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PlexinA1 signaling directs sensory axon segregation in the developing spinal cord: a role for proprioceptive axon exclusion in cutaneous afferent organization

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Summary

As different classes of sensory neurons project into the CNS, their axons segregate and establish distinct trajectories and target zones. One striking instance of axonal segregation is the projection of sensory neurons into the spinal cord, where proprioceptive axons avoid the superficial dorsal horn-the target zone of many cutaneous afferent fibers. PlexinA1 is a proprioceptive sensory axon-specific receptor for sema6C and sema6D, which are expressed in a dynamic pattern in the dorsal horn. The loss of plexinA1 signaling causes the shafts of proprioceptive axons to invade the superficial dorsal horn, disrupting the organization of cutaneous afferents. This disruptive influence appears to involve the intermediary action of oligodendrocytes, which accompany displaced proprioceptive axon shafts into the dorsal horn. Our findings reveal a dedicated program of axonal shaft positioning in the mammalian CNS, and establish a role for plexinA1-mediated axonal exclusion in organizing the projection pattern of spinal sensory afferents.

Keywords

semaphorin; plexin; sensory axon guidance; spinal cord; axonal segregation

Introduction

Information from the external world is transmitted to the central nervous system (CNS) by sensory neurons that convey distinct stimulus modalities. The neural circuits that process sensory information are established, in part, through the projection of the axons of sensory neurons to specific target zones. In many sensory systems the pattern of afferent projections is shaped by signals provided by cells in their target region (McLaughlin and O'Leary, 2005; Flanagan, 2006). There is also emerging evidence that interactions between sensory axons contribute to the patterning of sensory projections (Ebrahimi and Chess, 2000; Lee et al., 2003; Feinstein and Mombaerts, 2004; Komiyama et al., 2004; Reber et al., 2004). By extension, it is possible that the segregation, rather than association, of certain classes of axons also regulates afferent projection patterns. There are documented instances of axonal segregation in the development of vertebrate and invertebrate sensory systems (Scholes, 1979; Blagburn and Bacon, 2004), but it remains unclear if the exclusion of specific classes of axons is under active control, or has any role in the patterning of sensory projections.

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In the vertebrate somatosensory system, peripheral stimuli are conveyed by sensory neurons located within dorsal root ganglia (DRG) that flank the spinal cord. DRG neurons can be grouped into two major classes, those transducing proprioceptive and cutaneous sensory stimuli (Brown, 1981; Koerber and Mendell, 1992). Proprioceptive neurons convey information about the state of muscle contraction and limb position, whereas cutaneous neurons mediate a wide range of noxious and innocuous stimuli (Brown, 1981; Koerber and Mendell, 1992). The axons of these two sets of sensory neurons initially project along a common pathway in the dorsal roots, but on entering the spinal cord their axons segregate, and they pursue distinct paths to their target zones (Figure 1A). The trajectory of proprioceptive sensory neurons is notable in that their axonal shafts and collaterals avoid the superficial dorsal horn as they project to their ventral targets (Brown, 1981; Koerber and Mendell, 1992). In contrast, the axons of high-threshold cutaneous afferents project directly into the superficial dorsal horn, where they innervate target neurons (Figure 1A) (Brown, 1981; Koerber and Mendell, 1992). The mechanisms that establish the trajectory of proprioceptive axons have not been defined, and thus it remains unclear if their exclusion from the superficial dorsal horn is an important aspect of sensory afferent organization.

Genetic studies to define factors that regulate the targeting of sensory axons have revealed roles for several transcription factors (Arber et al., 2000; Zhong et al., 2006), but there is little information on guidance cues and surface receptors with more direct roles in assigning sensory axonal projection pattern. Studies of the path of proprioceptive and cutaneous axons have led to the proposal that discrete domains within the spinal gray matter express factors that shape sensory axonal trajectories through the local inhibition of axonal growth (Ozaki and Snider, 1997). Signals mediated by class 3 semaphorins (sema) and sensory axonal neuropilin (npn) receptors have been invoked as mediators of such repellant signals (Messersmith et al., 1995; Fu et al, 2000). However, genetic inactivation of class 3 semas in mice has yet to reveal a major role for these ligands in the patterning of sensory axonal trajectories (Behar et al., 1996; Taniguchi et al., 1997). Nevertheless, other classes of semas are expressed in the spinal cord (Cohen et al., 2005), and a second major class of sema receptors, plexins, is expressed by sensory and spinal neurons (Cheng et al., 2001; Cohen et al., 2005). Sema-plexin signaling has been shown to regulate the peripheral projection pattern of sensory neurons (Cheng et al., 2001; Suto et al., 2005; Yaron et al., 2005), raising the possibility of an additional role in establishing the central trajectories of spinal sensory afferents.

To begin to explore how the trajectory of sensory axons is established, we performed a screen to define surface receptors expressed selectively by proprioceptive sensory neurons. This screen identified plexinA1 as a proprioceptive axon-specific receptor, and revealed that two semas expressed by spinal cord cells, sema6C and sema6D, act as ligands for plexinA1. We find that the emergence of a *sema6C/6D*-sparse zone in the dorsal spinal cord is needed for the ingrowth of proprioceptive axon collaterals. And analysis of *plexinA1* mutant mice reveals that the loss of sema6C/6D-plexinA1 signaling elicits a dramatic defect in the positioning of proprioceptive axon shafts, which invade the dorsal horn and disrupt the organization of cutaneous sensory axons. These axonal interactions appear to be mediated by oligodendrocytes, which accompany displaced proprioceptive axon shafts into the superficial dorsal horn. Together, our results reveal an active program of axon shaft positioning in the mammalian CNS, and indicate that this program orchestrates the projection pattern of diverse classes of sensory neurons. More generally, they provide an insight into the puzzle of why developing nervous systems bother to exclude certain classes of sensory axons from target domains reserved for other sets of axons.

Results

Selective expression of *plexinA1* by proprioceptive sensory neurons

To identify receptors involved in establishing the trajectory of proprioceptive axons we examined 107 genes encoding putative transmembrane proteins for their profile of expression in e15.5 mouse DRG (Table S1). Of this set, 27 were expressed by subsets of DRG neurons (Table S1). These were analyzed further for expression in the DRG of *trkC* mutant mice, where proprioceptive sensory neurons are depleted (Klein et al., 1994), and in the DRG of *ngn1* mutant mice, where proprioceptive sensory neurons are enriched (Ma et al., 1999). The expression of one gene, *plexinA1*, was almost completely eliminated from the DRG of *trkC* mutants (Figures 1B, C; data not shown), and was enriched in the DRG of *ngn1* mutants (Figures 1B, D; data not shown).

To determine whether *plexinA1* is expressed by all proprioceptive sensory neurons, we compared the profile of *plexinA1* expression with that of *parvalbumin* (*Pv*), a marker of all proprioceptive sensory neurons (Honda et al., 1995; Arber et al., 2000). We found virtually complete coincidence in expression of *plexinA1* and *Pv* in e15.5 DRG neurons (Figures 1H-J), indicating that *plexinA1* is expressed by all proprioceptive neurons. During development, the expression of *plexinA1* by DRG neurons was first detected at e11.5, and by e13.5 expression was restricted to ~20% of DRG neurons, a profile that was maintained until at least p8 (Figures 1E-G; data not shown). Diffuse *plexinA1* expression was also detected throughout the gray matter of embryonic spinal cord (Figure 2A; data not shown). The selectivity of *plexinA1* expression prompted us to examine the patterns of expression of the eight other mouse *plexin* genes. *PlexinA2*, *plexinA3*, and *plexinD1* were each expressed by subsets of *Pv*-labeled proprioceptive neurons (data not shown), but were also expressed by other classes of DRG neurons (data not shown).

We also examined the pattern of expression of plexinA1 protein by DRG neurons. Little or no expression of plexinA1 was detected in sensory neuron somata, or in sensory axons in the dorsal roots, between e13.5 and p1 (data not shown). In contrast, plexinA1 was detected on the shafts of $TrkC^{+}$ proprioceptive axons within the dorsal funiculus, on rostrocaudally oriented axons branches within the dorsal columns (Figures 2D-F), and on axon collaterals in the dorsal spinal cord (Figures 2G-I). Thus, the axons of proprioceptive neurons express plexinA1 protein during the initial phase of their projection in the spinal cord. PlexinA1 expression was also detected on the axons of spinal neurons that project in the lateral funiculi and dorsal columns, and by cells in the intermediate and ventral spinal cord (Figure 2B; data not shown).

Sema6C and sema6D are ligands for plexinA1

To define ligands for plexinA1 we examined the binding of class 3 (sema3A, sema3C, sema3E and sema3F) and class 6 (sema6A, sema6B, sema6C, and sema6D) semas to plexins and npns (Figures 2J-M; data not shown). COS-7 cells transfected with *plexinA1*, *plexinA2*, *plexinD1*, *npn1*, or *npn2* plasmids, were exposed to the ectodomains (ecto) of semas conjugated to alkaline phosphatase (AP) (Figures 2J-M; data not shown). Binding of AP-sema6C^{ecto} and AP-sema6D^{ecto} was detected to *plexinA1*, but not to *plexinA2*, *plexinD1*, *npn1*, or *npn2*-transfected COS-7 cells (Figures 2K-M; data not shown). In contrast, AP-sema6A^{ecto}, AP-sema6B^{ecto}, and AP-sema3 proteins did not bind to *plexinA1*-transfected COS-7 cells (Figure 2J; data not shown). Thus, sema6C and sema6D interact selectively with plexinA1. The interaction of sema6D with plexinA1 has been reported (Toyufuku et al., 2004).

