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# Ectopic myelinating oligodendrocytes in the dorsal spinal cord as a consequence of altered semaphorin 6D signaling inhibit synapse formation

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## SUMMARY

Different types of sensory neurons in the dorsal root ganglia project axons to the spinal cord to convey peripheral information to the central nervous system. Whereas most proprioceptive axons enter the spinal cord medially, cutaneous axons typically do so laterally. Because heavily myelinated proprioceptive axons project to the ventral spinal cord, proprioceptive axons and their associated oligodendrocytes avoid the superficial dorsal horn. However, it remains unclear whether their exclusion from the superficial dorsal horn is an important aspect of neural circuitry. Here we show that a mouse null mutation of *Sema6d* results in ectopic placement of the shafts of proprioceptive axons and their associated oligodendrocytes in the superficial dorsal horn, disrupting its synaptic organization. Anatomical and electrophysiological analyses show that proper axon positioning does not seem to be required for sensory afferent connectivity with motor neurons. Furthermore, ablation of oligodendrocytes from *Sema6d* mutants reveals that ectopic oligodendrocytes, but not proprioceptive axons, inhibit synapse formation in *Sema6d* mutants. Our findings provide new insights into the relationship between oligodendrocytes and synapse formation in vivo, which might be an important element in controlling the development of neural wiring in the central nervous system.

**KEY WORDS: Semaphorin, Plexin, Spinal cord, Synapse formation, Oligodendrocyte, Mouse**

## INTRODUCTION

Glial cells, such as astrocytes, oligodendrocytes and Schwann cells, play important roles in neural function and in the development of neuronal circuitry in the mammalian nervous system. In addition to their passive and supportive roles, glial cells also guide synapse formation, influence synaptic function and mediate synaptic elimination (Eroglu and Barres, 2010). Substantial progress has been made toward elucidating the roles played by astrocytes in neural circuitry (Eroglu and Barres, 2010). Astrocytes secrete proteins, including thrombospondins, that induce neurons to form synapses (Christopherson et al., 2005), and through the action of the C1q protein, astrocytes also help to control synapse elimination (Stevens et al., 2007; Eroglu and Barres, 2010). In addition to astrocytes, Schwann cells also secrete synaptogenic factors, including transforming growth factor β (Feng and Ko, 2008).

Little is known about the roles of oligodendrocytes in synapse formation in the central nervous system (CNS), although many in vitro analyses have shown that oligodendrocytes contain inhibitors of axonal growth (Yiu and He, 2006). The activity of these axon growth inhibitors might partially explain why axons do not regenerate in the mammalian CNS (Yiu and He, 2006). Since oligodendrocytes inhibit axonal growth in vitro, the temporal and spatial control of oligodendrocyte positioning might also influence the proper development of axonal projections in vivo.

Different classes of sensory neurons in the dorsal root ganglia (DRG) convey peripheral stimuli to the CNS. DRG neurons can be grouped into two major classes of neurons: proprioceptive sensory neurons, which control the state of muscle contraction and limb position, and cutaneous sensory neurons, which mediate a wide range of noxious and innocuous stimuli (Brown, 1981; Koerber and Mendell, 1992). Proprioceptive sensory neurons are further subdivided mainly into group Ia and group Ib neurons (Brown, 1981). Group Ia sensory neurons project axons into ventral spinal cord and make direct monosynaptic connections with motor neurons, whereas group Ib sensory neurons make indirect connections with motor neurons through interneurons (Brown, 1981). Cutaneous sensory neurons can be subdivided into classes of neurons that project axons to different laminae within the dorsal horn. These neuron subtypes are marked by different molecular markers. For example, thinly myelinated cutaneous axons positive for calcitonin gene-related peptide (CGRP; Calca – Mouse Genome Informatics) terminate in lamina I and outer lamina II of the dorsal horn in the spinal cord (Lawson, 2002) (Fig. 1A), whereas non-myelinated cutaneous axons positive for isolectin IB4, a marker of some primary afferent C fibers, terminate in lamina II (Molliver et al., 1997; Fang et al., 2006) (Fig. 1A). Furthermore, vesicular glutamate transporter 1 (vGlut1; Slc17a7 – Mouse Genome Informatics) marks myelinated cutaneous afferents that terminate in laminae III-V and also marks heavily myelinated proprioceptive sensory axons that terminate in laminae V-VII and IX (Todd et al., 2003) (Fig. 1A). Since heavily myelinated proprioceptive axons enter the spinal cord medially and project to their ventral targets (Brown, 1981; Koerber and Mendell,

