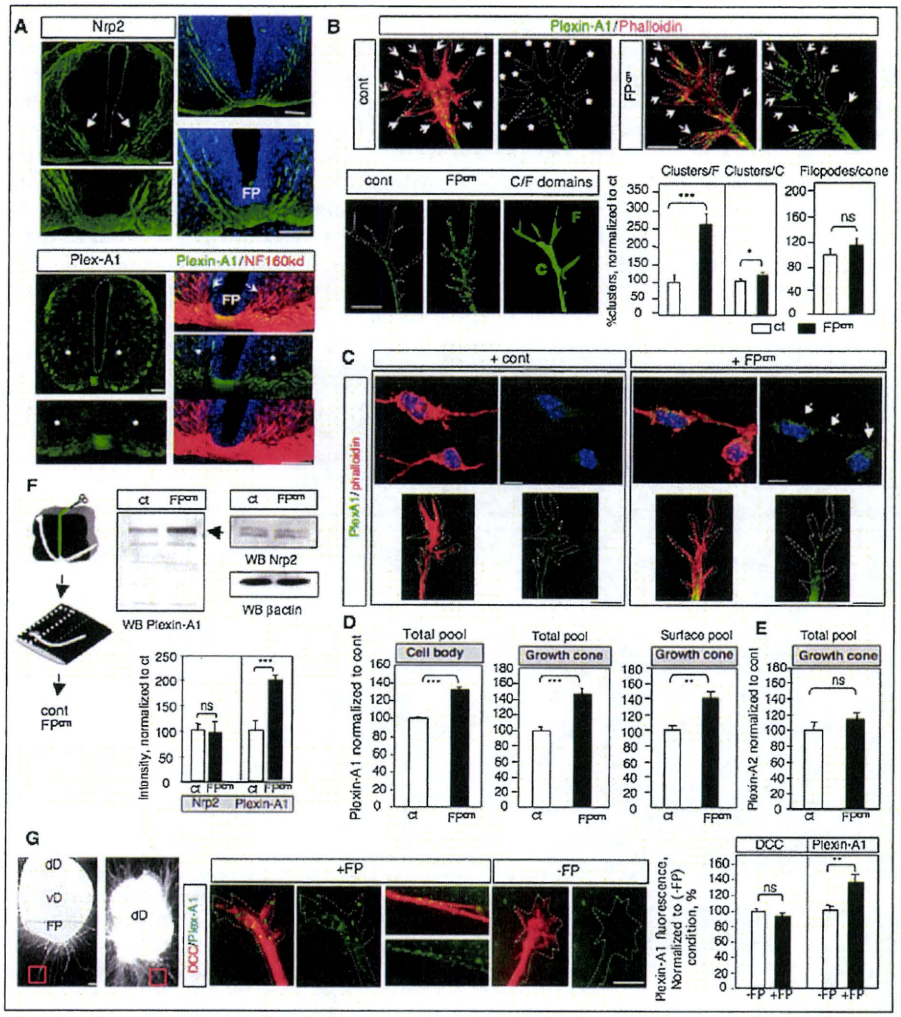

Figure 2. The Plexin-A1 level is increased by FP signals. (*A*) Immunolabeling of the Sema3B receptor subunits Nrp2 and Plexin-A1 (green) in E11.5 cross-sections with nuclear counterstaining (blue). Nrp2 is expressed on precrossing segments. Plexin-A1 is initially very low and is up-regulated in crossing and post-crossing segments. Bars, 100 μm. (*B*) Microphotographs illustrating the difference of Plexin-A1 filopodial distribution between control and FP^{cm} conditions (white arrows and stars). Counting of Plexin-A1 clusters in the central and peripheral growth cone domains. Treatment with FP^{cm} induces a strong increase of PlexinA1 clusters in the peripheral domain without modifying the number of filopodia per growth cone. Increase of Plexin-A1 clusters in the central domain is very modest. Bar, 15 μm. (*C*) Illustrations of Plexin-A1 immunolabeling (in green, phalloidin in red) in neuronal cultures showing Plexin-A1 increase in the FP^{cm} condition, in the soma, and in the growth cone. Bar, 15 μm. (*D*) The histograms summarize the quantitative analysis of Plexin-A1 fluorescence in the soma and the growth cones (total pool and cell surface) and its increase by FP signals. (*E*) Plexin-A2 fluorescence in the growth cones is not up-regulated by FP signals. (*F*) Western blot analysis of the Plexin-A1 level in fresh dorsal spinal tissue stimulated with control (cont) and FP^{cm}. (*Left* panel) Experimental procedure. (*Right* panel) Rise of the Plexin-A1 but not the Nrp2 level in Western blot. The histogram shows quantification of band intensity and the increase of Plexin-A1 but not Nrp2 in the FP^{cm} condition, compared with control. (*G*) Explants assays with spinal cord tissue in which the FP was removed or was not removed. Illustration and quantification of DCC and Plexin-A1 growth cone fluorescence in commissural axons that crossed or did not cross the FP. Bar, 15 μm. (dD) Dorsal domain of the spinal cord; (vD) ventral domain of the spinal cord.

confirmed that chick commissural neurons are unresponsive to Sema3B at the basal level, but acquire responsiveness to Sema3B upon exposure to chick FP^{cm} (Supplemental Fig. S6). To visualize the cell surface expression of Plexin-A1, the coding sequence was fused to gfp pHLuorin, a Ph-sensitive gfp that fluoresces in compartments with neutral pH such as the cell membrane, but very weakly in acidic intracellular compartments (Jacob et al. 2005). After transfection of the vector encoding Plexin-A1-Phluo in COS7 cells, the fluorescence was almost restricted to the cell surface, contrasting with a control egfp fusion membrane protein that was, in addition, detected in intracellular

compartments; Fig. 3A). Next, *Plexin-A1PhLuo* was electroporated at very low levels (0. 5 μg/μL) in the chick neural tube. Under these conditions, Plexin-A1-PhLuo was detected in dividing neuroepithelial cells and post-mitotic cells settling in the mantle (Fig. 3B). In commissural neurons, the fluorescence was visible in the soma and the proximal axon segment but was very weak in the precrossing axon segment, thus indicating that the Plexin-A1-PhLuo level was successfully repressed at the precrossing stage. Remarkably, intense fluorescence was detected in growth cones and axon segments after FP crossing (total of six embryos, 100% penetrance of the observation) (Fig. 3C). This compartmentalized pattern was never observed in embryos electroporated with egfp (total of six embryos) (Fig. 3D). To quantify these observations, *Plexin-A1-PhLuo* and *egfp* were coelectroporated with *rfp* (Fig. 3E). In the Plexin-A1-PhLuo condition, commissural growth cones were red prior to FP crossing, becoming yellow in the FP, while in the egfp condition, growth cones were already yellow before FP entry (Fig. 3E).

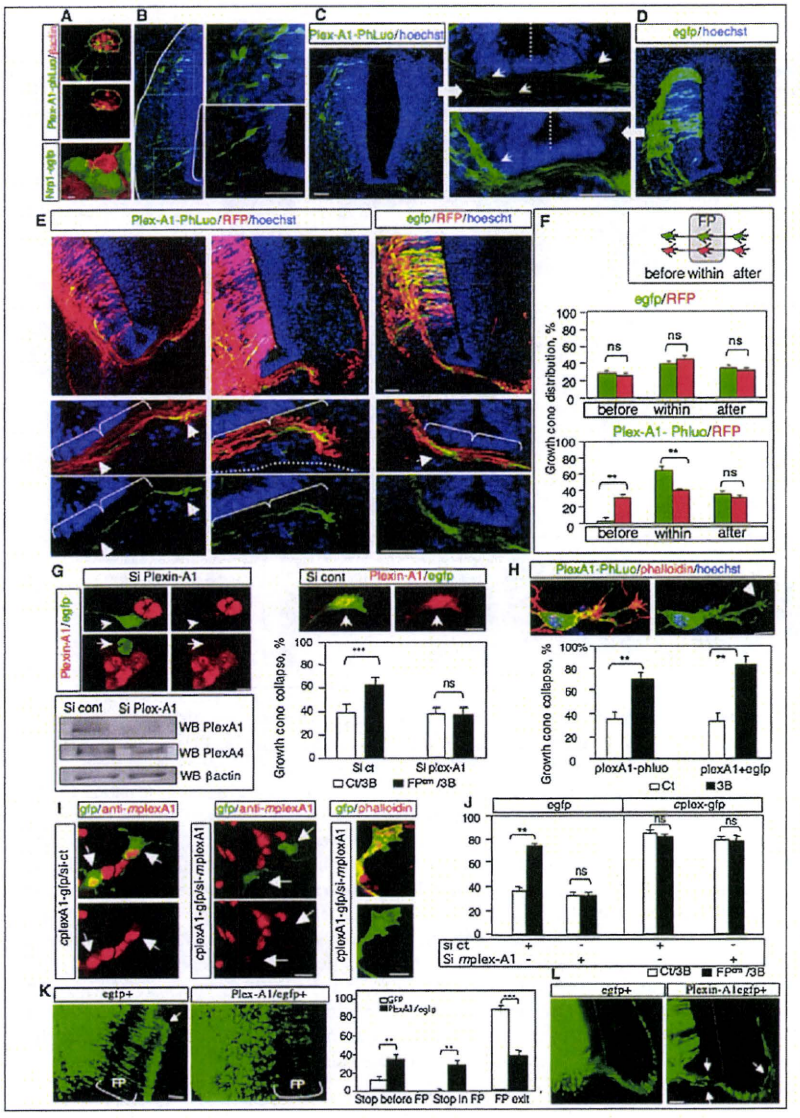

Figure 3. Plexin-A1 up-regulation in commissural growth cones during FP crossing and gain of response to Sema3B. (*A*) Two Z scans showing restricted Plexin-A1-PhLuo cell surface expression in COS7 cells. (*B–D*) Electroporation of *Plexin-A1PhLuo* or *egfp* in the chick neural tube. Plexin-A1-PhLuo is visible in polarized neuroepithelial cells, lateral post-mitotic soma, and initial axon segments, but is weakly detected along precrossing commissural axons, compared with egfp (white arrows). Note the bright fluorescence of Plexin-A1-PhLuo after FP crossing (*right* white arrow). (*E*) *Plexin-A1-PhLuo/RFP* and *egfp/RFP* coelectroporation. (*Left* panel) Yellow growth cones are detected before crossing in the egfp/RFP but not the Plex-A1-PhLuo/RFP condition. In the *right* panels, Plexin-A1-PhLuo green fluorescence is switched on upon FP crossing, unlike rfp, which is detected before FP entry. (*F*) Distribution of green and red growth cones before FP in-growth, in the FP, and after FP exit in the Plex-A1-PhLuo/RFP and egfp/RFP conditions, showing the extremely low number of green Plex-A1-PhLuo growth cones at the precrossing stage. Bar, 100 μm. (*G*) Microphotographs of siRNA-mediated knockdown of Plexin-A1 expression (red) in cultured dorsal spinal neurons labeled with gfp (green). Bar, 15 μm. Western blot showing the knockdown of Plexin-A1 but not Plexin-A4 by the si-*Plex-A1*, and the lack of effect of the si-control (si-*cont*). The histogram shows the loss of FP^{cm}-induced collapse responsiveness to Sema3B in the Plexin-A1 knockdown condition. (*H*) Plexin-A1-PhLuo overexpressed in commissural neurons is present at the soma and growth cone surface. Histogram showing that overexpressed Plexin-A1-PhLuo and vsv-Plexin-A1 both confer to commissural growth cones responsiveness to Sema3B. Bar, 15 μm. (*I*) Rescue of Plexin-A1 restores the sensitivity to Sema3B. Illustrations of combined siRNA and gfp-*Plexin-A1* cotransfection showing that mouse Plexin-A1 (*m*Plex-A1) was knocked down (labeled with mouse-specific anti-Plexin-A1) and replaced by chick gfp-Plexin-A1 (*c*Plex-A1, gfp label). *c*Plex-A1 is present in the growth cones. Bar, 20 μm. (*J*) The histograms show the results of a collapse assay and the rescue by *c*Plex-A1. (*K*) Illustrations and quantifications of DiI labeling in open book preparations after electroporation of Plexin-A1/egfp or egfp at high concentration in the chick embryo. Plexin-A1 but not egfp overexpression induced defects of FP crossing. (White arrow in the egfp condition) Post-crossing tract. Bar, 100 μm. (*L*) Gfp staining in cross-sections illustrating growth cone stalling at the FP entry in the Plexin-A1but not egfp condition. (White arrows) Stalling growth cones and aberrant post-crossing axon trajectories. Bar, 100 μm.

