

# FAK–MAPK-dependent adhesion disassembly downstream of L1 contributes to semaphorin3A-induced collapse

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Axonal receptors for class 3 semaphorins (Sema3s) are heterocomplexes of neuropilins (Nrps) and Plexin-As signalling coreceptors. In the developing cerebral cortex, the Ig superfamily cell adhesion molecule L1 associates with Nrp1. Intriguingly, the genetic removal of L1 blocks axon responses of cortical neurons to Sema3A *in vitro* despite the expression of Plexin-As in the cortex, suggesting either that L1 substitutes for Plexin-As or that L1 and Plexin-A are both required and mediate distinct roles. We report that association of Nrp1 with L1 but not Plexin-As mediates the recruitment and activation of a Sema3A-induced focal adhesion kinase–mitogen-activated protein kinase cascade. This signalling downstream of L1 is needed for the disassembly of adherent points formed in growth cones and subsequently their collapse response to Sema3A. Plexin-As and L1 are coexpressed and present in common complexes in cortical neurons and both dominant-negative forms of Plexin-A and L1 impair their response to Sema3A. Consistently, Nrp1-expressing cortical projections are defective in mice lacking Plexin-A3, Plexin-A4 or L1. This reveals that specific signalling activities downstream of L1 and Plexin-As cooperate for mediating the axon guidance effects of Sema3A.

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## Introduction

Class 3 semaphorins (Sema3s) are secreted guidance cues whose repulsive activity restricts the trajectory of a great

number of axon subsets to prevent their aberrant growth during the development of neuronal connectivity (He *et al*, 2002). Axonal receptors for Sema3s comprise binding subunits, the Nrps, complexed with signalling co-receptors, the Plexin-As, which mediate the guidance activity of Sema3s by activating various intracellular pathways, including RhoGTPases, CRMPs, mitogen-activated protein kinase (MAPK), PI3K and Src kinase families (Kruger *et al*, 2005). Ig superfamily cell adhesion molecules (IgSFCAMs) have also been found to contribute to Sema3 responses, but their function has remained elusive so far (Castellani and Rougon, 2002; Falk *et al*, 2005). The IgSFCAM L1 associates with neuropilin (Nrp)1 and was proposed to be a component of the Sema3A receptor (Castellani *et al*, 2000; Castellani, 2002; Itoh *et al*, 2004). Similarly, another member of the L1-CAM subgroup, NrCAM, was found to associate with Nrp2 and to be required for growth cone responses to Sema3B and Sema3F *in vitro* (Falk *et al*, 2005). In both cases, the interaction is mediated by the ectodomain of IgSFCAMs and Nrp proteins.

During the development of cortical connectivity, Sema3A is thought to guide axons at several levels, from their initial growth out of the cortical plate of the cerebral cortex, through the corpus callosum and during their navigation to subcortical targets in the internal capsule and at the entry of the spinal cord (Bagnard *et al*, 1998; Polleux *et al*, 1998; Castellani *et al*, 2000; Gu *et al*, 2003). Interestingly, the genetic removal of L1 also disturbs these cortical pathways, causing midline crossing defects and size reduction of the corpus callosum (Cohen *et al*, 1998; Demyanenko *et al*, 1999; Castellani *et al*, 2000; Dahme *et al*, 2007). Disorganization of fibres in the internal capsule and defective pyramidal decussation have also been reported in L1- $\gamma$  mice (Ohyama *et al*, 2004; Dahme *et al*, 2007). The contribution of Plexin-As to the guidance effects of Sema3A on cortical axons has not yet been investigated, but all plexin-A members are detected at the mRNA level in the developing cerebral cortex over the period of cortical neuron migration and axon extension (Murakami *et al*, 2001). Intriguingly, experiments conducted with explants from mice lacking L1 showed that cortical axons are unresponsive to Sema3A, indicating that Plexin-As cannot compensate for the lack of L1 *in vitro* (Castellani *et al*, 2000). These data lead to the question of the precise role of L1 in the Sema3A receptor and why Plexin-As cannot compensate their loss.

Four Plexin-A members have been identified and most of them are able to confer a morphological retraction to COS cells exposed to Sema3A when coexpressed with Nrp1, although the ligand binding affinity differs between the complexes (Togashi *et al*, 2006). Co-culture assays with DRG explants from mice lacking Plexin-A3 and Plexin-A4 have shown that Sema3A-responsive axons primarily utilize Plexin-A4 for their repulsive behaviour to Sema3A (Suto *et al*,

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2005; Yaron *et al*, 2005). However, cooperative effects exist between different Plexin-As as some DRG axons from Plexin-A4<sup>-/-</sup> mice only exhibited reduced sensitivity to *Sema3A* but were fully desensitized by additional genetic removal of Plexin-A3 (Yaron *et al*, 2005). In the context of the cerebral cortex, L1 and Plexin-A subunits could both be required in a common receptor to perform distinct and/or redundant roles. Alternatively, L1 could mediate *Sema3A* signalling and thus substitute to Plexin-As in these neurons. Finally, L1 and Plexin-As could also segregate in subpopulations of cortical axons to form distinct receptor complexes.

The goal of this study was to address these possibilities to gain insights into the role of L1 in the response of cortical neurons to *Sema3A*. We report that interaction of L1 but not Plexin-As with Nrp1 specifically mediates the activation of a focal adhesion kinase (FAK)-mitogen-activated protein kinase (MAPK) signalling pathway controlling a crucial process of the repulsive behaviour, the disassembly of adhesion points of the growth cones. We provide evidence from biochemical experiments, culture assays and analysis of mutant mice lacking Plexin-A3 that Plexin-A4 and L1 are coexpressed and initiate distinct signalling cascades that cooperate for mediating the growth cone responses to *Sema3A*. These data assign a specific function to L1 in *Sema3A* signalling during axon guidance and elucidate the lack of functional compensation for L1 by Plexin-A proteins in L1-deficient mice.

## Results

### ***FAK is required for the growth cone collapse response to Sema3A***

The first issue was to determine whether L1 shows signalling activity in the *Sema3A* receptor. The FAK acts downstream of integrin activation by extracellular matrix components and has a major role in the dynamics of focal adhesions (Mitra *et al*, 2005). Interestingly, FAK also participates in the signalling of guidance cues including *Sema3B* (Miao *et al*, 2000; Carter *et al*, 2002; Li *et al*, 2004; Liu *et al*, 2004; Ren *et al*, 2004; Falk *et al*, 2005) although its recruitment to Plexins has not been reported (Barberis *et al*, 2004). We investigated whether FAK participates in *Sema3A* signalling in cortical neurons and if so if it could be recruited by L1. Immunocytochemistry performed on E15 embryonic brain sections showed that L1, Nrp1 and FAK are expressed by post-mitotic neurons in the cortical plate of the developing cerebral cortex, consistent with some previous observations (Contestabile *et al*, 2003), and distributed over the cell body layer and the intermediate zone containing cortical efferents (Figure 1A). FAK and pFAK could also be detected in cultured cortical neurons (Figure 1A and data not shown). To address whether FAK mediates *Sema3A* signaling, different FAK variants were electroporated into cortical neurons. These included variants with a kinase-dead domain (FAK<sub>Δkin</sub>), a mutation on the Y397, which is the autophosphorylation site of FAK (FAK<sub>Y397</sub>), deletion of the FERM domain rendering FAK constitutively active (FAK<sub>ΔFERM</sub>; Cohen and Guan, 2005; Mitra *et al*, 2005) or wild-type FAK (FAK<sub>WT</sub>) as control. Collapse assays were performed on electroporated cortical neurons (Figure 1B, a total of 80 growth cones assessed per condition from 12 neonatal brains). Overexpression of FAK<sub>WT</sub> did not interfere with the collapse response observed for

wild-type neurons. FAK<sub>ΔFERM</sub> significantly increased the basal level of collapse observed under control conditions. Nevertheless, *Sema3A* application further increased the level of collapsed growth cones (Figure 1B). In contrast, FAK<sub>Δkin</sub> and FAK<sub>Y397</sub> forms had no detectable effects under control treatment but totally abrogated the collapse normally exerted by *Sema3A* (Figure 1B). Thus, FAK is required for *Sema3A* to collapse neuronal growth cones.

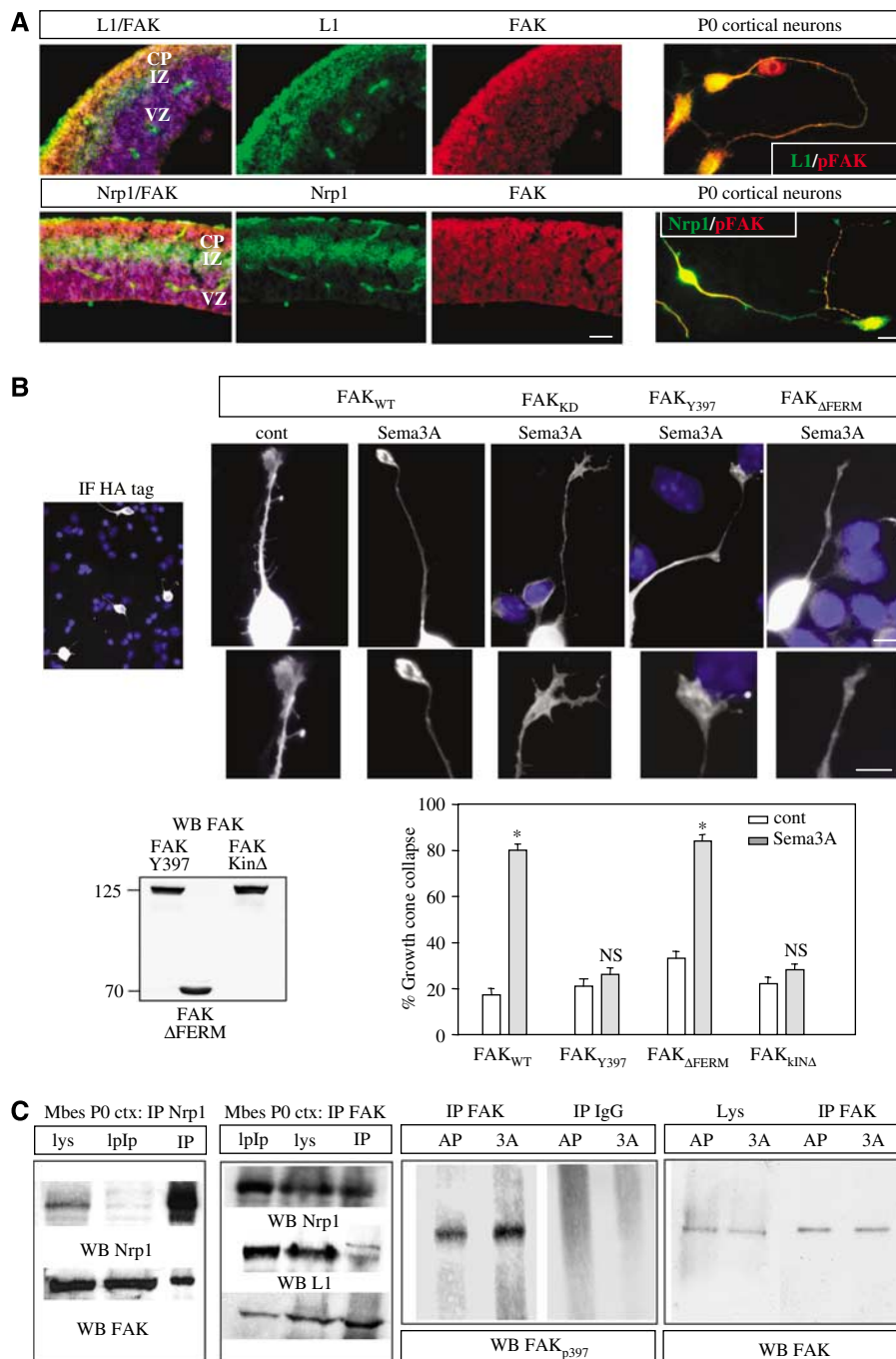
### ***FAK recruitment to the Sema3A receptor is mediated by L1/Nrp1 complex formation and is ligand-induced***

