Semaphorin 7A promotes axon outgrowth through integrins and MAPKs

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Striking parallels exist between immune and nervous system cellular signalling mechanisms. Molecules originally shown to be critical for immune responses also serve neuronal functions, and similarly neural guidance cues can modulate immune function. We show here that semaphorin 7A (Sema7A), a membrane-anchored member of the semaphorin family of guidance proteins previously known for its immunomodulatory effects, can also mediate neuronal functions. Unlike many other semaphorins, which act as repulsive guidance cues, Sema7A enhances central and peripheral axon growth and is required for proper axon tract formation during embryonic development. Unexpectedly, Sema7A enhancement of axon outgrowth requires integrin receptors and activation of MAPK signalling pathways. These findings define a previously unknown biological function for semaphorins, identify an unexpected role for integrins and integrin-dependent intracellular signalling in mediating semaphorin responses, and provide a framework for understanding and interfering with Sema7A function in both immune and nervous systems.

It is becoming increasingly clear that the nervous and immune systems display considerable overlap in their molecular repertoire. Molecules originally thought to be immune-cell-specific are now found also to perform critical neuronal functions, and vice versa[1–4]. These include molecules involved in crosstalk between these seemingly disparate systems, as well as cues with parallel functions. Importantly, these molecular relationships may provide valuable insights into function and disease in both systems.

Semaphorins constitute a large family of soluble and membrane-bound proteins first identified owing to their prominent contribution to repulsive axon guidance during development[5,6]. Although the roles played by semaphorins in the immune system are less well characterized, they support the possibility of a dual role for semaphorin family members in neural and immune functions[7,8]. The membrane-associated GPI (glycosylphosphatidylinositol)-linked semaphorin Sema7A (CDw108) was originally identified in a search for vertebrate homologues of virally encoded semaphorins[9,10]. Sema7A is expressed by a variety of lymphoid and myeloid cells[9–14], and also by several pre- and postnatal neuronal populations[10]. Like its viral counterpart SemaVB[15], Sema7A affects immune-cell function *in vitro*, including chemotaxis and cytokine production[14]. In addition, Sema7A defines the John–Milton–Hagen human blood group on erythrocytes, which has been implicated in a clinically benign autoimmune disorder[16]. Thus far, no neuronal function has been attributed to Sema7A. Sema7A and the viral semaphorins SemaVA and SemaVB bind to the receptor plexinC1 (VESPR, CD232) *in vitro*[15,17]. However, although plexinC1 is necessary for mediating the immunological effects of SemaVA[15] and although plexin receptors have been implicated in many semaphorin-mediated neuronal events[5,6], the functional significance of the interaction between Sema7A and plexinC1 remains unknown. We find here that Sema7A has pronounced effects on axon outgrowth, and that this activity is dependent upon integrin receptor, but not plexinC1, function. Therefore, integrin-mediated semaphorin signalling may be a general mechanism used in the development and function of both the nervous and immune systems.

Sema7A promotes axon outgrowth

To examine whether Sema7A serves a dual role by affecting both neuronal and immunological functions, we first analysed *Sema7A* expression in the nervous system by *in situ* hybridization. *Sema7A* is expressed in a variety of neuronal cell types and in glial cells during neural development as well as in the intact[10] and regenerating adult nervous system, revealing potential roles for this protein during development, plasticity and regeneration (Fig. 1a, c; data not shown).

To determine whether Sema7A, like other semaphorins, modulates axon guidance, we performed three-dimensional collagen matrix assays using 293-EBNA cells that secrete either a soluble, alkaline phosphatase (AP)-tagged, version of human Sema7A (AP-Sema7A)[10] or AP as a control. In the presence of an AP-Sema7A protein gradient, axons extending from explants derived from olfactory epithelium, olfactory bulb, cortex and dorsal root ganglia were more numerous and longer, while vomeronasal and spinal cord motor axons remained unaffected (Fig. 1d; data not shown).

Asymmetrical neurite growth in collagen matrix cultures (longer axons in proximal versus distal quadrants) hinted at attractive effects of Sema7A on axons. To study whether Sema7A steers axons and/or promotes axon growth, we analysed the trajectories of individual OB axons emerging parallel to AP-Sema7A-secreting cell aggregates[18] (Fig. 1e–h). Surprisingly, most of these fibres persisted on a linear course, showing no Sema7A-mediated chemotropic response (Fig. 1g). In contrast, Slit2, an OB axon repellent[19], reoriented axons away from the aggregate (Fig. 1h).

Because *in vivo* both GPI-anchored and soluble forms of Sema7A may exist[14], we next assessed the ability of full-length Sema7A to promote axon growth. OB explants grown on non-neuronal cell layers presenting soluble Sema7A or full-length, GPI-linked, Sema7A, displayed longer and more profuse axon outgrowth compared to control cell layers (Supplementary Fig. 1). This demonstrates that Sema7A can increase axon growth in both soluble and membrane-bound forms.

Sema7A is necessary for axon tract formation

The strong effect of Sema7A on OB axon growth suggests Sema7A may modulate the establishment of neuronal connectivity in the

central olfactory system. OB output neurons, that is, mitral and tufted cells, receive sensory inputs from the OE, processing this olfactory information before relaying it to the olfactory cortex[20]. At embryonic day (E)14 to E15 in rats, mitral cell axons initiate the formation of a discrete, caudally extending fibre bundle called the lateral olfactory tract (LOT). Around E17, collateral branches are elaborated from these projections which then invade the olfactory cortex[21]. Recent studies reveal some of the molecular mechanisms that regulate LOT axon pathfinding and branching[22–24]. However, factors controlling other aspects of LOT formation, including growth and positioning, remain unknown. Interestingly, it has been demonstrated that growth of the LOT relies on unidentified membrane-bound protein(s) presented by the presumptive LOT region and the olfactory cortex[25,26]. It is thus intriguing that at E15 *Sema7A* is expressed by OB neurons and, specifically, along the presumptive LOT trajectory (Fig. 2a, b). At E19, *Sema7A* expression persists in the OB and throughout the olfactory cortex (Fig. 2c, e; data not shown).

