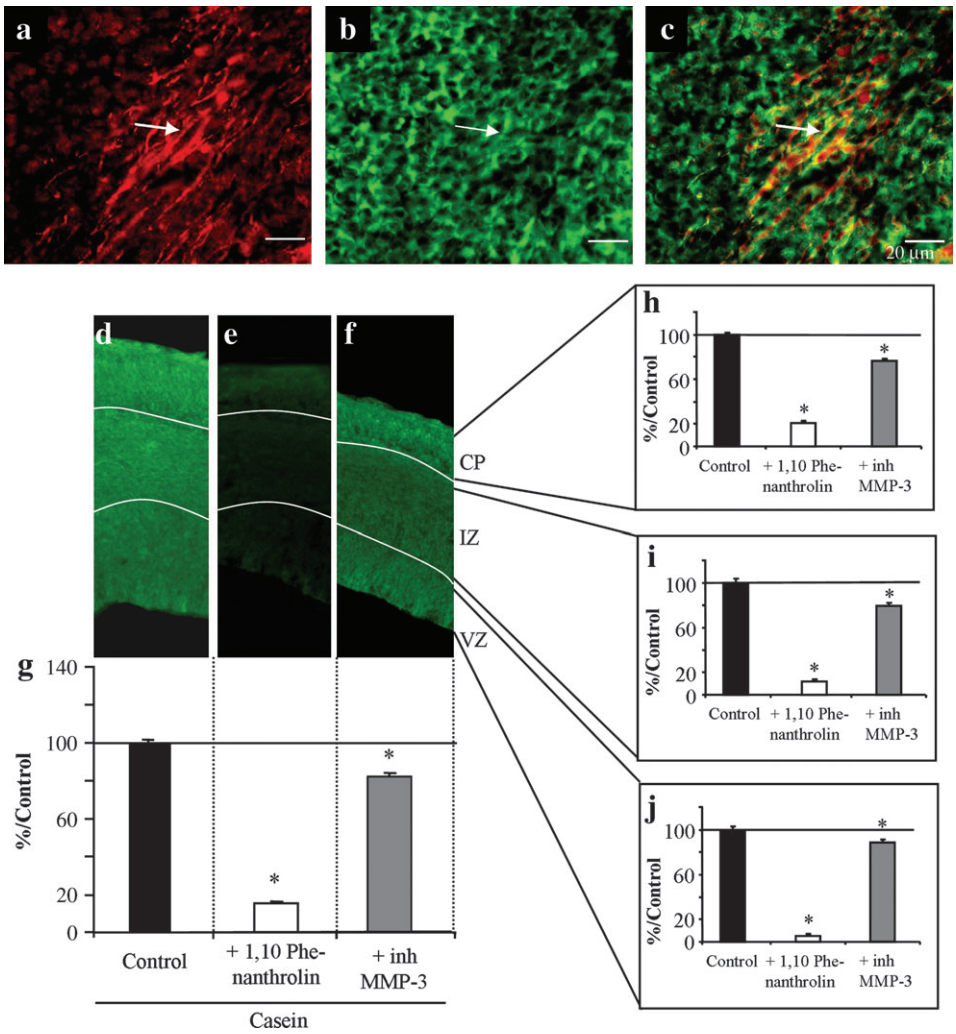

Figure 2. (*a–c*) Colocalization of MMP-dependent caseinolytic enzymatic activity (viewed by in situ zymography) with NF (viewed by immunocytochemistry) in E15 neocortex. (*a*) The immunostaining of the axons with anti-NF (*a*: white arrow shows an axon growing in the intermediate zone), (*b*) the corresponding zymographic signal, and (*c*) the overlay of the 2 images (*b*, *c*: white arrow indicates caseinolytic activity surrounding growing axon). (*d–j*) Quantification of the MMP activity revealed by in situ zymography performed with fluorescein-5-isothiocyanate (FITC)-conjugated casein. (*d–f*) Representative microphotographs of in situ zymography performed with FITC-conjugated casein on in (*d*) control condition, (*e*) in the presence of 1,10-phenantroline (general MMP inhibitor), and (*f*) in the presence of a selective inhibitor of MMP-3. (*g–j*) Quantification of the zymographic signals in the entire cortical plate (*g*) or in 3 different subregions (*h–j*). CP: cortical plate, IZ: intermediate zone, VZ: ventricular zone. $*P < 0.01$ Student's *t*-test.