Next, we investigated the mechanisms underlying the contribution of FAK to *Sema3A* effects. First, to assess whether FAK is recruited to the *Sema3A* receptor, neonatal cortical tissue was collected and then processed for membrane purification and precipitation experiments with antibodies to Nrp1. Western blot analysis showed that FAK co-precipitates with Nrp1 and L1 from this tissue (Figure 1C). To examine FAK activation, FAK was precipitated from control and *Sema3A*-stimulated fresh cortical tissue. Western blot analysis showed that *Sema3A* induced Y397 phosphorylation of FAK (Figure 1C). Second, to search for the partners of the *Sema3A* receptor that mediate the recruitment of FAK, 293 cells were transfected to express various combinations of receptor subunits (Figure 2A and D). The signalling properties of the L1/Nrp1 complex were compared with that of the prototypal *Sema3A* receptor Plexin-A1/Nrp1 (Toyofuku *et al*, 2005; Togashi *et al*, 2006). Interestingly, FAK co-precipitated with L1 from L1/Nrp1-expressing cells but not with Plexin-A1 from Nrp1/Plexin-A1-expressing cells. Likewise, when coexpressed with Nrp1, L1/FAK association could be observed by precipitation with antibodies to FAK and L1, whereas antibody to neither FAK nor to the VSV tag of Plexin-A1 could allow the detection of FAK/Plexin-A1 interaction (Figure 2A and B). This was not due to deficiency of Plexin-A1 construct, as expression of this construct with Nrp1 in COS7 cells conferred upon them a collapse response to *Sema3A* (data not shown).

These data suggest that FAK associates with L1. An alternative is that FAK is recruited by Nrp1, but only in an L1/Nrp1 complex and not when Nrp1 is complexed with Plexin-A1. This latter possibility was invalidated by the finding that an Nrp1/FAK interaction could not be detected in cells expressing only Nrp1 or Plexin-A1 (Figure 2C). Furthermore, co-precipitation of L1 with FAK from cells expressing a complex of L1 and a cytoplasmic deleted form of Nrp1 could be detected, which demonstrates that L1 is the subunit that triggers the recruitment of FAK (Figure 2C). Notably, we also observed that FAK/L1 association was almost undetectable in cells expressing L1 alone, indicating that the recruitment of FAK is conditional, based on the formation of the L1/Nrp1 complex (Figure 2D). Moreover, *Sema3A* treatment increased the recruitment of FAK to the L1/Nrp1 complex, as shown by precipitation with antibodies to L1 and FAK (Figure 2A and B).

### ***L1/Nrp1 complex formation triggers Sema3A-induced FAK activation***

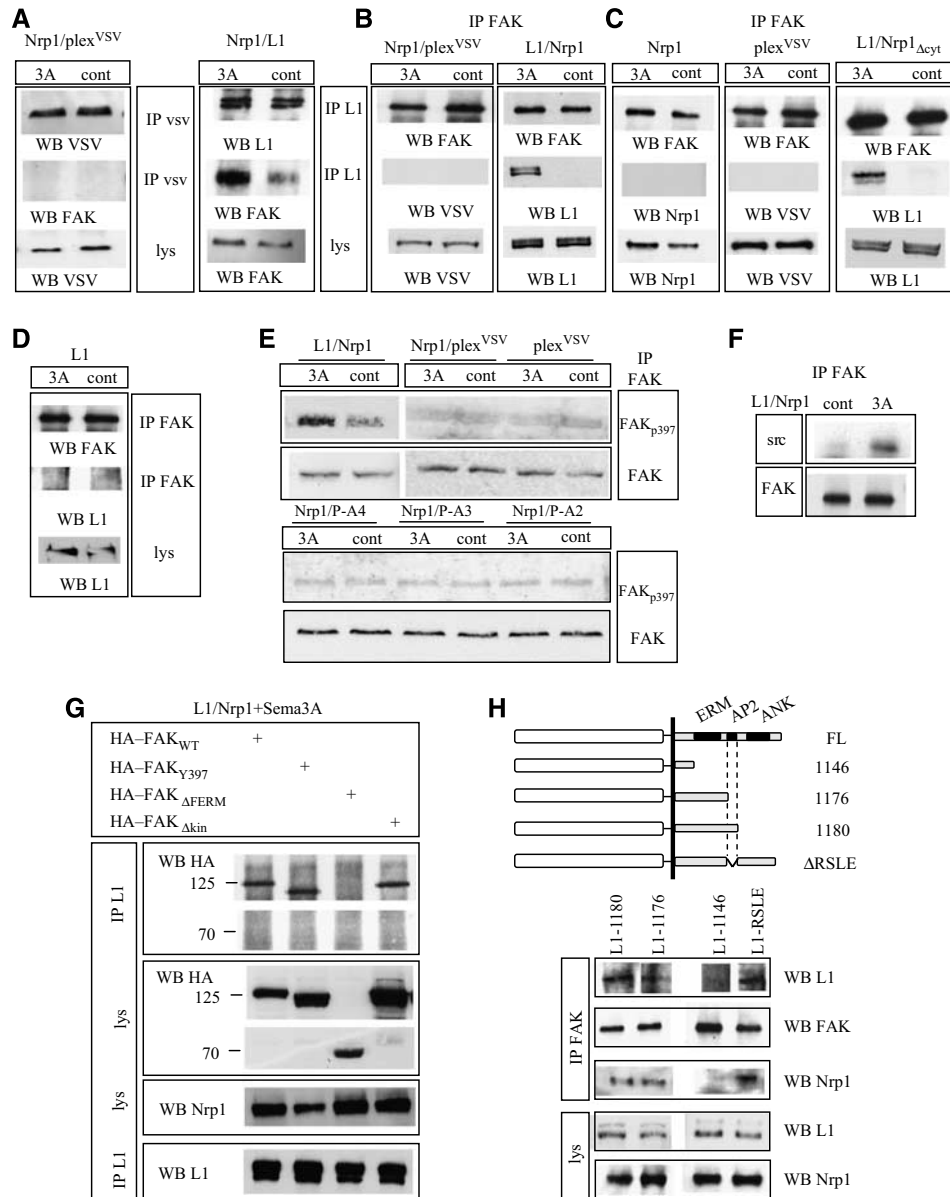
Although the recruitment of FAK to the *Sema3A* receptor was found to depend on L1 but not Plexin-A1, its activation could require Plexin-A in the complex or result from signalling events downstream of Plexin-A. The phosphorylation of



**Figure 1** FAK contributes to Sema3A-induced growth cone collapse. (A) FAK, L1 and Nrp1 are expressed by neurons residing in the cortical plate and detected in the intermediate zone where their efferents navigate to exit the cortex. Scale bar: 100 μm. Axonal localization of pFAK, L1 and Nrp1 in cultured cortical neurons is shown. Scale bar: 50 μm. (B) Microphotographs illustrating neurons labelled with antibodies to HA-tagged overexpressed FAK and magnification of the growth cone morphology under control and Sema3A-treated conditions. Overexpression of FAK<sub>Y397</sub> and FAK<sub>KinΔ</sub> but not FAK<sub>WT</sub> and FAK<sub>ΔFERM</sub> prevents the growth cone collapse. Western blot shows the expression of FAK proteins from the plasmids used in electroporation. The histogram shows the percentage of growth cone collapse under control and Sema3A-treated conditions showing that the response is abolished by FAK<sub>Y397</sub> and FAK<sub>KinΔ</sub>. (C) Western blot showing FAK precipitation with Nrp1 and L1 from the purified membrane fraction of neonatal mouse cortex. Sema3A stimulation of fresh cortical tissue induces Y397 FAK phosphorylation. lpIP: lysate post immunoprecipitation; NS, nonsignificant; \**P* < 0.001,  $\chi^2$  test.