To evaluate whether Sema7A stimulates formation of the LOT in its native environment, we placed AP-Sema7A-secreting cell aggregates adjacent to the presumptive LOT region in organotypic cultures of whole telencephalic hemispheres from E14 rat[23,25] (Fig. 2f, g). Consistent with our previous observations that Sema7A enhances OB axon length and number, the LOT diameter was significantly increased at the position just caudal to the OB where olfactory axons fasciculate to form the LOT (defined here as '0 μm') in the presence of Sema7A. This effect was also evident caudally in closer proximity to the olfactory cortex ('800 μm'). Furthermore, in the presence of Sema7A, LOT axons extended further into the olfactory cortex (Fig. 2h–j). However, the position of the LOT in the cultured telencephalon was unaltered, Sema7A did not affect collateralization of LOT axons, and individual LOT fibres

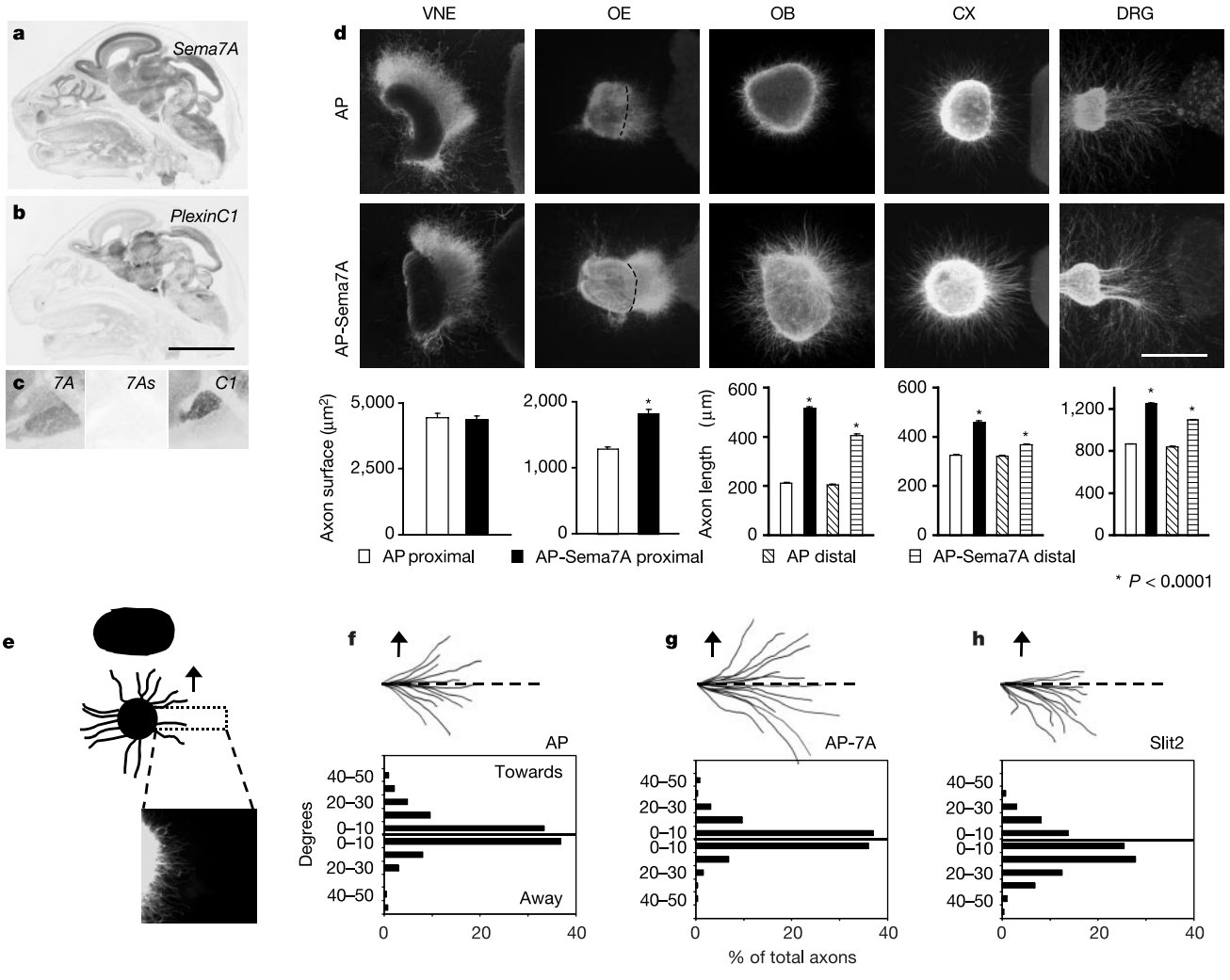

Figure 1 Sema7A promotes growth of central and peripheral axons. **a**–**c**, *In situ* hybridization[45] for *Sema7A* (**a**) and *plexinC1* (**b**) in adjacent sagittal sections through an E19 rat head. **c**, Higher magnifications of **a** and **b** show noticeable complementary expression in pituitary, whereas sense controls lack specific signals (*7As*; data not shown). **d**, E15 rat vomeronasal epithelium (VNE), olfactory epithelium (OE), olfactory bulb (OB), cortex (CX), and dorsal root ganglion (DRG) explants were cultured for 48–72 h in collagen matrix[22,33] next to alkaline phosphatase (AP)-transfected 293-EBNA cells or stable 293-EBNA cells secreting AP-Sema7A[10]. Axons were labelled using antibodies to class III β-tubulin or neurofilament (2H3) and results were quantified by measuring total axon surface (VNE, OE) or by measuring the length of the 20 longest axons in the proximal and distal quadrants (OB, CX, DRG)[30]. Total number of explants quantified from four to five independent experiments (n = AP, n = AP-7A): VNE (20, 20), OE (24, 24), OB (39, 39), CX (22, 17), and DRG (14, 15). Asterisk, $P < 0.0001$ compared with AP. **e**–**h**, Sema7A does not induce directional growth of OB axons. **e**, E15 rat OB explants were cultured as in **d**. The trajectories of individual axons emerging parallel to the cell aggregates were analysed[18]. **f**–**h**, On top are *camera lucida* drawings of representative axons for each condition. Histograms depict how many degrees axons deviate from a straight course. For each experimental condition, 8–10 individual axons were randomly selected and analysed per explant (n = 30 explants each). Scale bars: **a**, **b**, 3,500 μm; **c**, 1,080 μm; **d**, 140 μm, (VNE), 500 μm (OE, OB, CX), 1,000 μm (DRG); **e**, 390 μm; **f**–**h**, 170 μm.