cortical neurons. As seen in Figure 3, the expression of MMP-3 was maintained in culture and a strong proteolytic activity could be detected along the axons and at the level of the growth cones. Cells were grown on a neutral substrate of poly-L-lysine for 12 h in the presence of serum. During the following 24-h period of culture, cortical neurons were placed in serum-free medium and were subjected to various pharmacological treatments. In a first set of experiments, we analyzed axon outgrowth when the MMP activity was blocked by GM6001, a large spectrum inhibitor of MMPs. Axons were stained with an anti-NF antibody and measured using image analysis software. The results showed a dose-dependent decrease of axon length in the presence of GM6001 (Fig. 4*a*). The control experiments were performed with GM1006, the inactive analog of GM6001. To characterize the particular function of MMP-3 in cortical axons outgrowth, we used a selective pharmacological inhibitor (Fotouhi et al. 1994). The use of this MMP-3 inhibitor induced a 30% ($P < 0.01$, Student's *t*-test, results obtained in 4 independent experiments) reduction of axon length, a statistically significant decrease comparable with the one obtained with the large spectrum inhibitor of MMPs (Fig. 4*b*). Interestingly, axon length was not influenced by the addition of an MMP-2/9 inhibitor. Thus, these experiments showed that cortical axons require an MMP-dependent proteolytic activity to extend properly. This MMP-dependent effect is highly specific and is mostly due to MMP-3.

Sema3A and Sema3C Modulate the Expression of MMP-3

We previously showed that Sema3C is a chemoattractant for growing cortical axons in the developing cortical plate, whereas Sema3A induces axon retraction and repulsion (Bagnard et al. 1998). Hence, we analyzed the potential role of MMP-3 during cortical axon outgrowth in the presence of the growth inhibitory factor Sema3A and the growth-promoting factor Sema3C. To this end, we compared the potential variations of the level of expression of MMP-3 by western blot analysis after treatment with the 2 antagonistic semaphorins, Sema3C and Sema3A (Fig. 5). This approach revealed a significant increase of

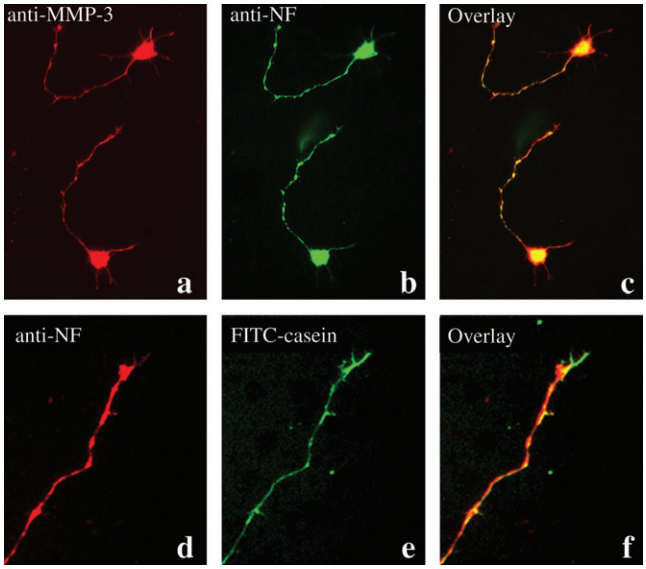

Figure 3. (*a–c*) Expression of MMP-3 in dissociated cultured cortical E15 neurons. Fluorescent images of double staining for MMP-3 (*a*), NF (*b*), and overlay (*c*) confirmed the expression of MMP-3 in axons. (*d–f*) Colocalization of MMP-dependent caseinolytic enzymatic activity (viewed by in situ zymography) with NF (immunocytochemistry) in E15 cultured neurons.