FAK was assessed with an antibody that selectively recognizes the pY397. Phosphorylation at Y397 could be detected in Sema3A-treated L1/Nrp1-expressing cells but not in cells expressing Plexin-A1 alone or complexed with Nrp1 (Figure 2E). The recruitment of FAK upon L1/Nrp1 complex formation was not sufficient to induce strong activation, as

pY397 FAK was only weakly detected in the untreated condition. As described above, cortical neurons express several Plexin-As other than Plexin-A1 that could share with L1 the capacity of recruiting and activating FAK. We thus examined this possibility by immunoprecipitation of FAK from Sema3A-treated-transfected HEK cells and found that none of the



**Figure 2** L1/Nrp1 mediates Sema3A-induced recruitment and activation of FAK. (A, B) FAK precipitates with L1 from L1/Nrp1-expressing 293 cells but not with Plexin-A1<sup>VSV</sup> (plex<sup>VSV</sup>) from Plexin-A1/Nrp1-expressing 293 cells. Sema3A treatment (3A) stimulates L1/FAK co-precipitation. The L1/FAK complex could be detected in the control condition by precipitation with anti-L1 but not anti-FAK antibodies. (C, D) FAK does not precipitate with Nrp1, plex<sup>VSV</sup> or L1 when expressed alone whereas it precipitates with L1 from cells expressing L1 complexed with Nrp1 truncated from its cytoplasmic domain (Nrp1<sup>Δcyt</sup>). (E) Sema3A induces FAK autophosphorylation detected with anti-p397 in cells expressing L1/Nrp1 but not Nrp1/Plex<sup>VSV</sup> or Plex<sup>VSV</sup>. None of the Plexin-A2-A3-A4/Nrp1 complexes mediate FAK phosphorylation upon Sema3A stimulation. (F) Sema3A stimulates FAK/Src association in L1/Nrp1-expressing cells. (G) Precipitation of L1 in Sema3A-stimulated HEK cells expressing L1, Nrp1 and FAK forms for detection of FAK. FAK<sub>Δkin</sub>, FAK<sub>Y397</sub>, FAK<sub>WT</sub> but not FAK<sub>ΔFERM</sub> co-precipitated with L1. The photographs show bands at 125 kDa for FAK<sub>Δkin</sub>, FAK<sub>Y397</sub> and FAK<sub>WT</sub> and at 70 kDa for FAK<sub>ΔFERM</sub>. (H) Precipitation of FAK in Sema3A-stimulated HEK cells expressing various truncated L1 forms lacking one or several functional domains of L1 and Nrp1 for the detection of L1. L1<sub>1176</sub>, L1<sub>1180</sub>, L1<sub>ARSLE</sub> but not L1<sub>1146</sub> co-precipitated with FAK. ERM: binding site to ERM proteins; AP-A: binding site for AP-2/chlatrin-dependent endocytosis; ANK: binding site to ankyrin.

Nrp1/Plex-A2, Nrp1-Plex-A3 and Nrp1/Plex-A4 complexes mediated FAK recruitment and activation (Figure 2E and Supplementary Figure 1). Thus, the L1/Nrp1 but not the Plexin-As/Nrp1 complex could mediate FAK phosphorylation. FAK<sub>Y397</sub> autophosphorylation creates a binding site for src (Mitra *et al*, 2005). Consistently, we found in L1/Nrp1-expressing cells that Sema3A stimulates FAK/src co-precipitation (Figure 2F).

To delineate the domains of FAK and L1 required for their association, HEK cells expressing L1/Nrp1 with FAK<sub>Δkin</sub>, FAK<sub>ΔFERM</sub>, FAK<sub>Y397</sub> or FAK<sub>WT</sub> were stimulated with Sema3A and processed for precipitation with antibodies to L1 (Figure 2G). Western blot analysis showed that the removal of FAK kinase domain and the Y397 mutation did not affect FAK recruitment to L1, whereas deletion of the FERM domain totally abolished it (Figure 2G). Next, we tested L1 constructs

having various cytoplasmic truncations removing key functional domains: L1-1146 truncated after the fourth cytoplasmic residue, L1-1176 lacking the binding domain for the cytoskeletal adaptor ankyrin and the AP-2/chlatryn-dependent internalization motif but still containing the binding site for ezrin-radixin-moesin (ERM) proteins, L1-1180 lacking only the Ank domain and L1-RSLE lacking only the RSLE motif (Cheng *et al*, 2005). FAK was precipitated from *Sema3A*-stimulated L1/Nrp1-expressing cells for western blot detection of L1. We found that L1-1180 but not L1-1146 could recruit FAK. FAK/L1 co-precipitation was also detected with L1-1176 and L1-RSLE. Thus, FAK recruitment to L1 requires a sequence comprising 1146–1176 amino acids, including the ERM binding domain but excluding the RSLE and ankyrin binding domains (Figure 2H).

### ***Sema3A*-induced FAK-dependent erk1/2 phosphorylation downstream of L1/Nrp1 complex**

FAK has complex roles during the turnover of adherent contacts. Recent work established that FAK initiates a signalling cascade leading to activation of the MAPK pathway, which controls the disassembly of adherent contacts (Webb *et al*, 2004). Interestingly, MAPK activation downstream of L1 also mediates some of the major functions of L1 in cell adhesion and axon growth (Kamiguchi and Lemmon, 2000). Moreover, MAPK activation is already linked to *Sema3A*-induced growth cone collapse, although the nature of the process affected by this signalling remains unclear (Campbell and Holt, 2003).

As expected, we could detect erk1/2 phosphorylation in neonatal cortical tissues stimulated with *Sema3A* (Figure 3A). We thus examined whether *Sema3A* could induce an MAPK signalling in L1/Nrp1-expressing cells. Stimulated cells were processed for immunoprecipitation and western blot analysis. *Sema3A* could trigger erk phosphorylation in cells expressing L1/Nrp1 but not Nrp1 alone (Figure 3A). To determine whether FAK signalling is required for MAPK activation, cells were first transfected with L1, Nrp1 and either egfp or dominant-negative FAK<sub>Δkin</sub>. We found that FAK<sub>Δkin</sub> but not egfp coexpression abolished *Sema3A*-induced erk1/2 phosphorylation (Figure 3B). Second, cells were transfected with Nrp1 and L1<sub>-1146</sub> mutant lacking FAK recruitment and in this condition *Sema3A*-induced erk phosphorylation was also abrogated (Figure 3C). Next, we examined whether constitutively active FAK although uncoupled to L1 is sufficient for inducing erk phosphorylation. Cells were transfected with L1, Nrp1 and either FAK<sub>WT</sub> or FAK<sub>ΔFERM</sub>. Western blot analysis showed that erk phosphorylation was still dependent on stimulation by *Sema3A* (Figure 3D). This ligand-dependent erk phosphorylation was not due to the recruitment of endogenous FAK to L1, as FAK<sub>ΔFERM</sub> overexpression resulted in loss of association of L1 with both FAK<sub>ΔFERM</sub> and endogenous FAK (Figures 2F and 3E). Thus, *Sema3A*-induced FAK recruitment and activation by L1 can be overcome by uncoupled active FAK, which is nevertheless insufficient for erk phosphorylation. Additional L1-dependent signalling induced by *Sema3A* might also occur to trigger erk phosphorylation. Consistent with this hypothesis, *Sema3A*-induced erk phosphorylation was lost in cells expressing Nrp1, FAK<sub>ΔFERM</sub> and the L1<sub>-1146</sub> mutant lacking almost all the cytoplasmic domain (Figure 3F).

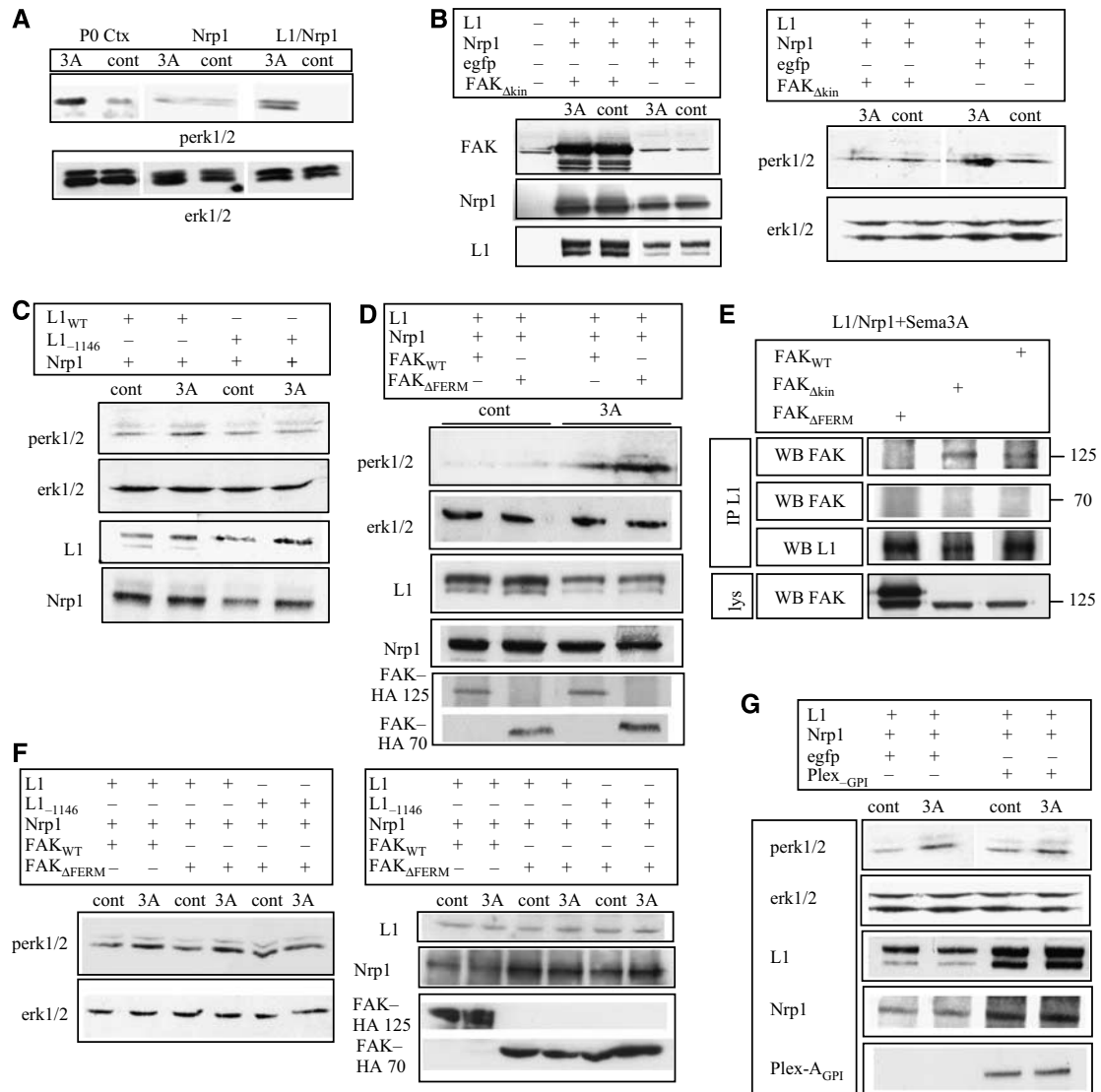
Finally, we examined whether the cascade downstream of L1 requires Plexin-As signalling because HEK cells express endogenous Plexin-As. However, erk1/2 phosphorylation was still detected in HEK cells transfected with L1/Nrp1 and dominant-negative truncated form of Plexin-A1 in which the cytoplasmic domain has been removed and replaced by a GPI anchor (Plex-A1<sub>GPI</sub>; Toyofuku *et al*, 2004; Figure 3G).