We counted the number of growth cones in the red and green channels before FP entry, within the FP, and after FP exit in the Plex-A1-PhLuo/rfp and egfp/rfp conditions (total of 73 sections from six embryos, 587 growth cones). The number of growth cones distributed equally before and after FP crossing in the egfp/rfp controls. In striking contrast, the number of green but not red fluorescent growth cones in the Plex-A1-Phluo condition was strongly shifted to the crossing and post-crossing categories (Fig. 3F). Thus, in vivo, the Plexin-A1 cell surface level is kept very low in commissural growth cones at the precrossing stage and is up-regulated during FP in-growth.

If the Plexin-A1 level is central to the regulation of growth cone responsiveness, then modifying Plexin-A1 levels should alter commissural growth cone behaviors. Accordingly, knockdown should invalidate the sensitization, and overexpression should mimic the effect of FP signals and confer responsiveness to Sema3B. First, Plexin-A1 was knocked down in cultured neurons by siRNA transfection. Efficiency and specificity were controlled by immunolabeling in cultured commissural neurons and in Western blot (Fig. 3G). Analysis of growth cone behaviors showed that those extending from neurons in which Plexin-A1 was silenced were unable to acquire responsiveness to Sema3B upon exposure to FP^{cm} (50 neurons analyzed per condition, two independent experiments) (Fig. 3G). Second, the Plexin-A1 level was increased by overexpression of Plexin-A1-PhLuo in cultured commissural neurons (Fig. 3H). Intense fluorescence was detected in the soma and growth cone compartment, demonstrating the ectopic accumulation of overexpressed Plexin-A1 at the growth cone surface. In this condition, the growth cones were found responsive to the collapsing effect of Sema3B in the absence of FP^{cm}. As control for Plexin-A1-PhLuo functionality, similar results were obtained after transfection of a vsv-tagged Plexin-A1 form (Supplemental Fig.S7A). Similar results were obtained in neurons isolated from the chick embryo (Supplemental Fig.S7B). We also performed rescue experiments (Fig. 3I). siRNA directed against endogenous mouse *Plexin-A1* (*m*PlexA1) was cotransfected with chick Plexin-A1-egfp fusion (*c*PlexA1), which escapes the silencing. Knockdown and overexpression was confirmed by immunolabeling with a Plexin-A1 antibody recognizing *m*PlexA1 but not *c*PlexA1, and gfp observations to detect *c*PlexA1. We observed in a collapse assay that *c*PlexA1 could rescue the sensitivity of neurons in which endogenous *m*PlexA1 was abolished (Fig. 3J). As observed in the overexpression-alone condition, the sensitization was no longer dependent on FP^{cm}. Thus, an artificial increase of the Plexin-A1 level mimics the effect of the FP^{cm} and is sufficient for conferring responsiveness to Sema3B. We also examined the consequences of knockdown of another Plexin-A, Plexin-A3, which appeared to be expressed by commissural neurons. Nevertheless, siRNA transfection, which efficiently and specifically extincted Plexin-A3, did not prevent commissural axon responses to Sema3B (Supplemental Fig. S7C).

We next assessed the consequences of in vivo Plexin-A1 overexpression. *Plexin-A1* and *egfp* constructs were overexpressed by electroporation in the chick neural tube (total of 24 embryos, 240 crystals). Given that Sema3B is produced at the midline, but also along the precrossing axon route, premature responsiveness should affect the growth of precrossing axons and their entry in the FP. The pattern of commissural projections was first examined by insertion of DiI in the dorsal domain of open book preparations (Fig. 3K). In embryos overexpressing Plexin-A1/egfp but not egfp alone, a significant proportion of commissural axons failed to reach and enter the FP (Fig. 3K; Supplemental Fig. S7D). Transversal sections were also performed to visualize the trajectory of individual $egfp^+$ axons. Consistent with the in toto DiI analysis, growth cones accumulated at the FP entry in the Plex-A1/$egfp^+$ but not $egfp^+$ condition (Fig. 3L). This phenotype was thus consistent with the acquisition of the precrossing commissural response to the FP-repellent Sema3B.

FP signals suppress proteolytic calpain1 activity to increase Plexin-A1 level

Next, we explored the metabolic pathways by which FP signals trigger increase of the Plexin-A1 level. We assessed activation of protein synthesis and, conversely, inhibition of proteasomal degradation, as both of these pathways were described to regulate guidance receptor levels at the midline (Brittis et al. 2002; Garbe and Bashaw 2004). We took a pharmacological approach in our culture assay, and quantified the Plexin-A1 level in the soma and the growth cone compartments. Application of MG132, a proteasome inhibitor, had no detectable effect on the Plexin-A1 level in both the soma and the growth cone (Fig. 4A). Surprisingly, inhibition of protein synthesis with anisomycin prevented the FP^{cm}-triggered gain of Plexin-A1 in the soma, but notably not in the growth cone (Fig. 4B,E). Thus, these pathways are unlikely to control the increase of Plexin-A1 levels by FP signals. We then assessed the possible implication of proteolytic processing by first applying a cocktail of inhibitors that blocks a panel of proteases, such as serine proteases, cysteine proteases, and metalloproteases (Fig. 4C). Interestingly, in the soma and the growth cone, this treatment increased Plexin-A1 and induced its accumulation in the filopodia in the control condition, fully mimicking the FP^{cm} condition. Moreover, combined pharmacological and FP^{cm} treatments did not produce additive effects, thus likely affecting a common pathway. We investigated the nature of the protease and found that specific inhibition of the cysteine protease calpain1 with ALLn (1 μM) recapitulated the effect of the cocktail (Fig. 4D,E). In contrast, Nrp2 level was not found increased by calpain1 inhibition (Supplemental Fig. S8A). The presence of calpain1 in commissural neurons was confirmed by immunolabeling (Fig. 4F). If calpain1 maintains Plexin-A1 at low levels, then its inhibition should confer responsiveness to Sema3B. Calpain1 was thus knocked down with siRNA transfected in neuronal cultures. Efficiency and specificity of the siRNAs were controlled in immunolabeling and Western blots (50 neurons per condition, two independent experiments) (Fig. 4G; Supplemental Fig.S8B). Analysis in collapse assays demonstrated

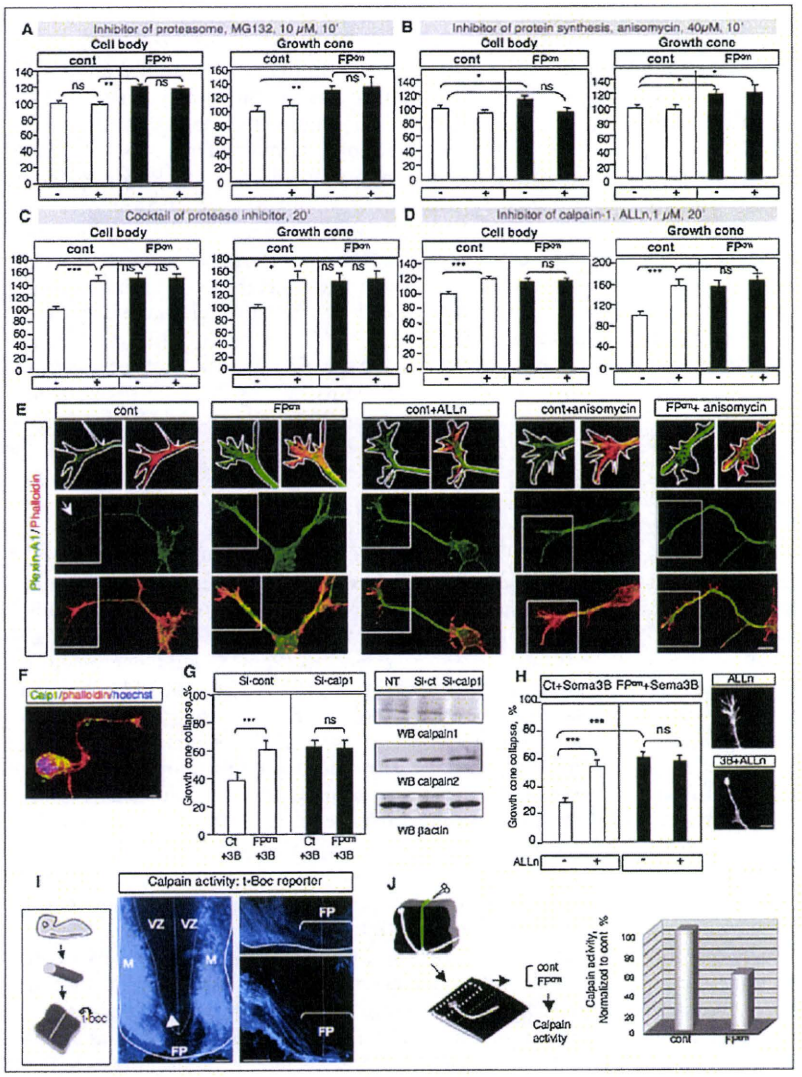

Figure 4. The Plexin-A1 level is regulated by a calpain1-dependent protease pathway. (*A*) Application of the proteasome inhibitor MG132 has no effect on Plexin-A1 levels, both in control and FP^{cm} conditions. (*B*) Inhibition of protein synthesis with anysomycin blocked FP^{cm}-induced Plexin-A1 up-regulation in the soma but had no effect in the growth cone. Note that applications were shortened to 10 min to limit cytotoxicity. FP^{cm}-induced increase of Plexin-A1 is attenuated but still significant. (*C*) A cocktail of protease inhibitors increased the Plexin-A1 level in the soma and the growth cone when applied in the basal condition, but not with FP^{cm}. (*D*) Inhibition of calpain1 had similar effects. (*E*) Illustrations of neuronal cultures showing that protein synthesis inhibition does not prevent FP^{cm}-induced increase of Plexin-A1 (green) in the growth cone. Illustrations of increase of Plexin-A1 and accumulation in the filopodia (green) induced by the calpain1 inhibitor ALLn as with the FP^{cm}, compared with control. Anisomicyn treatment does not prevent Plexin-A1 accumulation in the FP^{cm} condition. Soma and growth cones are delineated with phalloidin-TRITC. Bar, 15 μm. (*F*) Coimmunolabeling of calpain1 (green) and phallodin (red) in cultured commissural neurons. Bar, 15 μm. (*G*) Calpain1 knockdown experiments with siRNA. Immunoblots of neuroblastoma cell lysates showing the efficiency of the si-calp but not the si-control (si-cont) to silence endogenous calpain1. Calpain-2 level is not altered by the si-calp. The histogram shows acquisition of growth cone collapse response to Sema3B in the basal condition induced by calpain1 silencing. (*H*) Collapse assay showing that calpain1 inhibition with ALLn (1 μM) conferred responsiveness to Sema3B in the basal condition (control). Bar, 15 μm. (*I*) Endogenous calpain activity in unfixed chick spinal cord sections using t-Boc. The fluorescence is detected in the mantle (M) but not in the ventricular zone (VZ) and the FP. The magnification illustrates the sharp decrease of fluorescence in commissural axons entering the FP. Bar, 100 μm. (*J*) Measure of endogenous calpain activity in dorsal spinal tissue stimulated with FP^{cm}. The histogram presents the decreased rate of calpain1 activity in tissue treated with FP^{cm} normalized to control condition.

that application of the calpain1 inhibitor ALLn (1 μM) in the neuronal cultures conferred responsiveness to Sema3B, in the absence of FP^{cm} (number of growth cones per condition: 155 for control, 126 for control/ALLn, 123 for FP, and 127 for FP/ALLn; two independent experiments) (Fig. 4H). Knockdown of calpain1 with siRNA resulted in similar effects (Fig. 4H).