To determine if plexinA1 serves as a receptor for sema6C and sema6D in the developing spinal cord, we examined the binding of AP-sema6C^{ecto} and AP-sema6D^{ecto} to sections of spinal cord obtained from wild-type and *plexinA1* mutant mice (see below). We detected binding of

AP-sema6C^{ecto} and AP-sema6D^{ecto} to the dorsal and lateral funiculi of the spinal cord in wild-type mice at e13.5 (Figures 2O, Q), a pattern that matched sites of high level plexinA1 protein expression (Figure 2B). In contrast, there was no detectable binding of AP-sema6C^{ecto}, and a marked reduction in binding of AP-sema6D^{ecto}, to sections of spinal cord obtained from *plexinA1* mutant mice (Figures 2P, R). AP-sema3A, AP-sema3F, AP-sema6A^{ecto} and AP-sema6B^{ecto} bound in a similar pattern to spinal cord sections obtained from wild-type and *plexinA1* mutant mice (Figures 2S, T; data not shown). These data indicate that plexinA1 is a major target of sema6C and sema6D in the developing spinal cord.

Domains of *sema6C/6D* expression complement proprioceptive axon tracts

Proprioceptive sensory axons enter the spinal cord at the dorsal root entry zone (drez) and the parental axon shaft gives rise to branches that extend rostrally and caudally in the dorsal funiculus, avoiding the superficial dorsal horn (Figure 1A). With time, the rostrocaudally-oriented shafts and branches of proprioceptive axons shift progressively medially and reach the dorsal column. During their medial translocation, the branches of proprioceptive axons send off collaterals that circumvent the superficial dorsal horn as they project to their intermediate and ventral target zones (Figure 1A) (Brown, 1981). Throughout this developmental program, the shafts, branches and collaterals of proprioceptive axons are excluded from the superficial dorsal horn, the target region of high-threshold cutaneous afferent fibers.

To assess the role of plexinA1 signaling in the patterning of proprioceptive afferent projections, we compared the profiles of expression of *sema6C* and *sema6D* with the known trajectory of proprioceptive axons. We assessed proprioceptive axon trajectory by monitoring the pattern of GFP immunoreactivity in *TrkC::eGFP* BAC transgenic mice (Gong et al., 2003). At e11.5 to e12.5, *sema6C* was expressed by cells in the dorsal rim of the spinal cord (Figures 3A, E), whereas *sema6D* was expressed in a much broader dorsal domain (Figures 3B, F). At this stage, proprioceptive sensory axons have reached the drez (Ozaki and Snider, 1997), and are confronted by a dorsal domain of high level *sema6C/6D* expression (Figures 3A-H). By e13.5, the level of *sema6C* expression had decreased markedly (Figure 3I), and *sema6D* expression had cleared from a medial strip within the dorsal spinal cord (Figure 3J). Thus, the composite patterns of *sema6C* and *sema6D* at e13.5 reveal the emergence of a *sema6C/D*-sparse zone that coincides with the position of entry of proprioceptive axon collaterals (Figures 3I-L). By e14.5, *sema6C* expression was barely detectable (Figure 3M), and high level *sema6D* expression was restricted to an extreme dorsal rim that is flanked by the shafts of proprioceptive axons (Figure 3N). Together, these observations reveal a striking reciprocity in the patterns of *sema6C/6D* expression and the trajectory of proprioceptive axon shafts and collaterals.

The observation that proprioceptive axons avoid domains of *sema6C/6D* expression led us to explore two possible roles for plexinA1 signaling in the patterning of sensory axon projections. We asked first if the emergence of a *sema6C/6D*-sparse zone in the dorsal spinal cord is required for ingrowth of the collaterals of proprioceptive axons. And we next examined whether sema6C/6D-plexinA1 signaling normally constrains the trajectory of proprioceptive axon shafts, branches, or collaterals within the dorsal spinal cord.

Ectopic sema6C/6D suppresses proprioceptive axon collateral ingrowth

To assess whether the emergence of a *sema6C/6D*-sparse zone is necessary for the ingrowth of proprioceptive axon collaterals, we set out to fill this zone by ectopic expression of sema6C/6D throughout the dorsal spinal cord in the chick embryo. Analysis of the chick genome identified a *plexinA1* homolog, and a single *sema6C/6D* member with a sequence similar to that of mammalian *sema6D*. Expression of chick *plexinA1* in e8 to e11 DRG was restricted to neurons located in the ventrolateral region (Figure 3W; data not shown), the position of

proprioceptive sensory neurons (revealed by *runx3*expression; Figure 3X; Chen et al., 2006). There was also a complementary relationship between the path of proprioceptive axons (defined by TrkC expression) and domains of high-level *sema6D* expression (Figures 3Q-V). Thus, the inverse spatial relationship between *sema6C/6D* and proprioceptive axons is conserved in chick spinal cord.

We expressed *sema6C*^{ecto} or *sema6D*^{ecto} throughout the dorsal spinal cord of chick embryos at e4, prior to the entry of proprioceptive axons (Eide and Glover, 1997), and assessed domains of *sema* expression by co-expression of a *GFP* construct. The position of TrkC-labeled proprioceptive, and TrkAlabeled cutaneous, axons was analyzed at e11.0. Ectopic expression of *sema6C*^{ecto} or *sema6D*^{ecto} resulted in a ~70% suppression in the projection of proprioceptive axon collaterals into the dorsal spinal cord, assessed by quantitation of TrkC immunofluorescence (Figures 4C-G). No suppression of TrkC-labeled axon collateral entry was observed after expression of a control *GFP* construct, or of *sema6A*^{ecto} (Figures 4A, B, G). The actions of sema6C^{ecto} or sema6D^{ecto} appeared selective for proprioceptive axons, since their expression did not influence the projection of TrkA^{+} cutaneous axons into the dorsal spinal cord (Figures S2;4H). These findings provide evidence that the ingrowth of proprioceptive axon collaterals requires the emergence of a *sema6C/6D*-sparse zone in the dorsal spinal cord.

Defects in proprioceptive axon projections in *plexinA1* mutant mice

We next turned to the issue of whether the trajectory of proprioceptive axons is normally shaped by sema6C/6D-plexinA1 signaling. To assess this, we generated mice targeted with constitutive or conditional *plexinA1* mutant alleles (Figures S3A-B). Analysis of the spinal cord of constitutive *plexinA1* mutant embryos revealed the absence of plexinA1 immunoreactivity (Figures 2B, C), establishing that this allele encodes a null mutation. Mice homozygous for the constitutive and conditional *plexinA1* alleles were born at Mendelian frequencies, and were viable (data not shown).

In constitutive *plexinA1* mutant mice, the specification of proprioceptive and cutaneous neurons occurred normally, as assessed by the presence and number of Pv- and TrkA-labeled DRG neurons, analyzed from e14.5 to p1 (Figures S3C-F; S4A-D; data not shown). Since other plexinA proteins control the peripheral projection pattern of cutaneous sensory neurons (Cheng et al., 2001; Suto et al., 2005; Yaron et al., 2005), we examined whether there is a disruption in the peripheral projections of proprioceptive axons in *plexinA1* mutants. To assess this, we crossed a *Pv::eGFP* transgene (Dumitriu et al., 2006) into the constitutive *plexinA1* mutant background (Figures S3G-J). Analysis of GFP expression in *plexinA1* mutant mice at p1 indicated that the peripherally-directed axons of proprioceptive neurons projected normally, and formed specialized sensory endings with muscle spindles and Golgi Tendon Organs (Figures S3G-J; data not shown). Thus, the early differentiation and peripheral projection of proprioceptive sensory neurons appear unimpaired by the absence of plexinA1 signaling.

Proprioceptive axon collateral trajectory in plexinA1 mutants—We next examined whether the loss of plexinA1 signaling influences the central trajectory of proprioceptive neurons. To define the trajectory of proprioceptive axons before e14.0 we monitored GFP immunoreactivity in mice with a *TrkC::eGFP* allele crossed into constitutive *plexinA1* heterozygote or homozygous mutant backgrounds, and to define axonal projections after e14.5 we monitored expression of Pv.

In *plexinA1* mutants examined at e11.5 to e13.0, proprioceptive axons reached the drez (Figures S3K-M; data not shown), segregated from TrkA^{+} cutaneous axons (Figure S5), and branched rostrocaudally (Figures S3K-M; data not shown), in a pattern similar to that observed in wild-type and *plexinA1* heterozygote embryos. And in both *plexinA1* heterozygotes and mutants,

the collaterals of GFPlabeled proprioceptive axons entered the dorsal spinal cord at e12.5 to e13.5 and projected ventrally (Figures 5A-B; data not shown). Thus, plexinA1 signaling is not required for the early positioning of proprioceptive axon collaterals.

We examined whether the loss of plexinA1 signaling results in later defects in the projections of proprioceptive axon collaterals. A few Pv-labeled axons terminated in the superficial dorsal horn in postnatal *plexinA1* mutants (Figure 5N; data not shown), a domain normally devoid of proprioceptive axon terminals (Figure 5M). Nevertheless, analysis of the spinal cord of *plexinA1* mutants at p8 revealed that the collaterals of most proprioceptive axons projected to intermediate and ventral target zones in a pattern similar to that observed in wild-type mice (Figures 5O-P). Thus, plexinA1 signaling appears not to be involved in establishing the or ventral trajectory of the vast majority of proprioceptive axon collaterals.

Proprioceptive axon shaft positioning in plexinA1 mutants—The loss of plexinA1 signaling did, however, disrupt the organization of proprioceptive axon shafts. In *plexinA1* mutants analyzed from e12.5 to e16.5, the shafts of proprioceptive axons were displaced deep into the neuropil of the dorsal spinal cord (Figures 5A-H; data not shown). This early disorganization of proprioceptive axonal shafts persisted, and from p0 onwards transversely-orientated shafts of proprioceptive axons were observed to project through much of the superficial dorsal horn on their way to the dorsal columns (Figures 5I, J, M-P; data not shown). In *plexinA1* mutants analyzed at p0 to p8, over 95% of displaced proprioceptive axon shafts were located in the medial half of the superficial dorsal horn (Figure 5P; data not shown), and there was a ~95% decrease in the incidence of proprioceptive axon shafts within the medial half of the dorsal funiculus (Figures 5J; S6). These findings reveal that the loss of plexinA1 signaling erodes the normal exclusion of proprioceptive axon shafts from the superficial dorsal horn.