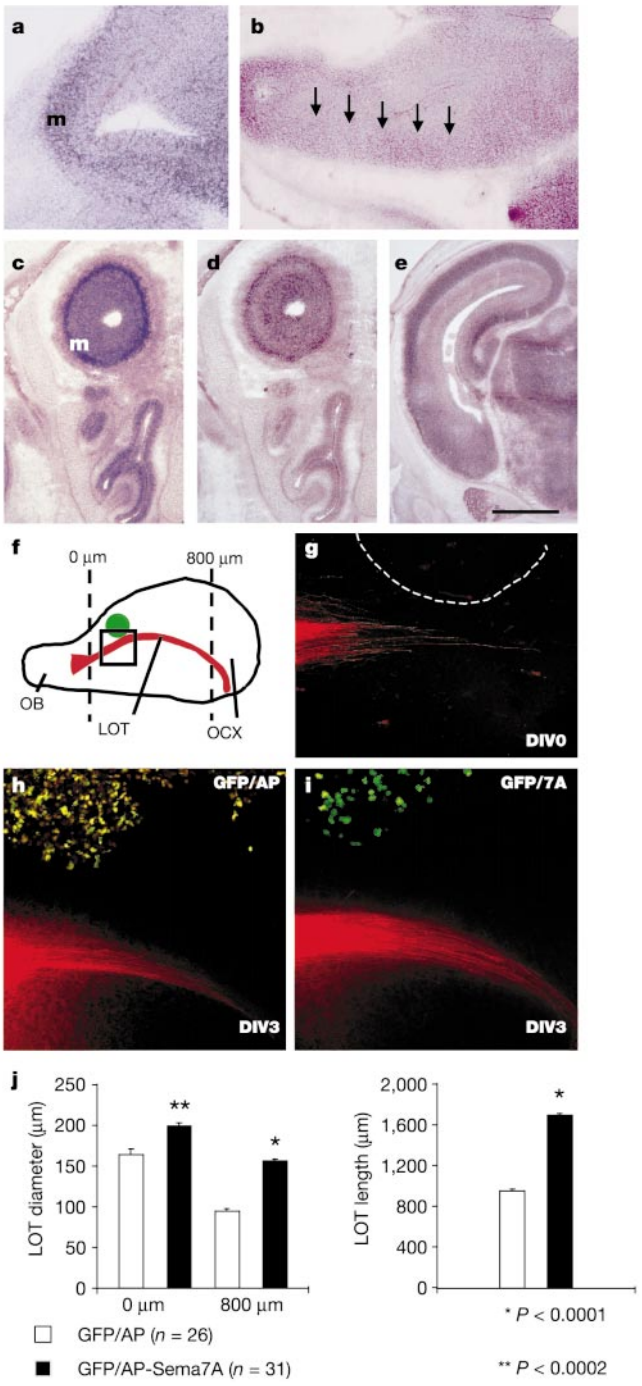

Figure 2 Sema7A stimulates lateral olfactory tract (LOT) formation. *Sema7A* (**a**–**c**, **e**) and *plexinC1* (**d**) messenger RNA expression in sagittal (**a**), horizontal (**b**), and coronal sections (**c**–**e**) through the E15 (**a**, **b**) and E19 (**c**–**e**) rat olfactory system. **b**, Arrows, *Sema7A* expression at the presumptive LOT position. m, mitral cells. Sense controls displayed no specific signals (not shown). **f**–**j**, Sema7A stimulates olfactory axon growth *in situ*. **f**, Green fluorescent protein (GFP)/AP (**h**) or GFP/AP-Sema7A (**i**) transfected cell aggregates were placed onto E14 rat whole-telencephalic cultures adjacent to the presumptive LOT position. After three days *in vitro* (DIV), the LOT was visualized by employing the lipophilic dye DiI (**g**–**i**)[23]. **g**, Boxed area in **f**. Around E14, OB axons exit the olfactory bulb and start to fasciculate to form the LOT. Dashed line, presumptive position cell aggregate. **h**–**j**, To determine LOT extension the lengths of the five longest axons was measured starting at 0 μm. *n*, total number of cultures that were quantified from five experiments. OCX, olfactory cortex. Asterisk, $P < 0.0001$ and double asterisk, $P < 0.0002$ compared with GFP/AP. Scale bars: **a**, 300 μm; **b**, 750 μm; **c**, **d**, 980 μm; **e**, 1,150 μm; **g**–**i**, 300 μm.

displayed no tropic responses to Sema7A aggregates (Fig. 2h, i; data not shown).

We then explored the *in vivo* role of Sema7A during neural development by generating *Sema7A* null mice (Fig. 3a–e) and analysing development of the LOT in E16 wild-type, *Sema7A*$^{+/-}$, and *Sema7A*$^{-/-}$ mice using retrograde DiI axon tracing[24] (Fig. 3f–n). *Sema7A* null mice were born at the expected mendelian frequencies from *Sema7A*$^{+/-}$ intercrosses and appeared grossly normal. At E16 in mice, the LOT has completely formed and olfactory axon collaterals start to elaborate into the olfactory cortex[24] (Fig. 3f, g). In line with our gain-of-function experiment which indicated that Sema7A can stimulate growth of the LOT (Fig. 2f–j), mice lacking Sema7A showed impaired LOT outgrowth (Fig. 3f–j). In *Sema7A*-deficient mice the LOT, visualized in E16 whole-mount brains, was abnormally narrow compared to heterozygous and wild-type littermates, whereas tract positioning in the telencephalon was unaltered (Fig. 3f–j). It is unlikely that cell death in the OB contributes to the reduction in the LOT projection, because both OB cytoarchitecture and mitral/tufted cell numbers were unchanged in *Sema7A*-deficient mice (data not shown). Surprisingly, length of the LOT as visualized in E16 whole-mount brains was not significantly different in control and mutant mice (+/+ and +/−, 2,033 ± 30 μm ($n = 43$); −/−, 1,918 ± 35 μm ($n = 24$); $P > 0.05$). However, evaluation of DiI tracing in coronal sections through E16 brains, which allows for more-sensitive visualization of both LOT axons and collateral branches, showed that the overall LOT projection was substantially reduced in size in *Sema7A* mutants (Fig. 3k–n; $n = 5$ +/+, $n = 5$+/−, and $n = 10$−/−). Remarkably, in many *Sema7A* mutants examined using coronal sections no LOT was detectable at the most caudal levels, suggesting that in the absence of Sema7A the majority of OB axons fails to project into the most caudal parts of the olfactory cortex by E16 (Fig. 3n). Branching of the LOT appeared to be reduced in *Sema7A* mutants (Fig. 3f–n), however, this is probably due to overall impaired growth of the LOT since Sema7A does not affect OB axon branching *in vitro* or *in situ* (data not shown). The ability of a (reduced) LOT to form in *Sema7A*-deficient mice indicates that Sema7A acts combinatorially with other growth promoters to control the establishment of LOT projections. Although OB axons fail to respond to most of the neurotrophic factors[26], laminin and fibronectin enhance olfactory axon extension *in vitro* and may contribute to LOT growth *in vivo*[23]. Overall, these experiments establish that Sema7A is an axon growth-promoting factor required for proper LOT development *in vivo*.