These data suggested that calpains might be active in commissural neurons to process Plexin-A1 at the pre-crossing stage but not in crossing axons, due to the action of the FP signals. To address this issue, we first investigated the pattern of calpain activity in the developing chick spinal cord (Fig. 4I). Spinal cords were isolated from HH25 (Hamburger and Hamilton 25) embryos, rapidly sectioned into 200-μm-thick slices; incubated for 15 min with the calpain activity reporter t-Boc, whose cleavage produces blue fluorescence (Robles et al. 2003); and observed immediately with a confocal microscope. As expected, intense fluorescence was detected in the mantle where post-mitotic spinal neurons reside. In contrast, any fluorescent staining was detected in the ventricular zone containing the progenitors and the FP. Notably, although the t-Boc fluorescence in cell populations along the commissural pathway prevented assessment of calpain activity in commissural axons in the dorsal and intermediate spinal cord domains, at more ventral positions,

t-Boc fluorescence was very clear in precrossing fibers, abruptly disappearing in crossing axon segments at all confocal *Z* positions (Fig. 4I).

Second, we measured endogenous calpain activity in fresh dorsal spinal cord tissue after exposure to control and FP^{cm} treatments using a biochemical approach that, unlike t-boc staining, allowed quantitative analysis. Consistent with our model, decrease of calpain activity by 52% was found in the samples stimulated with FP^{cm} compared with the controls, and the decrease could be reproduced in four independent experiments (Fig. 4J).

If calpain activity is required for precrossing commissural axon guidance, its inhibition should produce guidance defects at the FP. To address this issue, intraperitoneal injections of MDL28170 (or calpain inhibitor III), a calpain inhibitor that crosses the blood–brain barrier and efficiently inhibits calpain activity in the CNS (Markgraf et al. 1998; Mingorance-Le Meur and O'Connor 2009), were performed in pregnant mice at successive E10.5 and E11.5. Commissural axon trajectories were analyzed in open book preparations of E12.5 embryos (number of crystals/number of embryos: 73/six for vehicle and 204/nine for MDL28170) (Fig. 5A). In the vehicle-treated animals, commissural axons reached the FP in almost all cases, whereas in the MDL28170-treated animals, commissural axons stopped growing, turned in both directions, or stalled at the FP entry in 25% of the cases. The crossing and post-crossing trajectories of axons that could reach the FP were not deeply modified, and axons turned in an appropriate direction after FP crossing. Thus, calpain activity is required for commissural axons to reach and enter the FP, consistent with our finding that calpain activity silences precrossing commissural responsiveness to the FP cue Sema3B.

Finally, we examined whether Plexin-A1 is processed through direct or indirect action of calpain1. First, we performed an in vitro cleavage assay (Fig. 5B). *Plexin-A1* was translated in vitro in the presence of MetS35, and the product was incubated with purified active calpain1 and analyzed in Western blot. We found that Plexin-A1 is cleaved into two major fragments of apparent molecular masses of 55 kDa and 45 kDa. Inhibition of calpain1 activity by EDTA blocked the processing. Positive control of calpain activity was done with a known calpain target, the focal adhesion kinase (FAK). Second, Plexin-A1 tagged in Nter with Flag was transfected in COS7 cells. The cells were incubated by ALLn or control treatment, and Plexin-A1 band patterns were compared in Western blot (Fig. 5C). We observed two Plexin-A1 bands at ~70 kDa and 50 kDa in the control condition that were strongly reduced when calpain1 activity was inhibited with ALLn. Similar band patterns were observed using N-ter vsv-Plexin-A1 fusion and gfp-Plexin-A1 fusion (data not shown). Differences of masses between these two assays likely reflected post-translational modifications in cell lines. Thus, the Plexin-A1 extracellular domain is processed by calpains. Third, we examined whether such processed fragments could be detected in spinal dorsal

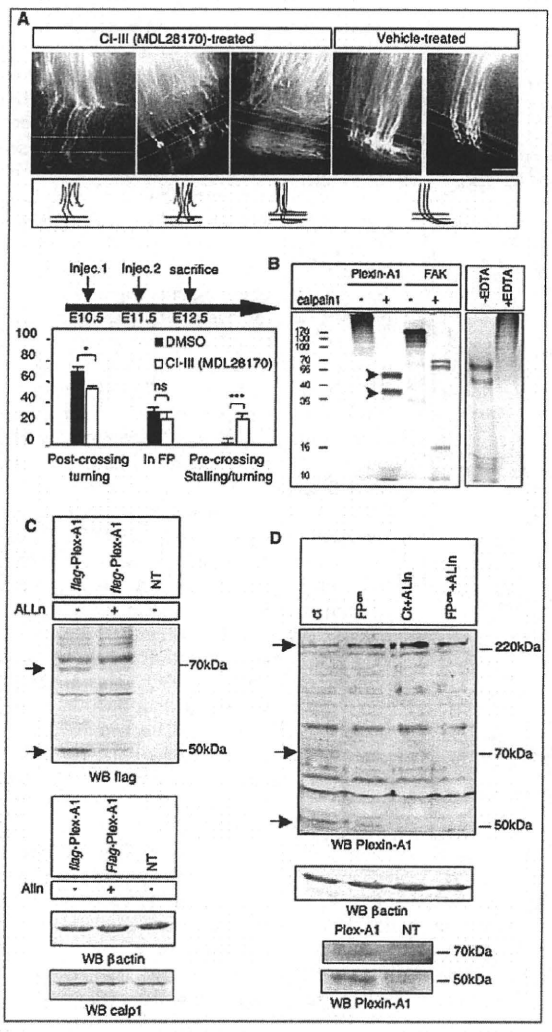

Figure 5. Calpain is required for precrossing commissural axon guidance and cleaves Plexin-1. (*A*) Illustrations and quantifications of DiI labeling in open book preparations. Inhibition of calpain activity induces significant defects of precrossing axon behaviors, with axons failing to enter the FP or turning before crossing. Bar, 100 μm. (*B*) Autoradiography of Western blot showing that in vitro translated Plexin-A1 is cleaved by active calpain1 into two main fragments. Positive control: FAK. Plexin-A1 is not processed when calpain1 is inhibited by EDTA. (*C*) Western blot showing that Flag-Plexin-A1 transfected in COS7 cells is processed by endogenous calpain1 into two fragments. The fragments are reduced by ALLn application. (*Right* panel) Western blot to detect calpain1 and βactin in the same samples. (*D*) Western blot showing Plexin-A1 processing in fresh dorsal tissue in control and FP^{cm} conditions. Inhibition of calpain1 by ALLn in the control condition induced accumulation of full-length Plexin-A1 and reduction of the processed fragments. The band patterns of control + ALLn and FP^{cm} are similar. ALLn application in the FP^{cm} condition does not modify the band pattern. (*Bottom* panel) Plexin-A1-processed fragments are detected with the same anti-PlexinA1 antibody in transfected COS cells but not in nontransfected control COS7 cells (NT).

tissue (Fig. 5D). Dorsal spinal cords were dissected from E12.5 embryos, and were treated with control and FP^{cm} in the presence and absence of ALLn. Plexin-A1 band patterns were compared in Western blot using an antibody recognizing Plexin-A1 extracellular epitopes. Notably, in the control condition, the full-length Plexin-A1 band was strongly increased by ALln, while conversely, the processed fragments that we found present in the samples were strongly decreased. As expected, the Plexin-A1 band pattern of FP^{cm} and control + ALLn was similar, and the presence of ALln in the FP^{cm} did not modify the band pattern (Fig. 5C). Thus, these experiments provided evidence that calpain1 activity in spinal commissural neurons cleaves Plexin-A1, and that this processing is suppressed by FP signals.

The FP cue NrCAM regulates Plexin-A1 level and commissural responsiveness to Sema3B

In the next step, we explored the nature of the active components mediating the gain of response to Sema3B. NrCAM was an interesting candidate due to its high and restricted expression in the FP, and its functional properties to regulate axon growth and guidance during the formation of various commissural tracts (Fig. 6A; Falk et al. 2005; Williams et al. 2006). Moreover, metalloprotease-mediated release of

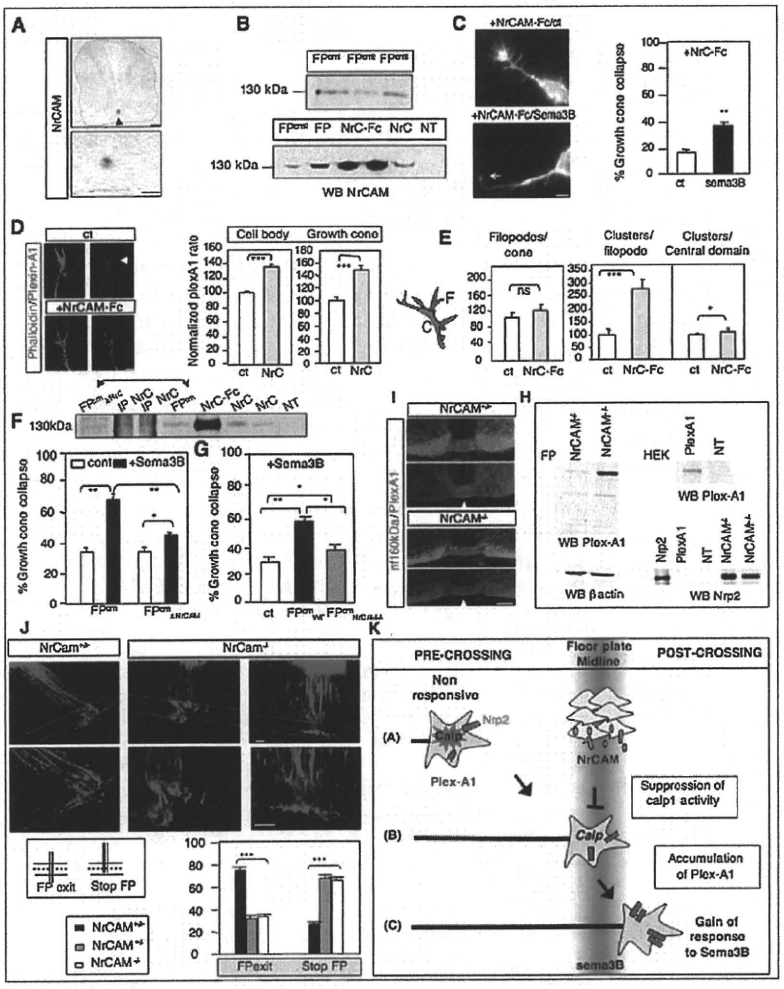

Figure 6. NrCAM is an active FP cue in the regulation of the Plexin-A1 level and acquisition of responsiveness to Sema3B. (*A*) In situ hybridization on E12.5 cross-sections showing mRNA transcript of *NrCAM* in the FP. (*B*) Western blot showing detection of NrCAM in four independent samples of FP^{cm} and in FP extracts (FP). (*C*) Collapse assay with chimeric NrCAM-Fc. Application of NrCAM-Fc combined with Sema3B allows commissural neurons to acquire responsiveness to Sema3B, while NrCAM-Fc, when applied alone, has no effect. (*D*) Application of NrCAM-Fc triggers a significant increase of Plexin-A1 levels in the soma and growth cones of cultured commissural neurons. (*E*) Plexin-A1 clusters accumulate in the growth cone peripheral domain upon exposure to NrCAM-Fc, while the number of filopodia per growth cone remains comparable. (*F*) NrCAM was biochemically depleted from the FP^{cm} through immunoprecipitation with anti-NrCAM antibody (compare bands pointed to by the arrow). The Western blot shows strong reduction of NrCAM detection in this sample ($FP^{cm}{}_{\Delta NrCAM}$) compared with native FP^{cm}. In functional collapse assays, the efficiency of $FP^{cm}{}_{\Delta NrCAM}$ to trigger responsiveness to Sema3B was significantly decreased, compared with native FP^{cm}. In the experiments using $FP^{cm}{}_{\Delta NrCAM}$, the control (cont) medium was also preincubated with an antibody to normalize possible nonrelevant effects of the biochemical depletion. (*G*) FP was isolated from the *NrCAM* mutant mouse line to produce FP^{cm}. This medium was significantly less efficient than the wild-type FP^{cm}. (*H*) Immunohistochemical labeling of E12.5 cross-sections showing a decrease of Plexin-A1 reactivity in the FP of *NrCAM*$^{-/-}$ embryos, compared with wild-type embryos. (*I*) The Plexin-A1 level in FP extracts from *NrCAM*$^{+/+}$ and *NrCAM*$^{-/-}$ embryos was compared in Western blot. NrCAM deficiency induced a strong decrease of the Plexin-A1 level, while the Nrp2 level remained constant. (*J*) Analysis of commissural projections in open book preparations from the *NrCAM* mouse line. The microphotographs and histogram show that *NrCAM* deficiency induced significant defects of FP crossing, with axons stalling in the FP. Bar:*A*,*H*,*J*, 100 μm; *C*,*D*, 15 μm. (*K*) Model for the mechanisms controlling the acquisition of Sema3B responsiveness at the ventral midline. (Panel *A*) At the precrossing stage, active calpain1 cleaves the signaling coreceptor Plexin-A1 and silences Sema3B responsiveness. (Panel *B*) During FP in-growth, commissural axons are exposed to FP signals that suppress calpain1 activity. (Panel *C*) Plexin-A1 is allowed to accumulate in the growth cones, thus sensitizing them to Sema3B. This guidance response participates in the FP exit.