Since *plexinA1* is also expressed by cells in the dorsal horn, we were concerned that defects in axon shaft positioning might reflect the loss of protein expression from spinal cord cells rather than sensory neurons. To assess this possibility, we examined proprioceptive axon trajectories in mice in which the *plexinA1* gene had been inactivated selectively in DRG neurons. To achieve this, we crossed a conditional *plexinA1* mutant line (*plexinA1*^{flox}; see Experimental Procedures) with an *Ht-PA::Cre* strain that inactivates loxP-flanked genes from DRG neurons, without disrupting expression in spinal cord neurons (Pietri et al., 2003) (Figures 5K, L). At p0, *Ht-PA::Cre*; *plexinA1*$^{flox/flox}$ mutant mice exhibited a pattern of proprioceptive axon shaft displacement that was similar to that observed in constitutive *plexinA1* mutants (Figures 5I-L). Thus, the elimination of *plexinA1* from proprioceptive sensory neurons is sufficient to induce defects in axon shaft positioning.

A secondary disruption of cutaneous axonal projections in *plexinA1* mutants

We next considered the consequences of the displacement of proprioceptive axonal shafts. Since the superficial dorsal horn is the site of termination of many high-threshold cutaneous axons, we examined whether the organization of cutaneous afferent projections is affected in *plexinA1* mutants.

We monitored the projection pattern of three classes of cutaneous sensory afferents that project to different laminae within the dorsal horn: unmyelinated sensory axons marked by the binding of isolectin IB4 and the expression of fluoride-resistant acid phosphatase (FRAP) (Nagy and Hunt, 1982; Molliver et al., 1997) (Figures 6A-D; S8A-H); small-caliber axons marked by expression of substance (SP) and calcitonin-gene-related-peptide (CGRP) (Lawson, 2002) (Figures 6I-L; S9), and thinly myelinated cutaneous axons defined by vGlut1 expression (Todd et al., 2003) (Figures 6E-H; S8I-P). In wild-type and *plexinA1* heterozygous mice analyzed at p0, the axons of $SP^+/CGRP^+$ and $IB4^+/FRAP^+$ sensory neurons have entered the spinal cord,

forming diffuse termination zones in the superficial dorsal horn that resolve into more discrete laminar patterns by p8 (Fitzgerald, 1987; Mirnics and Koerber, 1995; Lawson, 2002) (Figures S8A, C, E, G; S9A, C; 6A, C, I, K; data not shown). Similarly, vGlut1$^+$ sensory axons have entered deeper regions of the dorsal horn by p0, and this pattern sharpens by p8 (Figures S8I, K, M, O; 6E, G; data not shown).

In *plexinA1* mutants analyzed at p0, the pattern of projection of SP$^+$/CGRP$^+$, IB4$^+$/FRAP$^+$, and vGlut1$^+$ sensory afferents was similar to that in wild-type controls (Figures S8A-D, I-L; data not shown). From p5 onwards, however, we detected a progressive disruption in the pattern of IB4$^+$/FRAP$^+$ and vGlut1$^+$ axonal projections (Figures S8E-H, M-P; 6A-H, M-N). In *plexinA1* mutant mice analyzed at p8, the medial half of the superficial dorsal horn was associated with a zone of exclusion of IB4$^+$/FRAP$^+$ axons (Figures 6A-D; data not show). The domain of exclusion of IB4$^+$/FRAP$^+$ axons centered on the location of Pv$^+$ proprioceptive axon shafts, but typically extended beyond the vicinity of the axon shaft itself (Figures 6A-D; data not shown). Nevertheless, the lateral domain of the superficial dorsal horn of *plexinA1* mutants, which lacks proprioceptive axon shafts, exhibited a normal density of IB4$^+$/FRAP$^+$ axons (Figures 6A-D; data not shown). Similarly, there was a local annulus of exclusion of vGlut1$^+$ axons in the medial half of the dorsal horn, centered on the position of proprioceptive axon shafts (Figures 6E-6H). The spatial link between proprioceptive axon shafts and zones of exclusion of IB4$^+$/FRAP$^+$ and vGlut1$^+$ sensory axons (Figures 6A-H, M-N) suggests that the disruption of cutaneous axonal projections is a consequence of the displacement of proprioceptive axon shafts, rather than a disruption in the patterning of dorsal horn neurons. Thus, between p5 and p8, two distinct classes of cutaneous afferents that project to different domains of the dorsal horn are excluded from the vicinity of misplaced proprioceptive axon shafts.

In contrast, we did not observe exclusion of SP$^+$/CGRP$^+$ cutaneous afferents from the medial half of the superficial dorsal horn in *plexinA1* mutants, assayed at p8 (Figures 6I-L, M; S9; data not shown). Thus, only certain classes of cutaneous sensory axons that project to the superficial dorsal horn appear sensitive to the displacement of proprioceptive axon shafts.

Oligodendrocytes invade the superficial dorsal horn in *plexinA1* mutants

How does the displacement of proprioceptive axonal shafts lead to the disorganization of cutaneous projections? One possible scenario is that the presence of proprioceptive axons results in a local disruption in the cellular organization of the dorsal horn itself, which in turn disturbs the projection of cutaneous axons. Against this idea, we found that the density and distribution of dorsal horn neurons at p8, assessed by the pattern of NeuN$^+$ neuronal nuclei, was similar in wild-type and *plexinA1* mutant mice (Figures S10A-F). Moreover, the distribution and laminar organization of specific classes of dorsal horn neurons, defined by expression of calretinin, protein kinase CβII and protein kinase Cγ(Ren et al., 1993; Malmberg et al., 1997) was essentially unchanged in *plexinA1* mutants (Figures S10G-X), even in domains close to displaced proprioceptive axon shafts (Figures S10G-X).

The preservation of neuronal organization in the dorsal horn of *plexinA1* mutants does not exclude an alteration in the patterning of glial cells. During normal development, oligodendrocytes ensheath and begin to myelinate proprioceptive axons during the first few post-natal days (Schwab and Schnell, 1989; Woodruff and Franklin, 1998). In contrast, many high-threshold cutaneous axons that project to the superficial dorsal horn remain unmyelinated, and others begin the process of myelination only at a later stage (Schwab and Schnell, 1989; Woodruff and Franklin, 1998). Since oligodendrocytes are known to express factors that inhibit the axons of DRG neurons *in vivo* and *in vitro* (He and Koprivica, 2004), we considered the possibility that proprioceptive axons might disrupt cutaneous afferent projections through an intermediary action of oligodendrocytes.

To assess this, we monitored the temporal and spatial pattern of oligodendrocyte differentiation in the dorsal spinal cord of wild-type, *plexinA1* heterozygote, and mutant mice, assessed by expression of two definitive oligodendrocyte markers-*myelin associated glycoprotein* (*MAG*) and *myelin basic protein* (*MBP*) (Mikoshiba et al., 1991). In wild-type mice, very few MAG^+ and MBP^+ oligodendrocytes were detected in the dorsal spinal cord prior to p2, and these few were confined to the dorsal funiculus and dorsal columns (Figures 7A-B; S11A). From p3 onwards, there was a progressive increase in the number of MAG^+ and MBP^+ oligodendrocytes present within the dorsal funiculus and dorsal columns (Figures 7C-E; S11B-D), but very few were located in the superficial dorsal horn (Figures 7C-E; S11B-D). In contrast, in *plexinA1* mutants analyzed from p4 onwards, we observed a >10 fold increase in the number of MAG^+ and MBP^+ oligodendrocytes within the superficial dorsal horn, compared with heterozygote or wild-type controls (Figures 7E-H; S11D-E; data not shown). Over 95% of these ectopic MAG^+ and MBP^+ oligodendrocytes were found in the medial half of the superficial dorsal horn (Figures 7F, H; 8E). In addition, we found that MAG^+ and MBP^+ oligodendrocyte processes were tightly associated with displaced Pv^+ proprioceptive axonal shafts in the medial half of the superficial dorsal horn (Figures 7F, H; 8B, C; E; data not shown).

The spatial relationship between foci of ectopic oligodendrocytes and the positioning of $IB4^+/FRAP^+$ and $vGlut1^+$ sensory axons differed, however. We found that the presence of MAG^+ oligodendrocytes were associated with a broad zone of exclusion of $IB4^+/FRAP^+$ axons that often covered most of the medial half of the superficial dorsal horn (Figures 8A-C; data not show). In contrast, the zone of exclusion of $vGlut1^+$ axons was occupied almost entirely by MAG^+ oligodendrocytes, together with the cell bodies of dorsal horn neurons, assessed by NeuN expression (Figures 8D-G). Thus, there is a much more local displacement of $vGlut1^+$ axons.

Finally, we examined whether the ectopic positioning of oligodendrocytes in *plexinA1* mutants is a consequence of the loss of plexinA1 expression from oligodendrocytes themselves, or is an indirect response to the displacement of proprioceptive axonal shafts. In wild-type mice, MBP^+ oligodendrocytes did not express *plexinA1* over the first post-natal week (Figure S12), arguing against the idea that oligodendrocytes respond directly to sema6C/6D signals. Moreover, we detected misplaced MAG^+ oligodendrocytes in the vicinity of proprioceptive axonal shafts in the superficial dorsal horn of *Ht-PA::Cre*; $plexinA1^{flox/flox}$ mice (Figure S13), a situation in which the loss of *plexinA1* is restricted to DRG neurons. Thus, the mispositioning of oligodendrocytes appears to be a secondary consequence of the displacement of proprioceptive axon shafts.

Discussion

This study shows that sensory axons conveying one stimulus modality are actively excluded from target domains reserved for other functional classes of afferents. A program of sema6C/6D-plexinA1 signaling effectively excludes proprioceptive axon shafts, and their associated oligodendrocytes, from the superficial dorsal horn of spinal cord, and only by virtue of such exclusion can the projection of cutaneous afferents proceed in an organized manner. These findings provide a rationale for the segregation of different classes of primary afferent axons in the somatosensory system, and may offer insight into the significance of programs of axonal segregation and exclusion in other sensory systems.