### **L1-mediated FAK and MAPK activation is required for *Sema3A*-induced growth cone collapse**

To address whether L1-mediated MAPK pathway is required for cortical responses to *Sema3A*, we disturbed this activation. Two L1 serine residues, mutated in the L1-related human neurodevelopmental disease, have been found to alter the ability of L1 to trigger erk1/2 phosphorylation (Needham *et al*, 2001). The pathological substitution of the serines S<sub>1194</sub> and S<sub>1224</sub> by leucine prevented neither L1 cell surface expression nor interaction with Nrp1 (Supplementary Figure 1D). When coexpressed with Nrp1 in HEK cells, these mutated L1 constructs still mediated *Sema3A*-induced autophosphorylation of FAK. In contrast, erk1/2 phosphorylation was totally lost (Figure 4A). L1<sub>WT</sub>, L1<sub>-1146</sub>, L1<sub>S1194L</sub> and L1<sub>S1224L</sub> IRES EGFP vectors were overexpressed in cortical neurons in collapse assays. Overexpression of L1<sub>-1146</sub> but not L1<sub>WT</sub> totally blocked *Sema3A*-induced growth cone collapse (Figure 4B, a total of 320 cones from 12 neonatal brains). Similarly, the L1 serine mutations partially (for L1<sub>S1224L</sub>) and totally (for L1<sub>S1194L</sub>) abrogated *Sema3A*-induced growth cone collapse (Figure 4B, a total of 383 cones from 12 neonatal brains). To confirm that L1 signalling is required for the guidance of cortical axons, we took advantage of the previously developed slice overlay assay, assessing axon trajectory of dissociated cortical neurons grown on top of cortical slices. In normal conditions, about 65% of the axons are directed towards the white matter by *Sema3A* repulsive activity emanating from the pial side (Polleux *et al*, 1998). Using this assay, we found that overexpression of FAK<sub>Δkin</sub> and L1<sub>S1194L</sub> profoundly disorganized axon trajectory, as only 21 and 27% of axons were properly guided towards the white matter side, respectively (Supplementary Figure 2).

### **L1-mediated FAK–MAPK signalling controls the disassembly of paxillin + adhesion points induced by *Sema3A***

The nature of the process regulated by this L1-dependent FAK–MAPK activation found required for *Sema3A*-induced growth cone collapse remained undetermined. The FAK–MAPK cascade was shown to control focal adhesion disassembly in migrating cells (Webb *et al*, 2004). Moreover, other recent work described that *Sema3A* at a non-collapsing dose destabilizes adherent points of the growth cones, visualized by the focal adhesion marker Paxillin (Woo and Gomez, 2006). Paxillin is an adaptor protein recruited to dynamic focal contacts and has a crucial role in adhesion turnover. Interestingly for the present context, Paxillin forms a scaffold for activation of erk at focal contacts (Ishibe *et al*, 2003). It constitutively binds MEK. On phosphorylation by FAK in response to extracellular stimuli, Paxillin recruits inactive erk and active Raf, resulting in local erk activation. This prompted us to investigate first the links between L1 and Paxillin and second the possibility that L1-dependent

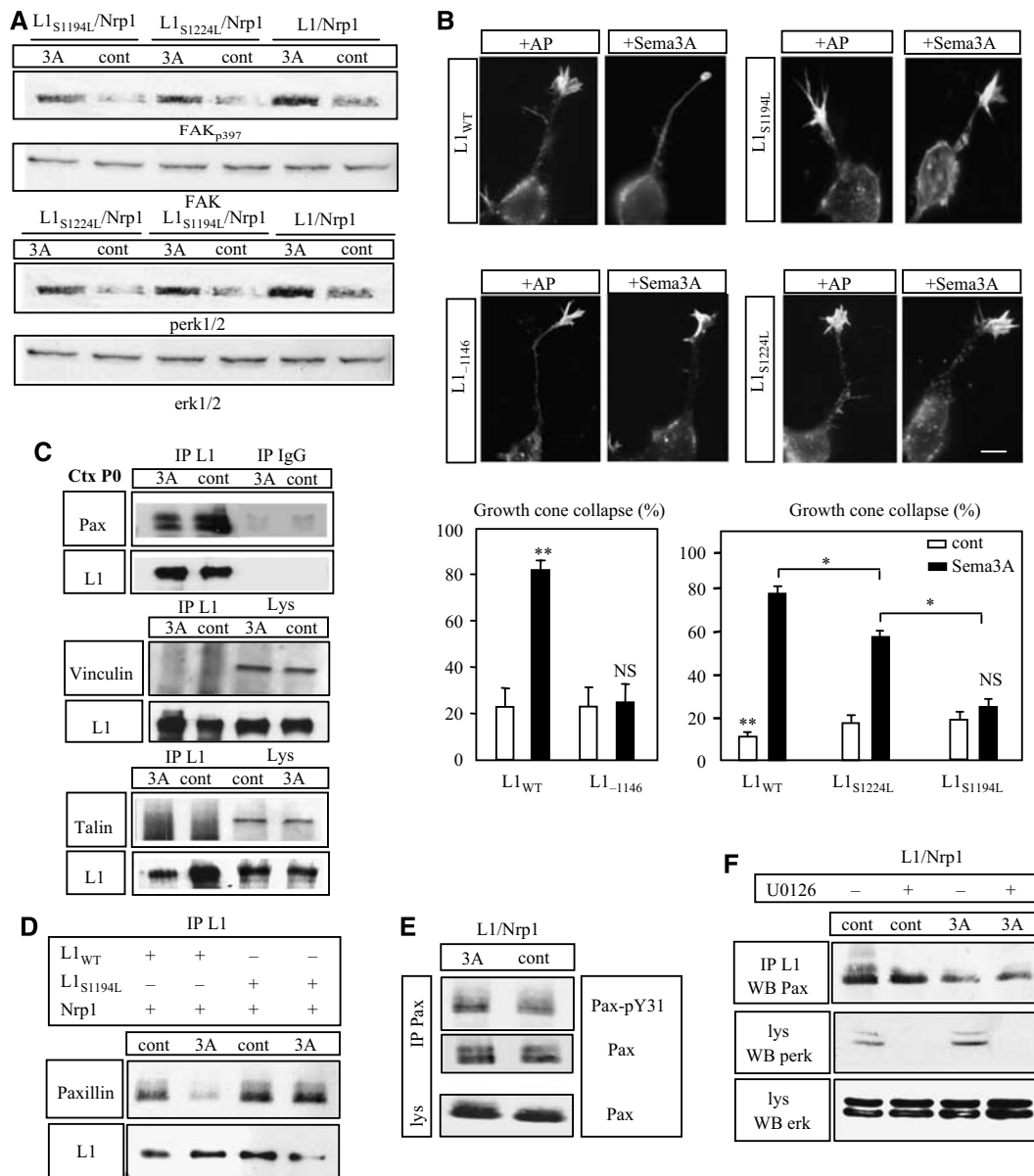


**Figure 3** L1/Nrp1 mediates Sema3A-induced erk phosphorylation. (A) Stimulation by Sema3A of fresh cortical tissue induces erk1/2 phosphorylation, which is reproduced in L1/Nrp1- but not Nrp1-expressing HEK cells. (B) Introduction of dominant-negative FAK<sub>Akin</sub> abrogates Sema3A-induced erk1/2 phosphorylation in L1/Nrp1-expressing cells. (C) Sema3A-induced erk phosphorylation is lost in cells expressing Nrp1 and L1<sub>-1146</sub> mutant lacking FAK recruitment. (D) Sema3A still triggers erk phosphorylation in cells overexpressing constitutively active FAK (FAK<sub>ΔFERM</sub>) even though this construct blocks the recruitment of endogenous FAK. (E) The blots show bands at 125 kDa (endogenous FAK, FAK<sub>WT</sub>, FAK<sub>Akin</sub>) and at 70 kDa (FAK<sub>ΔFERM</sub>). (F) Sema3A triggers erk phosphorylation in cells expressing L1<sub>WT</sub>/FAK<sub>WT</sub> and L1<sub>WT</sub>/FAK<sub>ΔFERM</sub> but not L1<sub>-1146</sub>/FAK<sub>ΔFERM</sub>. (G) Introduction of dominant-negative Plex<sub>GPI</sub> does not abrogate Sema3A-induced erk1/2 phosphorylation in L1/Nrp1-expressing cells.

FAK-MAPK activation mediates Sema3A-induced adhesion disassembly. First, fresh cortical tissues were stimulated with control and Sema3A treatment and L1 was immunoprecipitated for western blot analysis of Paxillin. Strong interaction of L1 with Paxillin but not other proteins of focal adhesions such as talin and vinculin was found in control condition, indicating that L1 might be associated with focal adhesion complexes (Figure 4C). Notably, this Paxillin-L1 association was strongly decreased by Sema3A (Figure 4C). Likewise, quantification of the bands showed that the ratio between precipitated L1 and Paxillin was reduced by 51 and 71% for each Paxillin band in the Sema3A condition compared to the control condition. Second, this Sema3A-induced regulation of Paxillin/L1 interaction could be fully recapitulated in L1/Nrp1-expressing cells (Figure 4D). Third, in these cells,

Sema3A could induce paxillin phosphorylation at Y31, a residue known to be phosphorylated by FAK (Mitra *et al*, 2005; Figure 4E). Next, we assessed whether the S1194L L1 mutation could interfere with the dynamic of L1/paxillin interaction. Strikingly, L1<sub>S1194L</sub> still bound Paxillin but was totally unable to release it upon Sema3A stimulation (Figure 4D). Thus, the S1194L mutation blocks both Sema3A-induced Paxillin release and erk phosphorylation. When L1/Nrp1-expressing cells were stimulated in the presence of the MEK inhibitor U0126 (10 μM), erk phosphorylation was totally abolished whereas but paxillin release from L1 was not prevented (Figure 4F). Thus, Paxillin release does not occur downstream of erk phosphorylation.