active NrCAM ectodomain has been reported, making it possible that soluble NrCAM contributes to the property of the FP^{cm}. We thus assessed the presence of NrCAM in the FP^{cm} by Western blot and could detect, with an antibody recognizing NrCAM extracellular epitopes, a 130-kDa NrCAM form corresponding to NrCAM ectodomain (Fig. 6B). We next assessed the functional properties of NrCAM ectodomain in our neuronal assay. Soluble NrCAM-Fc was applied to commissural neurons. While the level of collapse was very low in the presence of NrCAM-Fc alone, it significantly increased when NrCAM was applied with Sema3B, indicating that NrCAM-Fc could trigger responsiveness to Sema3B (Fig. 6C). As expected, NrCAM-FC application could also increase Plexin-A1 level in commissural cell bodies and growth cones, and the protein accumulated in the growth cone peripheral domain (Fig. 6D,E). Next, to determine whether NrCAM contributes to the property of the FP^{cm}, NrCAM was depleted from the medium by immunoprecipitation with an anti-NrCAM antibody. As controlled by Western blot, this procedure strongly reduced the NrCAM level in the FP^{cm} (Fig. 6F). This $FP^{cm}{}_{\Delta NrCAM}$ was applied to commissural neurons, and its efficiency in triggering growth cone responsiveness to Sema3B was significantly attenuated (Fig. 6F). We also assessed the consequence of *NrCAM* genetic ablation by producing FP^{cm} from *NrCAM*-null embryos. Consistently, the effect of the FP^{cm} was almost totally abrogated, indicating that NrCAM is a major active component of the FP^{cm} (Fig. 6G). To validate these data, we investigated whether NrCAM deficiency alters Plexin-A1 level in the FP. First, immunolabeling was performed on E12.5 $NrCAM^{+/+}$ and $NrCAM^{-/-}$ embryonic cross-sections. We observed a decrease of Plexin-A1 labeling in the FP of the *NrCAM*-null embryos compared with wild-type embryos (Fig. 6H). Second, FP tissue was dissected from $NrCAM^{-/-}$ and $NrCAM^{+/+}$ embryos originating from the same littermates, and the Plexin-A1 band pattern was analyzed in Western blot. The Plexin-A1 band was strongly decreased in the samples from $NrCAM^{-/-}$ embryos compared with those of the wild-type embryos, while, as control, Nrp2 level remained similar (Fig. 6I). By altering the Plexin-A1 level and subsequently the gain of response to Sema3B, NrCAM deficiency would then be expected to impair FP exit. Consistently, in open book preparations labeled with DiI, we found that a significant proportion of axon tracts stalled in the FP of the $NrCAM^{-/-}$ embryos instead of exiting the FP to turn rostrally, as observed in $NrCAM^{+/+}$ embryos (Fig. 6J).

Discussion

The present study identifies a pathway-dependent mechanism in vertebrates by which spinal commissural axons acquire sensitivity to a midline-derived repellent of the Semaphorin family. Sema3s share typical receptor organization in multimolecular complexes (Kruger et al. 2005). We found that precrossing commissural axons are maintained unresponsive to Sema3B by the protease calpain1, processing the signaling moiety of the Sema3B receptor complex, Plexin-A1, to prevent expression of the integral protein in commissural growth cones. Exposure to the local FP signal NrCAM suppresses this processing, enabling full-length Plexin-A1 accumulation in crossing commissural growth cones, thus switching on their responsiveness to Sema3B (Fig. 6K).

Semaphorin3B/Plexin-A1 signaling regulates guidance decisions at the ventral midline

Several ligand/receptor pairs regulate commissural axon guidance at the midline, playing synergic and complementary roles (Garbe and Bashaw 2004). First, Netrin/DCC and Shh/BOC attract commissural axons toward the FP (Okada et al. 2006). Repulsive Slit/Robo signaling ensures that axons exit the FP and do not cross the midline again. Nevertheless, genetic loss of Robo signaling (*Robo1* and *Robo2*) does not totally abolish FP exit, thus indicating that other cues participate in this process (Dickson and Gilestro 2006). Recent work identified SCF1 as an additional cue whose growth-promoting effect helps commissural axon exiting the FP (Gore et al. 2008). Ephrin-Bs are other candidates, particularly Ephrin-B3, which is expressed by the FP (Kadison et al. 2006). Zou et al. (2000) showed that important guidance decisions in the FP are mediated by the Semaphorin receptor Nrp2. We found that, in *Sema3B*-null mutant mice, commissural axons often stall in the FP or grow at aberrant directions after midline crossing. These defects are very similar to those observed in the *Nrp2* knockouts, thus showing that Sema3B might be the Nrp2 ligand required in vivo for proper commissural guidance at the midline. Nevertheless, despite their common expression at the ventral midline, Slits and Sema3B chemorepellents likely have specific roles, since the recrossing of commissural axons characterizing the loss of Robo/Slit signaling was not detected in the *Sema3B* knockouts. We could identify Plexin-A1 as the Plexin-A family member mediating Sema3B responses in spinal commissural axons. Likewise, Plexin-A1 is synthesized by commissural neurons, and its invalidation by siRNA abolishes the Sema3B-induced collapse response of their growth cones, normally triggered by FP signals. Moreover, *Sema3B*- and *Plexin-A1*-null embryos develop similar guidance defects of commissural projections at the FP.

Processing of guidance receptor by calpains: a mechanism for silencing Sema3B responsiveness at precrossing stage

In *Drosophila*, responsiveness of precrossing commissural axons to Slit is silenced through coupling of the Slit receptor Robo to Commissureless, and sorting for proteasome degradation (Dickson and Gilestro 2006). In vertebrates, a spliced variant of one of the three *Robo* gene products, Robo-3.1, appears as a functional equivalent of *Drosophila* Commissureless, preventing through a yet-undetermined mechanism Robo1 and Robo2 from mediating responsiveness to Slits at the precrossing stage (Sabatier et al. 2004; Chen et al. 2008). The present study

highlights a novel mechanism by which precrossing commissural responses are silenced, based on processing of guidance receptors.

An interesting aspect of this pathway is that it does not prevent ligand/receptor interaction as for the Robo/Slit pair in *Drosophila* and possibly in vertebrates, since Nrp2 sorting to the growth cone surface is not prevented. Rather, it precludes accumulation of full-length signaling moiety of the receptor complex specifically transducing Sema3B in these neurons. This mechanism is advantageous and well-suited to the Semaphorin signaling, as it enables the other receptor subunit, Nrp2, to engage in other complexes.

Calpains are calcium-dependent cysteine proteases, regulating various processes. For example, they play pivotal roles in cell motility and synaptic functions by cleaving components of adhesion complexes and neurotransmitter receptors (Carragher and Frame 2002; Wu and Lynch 2006). Their functions during neuronal development are less characterized, but calpains are present in neuronal growth cones, are activated by intracellular calcium transients to reduce growth cone motility, and also are found downstream from Semaphorin5B (To et al. 2007). Interestingly, calpains process rather than degrade proteins, and are seen as regulators of protein functions, modulating protein–protein interactions, phosphorylation state, distribution, and traffic (Carragher and Frame 2002). Our data indicate that calpain1 is active in commissural neurons and maintains the integral form of Plexin-A1 at very low levels at the precrossing stage by cleaving the Plexin-A1 extracellular domain, generating two fragments that can be detected in Western blot. This processing could take place before protein sorting to the cell membrane. Likewise, previous studies reported detection of calpain in the lumen of endoplasmic reticulum and golgi vesicles, thus being at an appropriate location for cleaving neosynthesized target proteins during their intracellular traffic (Hood et al. 2004, 2006). Alternatively, Plexin-A1 could be cleaved at the cell surface, since increasing evidence indicates that calpains are externalized and can be retained to the membrane surface through association with proteoglycans (Abe et al. 1998; Nishihara et al. 2001; Frangie et al. 2006; Peltier et al. 2006). Several of our results support an important role for calpain activity during precrossing commissural axon guidance. First, in the spinal cord sections, calpains were found active in spinal neurons and precrossing commissural axon segments. Second, inhibition of calpain activity in vivo resulted in strong defects of commissural axon behaviors before FP crossing: axons failing to enter the FP, turning before FP crossing, or even not reaching the FP. Interestingly, all of these defects were also induced by Plexin-A1 overexpression in the chick embryo, thus supporting that calpain-1 activity prevents Plexin-A1 expression at the precrossing stage. Whether this protease suppresses precrossing commissural responses to other midline repellents by processing guidance receptors other than Plexin-A1, such as Robo1/2 or Eph receptors, is an intriguing possibility that will be assessed in future studies.

Commissural axons acquire Sema3B responsiveness through a pathway-dependent mechanism, implicating local FP signals

Our results showed that suppression of calpain activity in the FP is instrumental for the gain of commissural responsiveness to Sema3B. Several data support this conclusion. First, in unfixed spinal cord sections, calpain were not active in FP cells or in the crossing axon tract. Second, we found that the FP tissue released signals that inhibit calpain activity in spinal tissue. Third, suppression of calpain activity could increase integral Plexin-A1 levels in acute dorsal tissues and cultured commissural neurons and induce responsiveness to Sema3B.

Commissural axons were found to acquire responsiveness to a variety of repellents, but whether this occurs through a pathway-dependent or pathway-independent mechanism remains unclear. In the *Xenopus* visual system, the temporal switch from attractive to repulsive behavior of retinal axons to Netrin1 does not depend on pathway experience (Shewan et al. 2002). Our data do not support the view that such an experience-independent mechanism operates to confer responsiveness to Sema3B at the midline. First, dorsal spinal neurons isolated from early E11 to late E13.5 were equally unresponsive to exogenous Sema3B application (Supplemental Fig. S3B). Second, in open book preparations, removing the FP was sufficient to abolish the repulsive behavior of commissural neurons to a focal source of Sema3B. Finally, neurons became sensitive to Sema3B upon exposure to FP^{cm}, independent from the developmental stage at which they were collected. Our data thus support that signals emanating from intermediate target cells play pivotal roles in the switch of responsiveness.