Sema6C/6D-plexinA1 signaling and the trajectory of proprioceptive axon collaterals

We have found that proprioceptive sensory neurons are defined by expression of plexinA1, that sema6C and sema6D are selective ligands for plexinA1, and that these two semas exhibit dynamic spatial patterns of expression as proprioceptive axons enter the dorsal spinal cord. These patterns can be reduced to two main features-a domain in which *sema6C/6D* expression

is low or absent, and a domain in which *sema6C/6D* is maintained at high levels. Our data indicate that both domains are critical for the establishment of proprioceptive axon trajectories, albeit in different ways. Thus, Sema6C and sema6D represent spatially-restricted repellant factors of the type invoked by Ozaki and Snider (1997) as determinants of the differential trajectory of sensory axons in the developing spinal cord.

What is the significance of the *sema6C/6D*-sparse domain? The path of the collaterals of proprioceptive axons follows the *sema6C/6D*-sparse domain that emerges at the time of initial axon collateral ingrowth. Preventing the emergence of this sparse domain, by ectopic sema6C/6D expression, reduces the ingrowth of proprioceptive axon collaterals without obvious impact on the entry of cutaneous axons. Thus, the emergence of a *sema6C/6D*-sparse domain in the dorsal spinal cord appears to be a necessary step in the initial ingrowth of proprioceptive axon collaterals.

These findings raise the inverse issue-whether domains of high level *sema6C/6D* expression constrain the trajectory of sensory collaterals. The domain of high level *sema6C/6D* expression that confronts proprioceptive axons as they arrive in the drez could serve to delay axon collateral ingrowth. One prediction of this idea is that elimination of sema6C/6D-plexinA1 signaling permits the precocious ingrowth of proprioceptive axon collaterals. However, elimination of plexinA1 signaling in proprioceptive neurons does not result in precocious axon collateral ingrowth from the drez. Thus plexinA1-independent signals regulate the timing of proprioceptive axon collateral ingrowth.

Proprioceptive sensory neurons express *plexinA2* as well as *plexinD1*, and cells in the dorsal spinal cord express *sema6A/6B* and *sema3E*, the respective ligands for these plexins (Gu et al., 2005; Suto et al., 2005; data not shown). It is possible therefore, that other sema-plexin interactions participate in the control of proprioceptive axon collateral ingrowth. In addition, recent studies have reported the expression of *netrin-1* by cells in the dorsal spinal cord (Watanabe et al., 2006), and analysis of netrin signaling mutants has revealed a role for this ligand and its receptor, DCC, in constraining the ingrowth and intraspinal projections of both proprioceptive and cutaneous afferents (Kawasaki et al., 2006; Watanabe et al., 2006). Mice deficient in slit-robo signaling also exhibit defects in the intraspinal trajectory of proprioceptive axons (Ma and Tessier-Lavigne, personal communication). Thus, genetic studies in mice argue that the composite trajectory of proprioceptive axons is established by the combined activities of sema, netrin, and slit signals. Studies in chick have suggested that two Ig-like proteins, TAG1/axonin1 and F11, control of sensory axon projection patterns (Perrin et al., 2001), but this idea has yet to receive genetic support.

PlexinA1 signaling and the positioning of proprioceptive axon shafts

The most prominent role for sema6C/6D-plexinA1 signaling in sensory neuron development appears to be in the control of proprioceptive axon shaft position. In *plexinA1* mutants we find a breakdown of the exclusion of proprioceptive axon shafts from the superficial dorsal horn, despite the lack of impact on axon collateral trajectory. Thus, independent guidance programs appear to regulate the positioning of proprioceptive axon shafts and the trajectory of their collaterals.

How might sema6C/6D-plexinA1 signaling control axon shaft position? The shafts of proprioceptive axons express plexinA1 as they traverse the dorsal edge of the spinal cord, suggesting an active program of sema6C/6D-plexinA1 signaling in axonal shafts. The idea that axonal shaft position is under active control has a precedent in *C. elegans* where the zig family of secreted Ig-domain proteins controls axon shaft position in the ventral nerve cord (Aurelio et al., 2002). In addition, studies on the interstitial branching of retinal ganglion axons in the tectum suggest a role for ephrin-ephA signaling in the control of axon shaft dynamics (Roskies

and O'Leary 1994; Yates et al., 2001). Our findings, however, do not exclude that plexinA1 signaling controls proprioceptive axonal shaft position indirectly. The status of plexinA1 signaling at the growth cones of sensory axons as they enter the spinal cord could induce the expression of a distinct axonal shaft receptor that controls axon shaft position (Figure 9A). But, regardless of the direct or indirect nature of plexinA1 signaling, our findings provide genetic evidence for an active program of axon shaft positioning in the mammalian CNS.

A role for sema signaling in shaping sensory axon projection patterns within the spinal cord has been invoked in previous analyses (Behar et al., 1996; Kitsukawa et al., 1997; Taniguchi et al., 1997). These studies focused primarily on the guidance of sensory axons by sema3 ligands and their npn receptors (Behar et al., 1996; Kitsukawa et al., 1997; Taniguchi et al., 1997). Yet the phenotype of mice carrying null mutations in genes encoding class 3 semas has not revealed pronounced defects in the central targeting of DRG axons (Behar et al., 1996; Taniguchi et al., 1997). Our findings indicate the involvement of sema signaling in the control of proprioceptive axon projection pattern, and suggest that sema6-plexinA signaling has a more prominent role than sema3-npn signaling in this process.

Proprioceptive axon shaft position influences the organization of cutaneous afferents

Proprioceptive axons have long been known to avoid the target zones of cutaneous afferents in the spinal cord (Brown et al, 1981; Ozaki and Snider, 1997), but the significance and molecular basis of this process of axonal segregation has remained unclear. Our analysis of *plexinA1* mutants reveals that the invasion of proprioceptive axon shafts into the superficial dorsal horn is accompanied by a dramatic disruption in the organization of cutaneous afferent projections.

How might proprioceptive axons influence the organization cutaneous afferents? In principle, proprioceptive axon shafts could secrete factors that disrupt the organization of cutaneous afferents. We cannot exclude this possibility, but favor the idea that these axonal interactions are attributable to an intermediary role of oligodendrocytes. In support of this view, we find that the displacement of proprioceptive axon shafts in *plexinA1* mutants is accompanied by the rerouting of oligodendrocytes into the superficial dorsal horn. We surmise that over the first few post-natal days of normal development, oligodendrocytes begin to form a tight association with the shafts of proprioceptive axons in the dorsal funiculus and dorsal columns (Figure 9B). The plexinA1 signaling program that excludes proprioceptive axonal shafts thus serves to ensure that oligodendrocytes are diverted away from the superficial dorsal horn over the period that cutaneous axons consolidate their laminar projection patterns. In the absence of plexinA1 signaling, displaced proprioceptive axonal shafts provide a substrate that directs the precocious invasion of oligodendrocytes into the superficial dorsal horn, in turn disrupting the organization of cutaneous afferent projections. A definitive assessment of this 'intermediary oligodendrocyte' hypothesis requires an analysis of cutaneous axon targeting in *plexinA1* mutants under conditions in which oligodendrocytes have been eliminated in a selective manner-currently a technical challenge.

The mispositioning of proprioceptive axons and oligodendrocytes appears to disrupt the stabilization, rather than initial projection, of cutaneous afferents into the superficial dorsal horn. We find that in *plexinA1* mutants, cutaneous sensory axons initially project into the superficial dorsal horn in an apparently normal manner, despite the presence of displaced proprioceptive axon shafts. But from p5 onwards certain classes of sensory axons fail to maintain their stereotypic termination pattern. There is a striking difference in the sensitivity of cutaneous sensory axons to the presence of displaced proprioceptive axon shafts and oligodendrocytes. $IB4^+/FRAP^+$ unmyelinated cutaneous axons that terminate in the superficial dorsal horn, and thinly-myelinated $vGlut1^+$ axons with deeper projections appear sensitive, whereas peptidergic afferents are apparently unaffected. Moreover, the nature of the disruption

of $IB4^+/FRAP^+$ and $vGlut1^+$ cutaneous axons differs. There is a local displacement of $vGlut1^+$ axons in the vicinity of ectopic oligodendrocyte foci, but a much more pervasive loss of $IB4^+/FRAP^+$ unmyelinated axons. Since both markers of this set of unmyelinated afferents are lost, it seems likely that these axons fail to maintain their terminal arbors in the vicinity of displaced proprioceptive axon shafts and oligodendrocytes. Nevertheless, we cannot exclude that this set of neuron activity maintains its axon terminal arborization pattern, but changes its molecular phenotype, down-regulating the expression of its defining markers.

Either a reorganization of terminal arbor, or a wholesale change in the phenotype of unmyelinated cutaneous afferents, might be expected to have profound consequences for sensory processing. There is a well-established somatotopic organization of inputs to the superficial dorsal horn: cutaneous afferents supplying discrete regions of the limb project to sagitally-oriented columns of target neurons in the dorsal spinal cord (see Schouenborg, 2004). Thus, the observed loss of high threshold $IB4^+/FRAP^+$ axons from specific mediolateral domains of the superficial dorsal horn may result in sensory deficits upon activation of cutaneous afferents within the corresponding peripheral receptive field. However, the receptive field that supplies an individual column of dorsal horn neurons is small (Schouenborg, 2004), and we observe considerable variability in mediolateral position of exclusion of high threshold. Thus, physiological demonstration of sensory deficits in *plexinA1* mutant mice is likely to be an arduous task.