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1992), proprioceptive axons and the associated oligodendrocytes avoid the superficial dorsal horn. However, it remains unclear whether their exclusion from the superficial dorsal horn is important for neural wiring.

To determine whether the exclusion of proprioceptive axons and their associated oligodendrocytes from the dorsal spinal cord has a role in neural circuit formation, we examined plexin A1 (*Plxna1*) mutant mice, which ectopically express both oligodendrocytes and proprioceptive axons in the dorsal horn (Yoshida et al., 2006). Plexin A1 is a receptor for semaphorins, which have various roles in neural development, including axon guidance (Yoshida et al., 2006; Tran et al., 2007). In *Plxna1* mutants, the ectopic positioning of oligodendrocytes and proprioceptive axons in the dorsal spinal cord disrupts the synaptic terminals of cutaneous afferents (Yoshida et al., 2006). Since both proprioceptive axons and oligodendrocytes are aberrantly expressed in *Plxna1* mutants (Yoshida et al., 2006), it remains unclear which cell type is causing the disruption in cutaneous axon terminals.

In this study we analyzed mutants of the plexin A1 ligands Sema6C and Sema6D. We found defects in proprioceptive axon and oligodendrocyte positioning in *Sema6d* mutants that were similar to those observed in *Plxna1* mutant mice. Deletion of oligodendrocytes using diphtheria toxin rescued the defects in synaptic disorganization in the superficial dorsal spinal cord of *Sema6d* mutants, which suggests that oligodendrocytes, but not proprioceptive axons, are involved in the disruption of synaptic organization. Interestingly, ectopic oligodendrocytes are likely to inhibit only synapse formation and not axonal projections in *Sema6d* mutants. Collectively, these data provide new insights into the roles of oligodendrocytes in neural circuit development in the CNS.

## MATERIALS AND METHODS

### Mice

The following mouse strains were used in this study: *Sema6c*, *Sema6d* (Takamatsu et al., 2010), *Plxna1* (Yoshida et al., 2006), *Avil-hPLAP* (Hasegawa et al., 2007), *Cnp-Cre* (Lappe-Siefke et al., 2003) and *lox-stop-lox-DTA* (*Rosa-DTA*) (Wu et al., 2006) mice.

### Tissue preparation

Animals were anesthetized and perfused with ice-cold 4% paraformaldehyde (PFA) in phosphate buffer (PB). Spinal cords, including surrounding tissues, were removed after perfusion and fixed in 4% PFA/PB for 2 hours on ice for immunofluorescence and alkaline phosphatase staining or overnight for in situ hybridization.

### Immunofluorescence and in situ hybridization

Digoxigenin (DIG)-labeled cRNA probes were used for in situ hybridization as described (Schaeren-Wiemers and Gerfin-Moser, 1993). In situ hybridization was performed on 16-20 μm cryosections according to standard protocols. For immunofluorescence, 200 μm vibratome sections were stained with the following antibodies: rabbit and goat anti-Pv (Swant), guinea pig anti-vGlut1 and vGlut2 (Chemicon), rabbit anti-CGRP (Peninsula Laboratories), mouse anti-NeuN (Chemicon), rabbit and chicken anti-Mbp (Chemicon, Aves Labs), mouse anti-PSD95 (Thermo Scientific), rabbit anti-Shank1 (Chemicon), mouse anti-βIII-tubulin (Tuj1, Convance) and mouse anti-SV2 (Developmental Studies Hybridoma Bank). Mouse anti-Mag antibody was kindly provided by M. Filbin (Mukhopadhyay et al., 1994). FITC-, Cy3- and Cy5-labeled secondary antibodies were purchased from Jackson ImmunoResearch. Images were scanned under an LSM510 confocal microscope (Zeiss).