Consistently, we could show that the Ig superfamily cell adhesion molecule NrCAM is an active FP component, regulating the Plexin-A1 level and acquisition of responsiveness to Sema3B. High levels of *NrCAM* transcripts were detected in the FP, and the protein was present in the FP^{cm}. In our neuronal assay, soluble NrCAM mimicked the FP^{cm}, triggering responsiveness to Sema3B and increase of the PlexinA1 level. In contrast, neither Netrin-1 nor Shh could recapitulate the gain of responsiveness to Sema3B conferred by the FP^{cm} (H Nawabi and V Castellani, unpubl.). Moreover, the biochemical or genetic depletion of NrCAM strongly altered the properties of the FP^{cm}. In vivo, strong decrease of the Plexin-A1 level in the FP of *NrCAM*-null embryos was found and significant amounts of axon tracts abnormally stalled in the FP. NrCAM is expressed by various developing neuronal projections, and several previous studies implicated it in the regulation of axon navigation, as receptors or coreceptors for environmental guidance cues (Lustig et al. 2001; Falk et al. 2005; Williams et al. 2006). Likewise, NrCAM interaction with Nrp2 was found to be required for axons to normally form the anterior commissure in the brain, and NrCAM expression is required by RGC axons to form proper patterns of ipsilateral/contralateral commissures in the visual system (Falk et al. 2005; Williams et al. 2006). Interestingly, NrCAM is highly expressed in specialized

glial structures in the ventral midline—not only in the spinal cord, but also in all upper floors of the CNS (Lustig et al. 2001)—but how NrCAM glial sources contribute to axon pathfinding remains unknown. The present work establishes a novel function for NrCAM as an intermediate target cue regulating the expression level of guidance receptors in the growth cones to control pathway choices at the ventral midline.

Materials and methods

Genotyping and vector cloning

Genotyping was performed as described in Falk et al. (2005), Yoshida et al. (2006), Takegahara et al. (2006), and Sakurai et al. (2001). Mouse Plexin-A1-PhLuorin was generated by introducing in Nter the coding sequence of the PhLuorin cloned from a vector encoding GABA A-PhLuorin-gfp (Jacob et al. 2005). Gfp was fused in Nter of the coding sequence of chick Plexin-A1.

Western blot

Spinal cords from E12.5 embryos were prepared in an open book configuration. Tissues were treated with control supernatant and FP^{cm} with or without ALLn (10 μM; Sigma) for 30 min. Dissected tissues were lyzed in 2× laemmli buffer. Cos7 cells transfected (Exgen, Euromedex) with Flag-plexinA1, vsv-Plexin-A1, and gfp-Plexin-A1 were treated for 1 h with ALLn (10 μM; Sigma). Samples were analyzed by Western blot using goat anti-Nrp2 (1/1000; R&D), rabbit anti-plexA1 (1/1000; AbCAM), anti-Calpain1 and anti-Calpain2 (1/500, Santa Cruz Biotechnologies), anti-NrCAM (1/1000; AbCAM), anti-vsv (1/1000; Sigma), anti-Flag (1/1000), and anti-βactin (1/1000; Sigma) antibodies.

Cocultures, explant cultures, cultures of dissociated neurons, and collapse assay

FPs were isolated from E12.5 embryos and cultured in three-dimensional plasma clots (Castellani et al. 2000) in B27-supplemented Neurobasal medium (Gibco). The supernatant was collected after 48 h. For coculture experiments, HEK 293 cells were transfected with plasmids encoding either Sema3B-Alcaline Phosphatase fusion protein or control Alcaline Phosphatase. Cell aggregates were cocultured with spinal cord open books as described in Falk et al. (2005). Cocultures and spinal cord explants with or without FP were grown for 24 or 48 h, fixed in 4% paraformaldehyde (PFA), and stained with phalloidin-TRITC or with an anti-neurofilament antibody, or with anti-DCC antibody (BD Bioscience) and anti-PlexinA1 antibody (Santa Cruz Biotechnologies). Axon outgrowth was quantified by measuring axon length using Image J software. Plexin A1 and DCC fluorescence levels were quantified with Image J software, as in Moret et al. (2007). For the collapse assay, dorsal spinal cord tissues from E11.5, E12.5, or E13.5 embryos were dissociated, and cells were plated into polylysin- and laminin-coated glass coverslips in Neurobasal supplemented with B27, glutamine (Gibco), and Netrin-1 (R&D) medium. After 1 or 2 d in vivo (DIV), neurons were incubated with control or FP^{cm} or different molecules for 30 min at 37°C. Then Sema3B-AP was added on cells for 30 min at 37°C. Cells were fixed in PFA 4%/1.5% sucrose and labeled with phalloidin-TRITC (1/500; Sigma). Collapsed growth cones were scored as in Falk et al. (2005). The following were used: protease inhibitor cocktail (Sigma), calpain inhibitor I, ALLn (1 μM; Sigma), actinomycin D (0.5 μg/mL; Sigma), MG132 (10 μM; Sigma), anisomycin (40 μM; Sigma), and Shh (2 μg/mL; Sigma). siRNA to Plexin-A1, calpain1, and control siRNA (Santa Cruz Biotechnologies) were transfected with Lipofectamin2000 according to the manufacturer's recommendations (Invitrogen). Statistical comparisons were done with Student's *t*-test for collapse assays and ANOVA for analysis of fluorescence level: (*) *P*-value < 0.05; (**) *P*-value < 0.01; (***) *P*-value < 0.001.

Immunohistochemistry, in situ hybridization, and t-Boc assay

Immunolabeling of neuronal cultures and sections was performed with antibodies anti-Plexin-A1 (1/100; Santa Cruz Biotechnologies, AbCAM, Chemicon), anti-Plexin-A2 (1/100; Santa Cruz Biotechnologies), anti-Nrp2 (1/100; R&D), anti-L1-CAM (1/100; Chemicon), anti-Ngn1 (1/100; Santa Cruz Biotechnologies), anti-Math1 (1/250; Chemicon), anti-Robo3 (1/100; R&D), and anti-calpain1 (1/100; Santa Cruz Biotechnologies). Nuclei were stained with bisbenzimide (Promega) and actin with TRITC-phalloidin. Twenty-microgram cryosections were prepared from embryos fixed in 4% paraformaldehyde, embedded in gelatin 7.5%/sucrose 15%, and incubated overnight at 4°C with antibodies anti-Nrp2 (1/100; R&D), anti-Plexin-A1 (1/100; AbCAM), and anti-Neurofilament (160 kDa, 1/100;RMO Zymed), and secondary antibodies Alexa 594, Alexa 488 (1/500; Invitrogen), and Fluoroprobe 546 (1/100) with bisbenzimide (1/1000; Promega). Chromogenic immunostaining and in situ hybridization were performed as described in Moret et al. (2007). For the t-Boc assay, 180 μM fresh cross-sections from embryonic spinal cords were incubated for 10 min with t-BOC (20 μM; Invitrogen). Staining was observed immediately using a confocal microscope.

Calpain inhibition in vivo

The calpain inhibitor III (in DMSO, diluted at 12.5 mg/kg; MDL28170, Calbiochem) was dissolved in saline solution (0.9% NaCl) and injected intraperitoneally into pregnant mice (Charles River) at 10.5 and 11.5 d post-coitum (dpc). The mice were sacrificed at 12.5 dpc, and the spinal cords from embryos were collected for open book preparations and DiI labeling. Controls were performed with DMSO-treated animals.

DiI staining on spinal cord open books

Spinal cords were prepared in an open book conformation and fixed in 4% PFA for 2 h. Small crystals of DiI (Invitrogen) were inserted in the dorsal part of one hemicord. Axon trajectories were observed using fluorescence microscopy after 48 h.

In ovo electroporation

In ovo electroporation of chick embryos (*Gallus gallus*, EARL Morizeau) was performed as described previously (Moret et al. 2007). Plasmids encoding Plexin-A1, egfp, or rfp were diluted at 2 μg/μL in PBS, and at 0.5 μg/μL for Plexin-A1-PhLuorin.

Analysis of calpain activity and in vitro cleavage assay

Isolated dorsal spinal cord fresh tissue was incubated for 30 min at 37°C with control and FP^{cm} supernatants, and was treated according to the manufacturer's instructions (Calbiochem). Calpain activity was measured by fluorogenic activity (Victor 3 multilabel counter, Perkin Elmer). Vectors encoding Plexin-A1 and FAK were transcribed and translated in vitro (TNT T7 kit, Promega) in the presence of Met^{S35}. The products were incubated with active calpain1 (Calbiochem), and were analyzed by

electrophoresis and autoradiography. EDTA was added in some experiments.

Statistical analysis

Statistics were done with ANOVA for quantification of Plexin-A1 levels and Student's *t*-test in all other cases. (***) $P < 0.0001$; (**) $P < 0.001$; (*) $P < 0.01$; (ns) not significant.

Acknowledgments

We thank J. Sanes and F. Rathjen for their gift of the Sema3B and NrCAM mouse lines, respectively; Camille Charoy and Cecile Herbreteau for their help; Geraldine Gouzer for her gift of plasmid; and the Center of Quantimetry of the University of Lyon1, Rockefeller. This work is supported by CNRS, Association Française contre les Myopathies (AFM), and ANR to V.C.; and AFM fellow to H.N. K.T. is supported by NIH grant RO1NS065048. Y.Y. is supported by NIH grant RO1NS065048.

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Clinical and Experimental Neuroimmunology 1 (2010) 33–45

REVIEW ARTICLE

Involvement of semaphorins and their receptors in neurological diseases

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Keywords
autoimmune diseases; immune regulation; neurological diseases; semaphorin receptors; semaphorins

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Received: 14 September 2009; revised: 9 October 2009; accepted: 15 October 2009.

Abstract

The immune and nervous systems have various common features in their functional characteristics. Both have an intricate network of synaptic connections and an exquisite communication system that enables intercellular signal transduction. Semaphorins were originally identified as guidance factors for developing neuronal axons. However, accumulating evidence indicates that several semaphorins called "immune semaphorins" are crucial for various phases of immune responses, from the initiation to the termination of inflammatory processes. Furthermore, it is becoming clear that immune semaphorins contribute to pathological immune responses in the central nervous system. Here, we review the present knowledge of the function of semphorins and their receptors in the immune system, and their involvement in the pathogenesis of neurological diseases. (Clin. Exp. Neuroimmunol. doi: 10.1111/j.1759-1961.2009.00004.x, January 2010)

Introduction

There are many links between the immune and nervous systems. Both are highly networked systems that interact with each other using shared molecules such as chemical mediators and cytokines.[1] The immune response is composed of a series of cell–cell contacts, including interactions between T cells and antigen-presenting cells (APC) such as B-cells, macrophages and dendritic cells (DC). Such cell–cell contact elicits the activation of immune responses, characterized by clonal expansion and development of effector functions of T cells in which the T cell receptor (TCR) forms a close contact with the cognate antigen peptide-major histocompatibility complex on the cell surface of APC. This structure is termed the "immunological synapse", which is similar to "neurological synapse".