Sema7A-mediated axon growth is plexinC1-independent

We next performed AP-Sema7A binding experiments[10,18] to visualize endogenous neuronal receptor(s) responsible for transducing Sema7A signals in olfactory neurons. In line with Sema7A effects on OB axons, AP-Sema7A avidly bound to cell bodies, axons and growth cones of dissociated OB neurons (Fig. 4a). Plexins and neuropilins have been shown to mediate semaphorin function in the nervous system[5]. Sema7A fails to bind neuropilin receptors[10,17]. However, *in vitro* binding studies[17], the strikingly complementary distribution of *Sema7A* and *plexinC1* in certain regions of the nervous system (Fig. 1a–c; data not shown), the strong *plexinC1* expression in the olfactory system (Fig. 2d) and the high levels of plexinC1 protein in dissociated OB neurons (data not shown) all support the possibility that plexinC1 serves as a neuronal Sema7A receptor. To test this hypothesis, we generated *plexinC1*-deficient mice and performed collagen matrix assays using tissues derived from mutant and wild-type mice. Remarkably, both AP-Sema7A binding to endogenous receptors and Sema7A-mediated axon growth of olfactory, cortical and sensory neurons were unaltered in the absence of plexinC1 (Fig. 4a–f; data not shown). By measuring OB axon length in the proximal quadrants of control and mutant cultures, we confirmed that wild-type and plexinC1-

deficient olfactory axons display identical responses to Sema7A as follows. (+/+): AP, 340 ± 7.9 μm (mean s.e.m.) ($n = 10$ explants) / Sema7A, 666 ± 13.8 μm ($n = 10$) versus (−/−): AP, 373 ± 7.1 μm ($n = 14$) / Sema7A, 651 ± 13.6 μm ($n = 12$). Consistent with these observations, formation of the LOT *in vivo* is intact in *plexinC1* mutant mice (data not shown). Thus, Sema7A-mediated axon growth is independent of plexinC1.

Sema7A axon outgrowth is mediated by integrins

Many neurons use members of the integrin family of cell surface receptors for responses to neurite growth promoting proteins[27]. Intriguingly, the semaphorin domain of Sema7A contains a known integrin-binding motif, that is, RGD[28] (amino acids 267–269), suggesting integrins might mediate Sema7A responses (Fig. 4g). To test this idea, we prepared neuronal cultures and manipulated integrin function. Dissociated neurons obtained from E15 or E17 OB displayed a dose-dependent growth-promoting response when grown on AP-Sema7A or Fc-dimerized Sema7A[10] but not on AP or AP-Fc control substrates (Fig. 4h, i, p; data not shown). In contrast, axon branching of both E15 and E17 OB neurons was unaffected by Sema7A treatment (data not shown). In addition, Sema7A did not influence the number (AP; 14.0 cells mm^{-2}, AP-7A; 12.8 cells mm^{-2}, $P > 0.05$) or survival (percentage of 'Hoechst 33258 dead' neurons[29]: AP; 0.7%, AP-7A; 1.0%, $P > 0.05$) of dissociated OB neurons. As expected, Slit2 or extracellular matrix component (laminin or fibronectin) substrates inhibited and promoted axon growth, respectively[19,23]. Sema3A (AP-3A), which has an overall domain structure similar to Sema7A except for its C terminus and lack of an RGD motif, did not modulate olfactory axon growth ($P > 0.05$) (Fig. 4p).