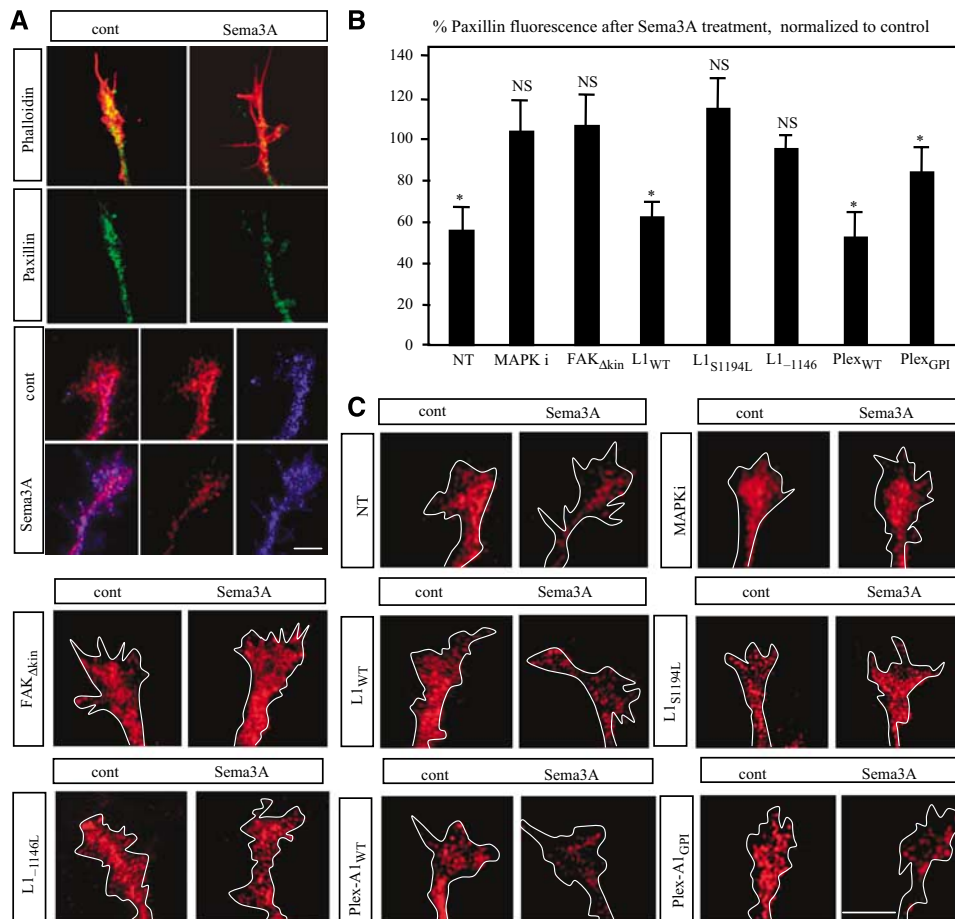
Overall, these data pointed to a model in which L1 couples the Sema3A receptor to Paxillin + focal adhesion points and



**Figure 4** L1 mutants interfere with Sema3A-induced erk phosphorylation, release of Paxillin/L1 interaction and collapse response. (A) The L1 serine mutations 1194 and 1224 abolished the Sema3A-induced erk1/2 phosphorylation in L1/Nrp1-expressing cells. Y397 FAK phosphorylation is still detected in cells expressing complexes of Nrp1 and L1<sub>S1194L</sub> and L1<sub>S1224L</sub>. (B) Collapse assays with cortical neurons overexpressing the L1 variants L1<sub>S1224L</sub>, L1<sub>S1194L</sub> and L1<sub>-1146</sub>. Microphotographs illustrate the loss of collapse response due to these overexpressions. Histograms depict the percentage of growth cone collapse showing defective responsiveness of neurons expressing L1<sub>S1224L</sub>, L1<sub>S1194L</sub>, L1<sub>-1146</sub> but not L1<sub>WT</sub>. \**P* < 0.001 with  $\chi^2$  test. Scale bar: 25  $\mu$ m. (C) Co-precipitation of L1 with Paxillin but not talin and vinculin, indicating that L1 complexes in the cerebral cortex contain paxillin. lpIP: lysate post immunoprecipitation. Paxillin/L1 co-precipitation is decreased by Sema3A. (D) Sema3A induces Paxillin release from L1 in L1/Nrp1-expressing cells. (E) L1/Nrp1 mediates Sema3A-induced paxillin phosphorylation at Y31. (F) Sema3A-induced release of Paxillin/L1 interaction is not prevented by the application of the MEK1 inhibitor U0126.

enables Sema3A to modulate their dynamics. To test this model, we first examined whether modulation of adherent points induced by low dose of Sema3A (0.1 nM, found inefficient to induce collapse, data not shown) could be detected in our culture model. Phalloidin and paxillin co-labelling was examined by confocal microscopy. As expected, punctate paxillin staining was distributed over the growth cone structures and was reduced by Sema3A application (Figure 5A). When Paxillin was co-labelled with Nrp1, Nrp1 labelling was qualitatively and quantitatively unchanged by low-dose Sema3A application, whereas paxillin staining was

profoundly reduced and no longer spread throughout the growth cone (Figure 5A and data not shown). Quantitative analysis of the fluorescence (see Materials and methods) confirmed that Sema3A significantly decreased Paxillin fluorescence (Figure 5B and C). FAK<sub>kin</sub> overexpression and application of the MAPK pharmacological inhibitor U0126 (5  $\mu$ M) could block the normal decrease of Paxillin (Figure 5B and C). Application of U0126 (5  $\mu$ M) in the control condition did not modify the basal Paxillin level. Next, L1<sub>WT</sub>, L1<sub>S1194L</sub> and L1<sub>-1146</sub> were co-electroporated in cortical neurons. The introduction of L1<sub>WT</sub> did not interfere with the



**Figure 5** The L1-dependent FAK–MAPK pathway mediates Sema3A-induced disassembly of paxillin+ adhesion points in cortical growth cones. **(A)** Confocal images of phalloidin and Paxillin staining in cortical growth cones showing Paxillin decrease induced by subcollapsing dose of Sema3A. The two lower panels illustrate that Paxillin (in red) but not Nrp1 (in blue) is decreased by Sema3A. **(B, C)** Quantitative analysis and illustrations of paxillin fluorescence in cortical growth cones. Pharmacological inhibition of dominant-negative FAK<sub>Akin</sub> and L1 variants abolish Paxillin decrease normally induced by low dose of Sema3A in non-transfected condition (NT). Paxillin decrease takes place in cells overexpressing PlexA1<sub>WT</sub> and dominant-negative Plex-A1<sub>GPI</sub>. Scale bars: 25  $\mu$ m.

Sema3A-dependent removal of paxillin whereas L1<sub>S1194L</sub> strikingly abolished it (Figure 5B and C). Thus, Sema3A-induced FAK and MAPK activation controls the disassembly of adherent points, and L1 mutants lacking FAK and MAPK activation disrupt this process. Plexins mediate the suppression of integrin-dependent adhesions (Serini *et al*, 2003; Barberis *et al*, 2004; Toyofuku *et al*, 2005). Thus, by preventing new contact formation or maturation, Plexin signalling should also trigger paxillin decrease in Sema3A-treated neuronal growth cones. As several plexin-As are expressed in cortical neurons, we thought that overexpression of cytoplasmic deleted Plexin-A1 form should favour the formation of non-functional Plexin–Nrp1 complexes, thus interfering with the signalling by all endogenous Plexin-As. We observed that Sema3-induced paxillin removal was very marked in cortical neurons overexpressing Plex-A1<sub>WT</sub>, whereas it was effectively weakened but still significant in neurons overexpressing Plex-A1<sub>GPI</sub>, consistent with still active signalling downstream of L1/Nrp1 (Figure 5B and C).

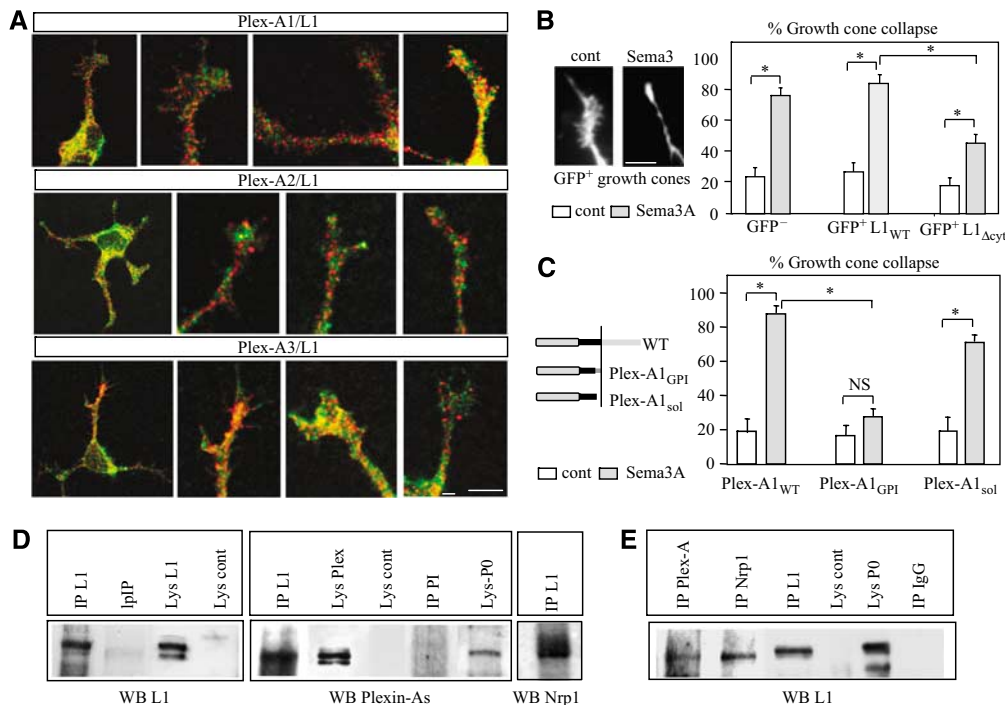
#### L1 and Plexin-As are coexpressed in cortical growth cones

To get further insights into the links between L1 and Plexin-As, we investigated their distribution to determine whether

they segregate in subsets or cortical neurons or rather colocalize in common pools. Co-labelling of L1 with three Plexin-A members was performed in cultured cortical neurons. We could not analyse the expression of Plexin-A4, owing to the lack of available antibodies. Interestingly, Plexin-A1, Plexin-A2 and Plexin-A3 were individually found coexpressed with L1 in cortical growth cones and thus did not label distinct pools of cortical neurons. However, they only partially colocalized within the growth cone (Figure 6).