Semaphorins, named for their analogy to the system of signaling flags used in maritime communications, are chemorepulsive factors required for guiding neuronal axons to appropriate targets. Since semaphorins were first described in the early 1990s, more than 20 types of these proteins have been identified (Fig. 1).[2–4] Although they were originally identified as axonal guidance factors,[4] semaphorins are currently known to have diverse and important functions in other physiological processes[5] including heart morphogenesis,[6] vascular growth,[7,8] tumor progression[9–11] and immune cell regulation.[12–14]

Semaphorins are secreted and membrane-associated proteins characterized by a conserved amino-terminal "Sema" domain. The semaphorins range in size from 400 to 1000 amino acid residues depending on additional C-terminal sequence motifs such as an immunoglobulin domain, thrombospondin domain or glycosylphosphatidylinositol (GPI) linkage site. On the basis of structural elements and amino acid sequence similarities, the family has been divided into eight subclasses. Invertebrate semaphorins are grouped into classes I and II, whereas classes III–VII are expressed in vertebrates. In addition, some DNA viruses encode functional semaphorin proteins. Semaphorins in classes I and IV–VII are membrane associated, whereas those in classes II, III and the viral semaphorins are secreted. Two groups of proteins, plexins and neuropilins, have been identified as the primary receptors for semaphorins.[15,16] Most

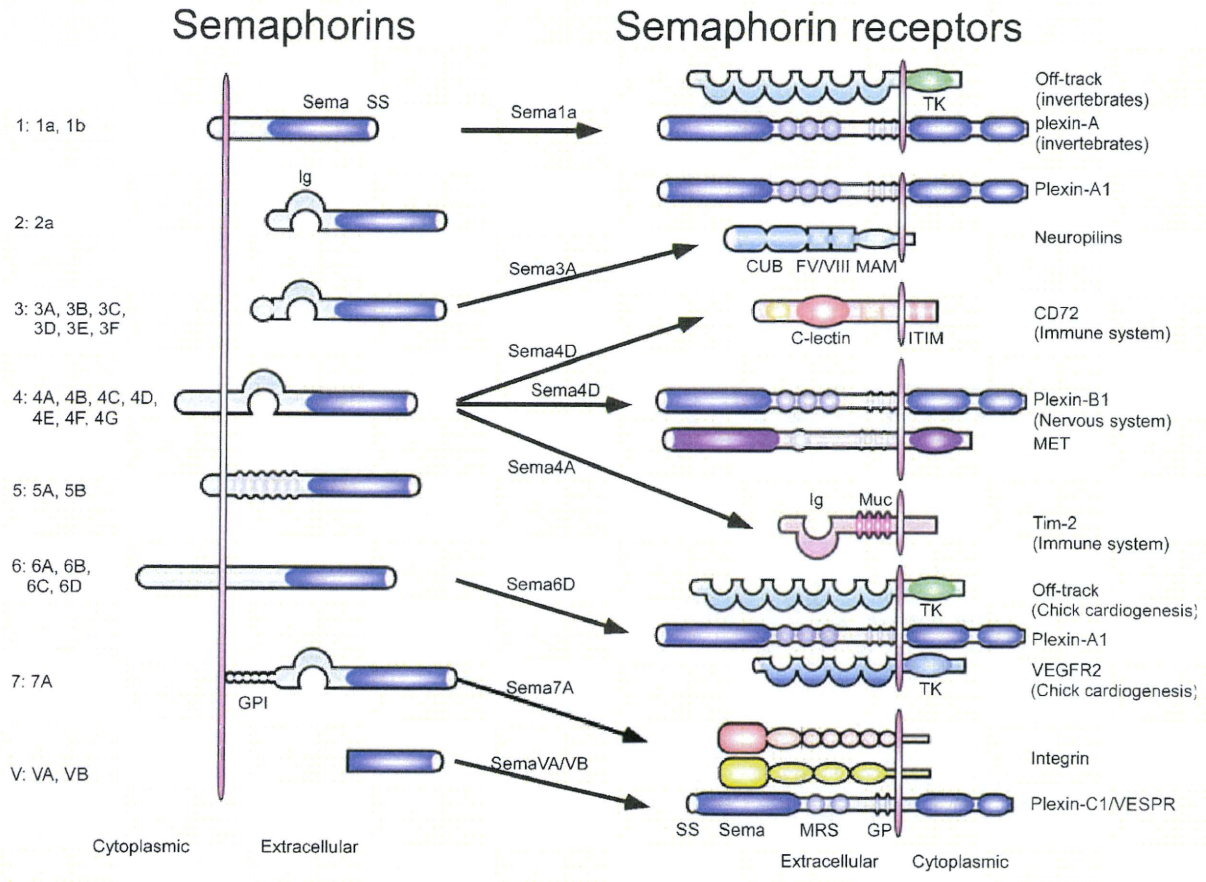

Figure 1 Semaphorins and their receptors. Class I and II semaphorins are found in invertebrates and class III–VII are vertebrate semaphorins. Classes II and III, and viral semaphorins are secreted, whereas class IV–VI are transmembrane. Class VII represents glycosylphosphatidylinositol-anchored proteins. The major semaphorin receptors are plexin family proteins. Plexins are categorized into four groups (A, B, C and D) and also carry sema domains. Another group of semaphorin receptors are neuropilins (neuropilin-1 and neuropilin-2), which form receptor complexes with plexin-A family members and exclusively bind to class III semaphorins. Plexins associate with several transmembrane molecules such as Met, Off-track and VEGFR2. In addition, in the immune system some semaphorins use non-plexin receptors such as CD72 and TIM-2.

membrane-bound semaphorins bind plexins directly, but class III semaphorins require neuropilins as obligate co-receptors.[17] However, recent reports have suggested that receptor usage by semaphorins is more complex than previously thought. For example, Sema3E signals independently of neuropilin through plexin-D1,[18] and Sema7A uses integrin receptors to exert its function in both the nervous and immune systems.[19,20] Some plexins further associate with various co-receptors to exert the diverse functions of semaphorins.[10,21,22] Additionally, in the immune system two molecules unrelated to plexins and neuropilins, CD72[23] and T cell immunoglobulin and mucin domain protein-2 (TIM-2),[24] functionally interact with Sema4D and Sema4A, respectively. The functions of semaphorins and their receptors have been shown by using gene-targeted mice (Table 1).

Understanding of the immunoregulatory functions of the semaphorin family has advanced considerably over the past several years. These semaphorins are currently called "immune semaphorins" (Fig. 2).[12,14] Furthermore, cumulative evidence shows that immune semaphorins are pathologically involved in various immune compromised diseases, including immune mediated-neurodegenerative disorders. In the present review, we will describe the most recent knowledge of their pathological involvement in neurological disorders.

Immune semaphorins

Sema4D: a semaphorin involved in B cell/DC activation

Sema4D, also known as CD100, is the first semaphorin protein of which immunoregulatory func-

Table 1 Phenotypes of semaphorin/semaphorin receptor/coreceptor-knockout mice

Knockout molecules	Phenotypes in non immune systems	Phenotypes in the immune system
Sema3A	Abnormalities in peripheral nerve projection	Enhanced T cell proliferation
Sema4A	Abnormalities in retinal formation	Impaired T cell priming Impaired helper T cell differentiation
Sema4D	Enhancement of motor activity	Impaired B cell activation and humoral immune responses Impaired T cell priming
Sema7A	Abnormalities in lateral olfactory tract formation	Impaired macrophage activation and inflammatory responses
Neuropilin-1	Abnormalities in the trajectory of efferent fibers of the peripheral nerve projection	Hyperproliferation of T cells (in Neuropilin-1sema-mutant mice)
Plexin-A1	Defective in the organization of cutaneous afferents	Impaired DC activation and T cell priming Impaired osteoclast development and develop osteopetrosis
Plexin-A4	Defective in the trajectory and projection of peripheral sensory axons	Hyperproliferation of T cells and enhanced T cell priming
Plexin-B1	Major defects has not been detected	Not reported
TIM-2	Not reported	Enhanced basal proliferation of T cells Dysregulated Th2 responses
CD72	Not reported	Enhanced B cell activation
Integrin α1β1	Develop hypocellular dermis	Inhibition of effector phase inflammatory responses
TREM-2	Not reported	Enhanced cytokine production by stimulation with TLR ligands
DAP12	Developmental arrest of oligodendrocytes and develop hypomyelinosis	Impaired T cell priming Impaired osteoclast development and develop osteopetrosis

tions were identified. In the immune system, the expression of Sema4D is detectable in resting T cells.[25,26] The basal expression of Sema4D in B cells and DC is very low, but it is considerably upregulated after cellular activation.[23]

Sema4D promotes B cell activation in the context of proliferation and antibody production.[23] Regarding the receptors for Sema4D, plexin-B1[16,23,27,28] and CD72[23] have been identified in the nervous and immune systems, respectively. CD72 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIM) in its cytoplasmic domain.[29,30] CD72 is known to function as a negative regulator of B cells through the recruitment of a tyrosine phosphatase SHP-1 to its phosphorylated ITIM.[31,32] Ligation of Sema4D to CD72 induces the dissociation of SHP-1 from CD72, resulting in the activation of B cells.[23] Sema4D-deficient mice display impaired antibody production.[33] implicating Sema4D in B cell activation.

In addition to the involvement of Sema4D in B-cell responses, it also plays a role in T cell responses through the activation of DC.[34] Sema4D expressed on T cells interacts with its cognate receptor on DC to promote the activation and maturation of DC, resulting in enhanced T cell activation. In fact, Sema4D-deficient mice display impaired generation of antigen-specific T cells.

Although Sema4D is a transmembrane protein, the extracellular region is proteolytically cleaved from the surface of activated lymphocytes by a metalloprotease-dependent process.[35] Sema4D is also cleaved from the surface of platelets by the metalloprotease ADAM17.[36] The elevation of the levels of soluble Sema4D protein is detectable in culture supernatants of activated lymphocytes and in the sera of either immunized or autoimmune mice.[35]

Sema4A: a semaphorin involved in both T cell activation and differentiation

Sema4A is another class IV semaphorin. Sema4A is expressed in a broad range of adult tissues, including the brain, lung and spleen. In the immune system, Sema4A is constitutively expressed on DC.[24] The expression of Sema4A is also detectable in activated T cells and T helper type 1 (Th1)-polarized cells.[37] DC-derived Sema4A and T cell-derived Sema4A play different roles during the course of T cell-mediated immunity. DC-derived Sema4A is crucial for antigen-specific T cell priming,[24] whereas T cell-derived Sema4A is involved in helper T cell differentiation.[37] Indeed, the critical involvement of Sema4A in the differentiation of helper T cells has been shown by the phenotypes of Sema4A-deficient mice. Sema4A-deficient mice show impaired responses to heat-killed *Propionibacterium acnes*, a Th1-inducing agent. Conversely, Sema4A-deficient mice show enhanced T helper type 2 (Th2) responses against infection of *Nippostrongylus brasiliensis*, a Th2-inducing intestinal

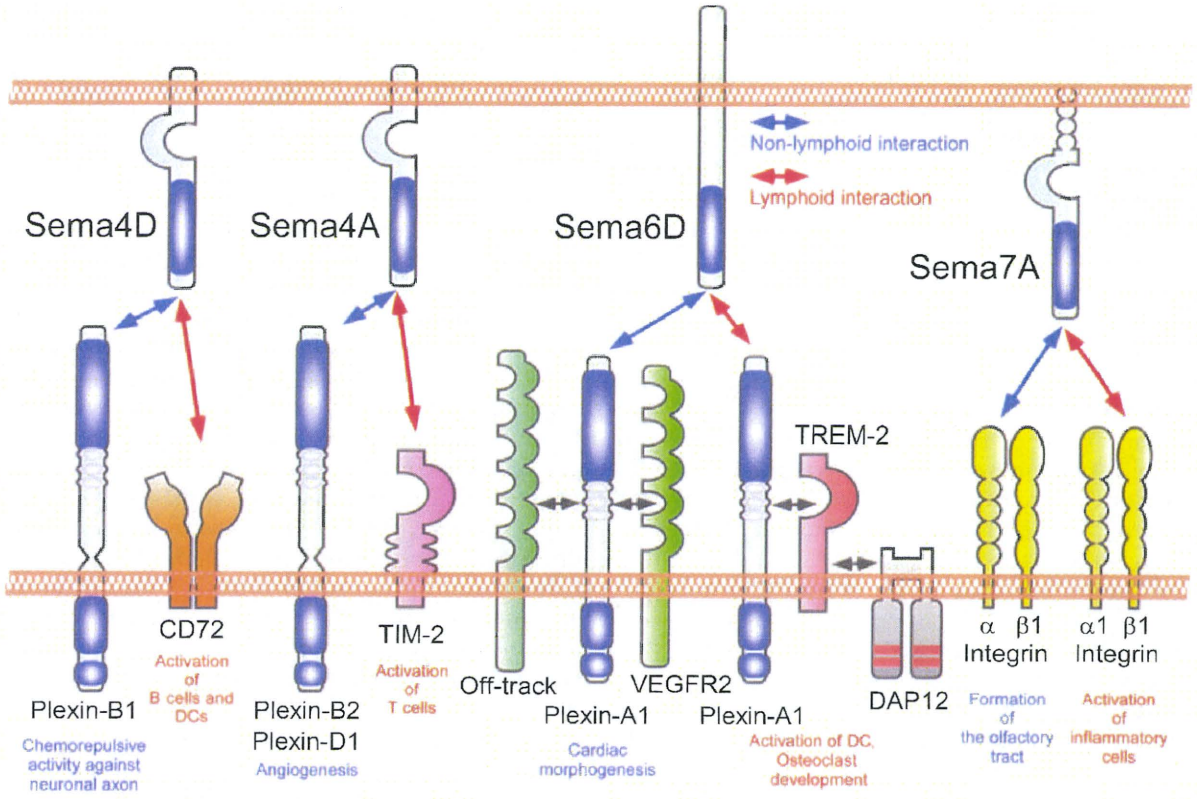

Figure 2 Representative immune semaphorins and their receptors in lymphoid and non-lymphoid cells. Although Sema4D binds to plexin-B1 in the brain and transduces chemorepulsive signals, plexin-B1 couples with Met in epithelial cells and induces Sema4D-mediated cell outgrowth. In the immune system, Sema4D uses CD72 as a functional receptor in B cells and dendritic cells (DC), and enhances the activation of B cells and DC. Sema4A binds TIM-2 and is involved in T cell activation and differentiation in the immune system. In the non-immune system, however, Sema4A recognizes plexin-B proteins and plexin-D1. Sema6D exerts different biological activities through plexin-A1, depending on its co-receptors. During chick embryogenesis, plexin-A1 differentially associates with Off-track and VEGFR2, and these receptor complexes have distinct functions in heart development. In the immune system, plexin-A1 forms a receptor complex with TREM-2 and DAP12 and, after Sema6D binds, this complex transduces signals that stimulate DC and osteoclasts. Sema7A uses β1 integrin as receptors in both the nervous and immune systems. In the immune system, Sema7A expressed on activated T cells stimulates macrophages through α1β1 integrin to promote inflammatory responses.

nematode.[37] Furthermore, Sema4A-deficient mice on a Th2-prone BALB/c background spontaneously develop atopic dermatitis (AD) (T.M. unpublished data), supporting the notion that Sema4A is involved in the regulation of Th1/Th2 development.