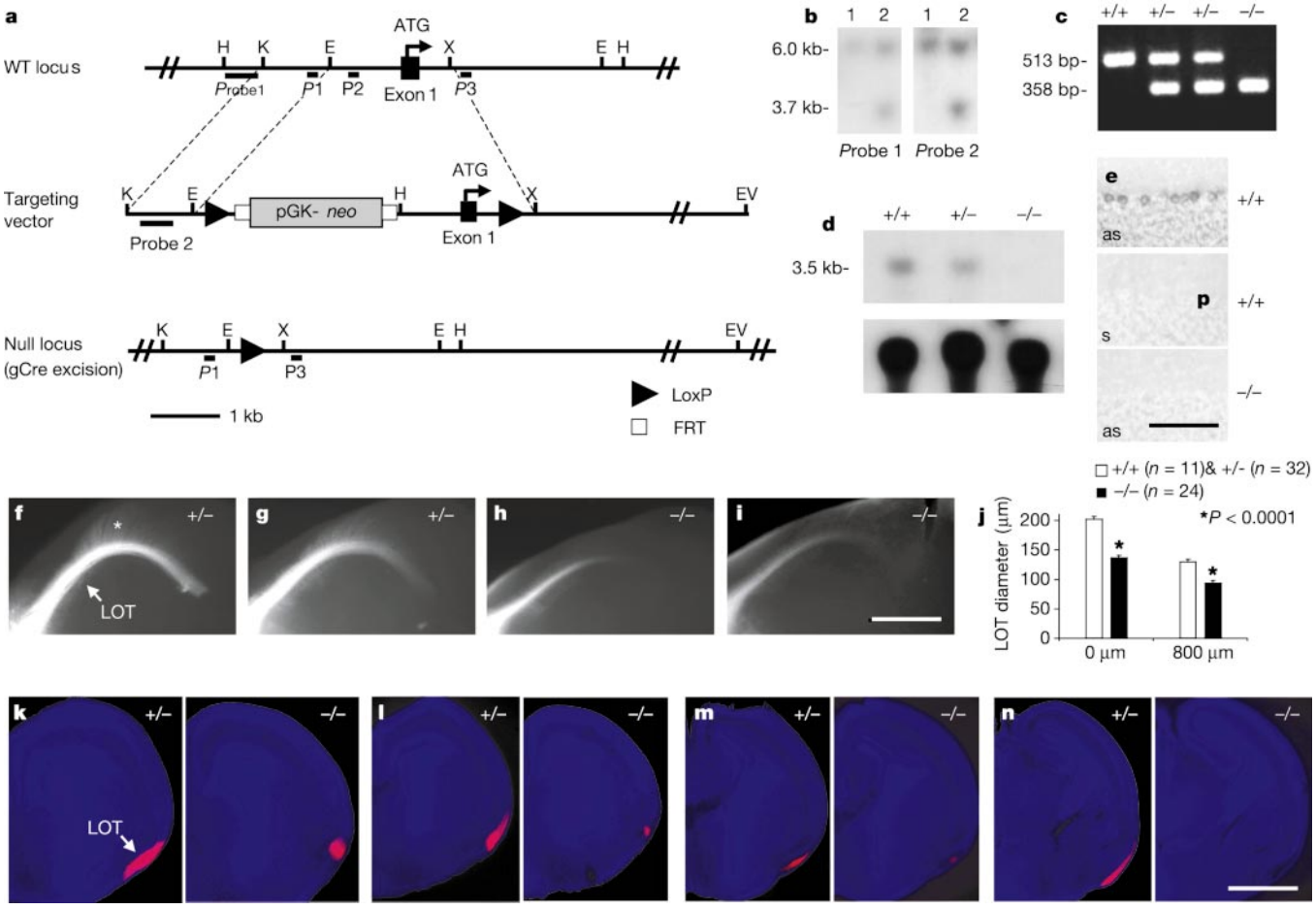

Figure 3 LOT formation is defective in *Sema7A* homozygous mutant mice. **a**–**e**, Generation of *Sema7A* mutant mice. **a**, Schematic structures of wild-type *Sema7A* gene, targeting vector, and mutant allele. The targeted exon encodes the amino-terminal 59 amino acids of Sema7A, which includes its entire signal sequence. Homologues recombination and subsequent Cre recombinase excision generates a mutant that lacks the first exon and an additional 1.1 kb of upstream DNA. The probes for Southern blot analysis and the polymerase chain reaction (PCR) primers (P1–3) used for genotyping are indicated. E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; K, *Kpn*I; X, *Xma*I. **b**, Southern blot analysis of control (1) and targeted (2) ES cells. Genomic DNA was digested with *Hind*III and subjected to Southern blot analysis with outer probe 1. Then the blot was stripped and reprobed using internal probe 2. **c**, PCR genotyping of wild-type, heterozygous, and *Sema7A* mutant P20 mice with primers P1, P2 and P3 (**a**). **d**, Northern blot analysis of 40 μg adult whole-brain RNA isolated from wild-type, *Sema7A* $^{+/-}$ and *Sema7A* $^{-/-}$ littermates using a rat Sema7A probe 3′ to the deleted region of *Sema7A* (base pairs 364–726 of coding region). The blot was reprobed with a GAPDH complementary DNA (lower panel). **e**, *In situ* hybridization of a coronal section from adult cerebellum using sense (s) and anti-sense (as) Sema7A probes (see **d**). p, Purkinje cell layer. **f**–**n**, LOT formation is inhibited in the absence of Sema7A. **f**–**i**, Ventral views of whole-mount brains showing the LOT in E16 *Sema7A* $^{+/-}$ (**f**, **g**) and *Sema7A* $^{-/-}$ littermates (**h**, **i**). OB is to the left. DiI tracing of the LOT was as described[24]. Asterisk indicates olfactory axon collaterals in the olfactory cortex. **j**, Quantification of LOT diameter determined from DiI-labelled E16 whole-mount brains as described in Fig. 2. No differences were found in LOT diameter between wild-type and heterozygous mice (data not shown). Asterisk, $P < 0.0001$. **k**–**n**, Matching 120 μm coronal brain sections from E16 *Sema7A* $^{+/-}$ and *Sema7A* $^{-/-}$ littermates labelled with DiI in the OB and counterstained with Hoechst[24] in rostral (**k**) -to-caudal (**n**) order. Scale bars: **e**,120 μm; **f**–**i**, 320 μm; **k**, 315 μm; **l**, 412.5 μm; **m**, 42 μm; **n**, 430 μm.

To examine whether integrins mediate Sema7A function, we first altered the putative integrin-binding RGD sequence in Sema7A to KGE (Fig. 4g), which is not recognized by integrins[30], and tested the ability of substrates composed of the mutated protein 7A(KGE) to enhance axon growth. OB axon outgrowth on 7A(KGE) substrates was similar to AP control ($P > 0.05$) (Fig. 4j, p).

Next, we used synthetic RGD-containing peptides as competitive inhibitors of the Sema7A RGD binding site to interfere with integrin signalling[28] (Fig. 4g, q). Treatment of OB neurons grown on Sema7A substrates with RGD peptide[31] dramatically reduced axon growth in a dose-dependent manner, whereas addition of a scrambled control peptide (P10)[30] had no effect (Fig. 4q; data not shown). We also assessed the ability of a 15-amino-acid RGD-containing peptide derived from the Sema7A sequence to interfere with Sema7A signalling (7A-RGD; Fig. 4g). Unexpectedly, treatment with this 7A-RGD peptide led to a further increase in OB axon length, whereas this additive effect was completely abolished after a single amino acid substitution was made in the RGD sequence (7A-RAD: Fig. 4g, q). Furthermore, the 7A-RGD peptide, which is an amino acid sequence unique to Sema7A, promoted axon growth from neurons growing on control AP substrates, demonstrating a role for these Sema7A residues in enhancing axon growth (Fig. 4k, l, q). The agonistic effects of 7A-RGD were blocked by mutating the RGD sequence (that is, 7A-RAD), and by addition of RGD peptide ($2.0\ \mathrm{mg\,ml^{-1}}$), thus demonstrating that this effect is RGD-dependent (Fig. 4q). Taken together, these results strongly suggest