#### Cytoplasmic deleted forms of L1 and Plexin-A1 disrupt growth cone collapse induced by Sema3A

Next, we introduced in cortical neurons L1 and Plexin-As cytoplasmic deleted forms to interfere with their signalling activities. First, wild-type and cytoplasmic deleted form of L1 were overexpressed with EGFP by electroporation of cortical neurons. We observed that overexpression of L1 <sub>$\Delta$ cyt</sub> but not L1<sub>WT</sub> significantly reduced Sema3A-induced growth cone collapse response (Figure 7B, a total of 254 growth cones scored from 8 neonatal brains). Second, overexpression of full-length Plexin-A1 did not abolish the collapse-inducing effect of Sema3A, whereas the Plex-A1<sub>GPI</sub> form totally blocked it (Figure 7C, a total of 240 growth cones scored from 8 neonatal brains). Overexpression of a secreted form of



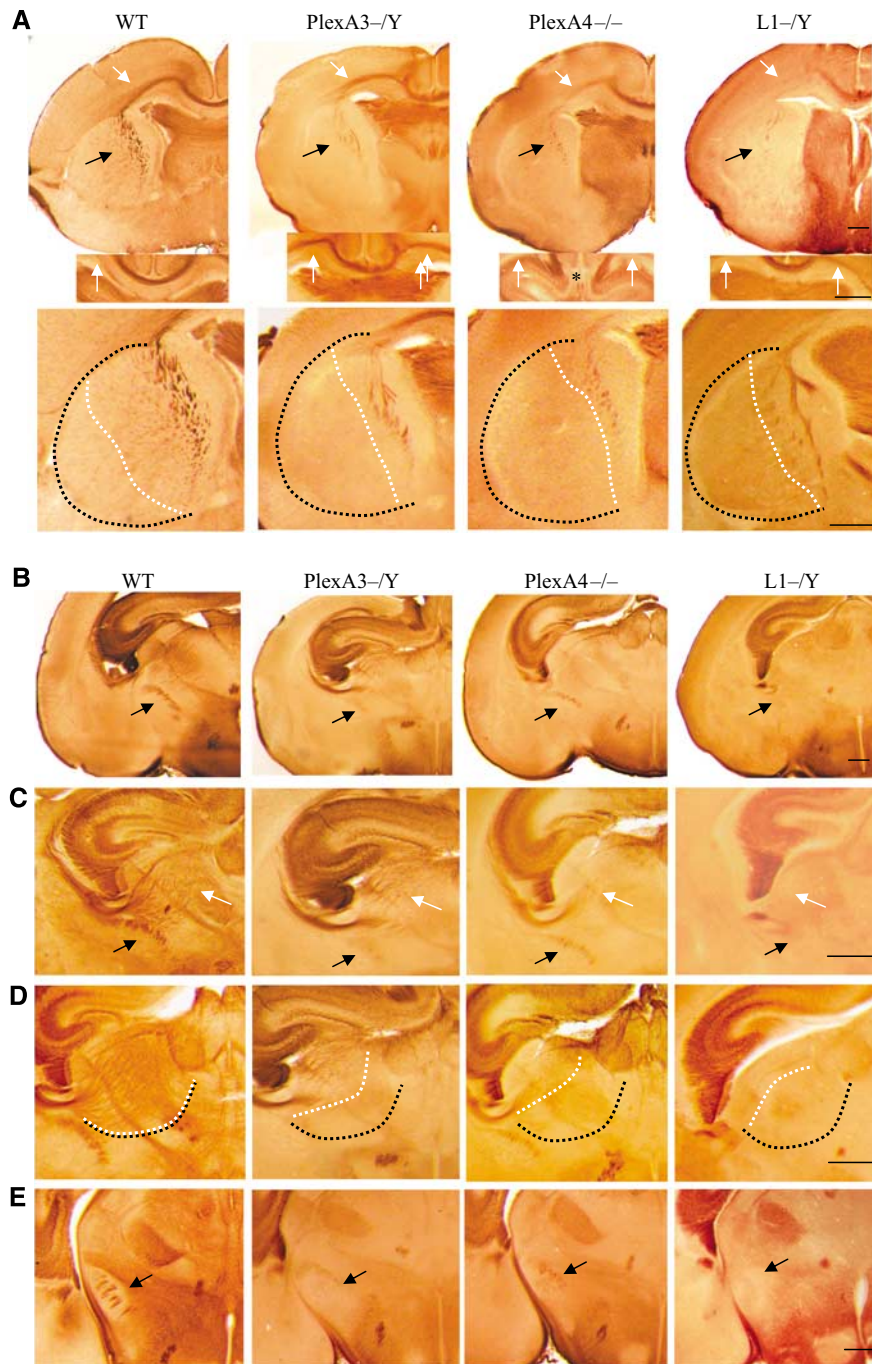
**Figure 6** Coexpression of Plexin-As and L1 in cortical neurons and dominant-negative effects of Plexin-A1 and L1 variants on Sema3A-induced growth cone collapse. (A) Confocal images of cortical neurons and their cortical growth cones, showing coexpression of L1 (in green) with Plexin-A1, Plexin-A2 and Plexin-A3 (in red). The distribution of L1 and Plexin-A proteins only partially colocalized both in the growth cone and the soma. Pictures illustrate cases of cortical growth cones with variable levels of Plexin-A/L1 colocalization. Scale Bar: 50  $\mu$ m. (B, C) Collapse assays with cortical neurons overexpressing L1<sub>WT</sub>, L1 <sub>$\Delta$ cyt</sub>, Plex-A1<sub>WT</sub>, Plex-A1<sub>GPI</sub> and Plex-A1<sub>sol</sub>. (B) Microphotographs illustrate the morphological collapse induced by Sema3A on growth cones, visualized by EGFP labelling. The histogram depicts the percentage of growth cone collapse and the significant reduction of responsiveness to Sema3A of neurons overexpressing L1 <sub>$\Delta$ cyt</sub> but not L1<sub>WT</sub>, compared to wild-type neurons. (C) Overexpression of Plex-A1<sub>GPI</sub> but not of Plex-A1<sub>WT</sub> or Plex-A1<sub>sol</sub> disrupted Sema3A-induced growth cone collapse. \* $P$ <0.001, with  $\chi^2$  test. Scale bar: 10  $\mu$ m. (D, E) Precipitation of L1 (D) and Plexin-As (E) from neonatal (P0) cortical tissues showing that L1 and plexin-A proteins are present in common complexes.

the Plexin-A1 ectodomain (Plex-A1<sub>sol</sub>) was in contrast inefficient (Figure 7C). Thus, both cytoplasmic truncated forms of L1 and Plexin-A interfere with Sema3A-induced collapse responses, indicating that L1 and Plexin-As might be jointly required. If so and although not directly interacting, L1 and Plexin-As might be present *in vivo* in common receptor complexes through association with Nrp1. Co-precipitation experiments performed on neonatal cortical tissue, using antibodies to L1 and a mixture of antibodies to Plex-A1, Plex-A2 and Plex-A3 to collectively detect these Plexin-As, confirmed this hypothesis (Figure 7D).

#### **Mice lacking Plexin-A3, Plexin-A4 or L1 have profound defects of Nrp1 + cortical projections**

If L1 and Plexin-As mediate together the guidance effects of Sema3A, the genetic removal of L1 or Plexin-As should thus impact the formation of cortical pathways. Cortical axons navigate into two main pathways, the corpus callosum and the internal capsule for reaching, respectively, cortical and subcortical targets. We thus examined these cortical pathways in mice lacking Plexin-A3, Plexin-A4 or L1. Because both Plexin-As and L1 are implicated in axonal responses to cues other than Sema3A, we focused our analysis on projections expressing Nrp1. In coronal sections of neonatal brains, Nrp1 + axons extending in the intermediate zone of the cerebral cortex and coursing in the internal capsule were strongly reduced in mice lacking Plexin-A3,