In the immune system, TIM-2-expression is induced on activated T cells.[24] Several lines of evidence support that TIM-2 serves as a functional receptor for Sema4A. The expression of TIM-2 is preferentially upregulated on T cells during Th2 differentiation. Administration of recombinant TIM-2 protein suppresses the development of experimental autoimmune encephalomyelitis (EAE) in SJL is suitable mice immunized with proteolipid protein-derived peptide by inhibiting the generation of Th1 cells.[38] Furthermore, TIM-2-deficient mice show exacerbated lung inflammation accompanied by dysregulated Th2 responses.[39] Taken together, it is tempting to speculate that Sema4A-TIM-2 interactions negatively regulate Th2 responses. However, there are some phenotypic differences between Sema4A-deficient and TIM-2-deficient mice. For example, T cells from TIM-2-deficient mice but not from Sema4A-deficient mice show enhanced basal proliferation. The observation raises the possibility that Sema4A and/or TIM-2 have other binding partners. Indeed, T cells express members of plexin-B proteins and plexin-D1, both of which have Sema4A-binding activities.[7]

Sema6D and plexin-A1: an interaction involved in T cell-dendritic cell interface and osteoclastogenesis

Plexins function as semaphorin receptors during the development of the nervous and cardiovascular

systems. Plexin-A1 functions have been extensively investigated in both the nervous and cardiovascular systems. Class III semaphorins bind a receptor complex formed by plexin-A1 and neuropilin-1. Additionally, plexin-A1 serves as a direct binding receptor for class VI semaphorins, Sema6C, and Sema6D.[21,40]

In the immune system, plexin-A1 is specifically expressed by DC. The function of plexin-A1 in DC is shown using an RNA interference system and analysis of plexin-A1 knockout mice. "Knockdown" of plexin-A1 in DC by short hairpin RNA impairs their ability to activate T cells *in vitro* and *in vivo*.[41] In addition, plexin-A1-deficient DC poorly stimulate antigen-specific T cells.[22] Furthermore, plexin-A1-deficient mice show impaired T cell-priming. These observations indicate that plexin-A1-expression in DC is required for the initial activation and efficient generation of antigen-specific T cells.[22] Additionally, plexin-A1 is involved in osteoclast differentiation, that is, plexin-A1-deficient mice develop osteopetrosis as a result of decreased bone reabsorption by defective osteoclastogenesis.[22]

Regarding the ligand of plexin-A1 in the immune system, Sema6D was identified as a putative ligand for plexin-A1 in the regulation of DC function.[22] The expression of Sema6D mRNA is detectable in T cells, B cells and natural killer (NK) cells. Recombinant Sema6D protein binds to and activates DC, and these activities are profoundly attenuated in plexin-A1-deficient DC.[22] These observations suggest that Sema6D-expression on T cells is involved in DC-activation. The expression of Sema6D is also observed in osteoclasts.[22] Recombinant Sema6D protein enhances *in vitro* osteoclastogenesis, suggesting that Sema6D-plexin-A1 might function in osteoclastogenesis in an osteoclast-autonomous manner. Plexin-A1 forms a receptor complex with the receptor-triggering receptor expressed on myeloid cell-2 (TREM-2) and the adaptor molecule DAP12 in DC and osteoclasts.[22] DAP12-deficient mice show impaired T cell responses and develop osteopetrosis,[42,43] and genetic mutations of human DAP12 or TREM-2 result in a bone fracture syndrome called Nasu-Hakola disease, supporting the idea that plexin-A1 physiologically associates with TREM-2/DAP12 complex.

Sema7A: a semaphorin involved in inflammatory responses

Sema7A, also known as CD108, is a membrane-associated GPI-linked protein. Sema7A transcripts are detectable in the embryonic nervous system and in adult tissues, including the brain, spinal cord, lung and secondary lymphoid organs.[44,45] In the nervous system, Sema7A has been shown to promote olfactory bulb axon outgrowth and is required for the proper formation of the lateral olfactory tract during embryonic development.[19] Plexin-C1 was initially identified as a receptor for Sema7A.[46] However, Sema7A contains an arginine–glycine–aspartate sequence that is a well conserved integrin-binding motif in its Sema domain, and it exerts its function through β1 integrin, not through plexin-C1.[19]

In the immune system, the expression of Sema7A is induced on activated T-cells,[45] and it is involved in T cell-mediated inflammatory immune responses.[20] Recombinant Sema7A protein stimulates monocytes/macrophages through α1β1 integrin, also known as very late antigen-1, inducing the production of proinflammatory cytokines.[20] Consistently, Sema7A-deficient mice are resistant to the development of inflammation, including hapten-induced contact hypersensitivity and experimental autoimmune EAE.[20] These observations indicate that interactions between Sema7A and α1β1 integrin is crucial for T cell-mediated macrophage activation at sites of inflammation.[20] Plexin-C1 is also expressed in macrophages. However, stimulation with recombinant Sema7A protein induces normal production of proinflammatory cytokines by plexin-C1-deficient macrophages (unpublished data). Therefore, at least for the T cell–macrophage interactions, α1β1 integrin but not plexin-C1 seems to be the predominant receptor for Sema7A. Furthermore, integrin-mediated signaling is a common mechanism for Sema7A-functions in both the nervous and immune systems.

Sema3A and plexin-A4: a semaphorin and its receptor required for negative regulation of T cell responses

Sema3A is the first semaphorin identified in vertebrates. Its function as an axon repellent has been well established. Sema3A directly binds to neuropilin-1, which induces activation of plexin-A proteins and the transduction of axon repulsive signals. Several lines of evidence suggest that Sema3A also functions in the immune system. The expression of Sema3A is detected in activated DC, T cells and some tumor cells. Sema3A inhibits spontaneous monocyte migration *in vitro*. In addition, Sema3A suppresses T cell proliferation by inhibiting actin cytoskeletal reorganization and downregulating MAPK signaling.[36,47] Furthermore, Sema3A-deficient T cells exhibit enhanced *in vitro* proliferative responses to anti-CD3 antibodies.[48] These observations suggest that Sema3A serves as a negative regulator of T cells.

Similar to other plexin-A proteins, plexin-A4 forms a receptor complex with neuropilin-1 to transduce class III semaphorin-mediated signaling or directly binds to Sema6A.[49] In the immune system, the expression of plexin-A4 is observed in various cells including T cells, DC and macrophages, but not in B and NK cells.[48] Plexin-A4-deficient T cells exhibit hyperproliferation and enhanced TCR signals on anti-CD3 stimulation.[48] Furthermore, plexin-A4-deficient mice show enhanced T cell priming and exacerbated T cell-mediated immune responses such as EAE.[48] Therefore, plexin-A4 might interact with Sema3A in the immune system and this interaction might negatively regulate T cell responses. However, it remains unclear how plexin-A4 negatively regulates T cells and whether other semaphorins are relevant to plexin-A4-mediated immune responses.

Neuropilin-1: a marker for regulatory T cells

As described above, neuropilin-1 was originally identified as a cell surface glycoprotein that acts as a class III semaphorin receptor. Neuropilin-1 is also known as human DC-specific antigen (blood DC antigen)-4, a specific plasmacytoid DC marker in humans, and was assigned CD304. In the immune system, the expression of neuropilin-1 was observed in DC and T cells.[50] Neuropilin-1 has been thought to be involved in the initiation of primary immune responses through a homophilic interaction at the contact sites between T cells and DC.[50] In addition, neuropilin-1 has been identified as a specific marker for $CD4^+CD25^+$ regulatory T (Treg) cells.[51] Indeed, neuropilin-1 is part of the group of forkhead box P3 (Foxp3)-inducible genes, including CD25, glucocorticoid-induced tumor necrosis factor receptor-related protein and cytotoxic T-lymphocyte antigen-4 (CTLA-4).[51]

More recently, one report suggested that neuropilin-1 in Treg cells contributes to the long contact between Treg cells and DC compared with the shorter contact between naive T cells and DC.[52] Treg cells made stable contact with DC that precedes the contact of naive T cells with DC, and this might lead to the inhibition of T-cell activation in the steady state. The finding that Treg cells are endowed with the ability to have long interactions with DC by neuropilin-1 supports the idea that neuropilin-1 might contribute to physical interaction between T cells and DC. However, it remains to be elucidated whether the long contact is mediated by a homophilic interaction such as semaphorins and neuropilin-1 or neuropilin-1-associating molecules such as plexins.

Semaphorins and neurological disorders

A large body of evidence has shown that the immune system is sometimes deleterious for the survival of neurons and the maintenance of central nervous system (CNS) integrity.[53] Once rapid and extensive neuronal death occurs as a result of infections, trauma, stroke or ischemic injury, CNS integrity is compromised and activated microglia or lymphocytes, especially T cells, inevitably come into contact with neurons. This accelerates degeneration of neurons concomitantly with increased expression of proinflammatory molecules. Neuroinflammation has been associated with a wide range of neurological disorders, including Alzheimer's disease, Parkinson's disease and multiple sclerosis (MS).[54,55] In accordance with their expression in the brain and their roles in immune responses, it is emerging that immune semaphorins and their receptors are crucially involved in the protection and/or progression of inflamed disorders in the CNS (Table 2). In this section, we will discuss the roles of immune semaphorins and their receptors in neurological disorders.

Multiple sclerosis, EAE and semaphorins

Multiple sclerosis is an immune-mediated chronic disease characterized by disseminated foci of inflammatory demyelination affecting the CNS. Although the pathogenesis of MS has not yet been elucidated, the conventional hypothesis assumes a T cell-mediated autoimmune reaction against unknown myelin antigens because there is an accumulation of activated T cells in nervous tissues, a hallmark of autoimmune diseases of the CNS. Myelin oligodendrocyte glycoprotein (MOG)-induced EAE is an established animal model of MS. EAE is, in part, mediated by neuroantigen-reactive T cells, especially $CD4^+$ T cells. In this model, inflammatory demyelination and axon loss as a result of a concerted T cell response are observed. Cumulative evidence shows that some immune semaphorins and their receptors play a role in the development of EAE through regulation of antigen-specific T cell immunity.