### Electron microscopy

Animals were anesthetized and perfused with ice-cold 4% PFA and 2.5% glutaraldehyde in PB. Spinal cords were removed and fixed in the same fixative overnight. Specimens were dehydrated and embedded in EMbed 812 (Ladd Research Industries). Sections were stained in uranyl acetate and lead citrate and viewed on a Hitachi H-7600 transmission electron microscope.

### Alkaline phosphatase staining

Tissue sections (20 μm) were collected on a cryostat and postfixed with 4% PFA/PB at room temperature for 1 hour, followed by incubation at 65°C for 4 hours in PBS. NBT/BCIP (Vector Laboratories) was used to detect alkaline phosphatase activity.

### Labeling DRG neurons with Dextran

Spinal cords were isolated and labeled with 20% fluorescein-Dextran (f-Dex) (3000 MW, Invitrogen) using tight-fitting glass capillaries at postnatal day (P) 7. Spinal cords were incubated overnight and then fixed with 4% PFA/PB for 2 hours.

### Quantification of vGlut1-positive synaptic terminals and *Mag*-positive oligodendrocytes

Vibratome sections (200 μm) were stained with vGlut1 antibody and viewed on an LSM510 confocal microscope (Zeiss). The number of vGlut1-positive synaptic terminals (in the black boxes, Fig. 3F,L) was quantified using the Imaris Spot tool (Bitplane) ($n$=3 animals for each genotype, two to eight images were analyzed for each animal). To quantify the number of *Mag*-positive oligodendrocytes, in situ hybridizations on 16 μm cryostat sections were analyzed using the Imaris Spot tool ($n$=3 animals of each genotype, eight sections per animal). Probabilities were determined by Student's $t$-test.

### Electrophysiology

We used the following methods as modified in previous studies (Mears et al., 2007; Takazawa et al., 2009).

#### Dissection of spinal cords

P5-8 mice were anesthetized on ice, perfused with cold artificial cerebral spinal fluid (ACSF), decapitated and transferred to a chamber with a cold circulating oxygenated (95% $O_2$, 5% $CO_2$) ACSF. ACSF comprised 252 mM sucrose, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1.25 mM $NaH_2PO_4$, 26 mM $NaHCO_3$, 10 mM glucose and 5 mM kynurenic acid, pH 7.4. The spinal cords were positioned in the recording chamber and perfused with oxygenated Krebs buffer comprising 117 mM NaCl, 3.6 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $NaH_2PO_4$, 1.2 mM $MgCl_2$, 11 mM glucose and 25 mM $NaHCO_3$, pH 7.4.

#### Intracellular recordings

Motoneurons were impaled with a glass micropipette and filled with 2 M potassium acetate, 0.5% Fast Green and 0.2 M lidocaine N-ethyl bromide (Sigma). Signals were acquired with an amplifier (Multiclamp 700B, Molecular Devices), an analog-to-digital converter (Digidata 1440A, Molecular Devices) and a data acquisition program (Clampex version 10, Molecular Devices) and analyzed with Clampfit software (version 10, Molecular Devices). Motoneurons were identified by antidromic responses from sciatic nerve stimulation. Only motoneurons with a resting potential below –55 mV were used for analysis. Sciatic nerves were stimulated with square pulses of 0.1 mseconds at 1.5 times the strength that evoked a maximal monosynaptic response at 1 Hz using a stimulus isolator unit (S88X dual output square pulse stimulator, SIU-C constant current stimulus isolation unit, Grass Technologies). For each motoneuron analyzed, 20 sequential traces were recorded for averaging off-line.