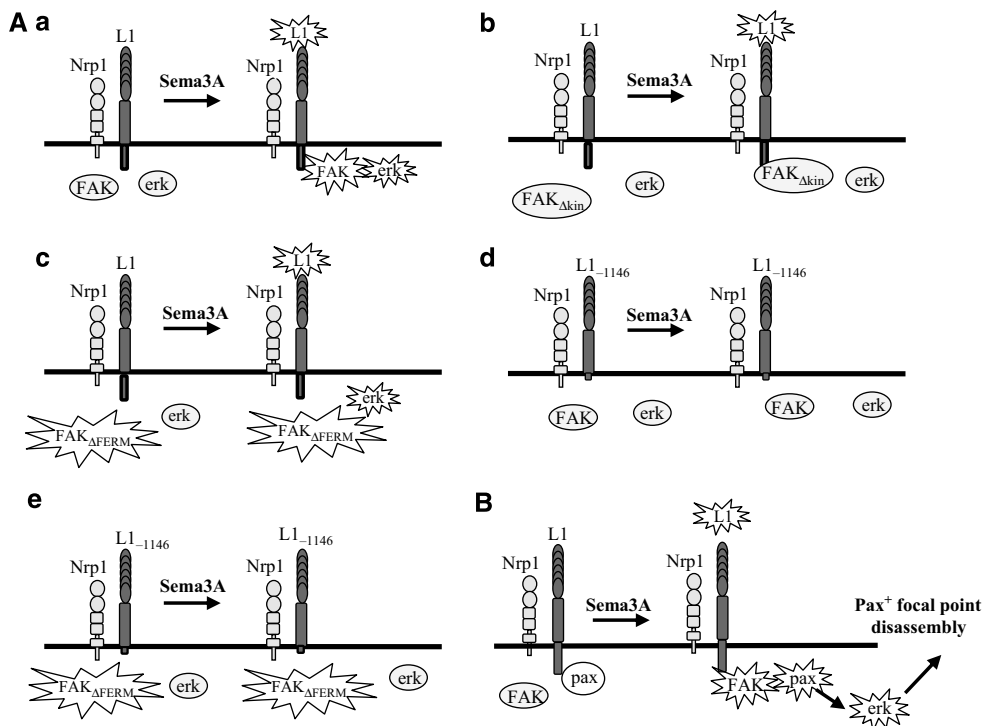
Plexin-A4 and L1 compared to wild type mice (Figure 8Aa). This reduction was particularly marked for laterally oriented axon bundles and also clearly detected in horizontal brain sections (Supplementary Figure 3). Remnant medial bundles were in addition abnormally oriented in mice lacking Plexin-A3 and L1 but not obviously in mice lacking Plexin-A4. In coronal and horizontal sections of wild-type mice, Nrp1 + axons of the corpus callosum are present at dorsal and anterior parts. This spatial organization was not modified to a greater extent in the different Plexin mutant genotypes, except that in mice lacking L1, Nrp1 + axons were absent in the anterior pole of the corpus callosum (Supplementary Figure 4). In addition, in Plexin-A4<sup>-/-</sup> mice but not Plexin-A3<sup>-/Y</sup> mice, Nrp1 + callosal axons prematurely stopped crossing the midline at caudal levels and accumulated on ipsilateral sides (Figure 8Aa). The corticospinal tract was also strikingly altered in these mutant mice. Nrp1 + corticospinal axons diverging from corticothalamic axons in the reticular thalamic nucleus were clearly detected in wild-type mice. Notably, this Nrp1 + diverging corticospinal tract was totally absent in Plexin-A3<sup>-/Y</sup> and L1<sup>-/Y</sup> mice, whereas it was still detected, although reduced, in Plexin-A4<sup>-/-</sup> mice (Figure 8Ab and c). Consequently, Nrp1 + corticospinal axons were also absent in the cerebral peduncle of mice lacking Plexin-A3 and L1 (Figure 8Ae). Finally, the innervation of the thalamus was also defective. Nrp1 + cortical axons innervating the dorsal thalamus were



**Figure 7** Defects of Nrp1+ cortical projections in mice lacking Plexin-A3, Plexin-A4 or L1. **(A)** Horizontal sections of neonatal brains immunolabelled with antibodies to Nrp1. The upper panels show reduction of Nrp1+ axons in the intermediate zone of the cerebral cortex (white arrows) and in the internal capsule (black arrows) in mice lacking Plexin-A3, Plexin-A4 and L1, compared with wild-type mice. The intermediate panels show that the dorsal positioning of Nrp1+ axons in the corpus callosum is maintained in different null mutants, but in Plexin-A4 knockout mice, axons prematurely stop crossing the midline (asterisk). The lower panels show a magnified image of the internal capsule and delineate the striatum (black lines) and the area occupied by Nrp1+ axon bundles (white lines) in each type of mouse. **(B, C)** Nrp1+ corticospinal axons (black arrows) diverging from Nrp1+ corticothalamic axons (white arrows) are detected in wild-type and Plexin-A4 null mice but are lost in Plexin-A3 and L1 null mutants. **(D)** Magnification showing the reduced area (white line) occupied by cortical axons innervating the dorsal thalamus (delineated by a black line) in Plexin-A3, Plexin-A4 and L1 null mutants compared with wild-type mice. **(E)** The Nrp1+ corticospinal tract in the cerebral peduncle is lost in mice lacking Plexin-A3 and L1, and reduced in mice lacking Plexin-A4 (black arrows). Scale bar: 1 mm.

almost absent in mice lacking Plexin-A4 and L1, and were only partially detected in mice lacking Plexin-A3 (Figure 8Ad). Thus, the genetic removal of Plexin-A3, Plexin-A4

and L1 leads to important alteration of cortical projections and L1 knockouts combined the defects of Plexin-A3 and Plexin-A4 knockouts.



**Figure 8** Model for Sema3A signalling downstream of L1. **(A)** Activation of FAK and MAPK. (a) Sema3A induces recruitment of FAK to L1, FAK and erk activation. Sema3A also triggers activation of L1, presumably involving the S1194 residue. (b) Kinase-dead FAK (FAK<sub>Akin</sub>) binds L1 upon Sema3A stimulation but erk phosphorylation is prevented. (c) Constitutively active FAK (FAK<sub>AFERM</sub>) prevents Sema3A-induced FAK recruitment to L1 but not erk phosphorylation, reflecting that FAK activity is required but not sufficient, and that other signalling triggered by Sema3A is also needed, for erk phosphorylation. (d, e) Consistently, L1-1146 having truncation of almost all the cytoplasmic domain is unable to mediate erk phosphorylation even in the context of activated FAK (FAK<sub>AFERM</sub>). **(B)** Model for the cascade triggering adhesion disassembly. Upon Sema3A stimulation, L1 in the L1/Nrp1 complex is modified, recruits FAK and releases Paxillin. These activations trigger erk phosphorylation leading to disassembly of Paxillin+ focal points.

## Discussion

### **Sema3A-induced FAK–MAPK signalling triggered by L1/Nrp1 interaction controls the disassembly of adhesion points of the growth cones**

From the initial observation that L1 and Nrp1 ectodomains interact and that neuronal growth cones lacking L1 are insensitive to Sema3A *in vitro* (Castellani *et al*, 2000), a recurrent question has been the nature of L1-dependent processes occurring during growth cone steering. The present findings show that L1 does not simply act as a binding partner for Nrp1 but controls important events of the growth cone collapse. Our results support a model in which L1/Nrp1 interaction activates a FAK–MAPK cascade triggering the disassembly of adhesion contacts, which is required for Sema3A-induced collapse behaviour to take place (Figure 8A and B).

Several data support this model. First, the Sema3A receptor complex is associated by L1 to a structural protein of dynamic focal adhesions, Paxillin, and this interaction is released by Sema3A. This coupling may thus provide a basis for Sema3A to suppress adhesion points during growth cone steering. Second, Sema3A stimulates Y397 FAK, Y31 Paxillin and erk1/2 phosphorylation in L1/Nrp1-expressing cells, a cascade initially described to control the disassembly of adhesion sites at the leading edge of protrusions in migrating cells (Webb *et al*, 2004). Consistently, subcollapsing dose of Sema3A elicits the release of Paxillin+ adhesions in cortical growth cones. Pharmacological inhibition of

MAPK and overexpression of inactive FAK variant, L1<sub>-1146</sub> and L1<sub>S1194L</sub> mutants deficient for FAK and/or MAPK activation all abolish Sema3A-mediated disassembly of Paxillin+ adhesions. Finally, our data indicate that this L1-dependent removal of Paxillin+ dynamic adhesions is needed for Sema3A-induced guidance response, as overexpression of inactive FAK and L1 mutants also blocks Sema3A-induced growth cone collapse of cortical neurons and impairs the trajectory of cortical axons in slice overlay assays.

Several issues remain open concerning the mechanisms by which L1 in the Sema3A receptor couples FAK and erk activation to control adhesion disassembly. Our data indicate that FAK signalling is necessary but not sufficient for erk activation. First, overexpression of inactive FAK blocks erk phosphorylation but constitutively active FAK does not mimic Sema3A, as erk phosphorylation remains ligand-dependent. This is also reflected in collapse assays, as kinase-dead FAK abolishes Sema3A-induced growth cone collapse whereas active FAK only induces modest increase of collapse in the absence of Sema3A. Second, FAK but not erk phosphorylation can be detected in cells expressing L1<sub>S1194L</sub> instead of L1<sub>WT</sub>, suggesting that FAK is not the only trigger of erk phosphorylation. The cytoplasmic domain of L1 contains several tyrosine and serine residues, which are phosphorylated upon stimulation by extracellular cues (Kamiguchi and Lemmon, 2000). S1194 phosphorylation has not been described so far but given the pathogenic consequences of its mutation, this residue might be critical for L1 functions. Likewise, integrin-dependent stimulation of cell migration

by L1 was found impaired by this mutation (Thelen *et al*, 2002). We do have evidence that L1 becomes serine phosphorylated upon Sema3A stimulation in an S1194-dependent manner (unpublished observations). Additional work will be needed for identifying the pathways controlling this phosphorylation. Interestingly, here we found that in addition to preventing erk phosphorylation, the S1194L mutation disrupts Sema3A-induced Paxillin release. Previous work established that upon phosphorylation by FAK, Paxillin recruits inactive erk and triggers its activation at focal contacts and that Paxillin acts upstream of erk phosphorylation in adhesion disassembly in cell protrusions (Ishibe *et al*, 2004; Webb *et al*, 2004). Thus in the present context, erk phosphorylation could be controlled by Paxillin, upon its release from L1. Alternatively, FAK activation and paxillin release could enable L1 to recruit ranBPM, a signalling molecule that interestingly can trigger erk phosphorylation and is also downstream of Plexin-A in the Sema3A response (Cheng *et al*, 2005; Togashi *et al*, 2006). Future study will also focus on the mechanisms by which adhesion disassembly is achieved. It could result from actinomyosin contraction triggered by the myosin-like chain kinase downstream of FAK-MAPK signaling, which can break adherent contacts and could also involve RHOA kinase signalling (Crowley and Horwitz, 1995; Mitra *et al*, 2005).

Intriguingly, FAK has been implicated as a positive and negative regulator of axon growth and branching and, in the context of axon guidance, it participates in both attractive and repulsive guidance effects (Li *et al*, 2004, 2006; Liu *et al*, 2004; Rico *et al*, 2004; Falk *et al*, 2005; Parri *et al*, 2007). As in cell protrusions, FAK continuously relocalizes from disassembled adhesion points to newly formed ones in advancing growth cones and, possibly, remodelling of adherent contacts might be required for coupling adhesion turnover and growth cone steering. Whatever the attractive or repulsive nature of the guidance effects, polarization of adherent points could be correlated with the direction of the growth cone steering. Alternatively and owing to its multifunctional nature, FAK could recruit different signalling effectors to control events that would be specific for attractive and repulsive effects.