Sema4D and EAE

As described earlier, Sema4D expressed on T cells is crucially involved in the initial activation of T cells through maturation of DC.[34] When Sema4D-deficient mice are immunized with a MOG-peptide in Freund's complete adjuvant (CFA), they show attenuated development of EAE. $CD4^+$ T cells from the draining lymph nodes of immunized Sema4D-

Table 2 Immune semaphorins, their receptors and neurological diseases

Semaphorins/receptors	Expression in the immune system	Binding partner	Immunological activities	Related neurological diseases
Sema3A	ND	Plexin A proteins	Inhibition of monocyte migration Inhibition of T cell activation	Alzheimer's disease Atopic dermatitis
Sema4A	Dendritic cells Activated-T cells Th1 cells	Plexin B proteins Plexin-D1 TIM-2	T cell activation Promotion of Th1-differentiation	EAE Atopic dermatitis
Sema4D	T cells Activated-B cells Dendritic cells	Plexin-B1 CD72	B-cell activation DC-activation	EAE HAM
Sema5A	ND (Oligodendrocytes)	ND	ND	Parkinson's disease
Sema6D	T cells B cells NK cells	Plexin-A1	DC-activation	
Sema7A	Acticated-T cells	Plexin-C1 Integrin α1β1	Monocyte/macrophage-activation	Contact hypersensitivity EAE
Neuropilin-1	T cells Treg cells	Class III semaphorins		Alzheimer's disease
Plexin-A1	Dendritic cells (Osteoclasts)	Class VI semaphorins	DC-activation	EAE
Plexin-A4	T cells Dendritic cells Macrophages	Class VI semaphorins	Inhibition of T-cell activation	EAE
Plexin-B1		Class IV semaphorins		
TIM-2	Acticated-T cells Th2 cells	Sema4A	T-activation	EAE Airway atopy
CD72	B cells Dendritic cells	Sema4D	B cell activation DC-activation	
Integrin α1β1	Monocytes Macrophages	Sema7A	Monocyte/macrophage-activation	EAE

deficient mice exhibit impaired antigen-specific T cell responses, particularly the generation of cytokine-producing effector cells, after *in vitro* restimulation with antigen. These observations indicate the involvement of Sema4D in the pathogenesis of EAE during the interaction between T cells and DC.

Sema4A and EAE

Sema4A expressed on DC is involved in the initial activation of T cells.[24] Development of MOG-induced EAE in wild-type mice can be suppressed by intravenous injection of anti-Sema4A monoclonal antibody concurrently with MOG immunization.[24] Infiltration of mononuclear inflammatory cells into the spinal cord is diminished in anti-Sema4A antibody-treated mice, in which MOG-peptide-specific responses of $CD4^+$ T cells isolated from the draining lymph nodes are greatly decreased. Thus, blocking Sema4A with anti-Sema4A monoclonal antibody inhibits generation of MOG-peptide-specific $CD4^+$ T cells, leading to attenuated development of EAE.[24] T helper type 17 (Th17) cells, $CD4^+$ T cells that secret IL-17, play a critical role in inflammatory pathology in autoimmune diseases, including MS. Because the function of Sema4A is shown to be important for helper T cell differentiation,[37] it is plausible that Sema4A is involved in both the priming and effector phases of EAE through regulation of Th17 cell development even though the relevance of Sema4A in the development of Th17 cells has not been clarified. Ongoing studies will clarify the involvement of semaphorins in the development of Th17 cells and the pathogenesis of EAE.

Sema7A and EAE

Sema7A is involved in T cell-mediated inflammation through the activation of peripheral macrophages.[20] When Sema7A-deficient mice are immunized with MOG-peptide in CFA, the T cells are primed normally and generate MOG-peptide-specific $CD4^+$ T cells. However, these mice are resistant to EAE development. $CD4^+$ T cells from MOG-immunized Sema7A-deficient mice fail to induce EAE when they are transferred into naïve wild-type mice. In addition, MOG-peptide-primed $CD4^+$ T cells from wild-type mice fail to induce EAE on transfer into

α1 integrin-deficient mice. Furthermore, Sema7A on antigen-primed effector T cells plays a role in the induction of inflammation in EAE through interaction with α1β1 integrin, and contribute to the exacerbation of EAE.[20] These findings show the pathological involvement of Sema7A in the effector phase of EAE, of which functional sites seem to be different from those of class IV semaphorins.

Plexin-A1 and EAE

Plexin-A1 expressed on DC is involved in the generation of antigen-specific T cells.[22] Immunization of plexin-A1-deficient mice with MOG-peptide in CFA results in impaired development of EAE in accordance with impairment of MOG-peptide-specific $CD4^+$ T cell responses.[22] Consistent with the finding that DAP12 associates with plexin-A1, DAP12-deficient mice exhibit attenuated development of MOG-induced EAE and impaired generation of MOG-specific T cells.[42,56]

Plexin-A4 and EAE

Plexin-A4 is also involved in the pathology of EAE. As described above, plexin-A4 negatively regulates T cell immunity.[48] In accordance with *in vitro* hyper responses of plexin-A4-deficient $CD4^+$ T cells, plexin-A4-deficient mice exhibit enhanced generation of antigen-specific-T cells and exacerbated EAE when they are immunized with MOG-peptide in CFA.[48] On transfer to naïve wild-type mice, $CD4^+$ T cells from MOG-immunized plexin-A4-deficient mice can induce a more severe EAE than cells from wild-type mice.[48] Collectively, these observations indicate that plexin-A4 is involved in the pathology of EAE, although its contribution is precisely the opposite to that of plexin-A1. It is interesting to determine why these closely related molecules, plexin-A1 and plexin-A4, play opposite functions in T cell immunity; plexin-A1 expressing DC are required for the induction of effective T cell immunity, whereas plexin-A4 expression by T cells is required for the regulation of excess T cell responses. Further studies are required to clarify the detailed mechanism underlying the opposing functions of these two molecules.

Parkinson's disease and semaphorins

Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra and the presence of neuronal intracellular Lewy bodies. The clinical features of Parkinson's disease are resting tremor, rigidity, bradykinesia and postural instability. Recently, a link between Sema5A and Parkinson's disease has been reported. Sema5A is a member of class V semaphorin proteins. The extracellular domain of Sema5A contains seven thrombospondin type-1 repeats following the Sema domain.[57] In the nervous system, Sema5A is expressed by oligodendrocytes and inhibits axonal growth,[58] and has been shown to be essential for the development of the extra-embryonic tissues and the cardiovascular system.[59,60] A high resolution whole genome association study showed that a single-nucleotide polymorphism within SEMA5A (rs7702187) is associated with Parkinson's disease.[61] In addition, polymerase chain reaction-restriction fragment length polymorphism analysis showed an association of Sema5A haplotypes with Parkinson's disease risk in the Chinese Han population, although the analysis showed no significant association of variant genotypes of Sema5A with the risk of Parkinson's diseases in the population.[62,63] Not only genetic factors but also environmental factors have been suggested to be risk factors for Parkinson's disease. Further studies are needed to understand the exact mechanism of the pathogenesis of Parkinson's disease.

Alzheimer's disease and semaphorins

The neurodegeneration of selectively vulnerable hippocampal CA1 and subicular pyramidal neurons is a hallmark of the earliest pathogenesis of Alzheimer's disease. Neurodegenerative changes in hippocampal CA1 and subiculum are observed during the incipient phases of Alzheimer's disease, and progress during further pathogenesis of the disease. The relationship between Alzheimer's disease and semaphorins was first reported in 1999, in which the altered expression pattern of class IV semaphorins was reported in the brains of patients with Alzheimer's disease.[64] Several lines of evidence suggest the association of semaphorin-plexin signaling with Alzheimer's disease. Progressive accumulation of Sema3A proteins was detected in hippocampal CA1 and subicular neurons in Alzheimer's disease.[65] In addition, phosphorylated microtubule associated protein 1B, collapsin-response mediator protein-2 (CRMP-2), plexin-A1 and plexin-A2 were also detected in the hippocampus of patients with Alzheimer's disease.[65] CRMP-2 has been identified as an intracellular signaling molecule in the Sema3A signaling pathway. Phosphorylated CRMP-2 was detectable in neurofibrillary tangles in Alzheimer's disease,[66–68] and stimulation with Sema3A enhanced the levels of the phosophorylated form of CRMP-2.[69] Collectively,

these observations suggest that aberrant Sema3A signaling might contribute to the degeneration of neurons in the CA1 field of the hippocampus of Alzheimer's disease patients.

Amyotrophic lateral sclerosis and semaphorins

Amyotrophic lateral sclerosis (ALS) is a devastating disease characterized by progressive neurodegeneration of motor neurons in the brain and spinal cord.[70] The loss of motor neurons results in paralysis of voluntary muscles and eventual death by respiratory failure within 1–5 years of the disease. A direct relationship between Sema3A and ALS was recently reported. Transgenic mice that express a dominant gain-of-function mutant of superoxide dismutase-1 ($SOD1^{G93A}$), a mouse model for ALS, displayed a marked increase of Sema3A expression in terminal Schwann cells (TSC).[71] This increase was limited to TSC on a specific subset of muscle fibers known as fast-fatigable or type IIb and IIx muscle fibers. This subtype of muscle fiber has been characterized by its inability of stimulate nerve sprouting after injury and is the first muscle subtype that is lost in ALS. Although an increased expression of Sema3A was also found in adult TSC,[71] developmental expression changes might be involved in inappropriately repelling motor axons away from the neuromuscular junctions and predispose patients to ALS.

Human T lymphotropic virus type 1 associated myelopathy and semaphorins

Myelopathy associated with human T lymphotropic virus type 1 infection (tropical spastic paraparesis/HTLV-1-associated myelopathy) is a neuroinflammatory disease characterized by inflammation with white matter damage and axonal degeneration in the brain and spinal cord. Both infiltrating T cells and inflammatory mediators are suspected to participate in the pathogenic mechanisms of HAM. Soluble forms of Sema4D have been reported to be increased in the cerebrospinal fluid and spinal cords of patients with HAM.[72] Activated T cell-derived Sema4D induces apoptotic cell death of multipotent neural progenitors and immature oligodendrocytes, both of which are required for re-myelination and neuronal integrity.[72] These observations suggest that Sema4D might function in the deleterious cross talk between T cells and neuronal cells during neuroinflammation, thus playing a role in demyelination or inhibiting re-myelination in neuroinflammatory diseases such as HAM.

Neuroinflammation, other neurological disorders and semaphorins

Sema3A, neuropilin-1 and plexin-A1 play roles in T cell responses and T cell-mediated immunity.[22,48,50,52] Recent studies also showed the involvement of these molecules in the protection of neurons through interactions between neurons and microglia. In the rat CNS, stress signals induced the upregulation of Sema3A in neurons while activation of microglia induced upregulation of plexin-A1 and neuropilin-1.[73] Culture with recombinant Sema3A induced apoptosis-mediated cell death of microglia and a similar result was obtained on activated microglia on coculture with stressed neurons which produce Sema3A.[73] It has been shown that the interaction with apoptotic neurons induces microglial cells to release neuroprotective agents, such as anti-inflammatory cytokines and growth factors, while inhibiting the production of nitric oxide and proinflammatory cytokines.[74] Thus, these observations indicate that Sema3A expressed by stressed neurons might serve to protect them from further damage by microglia through the promotion of microglial cell death.

Interestingly, contributions of Sema3A on the treatment of AD have been reported. In AD patients, C-fibers in the epidermis increase and sprout, inducing hypersensitivity, which is thought to aggravate the disease. Administration of recombinant Sema3A to the skin lesions of NC/Nga mice, an animal model of AD, resulted in the improvement of skin lesions and attenuation of the scratching behavior in NC/Nga mice.[75] Histological examination showed a decrease in epidermal thickness and the density of invasive nerve fibers in the epidermis.[75] In addition, infiltration of immune cells, such as mast cells and $CD4^+$ T cells, also decreased.[75] These observations suggest an alleviative effect of Sema3A on AD. Because the interruption of the itch–scratch cycle likely contributes to the improvement of the atopic-dermatitis lesions, Sema3A will become a good pharmacological target for treatment of AD patients.

Regarding co-receptors of plexin-A1, dysfunction of TREM-2 or DAP12 is known to be the cause of Nasu-Hakola disease.[43,76,77] Nasu-Hakola disease is characterized by a combination of bone fractures and psychotic symptoms similar to schizophrenia, rapidly progressing to presenile dementia. A recent study has identified a role for TREM-2 expression by microglia in Nasu-Hakola disease.[78] In the nervous system, the expression of TREM-2 was detectable in microglia. Knockdown of TREM-2 in microglia by