## RESULTS

### Defects in proprioceptive axon positioning in *Sema6d* but not *Sema6c* mutant mice

Both Sema6C and Sema6D bind to plexin A1 and are expressed in the dorsal spinal cord (Toyofuku et al., 2004; Yoshida et al., 2006). To determine whether these semaphorins are functional ligands for plexin A1 in the spinal cord, we examined axonal projections of proprioceptive sensory neurons in the DRG using the proprioceptive marker parvalbumin (Pv), a marker of all

proprioceptive sensory neurons (Honda, 1995; Arber et al., 2000), in *Sema6c* and *Sema6d* single mutants, and in *Sema6c*; *Sema6d* double mutants (Takamatsu et al., 2010). Although there were no obvious defects in proprioceptive axonal projections in *Sema6c* mutants compared with control mice at postnatal day 0 (P0) (Fig. 1B,C), we observed defects in Pv-positive proprioceptive axon positioning in *Sema6d* mutants (Fig. 1D) that were similar to those observed in *Plxna1* mutants (Fig. 1E) (Yoshida et al., 2006). *Sema6c*; *Sema6d* double mutants were equally defective in proprioceptive axon positioning as *Sema6d* single mutants (Fig. 1D; see Fig. S1 in the supplementary material). Similar to *Plxna1* mutants (Yoshida et al., 2006), we did not find any obvious defects in TrkA-positive cutaneous axonal projections in any of the semaphorin mutants at P0 (data not shown; TrkA is also known as Ntrk1 – Mouse Genome Informatics). Thus, given the similarity in axonal phenotypes between *Plxna1* and *Sema6d* mutants, Sema6D appears to be a functional ligand for plexin A1 in the dorsal spinal cord.

We then turned to the issue of whether proprioceptive axons in *Sema6d* mutants can make synapses with motor neurons in the ventral spinal cord. Our anatomical analysis involved the examination of vGlut1 expression, where it is only expressed by proprioceptive sensory axons in the ventral spinal cord (Todd et al., 2003). We used vGlut1 as a marker to detect proprioceptive synapses on motoneurons in the ventral spinal cord for the following reasons. First, when *Er81* (*Etv1* – Mouse Genome Informatics), an ETS transcription factor expressed by proprioceptive sensory neurons, is knocked out, the resulting mutants have no ventral axonal projections of proprioceptive