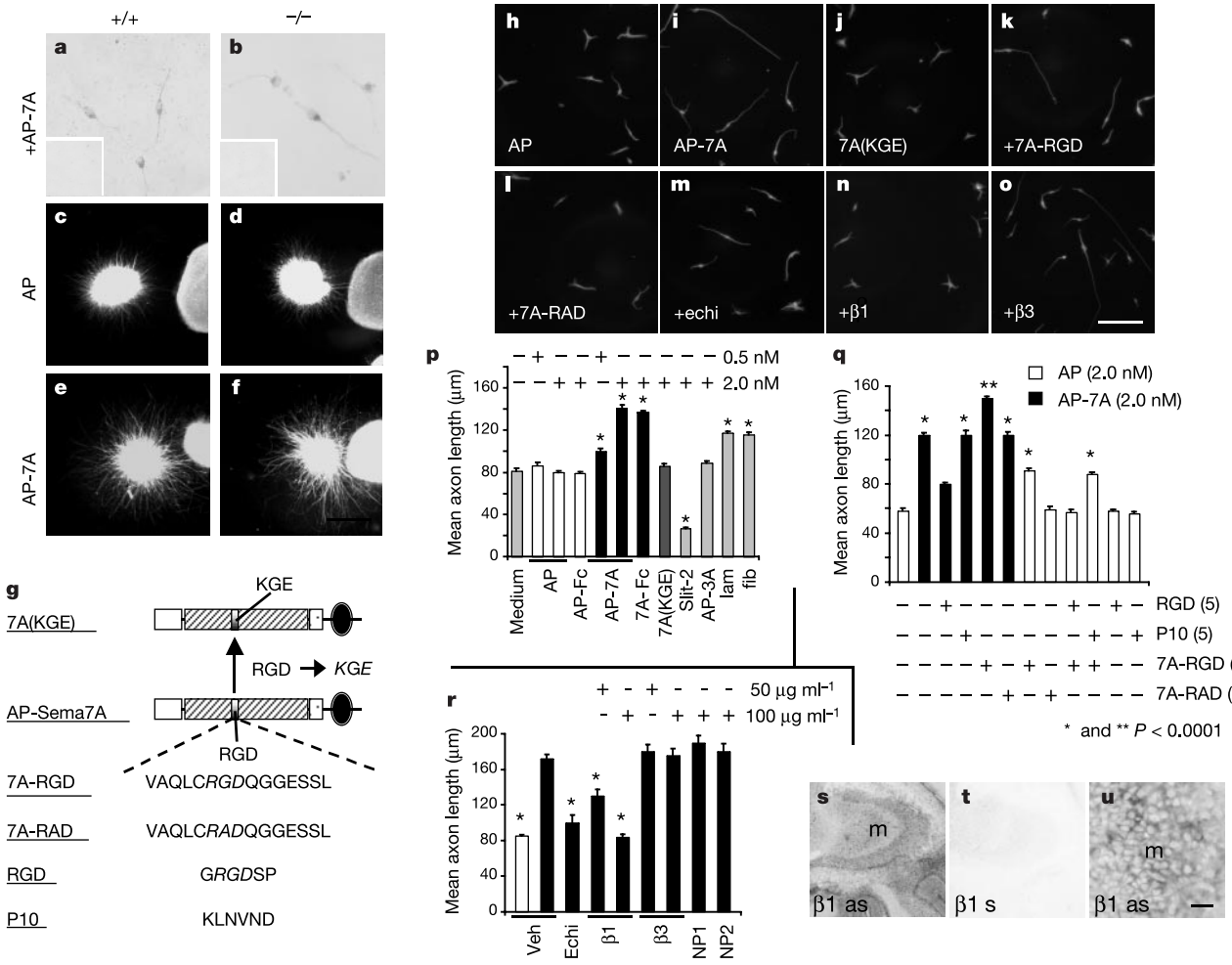

Figure 4 Integrins mediate Sema7A signalling. **a**–**f**, Sema7A enhances axon growth in a plexinC1-independent manner. **a**, **b**, AP-Sema7A binding[10] to E14 dissociated OB neurons from wild-type (**a**) or *plexinC1* $^{-/-}$ (**b**) mice. Insets show AP controls. **c**–**f**, Collagen matrix assays (see Fig. 1d) using E14 OB explants from wild-type (**c**, **e**) or *plexinC1* $^{-/-}$ (**d**, **f**) mice. **g**–**u**, RGD-sensitive β1-subunit-containing integrins mediate axon growth promotion by Sema7A. **g**, Schematic drawings of soluble, AP-tagged (AP-Sema7A)[10] and RGD-mutated, AP-tagged (7A(KGE)) Sema7A constructs, and synthetic peptides used in competition studies. **h**–**r**, Site-directed mutagenesis of the Sema7A RGD sequence, RGD peptides, and integrin function-blocking antibodies neutralize Sema7A axon growth. Dissociated neurons derived from E15 rat OB were cultured for 2–3 days on substrate-coated coverslips[23], fixed and processed for immunocytochemistry with anti-β tubulin or anti-tau (not shown) antibodies. **h**–**o**, Representative examples of neurons cultured on AP- (**h**, **k**, **l**), AP-Sema7A- (**i**, **m**–**o**) or 7A-KGE substrates (**j**) in the absence (**h**–**j**) or presence of peptides (**k**, **l**), inhibitor (**m**), or antibodies (**n**, **o**). **p**–**r**, Axon-length quantification[30] of neurons cultured on various substrates (**p**), on AP- or AP-Sema7A substrates in the presence of different synthetic peptides or combinations thereof (peptide concentrations in $\mathrm{mg\,ml^{-1}}$ are in parentheses) (**q**), or on AP- and AP-Sema7A substrates and treated with the β1/β3-specific integrin inhibitor (echistatin (Echi); 1.0 μM) or function-blocking antibodies to β1, β3, neuropilin (NP)-1 or NP-2 (**r**). Final concentration of fusion protein substrates is 0.5 or 2.0 nM. Veh, vehicle. Data represent means ± s.em. of at least four independent experiments. Asterisk, $P < 0.0001$ compared with AP (**p**, **q**) or AP-7A (**r**). Double asterisk, $P < 0.0001$ compared with AP-7A. **s**, **u**, *β1* expression in the E18 rat olfactory system. **t**, Sense control. fib, fibronectin; lam, laminin; m, mitral cell. Scale bars: **a**, **b**, 50 μm; **c**–**f**, 300 μm; **h**–**o**, 170 μm; **s**, **t**, 290 μm; **u**, 70 μm.

that an RGD-sensitive integrin is essential for mediating the axon growth-promoting responses of Sema7A.

Integrins are functional heterodimers composed of α and β subunits. There are 18 α and 8 β subunits, and they associate to form 24 different integrin receptors with differing ligand specificities[27]. Several α and β subunits expressed in the nervous system can form RGD-recognizing integrins[27,28]. The disintegrin echistatin, a viper-venom-derived RGD peptide that specifically inhibits β1- and β3-containing integrins[32], dramatically reduced Sema7A's effect on OB axon growth (Fig. 4m, r). To discriminate between β1- and β3-containing integrins, we used function-blocking antibodies which completely inhibit the activity of these subunits[31]. The blockade of β1-containing receptors with anti-β1 monoclonal antibodies (mAbs) neutralized axon growth promotion by Sema7A in a dose-dependent manner (Fig. 4n, r). In contrast, anti-β3 mAbs and anti-neuropilin-1 and -2 function-blocking antibodies[33] had no effect ($P > 0.05$) (Fig. 4o, r). Consistent with these results, robust levels of *β1* transcripts are observed in Sema7A-responsive neurons including mitral cells (Fig. 4s–u; data not shown). Thus, a β1-subunit-containing integrin receptor mediates the growth-promoting effects of Sema7A on axons.