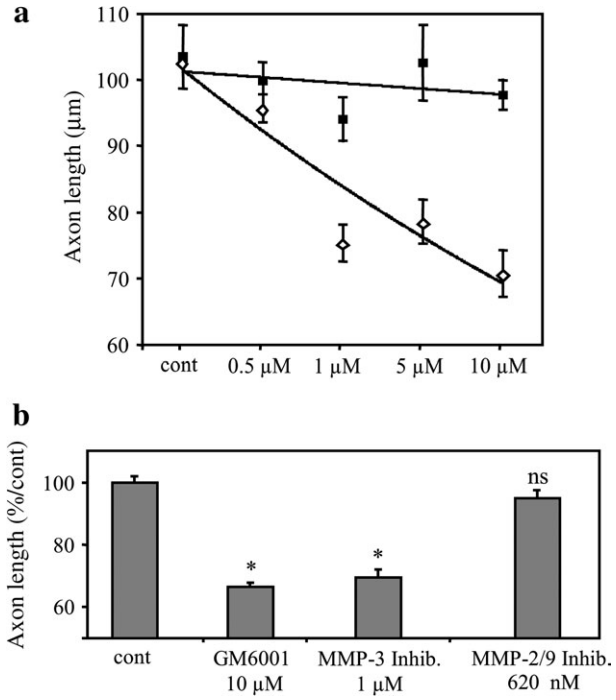

Figure 4. MMP-dependent axonal outgrowth. (*a*) Cortical neurons were grown in the presence of the large spectrum MMP inhibitor GM6001 (open squares) or its inactive control (GM1006, dark squares). (*b*) Cultures were treated with potent inhibitors of MMP-3 or MMP-2/9. (*$P < 0.01$ Student's *t*-test, ns not significant).

MMP-3 expression (+18.4 ± 3.9%, $P < 0.01$, Student's *t*-test) when cortical neurons were treated with Sema3C (Fig. 5*a*). Interestingly, this increase of MMP-3 expression was abolished in the presence of a function blocking antibody directed against NRP2, the functional receptor of Sema3C (Chen et al. 1997) (Fig. 5*b*). On the other hand, consistently with the induction of an axonal growth inhibition we detected a decrease of MMP-3 expression (–34.0 ± 4.4%, $P < 0.01$, Student's *t*-test, results obtained in 3 independent experiments) when cortical neurons were treated with Sema3A (Fig. 5*c*).

Sema3A and Sema3C Modulate the Enzymatic Activity of MMP-3

As previously mentioned, it is crucial to monitor the enzymatic activity of the proteins to fully address the potential function of MMPs. Thus, we performed a gel zymography to verify the existence of an MMP-3 activity in our culture system. A seen in Figure 6(*a*), we were able to detect MMP-3 activity in conditioned medium obtained from cultures of cortical explants. The positive control was performed with a purified MMP-3 and negative control was done with nonconditioned culture medium. To further validate the existence of a MMP-3 activity in our model and to monitor the modulation of activity in response to semaphorins, we took advantage of an ELISA-based activity assay. This very sensitive and specific method allowed us to confirm a basal level of MMP-3 activity in cortical neurons under control conditions. Strikingly, the addition of Sema3C induced a 2- to 3-fold increase of MMP-3 activity (+60.9 ± 18.9%, $P < 0.01$, Student's *t*-test). As expected from the western blot analysis, the function blocking antibody to NRP2 or Sema3A treatments significantly reduced MMP-3 activity (–100.9 ± 16.8%, –115.9 ± 14.6% respectively compared with Sema3C treatment, $P < 0.01$, Student's *t*-test, results obtained in 4 independent experiments). Thus, both the expression and activity of MMP-3 are modulated by semaphorins (Fig. 6*b*). The chemoattractant Sema3C increased expression and activity of MMP-3, whereas the chemorepellent Sema3A decreased expression and activity of MMP-3.