At a molecular level, it remains unclear how oligodendrocytes influence cutaneous axon targeting. Several oligodendrocyte-associated proteins, notably MAG, Nogo-A and OMgp have been shown to inhibit the axons of DRG neurons (He and Koprivica, 2004). The temporal profile of expression of these inhibitory proteins may help to explain the sequence of interactions that occurs between proprioceptive sensory axons, oligodendrocytes, and cutaneous axons. In *plexinA1* mutants, we detect MAG^+ oligodendrocytes in the superficial dorsal horn only after p4, the time that the disorganization of $IB4^+/FRAP^+$ and $vGlut1^+$ cutaneous sensory axons becomes evident. Thus, the relatively late onset of expression MAG and other oligodendrocyte inhibitor proteins (data not shown) could contribute to the late clearance of cutaneous axons from the dorsal horn of *plexinA1* mutants. In addition, DRG neurons acquire sensitivity to the inhibitory actions of oligodendrocyte-associated proteins only after p4 (DeBellard et al., 1996). An intrinsic temporal program of neuronal sensitivity to oligodendrocyte-derived signals could, therefore, also contribute to the late impact on cutaneous afferent organization.

The involvement of oligodendrocyte inhibitory proteins as intermediary signals may help to explain why only some classes of cutaneous axons are sensitive to the presence of displaced oligodendrocytes. In vitro studies have show that the axons of the $IB4^+/FRAP^+$ cutaneous sensory neurons are more sensitive to the inhibitory actions of oligodendrocyte factors, such as Nogo66, than are $IB4^-/FRAP^-$ cutaneous neurons (Park et al., 2005). Moreover, Nogo receptor expression is restricted to a subset of DRG neurons (Hunt et al., 2002). We speculate, therefore, that the sensitivity of sensory axons to proprioceptive axon shafts and/or oligodendrocytes correlates, in an inverse manner, with the extent or timing of myelination of sensory axons-cutaneous afferents with little or late myelination exhibiting greater sensitivity to the presence of displaced oligodendrocytes.

More broadly, our findings provide genetic evidence that an active program of axon exclusion coordinates the projection pattern of different functional classes of primary sensory neurons. Selective affinities between subsets of sensory axons have previously been implicated in the establishment of afferent projection patterns in other sensory systems. Our findings emphasize that axonal segregation, as well as association, can contribute to the patterning of sensory afferent projections.

Experimental Procedures

In situ hybridization and immunocytochemistry

Digoxigenin (DIG)-labeled *cRNA* probes were used for in situ hybridization as in Schaeren-Wiemers and Gerfin-Moser (1993). Dual color fluorescence in situ hybridization histochemistry was performed as described (Price et al. 2002).

Rabbit anti-plexinA1 antibodies were generated against the 16 C-terminal amino acids of mouse plexinA1. Other antibodies were: rabbit and goat anti-Pv (Swant); sheep anti-GFP (Biogenesis); rabbit anti-GFP (Molecular Probes); guinea pig anti-vGlut1 (Chemicon); mouse anti-NeuN (Chemicon); rabbit anti-calretinin (Swant); goat anti-PKCβII (Santa Cruz); rabbit anti-PKC_{γ} (Santa Cruz); rabbit anti-CGRP (Peninsula Lab); rabbit anti-SubP (Neuromics); rabbit anti-MBP (Chemicon); rat anti-laminin B1 (Chemicon); rabbit anti-TrkA and anti-TrkC (Lefcort et al., 1996). Immunocytochemistry was performed as described (Kania et al., 2003). FRAP histochemical staining was performed as described (Nagy and Hunt, 1982).

Generation of *plexinA1* mutant mice

A constitutive *plexinA1* targeting vector was constructed using a 4 kb NotI-EcoRV fragment and a 10 kb 5′ region upstream of the first methionine. A loxP-PGKneo-triple pA signal-loxP and a farnesylated eGFP-pA cassettes were inserted between the two arms. A linearized targeting construct was electroporated into 129/Sv/Ev ES cells. Cells were selected with G418 and screened by Southern blot using an EcoRVI-EcoRI fragment as a probe, generating 12 kb wild-type and 7 kb mutant bands. A second probe outside the 5′ long arm was used to confirm homologous recombination.

A conditional *plexinA1* targeting vector was constructed using an 8 kb 5′ region, 2 kb SphI-XhoI, and 4 kb NotI-EcoRV fragments. A 2 kb SphI-XhoI was flanked by loxP sites. Cells carrying the targeted mutation were injected into C57BL/6J blastocysts. Chimeric offspring were mated with C57BL/6J mice. Germ-line transmission of the mutant allele was determined by Southern blot of genomic DNA. Homozygosity for the *plexinA1* knockout allele was confirmed by Southern blot and immunocytochemistry. Mutant mice were genotyped using oligonucleotides: 5'-CCTGCAGATTGATGACGACTTCTGC-3'(*plexinA1* 5'), 5'-TCATGCAGACCCAGTCTCCCTGTCA-3'(*plexinA1* 3'), 5′-ATGGTGAGCAAGGGCGAGGA-3'(*GFP* 5'), and 5'-TTACTTGTACAGCTCGTCCA-3'(*GFP* 3'). All experiments involved analysis of mice derived from heterozygous 129/Sv X C57BL/B6J intercrosses.

Transgenic mice

TrkC::eGFP, Pv::eGFP and *Ht-PA::Cre* mice have been described (Gong et al., 2003: Dumitriu et al., 2006; Pietri et al., 2003).

Sema-AP fusion protein binding

AP-fusion protein binding to COS-7 cells and tissue sections, and quantitative cell surface binding were performed as in Gu et al. (2005).

In ovo electroporation

Gene expression in chick was achieved by *in ovo* electroporation at e4 (Momose et al., 1999; Chen et al., 2006), and analyzed at e11. To quantify axonal projections in mouse, cryostat sections were labeled with anti-TrkA and anti-TrkC antibodies, and then Cy3-conjugated secondary antibodies. Optical sections were imaged using a confocal microscope.

Analysis of proprioceptive and cutaneous axonal projections

In mice, >30 sections were obtained from embryos at cervical and lumbar spinal cord (in register with the limbs) and similar findings were obtained at both levels. Regions of the chick spinal cord shown in Figure 4B for TrkC and Figure S2B for TrkA were framed and processed using Adobe Photoshop. Pixel counts were expressed as percentage of axonal fluorescence of control spinal cord (see Kania et al, 2003 for details).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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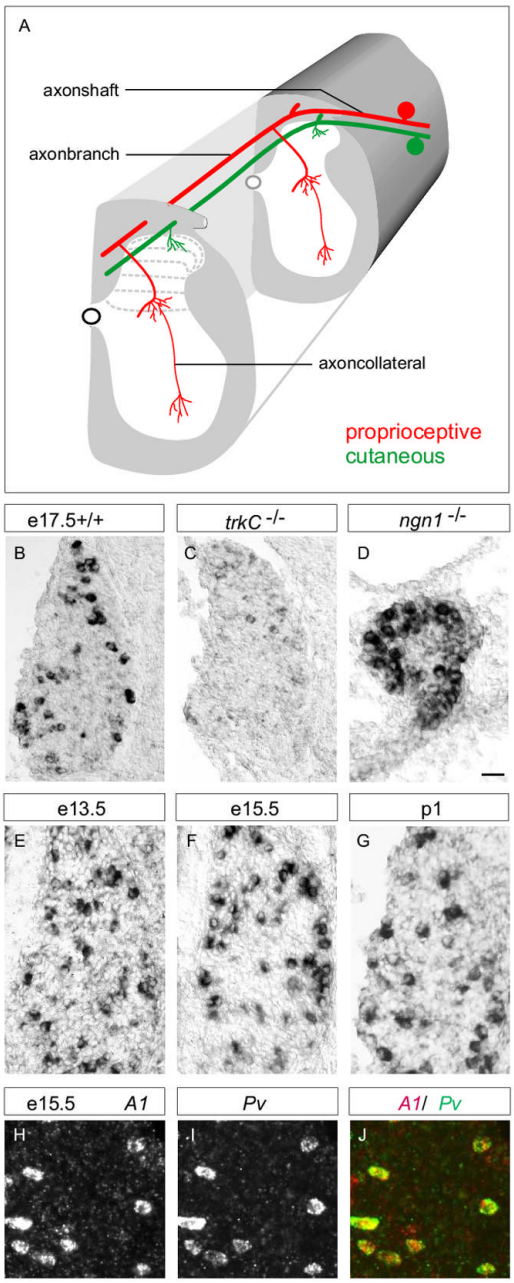

Figure 1.

Expression of *plexinA1* in developing mouse DRG

(A) Axonal projections of cutaneous (green) and proprioceptive (red) DRG neurons. Sensory axons arrive at the dorsal root entry zone, and axon shafts give rise to branches that extend rostrocaudally and to collaterals that project to different target zones in the spinal gray matter.

(B-D) Expression of *plexinA1* in e17.5 DRG from wild-type (B), *trkC*$^{-/-}$(C), and *ngn1*$^{-/-}$(D) mouse embryos.

(E-G) Developmental patterns of expression of *plexinA1* in mouse DRG.

(H-J) Localization of *plexinA1* (H) and *Pv* (I) in e15.5 mouse DRG.

Scale bar for B-D = 50μm.