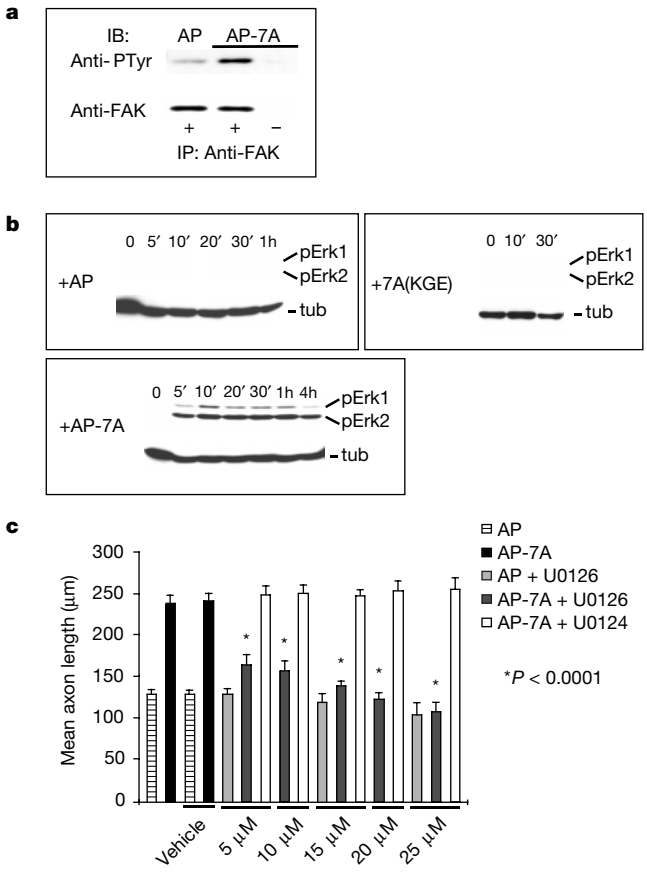

Figure 5 Integrin-induced MAPK signalling is necessary for Sema7A-mediated axon growth. **a**, Sema7A increases phosphorylation of FAK. E15 dissociated OB neurons were grown on 0.02% poly-L-lysine for 16 h, treated with AP- or AP-Sema7A-conditioned medium (20 nM) for 30 min, and subjected to immunoprecipitation (IP) using anti-FAK antibodies (+) or rabbit IgG (−)[46]. The samples were immunoblotted with anti-phosphotyrosine antibodies. After stripping, the blots were reprobed with anti-FAK to control the levels of IP-ed FAK[46]. Experiments were done three times with similar results. **b**, Sema7A induces ERK1/2 phosphorylation in an RGD-dependent manner. E15 dissociated OB neurons were treated with AP-, AP-Sema7A- or 7A(KGE)-conditioned medium (20 nM) for 5 min to 4 h as described above, and subjected to immunoblotting with an antibody specific for the phosphorylated forms of ERK1 and ERK2. The immunoblot was later stripped and reprobed for α-tubulin to normalize for protein amounts[29]. Experiments were done three times with similar results. **c**, The MEK inhibitor U0126 neutralizes axon growth responses by Sema7A. Dissociated OB neurons grown on AP- or AP-Sema7A substrates (2.0 nM) were untreated or treated with vehicle (0.1% DMSO), U0126 (5–25 μM), or negative control U0124 (5–25 μM). Quantification of axon length was determined as described[30] from three independent experiments. Asterisk, $P < 0.0001$ compared with AP-7A (untreated, vehicle or U0124).

Sema7A activates FAK and MAPKs

Ligand-induced integrin clustering leads to the activation and recruitment of intracellular signalling and structural proteins to focal adhesion sites[27]. The non-receptor protein kinase focal adhesion kinase (FAK) plays a central role in β1 integrin-dependent signalling. FAK is rapidly phosphorylated upon ligand binding, leading to the activation of cytoskeleton-linked proteins responsible for signal propagation downstream of integrins. To determine whether Sema7A elicits integrin-dependent signalling responses, we treated OB neurons with AP- or AP-Sema7A-conditioned medium and assessed tyrosine phosphorylation of FAK. AP-Sema7A increased FAK phosphorylation in neurons, confirming a role for integrin-associated signal transduction machinery in propagating Sema7A signals (Fig. 5a).

An important function of FAK is the regulation of mitogen-activated protein (MAP) kinase pathways[34]. MAPKs have been implicated in neurite growth stimulated by cell adhesion molecules and neurotrophic factors[35,36]. Interestingly, MAPK signalling also underlies neurite outgrowth of PC12 cells by the secreted semaphorin Sema3E[37] and the potentiation of neurotrophin-mediated PC12 neurite outgrowth by the transmembrane semaphorin Sema4D[38]. To investigate whether MAPK cascades are involved in neuronal Sema7A signalling, dissociated OB neurons were stimulated with AP- or AP-Sema7A-conditioned medium, after which phosphorylation of the MAPKs extracellular regulated kinases (ERK) 1 and 2 was assessed. Sema7A caused a rapid and sustained increase in the phosphorylation of ERK1/2, whereas AP had no effect (Fig. 5b, Supplementary Fig. 3). In contrast, treatment with 7A(KGE) failed to trigger ERK1/2 phosphorylation, showing that the integrity of the RGD motif, and thus interactions with integrins, are essential for activation of MAPK signalling by Sema7A (Fig. 5b). To determine whether ERK activation is required for Sema7A function, we cultured neurons in the presence of the U0126, a specific inhibitor of MAPK kinases (MEK)-1 and MEK-2 that blocks ERK1/2 phosphorylation. U0126 blocked Sema7A-mediated axon growth stimulation in a dose-dependent manner, whereas the related but inactive compound U0124 had no effect (Fig. 5c). Therefore, MAPK-dependent signalling mechanisms are required for axon outgrowth stimulation by Sema7A.

Discussion

These studies describe a new biological function for semaphorins, establish an unexpected role for integrins as mediators of semaphorin responses, and provide insight into the molecular basis of semaphorin-dependent intracellular signalling. Mutation of the Sema7A integrin-binding motif and RGD blocking peptides, both commonly used methods to abrogate interactions between integrins and their ligands[28,30–32], blocks Sema7A-mediated axon growth. This suggests that Sema7A signalling in neurons may involve direct interactions of Sema7A and a β1-integrin(s). However, the requirement for β1-integrins in mediating Sema7A-induced axon growth does not exclude the possibility that other molecules are involved in mediating Sema7A functions. Integrins are known to associate with a large number of distinct proteins[27]. Thus, β1-integrins may constitute obligate co-receptors which associate with unidentified Sema7A-binding proteins. All neural semaphorin signalling has been assumed to be mediated by multimeric receptor complexes containing plexins as obligatory signal transducing subunits[5].