The Growth Promoting Effect of Sema3C Requires MMP-3 Enzymatic Activity

To explore the biological consequence of the modulation of MMP-3 expression and activity in response to semaphorins, we performed coculture experiments in which cortical explants were cocultured with cell aggregates secreting recombinant Sema3C or Sema3A. Under these conditions, numerous long axons extended preferentially from the side directly facing the cell aggregate expressing Sema3C whereas in control cultures (aggregates of mock transfected cells), axon outgrowth appeared radial and symmetric without any growth preference. Moreover, fibers were growing away from the Sema3A-secreting cell aggregates (Bagnard et al. 1998). Strikingly, addition of GM6001 suppressed the asymmetric growth triggered by Sema3C (Fig. 7*a*), leading to a radial outgrowth comparable with that observed in control cultures (cortical explants facing control cell aggregates in the presence of GM6001). In this assay, the concentration of MMP inhibitors (10 µM maximum) was chosen in order to maintain a reasonable axon extension (around 70 µm) to be certain that the lack of preferential growth is not due to a total loss of growth capacity but to the suppression of the growth-promoting effect dependent on an additional MMP activity triggered by Sema3C. Indeed, the use of the specific MMP inhibitors revealed that MMP-3 but not MMP-2/9 was involved in the asymmetric growth of cortical axons in response to Sema3C gradients (Fig. 7*a,b*). Moreover, the repulsing activity of Sema3A was still observed in the presence of

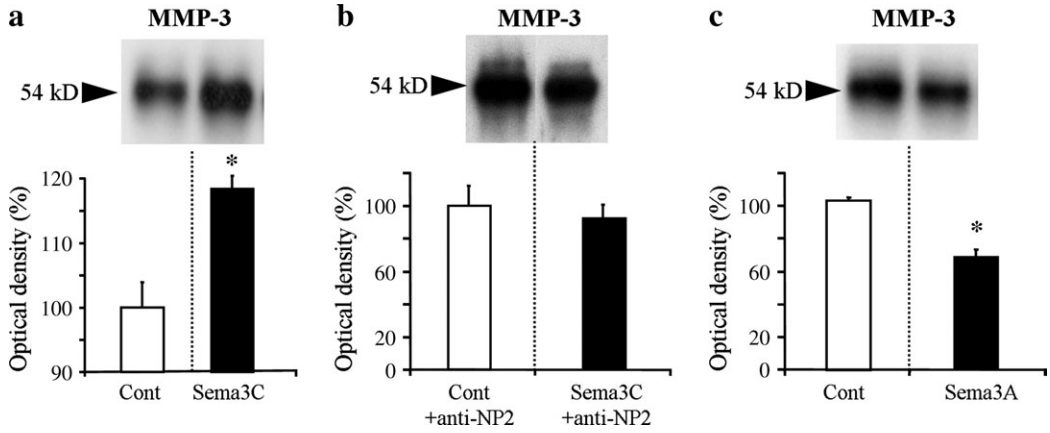

Figure 5. Western blotting analysis of MMP-3 expression in conditioned media obtained from coculture experiments. (*a*) The expression of MMP-3 was increased in the presence of Sema3C. (*b*) Addition of a function blocking antibody against NRP2, the ligand-binding subunit of Sema3C receptor, suppressed the increase of MMP-3 expression. (*c*) In presence of the inhibitory Sema3A, the level of MMP-3 expression was decreased (*$P < 0.01$ Student's *t*-test/control, $n = 3$/condition).