#### **Interaction of Nrp1 with L1 but not Plexin-A mediates Sema3A-induced FAK signalling**

We found that FAK does not constitutively associate with Plexin-A, Nrp1 or L1 individual subunits but only with L1 in the L1/Nrp1 complex. Sema3A strengthens this recruitment and also induces the phosphorylation of FAK and its downstream target paxillin. Thus, this strongly suggests that we have not identified a general binding partner for L1 but rather a specific signalling effector of L1 function in the Sema3A receptor. Notably, we failed to detect any interaction between Plexin-As and FAK, consistent with previous work (Barberis *et al*, 2004). Overexpression of a truncated Plex-A1 totally disrupted Sema3A-induced growth cone collapse, probably due to the loss of Plexin signalling to actin dynamics. However, removal of adhesion sites of growth cones still occurred, presumably reflecting L1 signalling. It should be noted from this condition however that the loss of paxillin was less pronounced than when the integrity of Plexin signalling was preserved. Thus, the activation of the FAK cascade likely specifically relies on the L1 subunit in the Sema3A receptor but the final downregulation of adhesion

sites might be controlled by coincident Plexin and L1 signalling. Several previous works indeed reported that Plexin signalling suppresses integrin-dependent adhesions (Barberis *et al*, 2004; Serini *et al*, 2004; Garrity, 2005; Toyofuku *et al*, 2005). It is thus plausible that the Sema3A receptor initiates a Plexin signalling to inhibit the formation and/or the maturation of new adhesion points, and an L1 signal to disassemble existing contacts, with both effects converging on the downregulation of adherent points.

#### **Joint requirement for L1 and Plexin-As in the development of cortical pathways**

Immunolabelling revealed that cortical growth cones coexpress L1 with at least one Plexin-A and segregation of cortical neurons in pools expressing either a Plexin-A or L1 was not found, consistent with joint requirement for at least one Plexin-A member with L1 in the same cortical neurons. Interestingly, association of L1 and Plexin-A proteins could be detected in the developing cerebral cortex and, in brain sections, L1 was also found expressed by cortical fibres whose trajectory was disorganized in mice lacking Plexin-A3 and Plexin-A4. We cannot exclude that L1 and Plexin-As segregate in subsets of cortical axons projecting to common targets, but it is however noticeable that a majority of cortical neurons that were isolated from the whole cerebral cortex were sensitive to the dominant-negative effects of both truncated L1 and Plexin-A1 overexpression. Analysis of mouse models provides substantiate this conclusion, as the consequences of genetic removal of L1 and Plexin-A3 or Plexin-A4 show striking similarities in the reduction of Nrp1-expressing fibres along cortical pathways. Likewise, the genetic removal of Plexin-A3, Plexin-A4 or L1 leads to drastic reduction of Nrp1 + axons in the intermediate zone of the cerebral cortex and the internal capsule. Moreover, both Plexin-A3 and L1 knockout mice lack Nrp1 + corticospinal axons, whereas Plexin-A4 and L1 knockout mice show common strong reduction of Nrp1 + cortical axons innervating the dorsal thalamus. These data thus strongly suggest that Plexin-A3 and Plexin-A4 act with L1 to specify the trajectory of Nrp1 + axons. It is also clear from this analysis that the defects are more marked in L1 knockouts than in Plexin-A3 and Plexin-A4 knockouts. This could be due to compensation for the loss of Plexin-A3/A4 by other Plexin-As. Alternatively, the genetic removal of L1 could alter another important signalling for the guidance of Nrp1 + axons. Conversely, some of the defects observed in the L1-/- mice are unlikely due to loss of Sema3A responses, as abnormal hyper-fasciculation of axon bundles expressing DCC but not Nrp1 was observed in the internal capsule. Similarly, we cannot exclude that additional Plexin-A-dependent signalling also accounts for the defects described here, as Plexin-As also contribute to the response to other semaphorins. Overall, the observation that different Plexins are used by different axons to mediate the response to a given semaphorin has a precedent in the finding that Sema3F effects require Plexin-A3 in hippocampal fibres but Plexin-A4 in axons forming the anterior commissure (Cheng *et al*, 2001; Suto *et al*, 2005; Yaron *et al*, 2005).

Altogether, these data support the idea that L1 and Plexin-A might be joint components of common Sema3A receptors. However, given their only partial colocalization in cortical growth cones, an additional possibility is that partitioning of some Plexin-A and L1 pools in physically separated

membrane domains of the growth cone could also underlie their recruitment to different receptor complexes and the initiation of distinct signalling cascades at distinct sites of the same growth cone. Future direction will focus on a detailed analysis of spatio-temporal dynamics of L1, Plexin-As and their downstream effectors during growth cone steering. Finally, independent activation of the Plexin-A/Nrp1 and L1/Nrp1 complexes to modify growth cone adhesion properties without inducing collapse could also enable Sema3A to control processes other than growth cone steering during axon navigation, such as axo-axonal and axon-substrate interactions.

## Materials and methods

### Immunohistochemical analysis of Plexin-A3/A4 and L1 mutants

Genotypes were determined by PCR as described by Itoh *et al* (2004) for L1 mice and by Yaron *et al* (2005) for Plexin-A3 and Plexin-A4 mice. Free-floating 60- $\mu$ m-thick horizontal and coronal sections from neonatal brains were incubated overnight with antibodies to DCC (Oncogen, 1:100), to L1 (Hybridoma Bank, 1:50) or to Nrp1 (R&D, 1:50) and processed for DAB labelling as described by Falk *et al* (2005).

### cDNA construction

Point mutations in L1 were generated by the Quickchange site-directed mutagenesis kit (Promega) according to the manufacturer's specifications and were confirmed by DNA sequencing. Mutations were produced by using pcDNA3.1-L1 as template and the following primers: for mutation S<sub>1194L</sub>, 5'-GGCAGCAGCCAGCCAT TGCTCAACGGGACATC-3' and 5'-GATGTCCCCGTTGAGCAATGGCT GGCTGGCTGCTGCC-3'; for mutation S<sub>1224L</sub>, 5'-GTTCAACGAGGA TGGTTTGTTCATTGGCCAGTACAG-3' and 5'-CTGTACTGGCAATGA ACAACCATCCTCGTTGAAC-3'. The ires EGFP L1 construct was cloned by PCR amplification for the ires EGFP (pIRES2-EGFP) with the primers 5'-ATCTCGAGCAAATGTATGGCTGATTATGATC-3' and (5'-GAGGTCTATATAAGCAGAGCTGGTTAGTG-3'. The amplified fragment and L1 constructions were digested by *Xho*I and ligated.

### Electroporation of neuronal cell cultures, collapse and slice overlay assays

P0 mouse cortices were dissected, dissociated and electroporation was performed with Nucleofector (Amaxa). Transfected cells were plated onto polylysine-coated coverslips and grown for 24 h in DMEM/F12 medium supplemented with B27, Glutamax and antibiotics. Collapse assays were performed as described by Castellani *et al* (2004) and slice overlay assays as described by Polleux *et al* (1998). Briefly, 250 thick cortical slices were incubated in laminin-coated inserts. Dissociated cortical neurons were electroporated with Nucleofector and placed on top of the slices. After 1 DIV, the cultures were fixed and processed for analysis.

### Biochemistry and immunocytochemistry

The following antibodies were used: anti-phosphoserine (Sigma), anti-ERK15C165 and anti-phospho-ERK(E-4) (Santa Cruz),

anti-FAK(A-17) (Tebu), anti-phospho-FAK Y397 (Cell Signaling), anti-VSV (Sigma), anti-HA clone 12CA5 (Roche), anti-paxillin (BD Transduction Laboratories) and anti-phospho-Y31 Paxillin (Biosource). The pharmacological inhibitor U0126 (Calbiochem) was added 30 min before control and Sema3A treatments. HEK 293T cells were transfected with vectors encoding L1, L120V, L1 $\Delta$ cyt, L1<sub>S1194L</sub>, L1<sub>S1224L</sub>, Nrp1 and Nrp1 $\Delta$ cyt with Exgene (Fermentas). Cells were lysed with immunoprecipitation buffer (HEPES, 25 mM; EDTA, 5 mM; MgCl<sub>2</sub>, 1 mM; PMSF, 2 mM; glycerol, 10%; and Triton X-100, 1%; pH 7). For biochemistry on fresh tissue, brains from P0 embryos were dissected out, cut into small pieces and stimulated with Sema3A and control supernatants as described by Falk *et al* (2005). Tissues were lysed for 1 h in a mixture containing 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium  $\beta$ -glycerol phosphate, 0.1 mM PMSF and 1  $\mu$ g/ml each of aprotinin, pepstatin and leupeptin. In some experiments, purification of the membrane fraction was carried out as described by Falk *et al* (2005). For immunocytochemistry, cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT) and permeabilized or not with 0.02% Triton X-100 in PBS for 15 min at RT. Cells were incubated with primary antibodies for 2 h at RT followed by incubation with secondary antibodies for 1 h at RT. Immunohistochemical labelling of brain sections was performed according to Falk *et al* (2005). Polyclonal rabbit antibodies to Plexin-A1, Plexin-A2 and Plexin-A3 (Santa Cruz, 1:100) were used for immunodetection in neuronal cultures.

### Quantitative analysis of Paxillin staining

Cultures of cortical neurons were treated with Sema3A-AP or AP supernatants for 20 min, fixed with 4% paraformaldehyde and processed for triple immunolabelling to detect Paxillin, Nrp1 and the electroporated construct by its tag. Growth cones were imaged with a microscope (Axiovert 200M, Zeiss). Images were captured under constant parameters with a Coolsnap CCD camera (Photometrics, Evry, France) under non-saturating exposure conditions and analysed with the ImageJ software (National Institutes of Health, USA). The growth cone surface was delineated with Nrp1 labelling. After background subtraction, the intensity of Nrp1 and paxillin staining was measured within the outline and the mean intensity per pixel was calculated. Intensity values were normalized to the respective control experiments.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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