However, Sema7A-mediated axon growth is likely to be plexin-independent since plexinC1 is not required for this effect and Sema7A fails to bind vertebrate plexins other than plexinC1[17] (Z. He and L. Tamagnone, personal communications; data not shown). Intriguingly, semaphorin proteins belonging to different classes posses evolutionarily conserved integrin-binding motifs (Sema3C, Sema4G) suggesting that additional semaphorin–integrin interactions may occur. Furthermore, several other guidance cues with neurite-outgrowth-promoting activities contain integrin-binding motifs (including netrin-1 and certain class A ephrins)[39,40]. Therefore, integrin-mediated signalling mechanisms may be critical in mediating positive effects of axon guidance cues on axons.

Sema7A is expressed by several different classes of lymphoid and myeloid cells and is a potent immunomodulator[13,14]. Host-lymphocyte Sema7A is incorporated in the HIV-1 envelope during the budding stage, and it is suspected to be involved in HIV-1 infection[41]. Sema7A is also present in microglia, the principal immune effector cells of the nervous system, which are postulated to arise from blood monocytes[14]. Since sema7A is an autocrine monocyte activator[14] and *Sema7A* expression is upregulated following spinal cord injury (data not shown), Sema7A may participate in post-injury neural immune responses. The discrepancy between the biological activity of Sema7A during monocyte activation (0.1–1 pM) (ref. 14) and the affinity of Sema7A for plexinC1 ($K_D = 2.1$ nM)[17] suggests plexinC1 might not mediate all aspects of immunological Sema7A function. Given their role as mediators of neuronal Sema7A functions, integrins seem likely candidates to mediate the immunomodulatory effects of Sema7A. This is supported by the fact that integrins control several aspects of immune function[42], the observation that Sema7A induces monocyte aggregation, suggesting an interaction with cell surface adhesion proteins such as integrins[14], and expression of integrin receptors by monocytes and microglia[42,43]. Thus, Sema7A and the signalling machinery it activates may constitute targets for intervention in pathological immune responses and neural regeneration. □

Methods

Reagents and antibodies

Blocking peptides (JHU, HHMI Biopolymer lab); Echistatin, anti-α- or β-tubulin mAbs (Sigma); laminin, fibronectin (40 μg ml^{-1}; Gibco); U0126, U0124, anti-pERK (Cell Signaling); DiI, Hoechst 33258 dye (Molecular Probes); hamster anti-rat β1 mAb (Clone Ha2/5), mouse anti-rat β3 mAb (Clone F11, Pharmingen); anti-neurofilament mAb (2H3; Developmental Studies Hybridoma Bank); rat anti-mouse plexinC1 mAb (m653; Amgen); anti-tau mAb (Chemicon); rabbit anti-FAK (A-17; Santa Cruz); anti-phosphotyrosine (4G10; Upstate Biotechnology).

Cell and tissue culture

293-EBNA cells were cultured and transfected as described[10,33]. Inhibitors, blocking antibodies and peptides were added to the culture medium which was refreshed each day. Conditioned media were prepared as described[23] and concentrations of fusion proteins were determined by western blotting and soluble AP assays. Substrate-coated coverslips were prepared with recombinant proteins or conditioned media from transiently or stably transfected 293 EBNA cells[23,30]. Routinely, a 10% adsorption of input protein to poly-L-lysine-coated coverslips was observed[30] (data not shown). Therefore, 5 and 20 nM of input protein correspond to 0.5 and 2.0 nM final concentrations (Figs 4 and 5). Neuronal survival was assessed by employing Hoechst 33258 chromatin stains[29]. All axon and tract measurements and cell counts were performed using a CCD camera and Openlab software. Data were plotted as a mean ± s.e.m. and analysed with ANOVA or paired *t*-tests.

Site-directed mutagenesis and plasmid constructs

The four-primer method[30] was employed to mutate the RGD sequence of AP-Sema7A to KGE using the following primers; 5′-CGGGATCCCACCTAAGGAGCGGACCCCGC-3′, 5′-CCCTCGAGCAGCAGGTGCTCGGCCATG-3′, 5′-CAGGGTGGGGAAAGTTCACTG TCAGTC-3′, and 5′-CCCCACCCTGCTCCCCCTTGCAC-3′ (mutations are in italics).

The following DNA constructs were used: pEGFP-N1 (Clontech); pAP-TAG4 (Genhunter); pCIneo-plexinC1, which encodes full-length mouse plexinC1; pB-rSema7A, which encodes base pairs 364–726 of rat Sema7A; pAP-Sema3A, pEX-AP-Sema7A and pEX-Sema7A-Fc have been described[10,33]; pBSK-β_{OL} encoding rat β1 integrin, pSECtag-hSlit2, encoding myc-tagged full-length human Slit2, pCMV-CDw108, encoding mouse Sema7A, and pcDNA-AP-Fc/myc were provided by L. H. Rome, M. Tessier-Lavigne, A. Yamada and R. J. Giger, respectively.

Generation of *Sema7A* and *plexinC1* deficient mice

A BAC containing a 5′ portion of the Sema7A gene was obtained from Incyte Genomics. BAC DNA fragments and a *neo FRT/loxP* cassette (provided by K. Takamiya and R. Huganir, Johns Hopkins School of Medicine) were used to generate a conditional *Sema7A* targeting vector using standard recombinant DNA techniques (Fig. 3a). Following electroporation of the *Sema7A*-based targeting vector, targeted 129-derived ES clones were identified and injected into C57BL/6 blastocysts. Resulting male chimaeras were bred to C57BL/6 females to generate heterozygous mice carrying the mutation. *Sema7A* null mice were obtained by crossing F_1 heterozygous mice with mice that express Cre recombinase in the germ-cell lineage[44]. The resulting *Sema7A* mutation was then moved into a C57BL/6 background by three successive backcrosses. PCR genotyping was performed using the following primers (Fig. 3a); P1, 5′-GGCCACAGGATTCAGTGCA GGCC-3′; P2, 5′-CCCACAGACCCAGACATACTGAGC-3′; P3, 5′-CCCGCGTGCCAGC AGAGCTCGC-3′.

For details on the generation of *plexinC1* null mice see Supplementary Fig. 2.

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