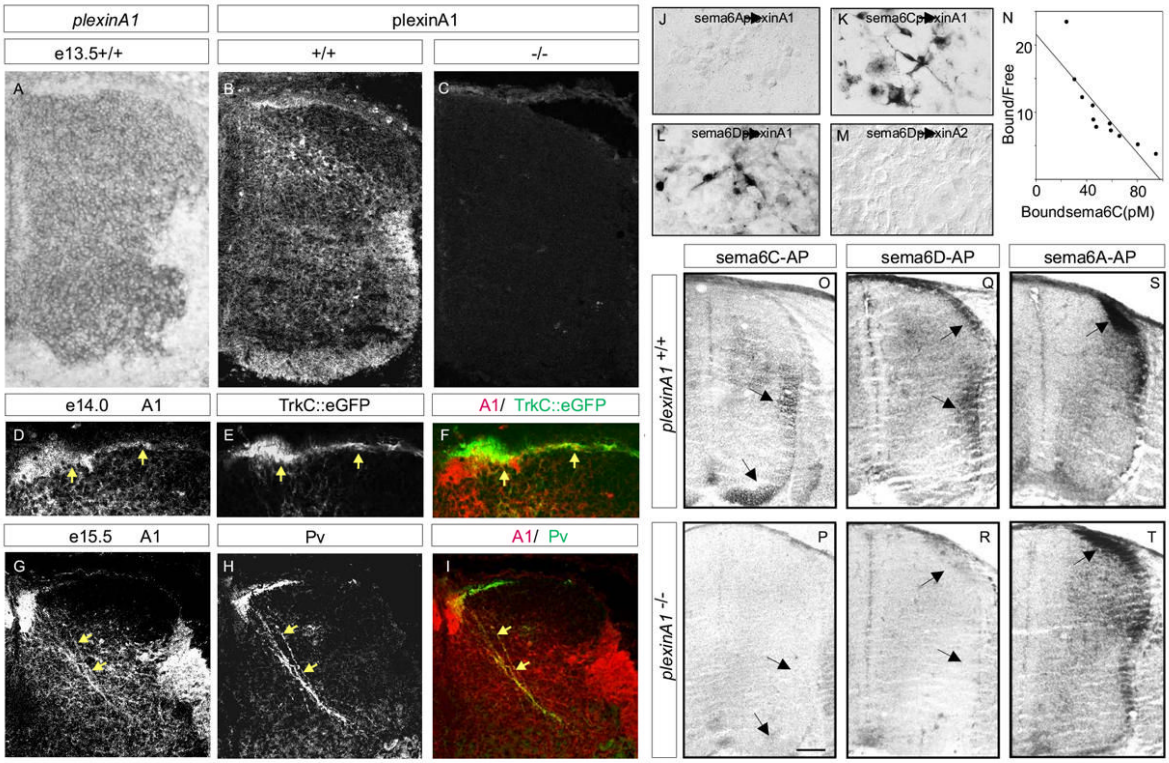

Figure 2.

Sema6C and sema6D binding to plexinA1

(A) Expression of *plexinA1* mRNA in e13.5 mouse cervical spinal cord.

(B, C) Expression of plexinA1 protein in e13.5 wild-type (B) and *plexinA1* $^{-/-}$ (C) cervical spinal cord.

(D-I) Expression of plexinA1 (D, G), eGFP (E), and Pv (H) protein in e14.0 (D-F) and e15.5 (G-I) spinal cord in *TrkC::eGFP* (D-F) and wild-type (G-I) mice. PlexinA1 is expressed in a ventral region of the dorsal columns (G-I) that lacks Pv expression, probably the axons of post-synaptic dorsal column neurons. (J-M) AP-sema6C and AP-sema6D bind to COS-7 cells expressing plexinA1.

(N) AP-sema6C-plexinA1 Scatchard binding analysis: the Kd (± sem) is 4.1 ± 0.2nM.

(O-T) Binding of AP-semas to sections of e13.5 spinal cord. AP-sema6C and AP-sema6D binding to spinal cord sections from e13.5 wild-type (O, Q) and *plexinA1* mutant (P, R) mouse embryos. AP-sema6A binding in spinal cord sections from wild-type (S) and *plexinA1* mutant (T) embryos.

Scale bar for O-T = 100μm.

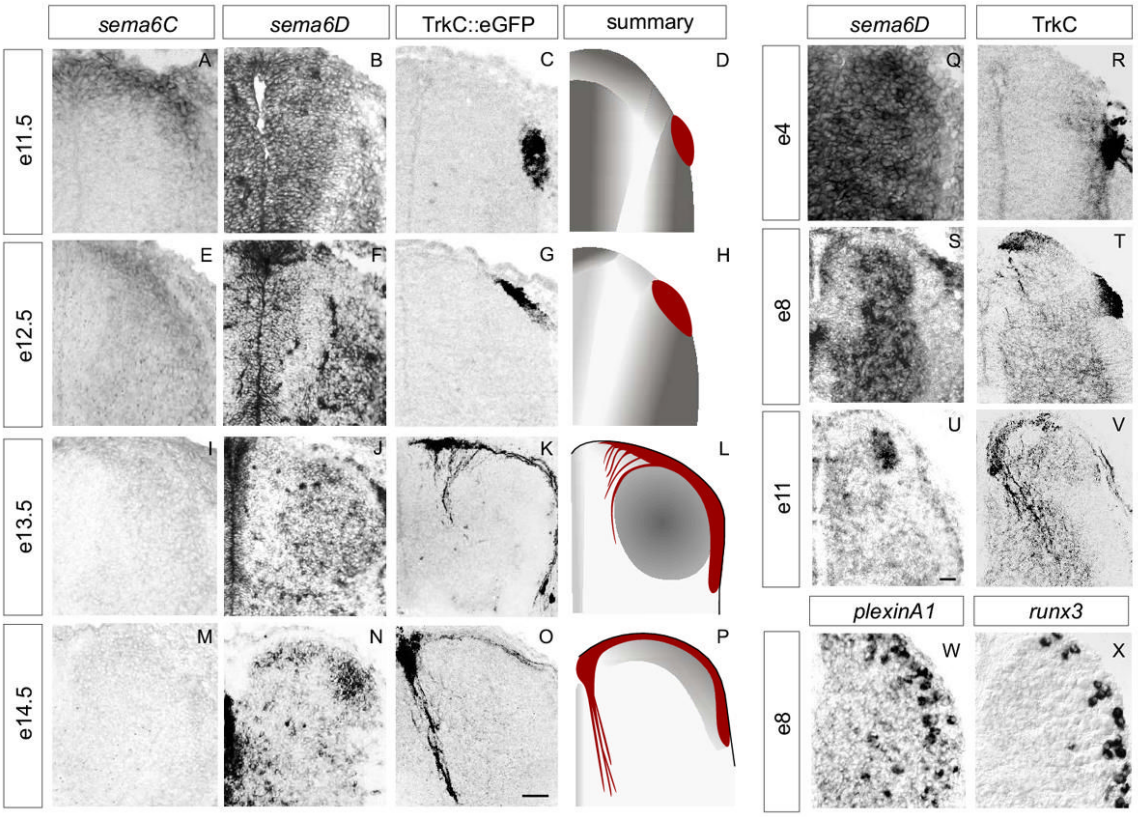

Figure 3.

Expression of *sema6C* and *sema6D* in the developing spinal cord

(A-P) Expression of *sema6C* and *sema6D* in e11.5 (A-B), e12.5 (E-F), e13.5 (I-J), and e14.5 (M-N) mouse spinal cord. (C, G, K, and O) GFP immunoreactivity in *TrkC::eGFP* BAC transgenic mice marks proprioceptive axons. (D, H, L, and P) Summary of *sema6C* and *sema6D* expression, and proprioceptive axon trajectory. Gray regions indicate domains of *sema6C*/*6D* expression. Red region indicates position of proprioceptive axon shafts and collaterals. *Sema6D*, but not *sema6C*, is expressed by DRG neurons (Figure S1).
Scale bar = 50μm.

(Q-V) Expression of *sema6D* and TrkC in e4 (Q-R), e8 (S-T), and e11 (U-V) chick spinal cord.
(W-X) Expression of *plexinA1* and *runx3* in e8 chick DRG.
Scale bar = 50μm.

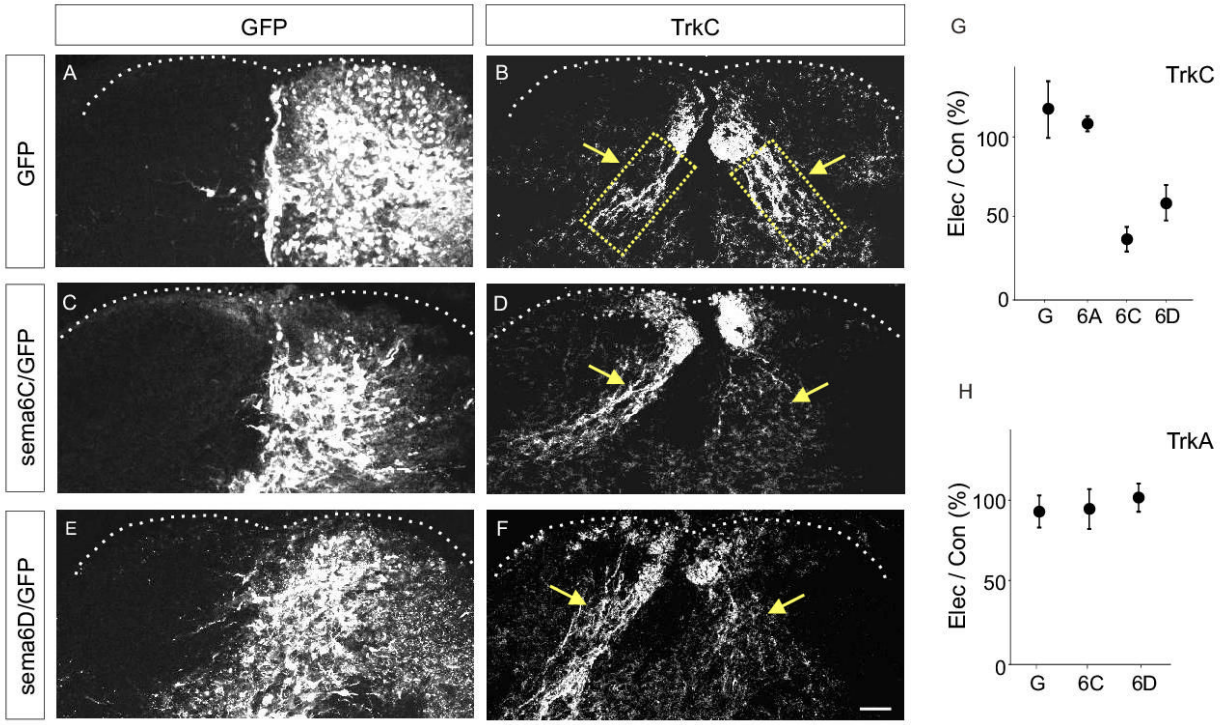

Figure 4.

Sema6C^{ecto} and sema6D^{ecto} suppress proprioceptive axon collateral projections

(A-F) Proprioceptive axon projections, defined by TrkC after expression of GFP, sema6C^{ecto}, and sema6D^{ecto} in chick brachial spinal cord.