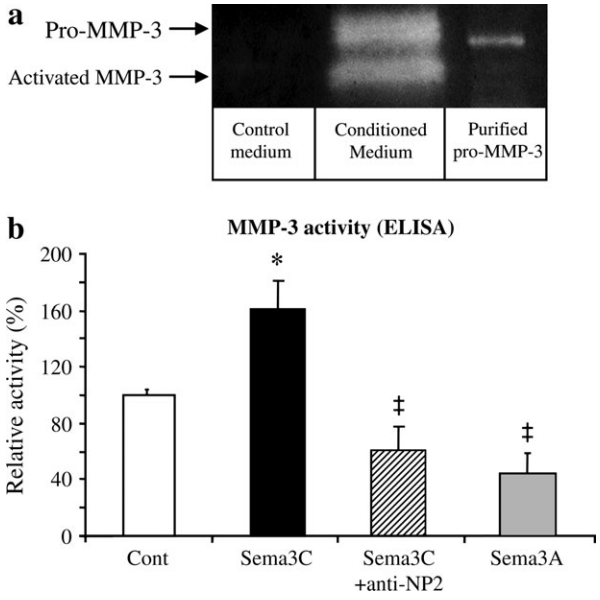

Figure 6. MMP-3 enzymatic activity assays. (*a*) Gelatin gel zymography were performed using control culture medium (nonconditioned culture medium as negative control), conditioned culture medium incubated with E15 neocortical tissue blocks or purified pro-MMP-3 (positive control). (*b*) Determination of MMP-3 enzymatic activity by ELISA assay. MMP-3 activity was determined in culture medium from cortical explants in control condition, in the presence of Sema3C, Sema3C, and anti-NRP2 function blocking antibody or when neurons were exposed to Sema3A gradients (*$P <$ 0.01 Student's *t*-test/control, ‡$P < 0.01$ Student's *t*-test/Sema3C condition, $n = 4$/condition).

all MMP inhibitors (Fig. 7*c*) thereby strengthening the existence of a specific functional link between MMP-3 and Sema3C to trigger cortical axons growth promotion.

Sema3C-Dependent Cortical Axon Orientation Requires MMP-3 Activity

To further characterize the importance of MMP-3 to exert the chemoattractant effect of Sema3C, we performed camera lucida drawing of axons growing perpendicularly to the gradients. Under these conditions, it is possible to measure the turning angle of growing axons and thereby analyze the guidance effect of the semaphorins (Fig. 8*a*). Our results show that in control experiments done with mock-transfected cell aggregates, axons exhibited no preferential direction of growth as seen by the equal distribution of turning angles in the attraction and repulsion quadrant (Fig. 8*b*). Strikingly, in the presence of the selective MMP-3 inhibitor axons were not preferentially oriented toward the source of Sema3C compared with what was observed without MMP-3 inhibitor (Fig. 8*c*). In the case of Sema3A, axons were growing away from the source of Sema3A with or without MMP-3 inhibitor (Fig. 8*d*). As expected, in all cases examined in the presence of MMP-3 inhibitor, axons were shorter. Thus, MMP-3 is required to induce the chemoattractant effect of Sema3C both in term of growth promotion and growth orientation but is not necessary to induce Sema3A chemorepulsive effect.

Discussion

The molecular cascade mediating semaphorin signaling is being well understood (Castellani and Rougon 2002; Huber et al. 2003). It is currently thought that semaphorins require the formation of a receptor complex including neuropilins, the ligand-binding subunits that subsequently recruit various additional receptors with signaling functions. This signaling cascade ultimately modulates the dynamic of the cytoskeleton in order to ensure extension or retraction of the growing processes. The molecular partners participating in these intracellular cascades that mediate the repulsion of growth cones are well characterized. In contrast, those involved in promoting axon growth in response to guidance cues remain to be identified. Our study was designed to show whether a member of the MMP family (MMP-3) participates in semaphorin signaling during cortical development. The importance of proteinases during axon elongation is well known (Muir 1994; Zuo et al. 1998; Webber et al. 2002) but few studies analyzed their role in response to guidance cues so far. In fact, it has been shown that the signaling of members of the Ephrin family (Hattori et al. 2000) and Netrins (Galko and Tessier-Lavigne 2000) requires a metalloproteinase activity to modulate their function. In this case, the metalloproteinase activity induced ligand or receptor cleavage which directly modulated signal transduction of these guidance factors. Evidence for an involvement of proteinases in axon guidance in vivo comes from a study showing that the metalloproteinase Kuzbanian regulates Slit-Robo signaling at the midline of the *Drosophila* CNS during growth cone and cell