(G-H) Quantitation of axonal TrkC (G) and TrkA (H) immunofluorescence in the spinal cord. Plot indicates ratio of axonal TrkC and TrkA immunofluorescence in electroporated (Elect) and control (Con) spinal cord. The region used for quantitation for TrkC and TrkA is shown in yellow boxes in (B) and (Figure S1B).

Results from: TrkC: n= 9 (GFP), 10 (sema6C^{ecto}), and 12 (sema6D^{ecto}) embryos; TrkA: n= 8 (GFP), 8 (sema6C^{ecto}), and 8 (sema6D^{ecto}) embryos.

Scale bar = 50μm.

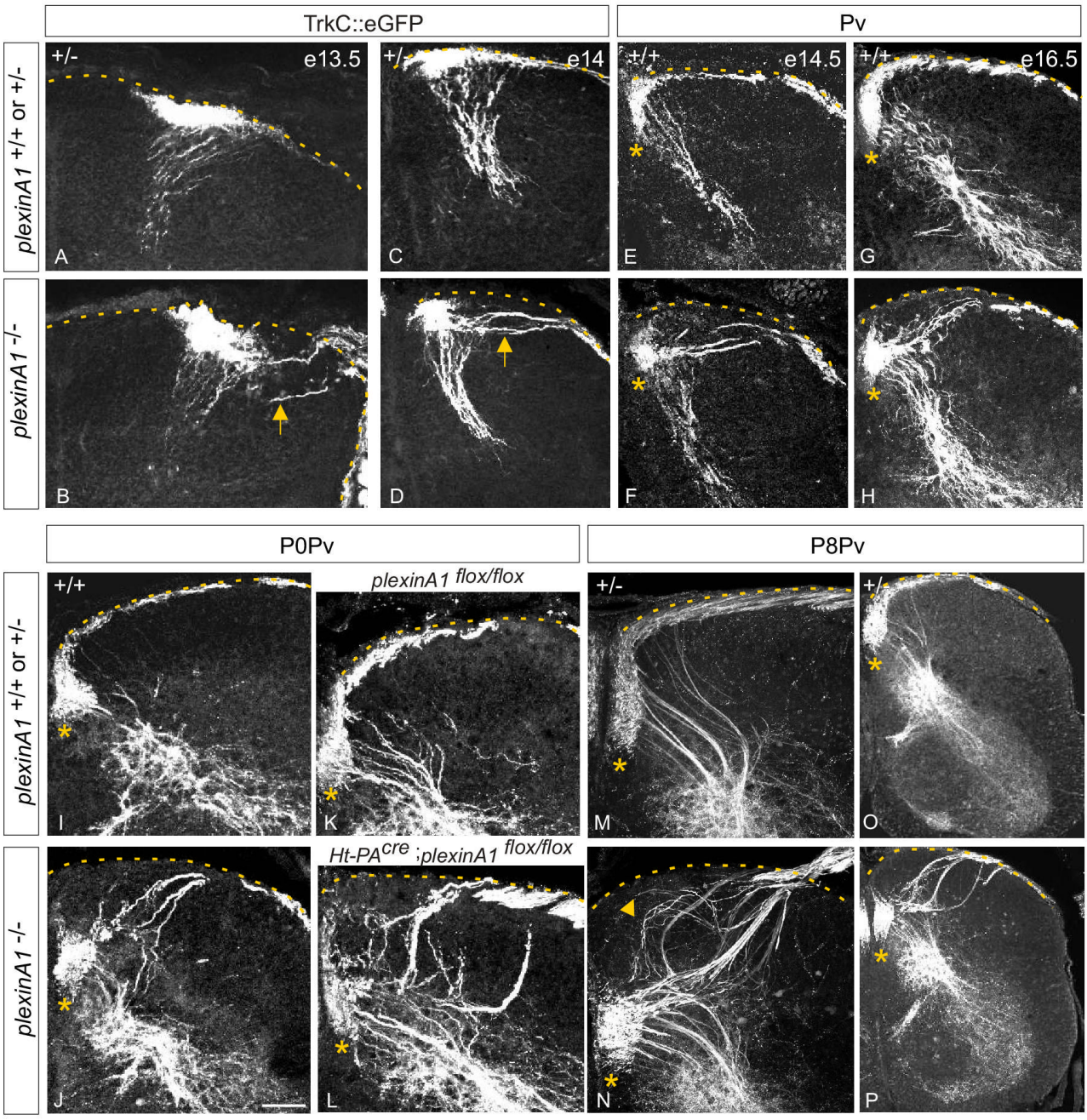

Figure 5.

Proprioceptive axon projections in the spinal cord of *plexinA1* mutant mice

(A-B) Proprioceptive axon shaft positioning revealed by eGFP immunoreactivity in e13.5 *plexinA1*$^{-/-}$; *TrkC::eGFP* mice.

(C-D) Position of proprioceptive axon shafts, determined by eGFP immunoreactivity in e14.0 *plexinA1*$^{+/-}$; *TrkC::eGFP* and *plexinA1*$^{-/-}$; *TrkC::eGFP* mice. A similar phenotype is observed after *sema6D* siRNA expression in chick spinal cord (Figure S7), arguing that the axonal immunoreactivity in *plexinA1* mutants reflects the loss of sema6C/D signaling.

(E-P) Axon shafts of Pv^{+} proprioceptive neurons in e14.5, e16.5, p0, and p8 dorsal lumbar spinal cord. Similar findings were made at cervical spinal cord. (*) indicates the position of the

dorsal funiculus. Arrowhead indicates aberrant dorsal termination of proprioceptive sensory axons in *plexinA1*$^{-/-}$ mice. Number of mice analyzed at each stage: n= 5 (e13.5, +/-), 5 (e.13.5, -/-), 2 (e14.0, +/-), 2 (e14.0, -/-), 3 (e14.5, +/+), 3 (e14.5, -/-), 2 (e16.5, +/+), 2 (e16.5, -/-), 12 (p0, +/+), 12 (p0, -/-), 15 (p8, +/-), and 17 (p8, -/-).
Scale bar = 50μm.

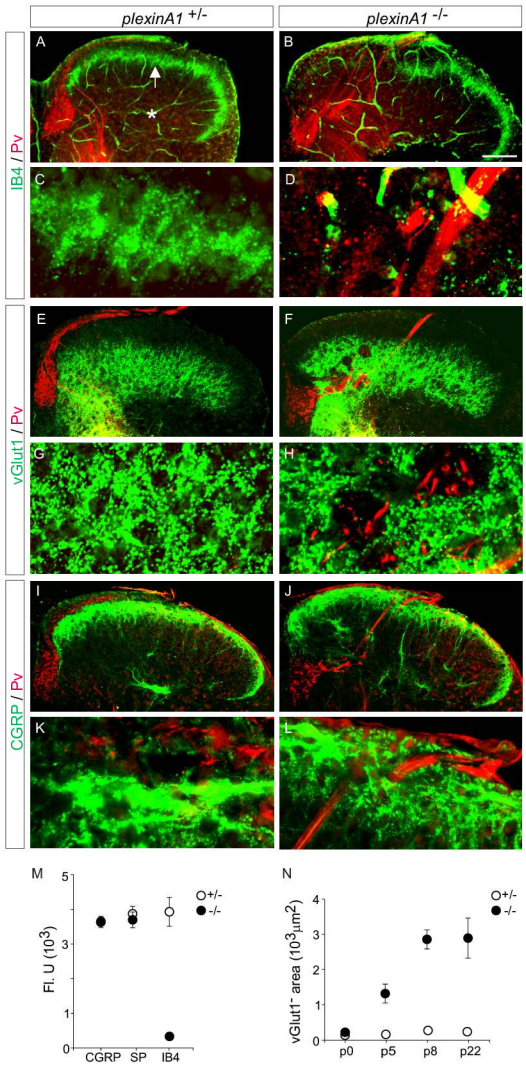

Figure 6.

Organization of cutaneous afferent projections in *plexinA1* mutant mice

(A-D) IB4-binding (green) and Pv expression (red) in p8 *plexinA1*$^{+/-}$(A and C) and *plexinA1*$^{-/-}$(B and D) mice.

(C and D) show higher magnification panels from the medial region of the superficial dorsal horn from A and B. IB4-binding detects both cutaneous sensory axons (allow) and blood vessels (*)

(E-H) Expression of vGlut1 (green) and Pv (red) in p8 dorsal horn in *plexinA1*$^{+/-}$(E and G) and *plexinA1*$^{-/-}$(F and H) mice. (G and H) are higher magnification panels from E and F.

(I-L) Expression of CGRP (green) and Pv (red) in p8 dorsal horn in *plexinA1*$^{+/-}$(I and K) and *plexinA1*$^{-/-}$(J and L) mice. (K and L) are higher magnification panels from I and J.

Images representative of analysis of 10 heterozygous *plexinA1* and mutant mice.

We detected no change in the central projections of TrkA^{+} cutaneous afferents monitored at e14.5 and p1 (see Figures S4E-H).

Scale bar = 100μm.

(M) Quantitation of $CGRP^+$, SP^+ axons, and IB4-binding in a 100μm × 50μm region of the medial half of the superficial dorsal horn that contains displaced Pv^+ proprioceptive axonal shafts, at p8 (Fl. U indicates normalized fluorescence intensitity; see Experimental Procedures). (N) Quantitation of the area of vGlut1-deficient regions in the vicinity of Pv^+ proprioceptive axons at p0, p5, p8, and p22.

The number of $IB4^+$ sensory neurons in p8 lumbar (L1-L3) DRG was similar in *plexinA1*$^{+/-}$(22.7 ± 1.9/15μm section, n=35 sections from 5 mice) and *plexinA1*$^{-/-}$mice (24.6 ± 1.9/15μm section, n=35 sections from 5 mice).