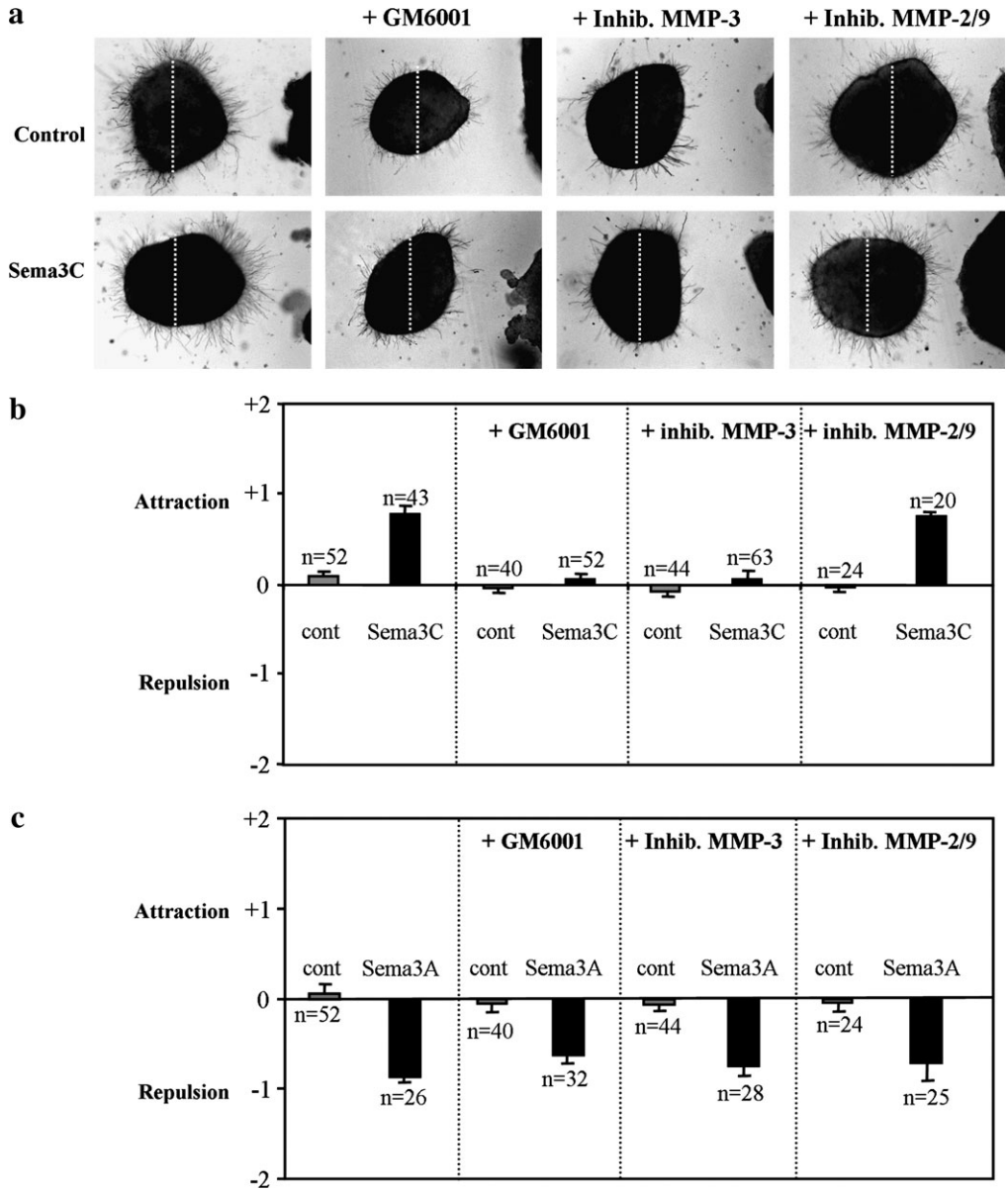

Figure 7. Coculture experiments. (*a*) E15 cortical explants were grown in front of control cell aggregates or Sema3C-secreting cell aggregates with or without the large spectrum MMP inhibitor GM6001 or the selective inhibitors of MMP-3 or MMP-2/9. (*b*, *c*) Migration index measured in the different culture conditions. A positive index reflects chemoattraction, whereas a negative index indicates chemorepulsion (n = number of explants).