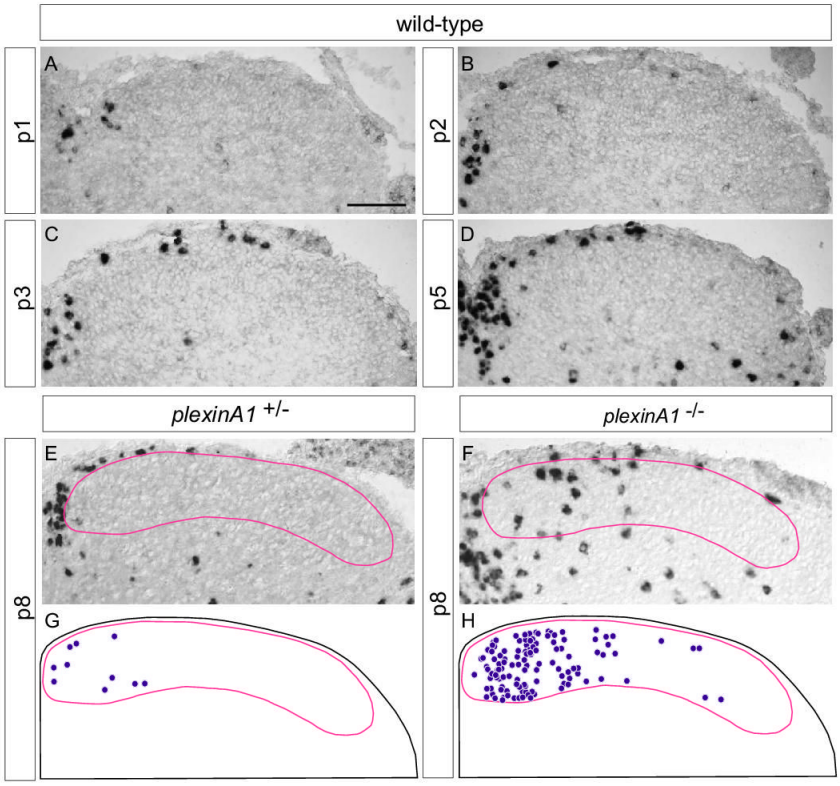

Figure 7.

Oligodendrocytes invade the superficial dorsal horn in *plexinA1* mutantmice

(A-D) Expression of *MAG* in the dorsal spinal cord of wild-type mice from p1 to p5.

(E and F) Expression of *MAG* in the dorsal spinal cord of p8 *plexinA1*$^{+/-}$(E) and *plexinA1*$^{-/-}$(F) mice.

(G and H) Quantitation of *MAG*$^{+}$ cells in superficial dorsal horn (red contour) in p8 *plexinA1*$^{+/-}$(G) and *plexinA1*$^{-/-}$(H) mice. Images representative of analysis of 26 sections of 6 *plexinA1* heterozygote and 6 mutant mice (G and H).

Scale bar = 100μm.

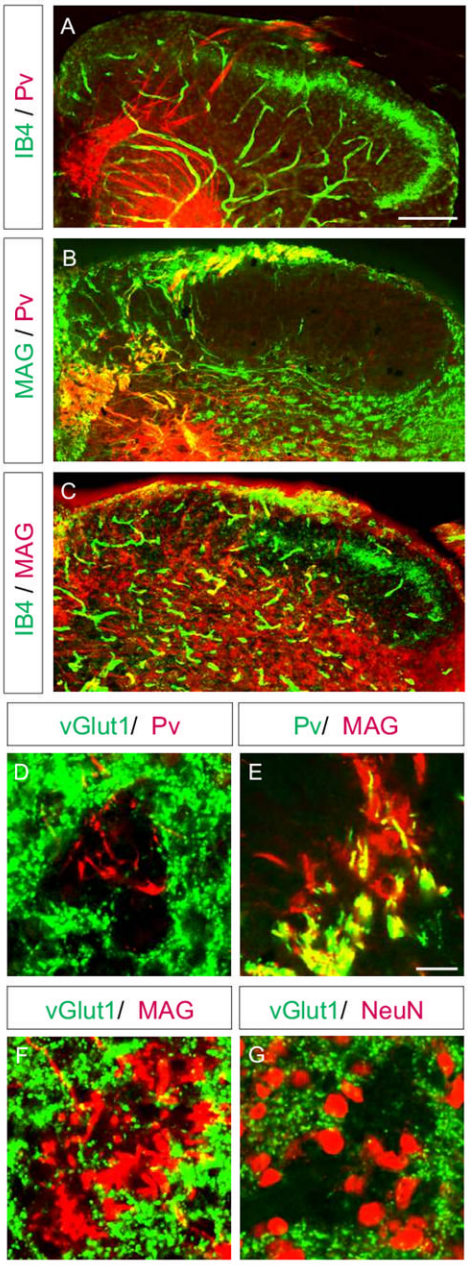

Figure 8.

Foci of ectopic oligodendrocytes coincide with regions of cutaneous afferent disorganization in the dorsal horn of *plexinA1* mutant mice

Images in (A-G) show spatial relationship between markings of cutaneous and proprioceptive axons and oligodendrocytes in p8 *plexinA1* mutant mice, Panel G shows $NeuN^{+}$ neuronal nuclei.

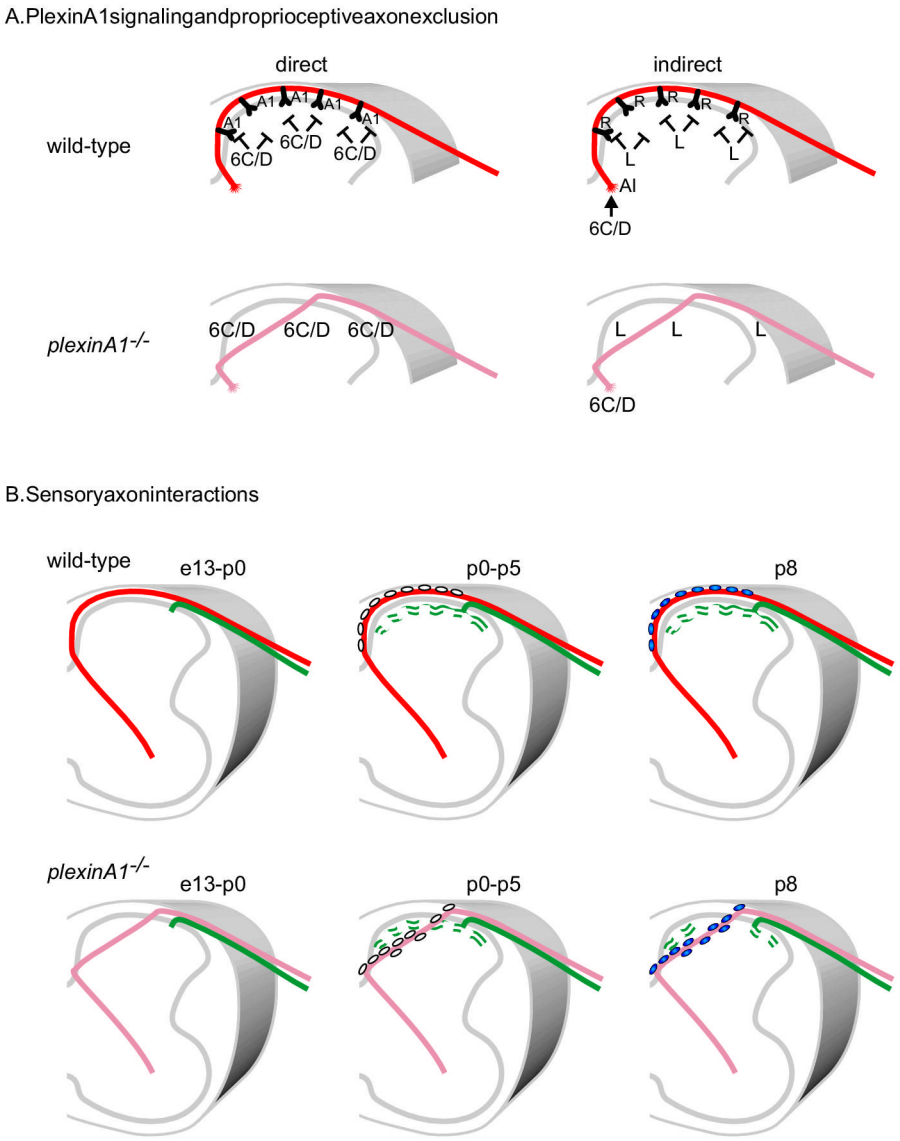

Figure 9.

PlexinA1 signaling and the coordination of sensory afferent projection pattern in the developing spinal cord

(A) Two possible ways in which plexinA1 signaling might influence the position of proprioceptive axon shafts (red) in the dorsal spinal cord. In a direct pathway, sema6C/6D signals provided by dorsal spinal cord cells act on the shafts of proprioceptive axons to confine them to the superficial rim of the spinal cord. The loss of plexinA1 renders proprioceptive axons insensitive to the actions of sema6C/6D, and axon shafts invade the dorsal horn. In an indirect pathway, sema6C/6D signaling acts via plexinA1 on the growth cones of proprioceptive axons to induce expression of a distinct axonal receptor [R], which responds to a ligand [L] expressed by dorsal spinal cord cells to drive axonal shaft exclusion. Both models argue for active control of axonal shaft position.

(B) Oligodendrocytes as probable intermediaries in the interaction between proprioceptive and cutaneous sensory axons. In wild-type embryos, proprioceptive sensory axons (red) are excluded from the dorsal horn from e13 onwards, by virtue of expression of plexinA1. At post-

natal stages oligodendrocytes (ovals) present in the superficial rim of the dorsal spinal cord associate with proprioceptive axons and are directed along axonal shafts away from the superficial dorsal horn. The exclusion of oligodendrocytes permits cutaneous sensory axons (green) to form afferent projections within the superficial dorsal horn. In the absence of plexinA1 signaling, maturing oligodendrocytes track along displaced proprioceptive axonal shafts into the superficial dorsal horn. The presence of oligodendrocytes prevents the stabilization of cutaneous sensory axon terminal arbors. For further details, see Discussion.