migration (Schimmelpfeng et al. 2001). Similarly, it has been shown that MMPs are crucial for the guidance of retinal ganglion cell axons in the developing visual system of *Xenopus laevis* (Webber et al. 2002; Hehr et al. 2005). Our results provide additional evidence for an important role of MMPs in axon guidance by demonstrating that MMP-3 expression and activity can be modulated by guidance factors of the semaphorin family. The chemoattractant Sema3C induced the expression and activity of MMP-3, whereas the inhibitory Sema3A decreased the expression and activity of MMP-3, thereby modifying the growth capacity of cortical axons. Thus, axons extend their growth cones in response to growth-promoting or growth-inhibitory guidance signals by a direct modulation of their proteolytic capacity. This is in agreement with the classical view of the role of MMPs digesting extracellular matrix components to favor the elongation of axons through their environment. We cannot exclude that MMP-3 also modulates the signaling cascade of semaphorins by cleavage of ligands or receptors as shown for the Ephrins (Hattori et al. 2000) or Netrins (Galko and Tessier-Lavigne 2000). Nevertheless, using western blot analysis, we failed to detect any MMP-3–dependent cleavage of some of the major receptors of semaphorins (including neuropilin-1 and 2, plexine A1 or L1) (data not shown). Hence, we propose that the induction of MMP-3 at the level of growth cones exposed to the chemoattractant Sema3C is sufficient to favor and stabilize the direction of growth toward the source of the positive signal. The triggering of additional proteolytic activity would be the driving force of the growth-promoting effect of Sema3C. This growth-promoting effect is exerted at the level of extension capacity (length of axons) and growth orientation (direction of growth). Future studies will investigate the intracellular pathways leading to the modulation

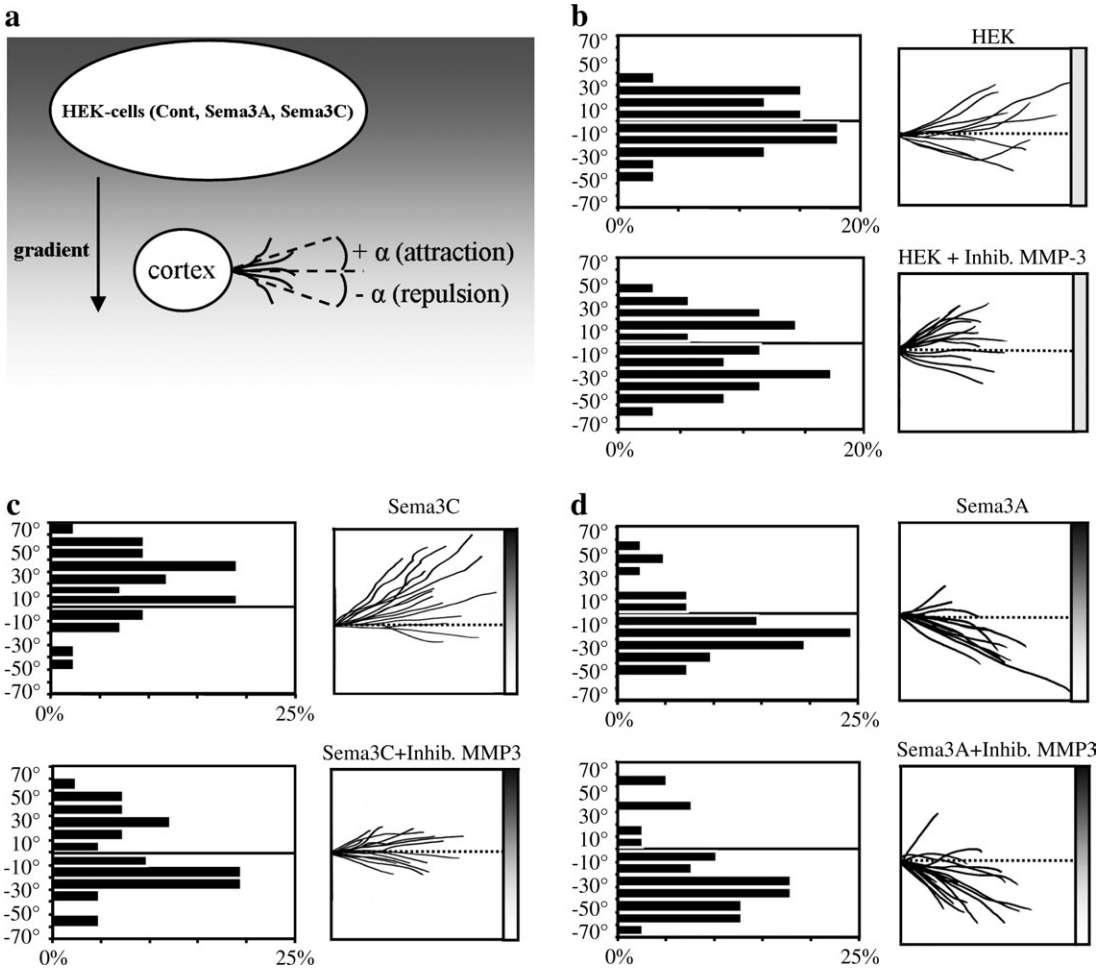

Figure 8. Turning angle analysis. (*a*) Schematic representation of turning angle measurement. (*b–d*) The histograms depict how many degrees cortical axons extending perpendicular to the gradients produced by cell aggregates deviate from a straight course with or without MMP-3 specific inhibitor in control conditions (*b*), in the presence of Sema3C-secreting cells (*c*) or in the presence of Sema3A gradients (*d*).

of MMP-3 expression in response to Sema3C. In particular, we will analyze how MMP-3 may interact with other MMPs (Basbaum and Werb 1996) or with crucial elements of the signaling cascade of semaphorins such as Rho GTPases (Rohm et al. 2000; Vikis et al. 2000), cGMP (Song et al. 1998; Song and Poo 1999; Polleux et al. 2000), or MAP kinase (Campbell and Holt 2003; Pasterkamp et al. 2003; Bagnard et al. 2004; Schwamborn et al. 2004). Our results not only provide new data about the signaling cascades activated by semaphorins but also give new insight into the molecular mechanism governing cortical development. The expression pattern of MMP-3, together with the proteolytic activity detected at the tip of growing axons in the intermediate zone are consistent with our previous model proposing a role of Sema3C gradient to attract and direct efferent cortical axons toward the lower part of the intermediate zone (Bagnard et al. 1998). Although MMP-3 has been involved in many pathological situations in the adult brain, our results now demonstrate a physiological role of MMP-3 during embryonic brain development by ensuring appropriate growth response to Sema3C. Recent studies have implicated MMPs in postnatal cerebellum development (Vaillant et al. 1999). Moreover, it was shown that MMP-9 is involved in the granular cell axon outgrowth (Vaillant et al. 2003). Our results show the existence of a cell type specificity of the MMPs required for proper axonal outgrowth because we found a major implication of MMP-3 during cortical axon outgrowth. Hence, as shown here, the quantitative analysis of MMP activity in situ revealed that many members of this family are probably involved in the cortical development. This might represent the diversity of functions required ranging from cell proliferation, cell migration to cell differentiation, all of these functions being ensured by MMPs (Werb 1997). They might also reveal a high level of redundancy or compensation in order to achieve these extremely important roles. Thus, it will be necessary to analyze the putative function of many other members of the MMPs' family during cortical development.

Supplemental Data

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

Notes

This work was supported by grants from the FRM (#INE20011109006), BQR-ULP (#BE/CH/2001-225V), and ACI JC (#5327) to D.B. B.G. is supported by Neurex. Special thanks to S. Dirrig-Grosch for help with confocal microscope analysis performed in the "Plateforme imagerie" of IFR37. Thanks to P. Stemmer who did part of the cortical neurons growth analysis. We thank A. W. Püschel for providing the Sema3A and

Sema3C expressing cell lines and comments to the manuscript and P. Anglard for critical reading of the manuscript. *Conflict of Interest*: None declared.

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