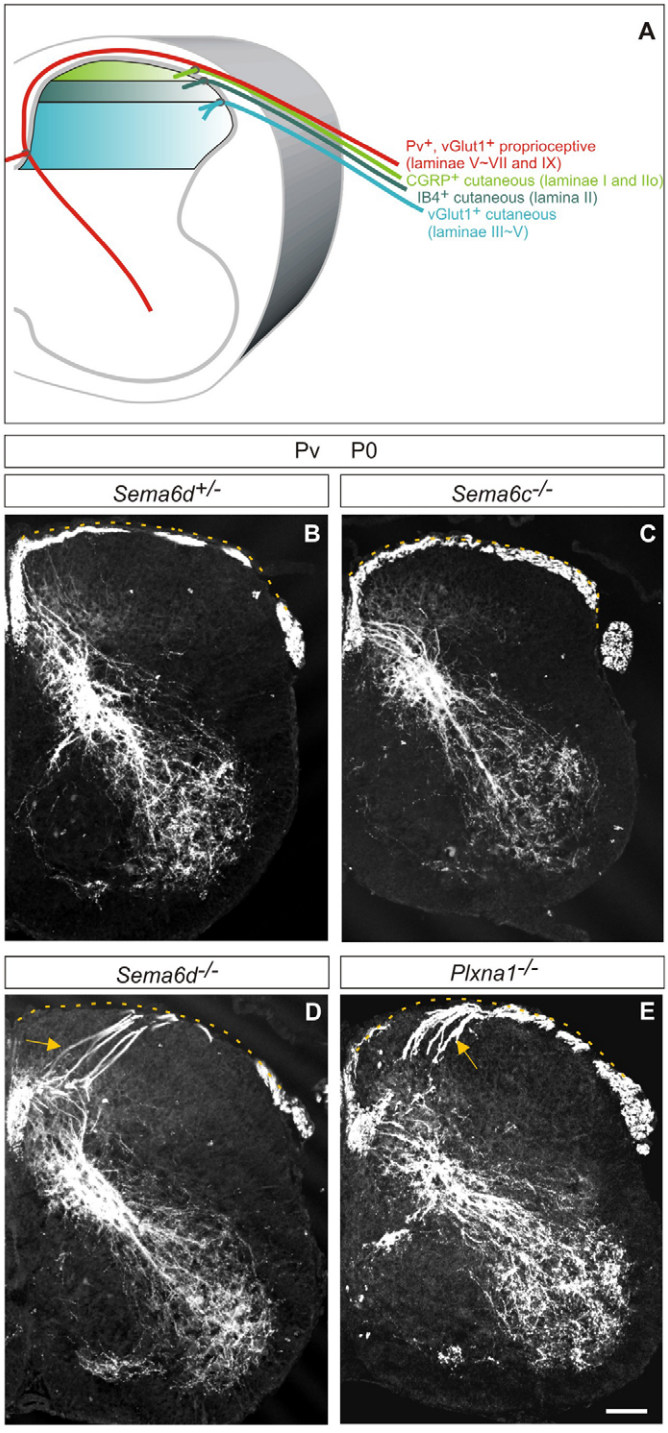


**Fig. 1. Proprioceptive axonal projections in the spinal cord of *Sema6c*$^{-/-}$, *Sema6d*$^{-/-}$ and *Plxna1*$^{-/-}$ mice.** (**A**) Illustration of the proprioceptive and cutaneous axons in the mouse spinal cord. (**B-E**) Positions of axonal shafts of proprioceptive sensory neurons determined by Pv expression in P0 spinal cords. (D,E) Defects in proprioceptive axonal shaft positioning (arrows) were detected in *Sema6d*$^{-/-}$ and *Plxna1*$^{-/-}$ mice. Scale bar: 50 μm.

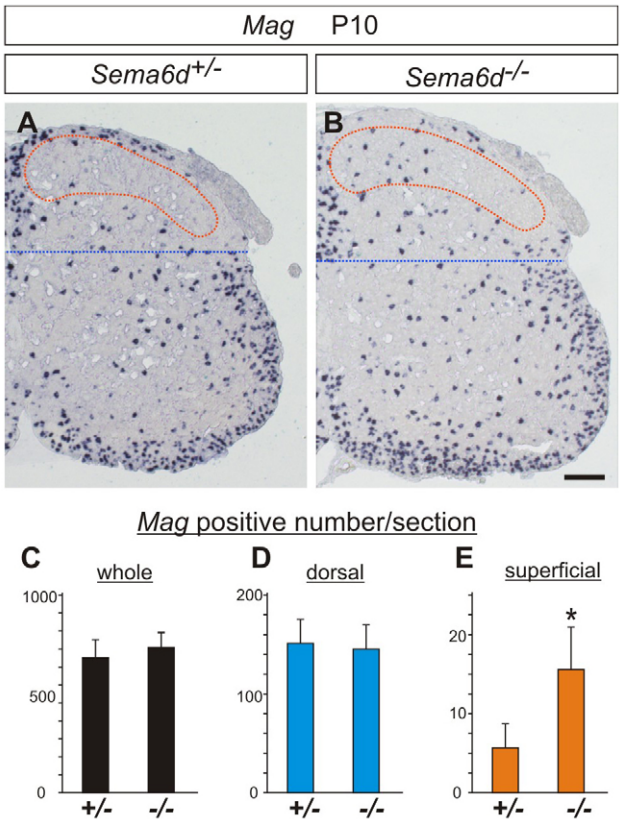


**Fig. 2. Oligodendrocytes invade the superficial dorsal horn of *Sema6d*$^{-/-}$ mice.** (**A**,**B**) Expression of *Mag* in the spinal cord of *Sema6d*$^{+/-}$ (A) and *Sema6d*$^{-/-}$ (B) mice at P10. The superficial dorsal horn is outlined in red; the blue dotted line indicates the border between the dorsal horn and the rest of the spinal cord. (**C-E**) Number of *Mag*-positive oligodendrocytes in the whole spinal cord (C), dorsal half (D) and superficial dorsal horn except dorsal funiculus (E) of *Sema6d*$^{+/-}$ and *Sema6d*$^{-/-}$ mice. *, $P<0.05$. Error bars indicate s.d. Scale bar: 100 μm.

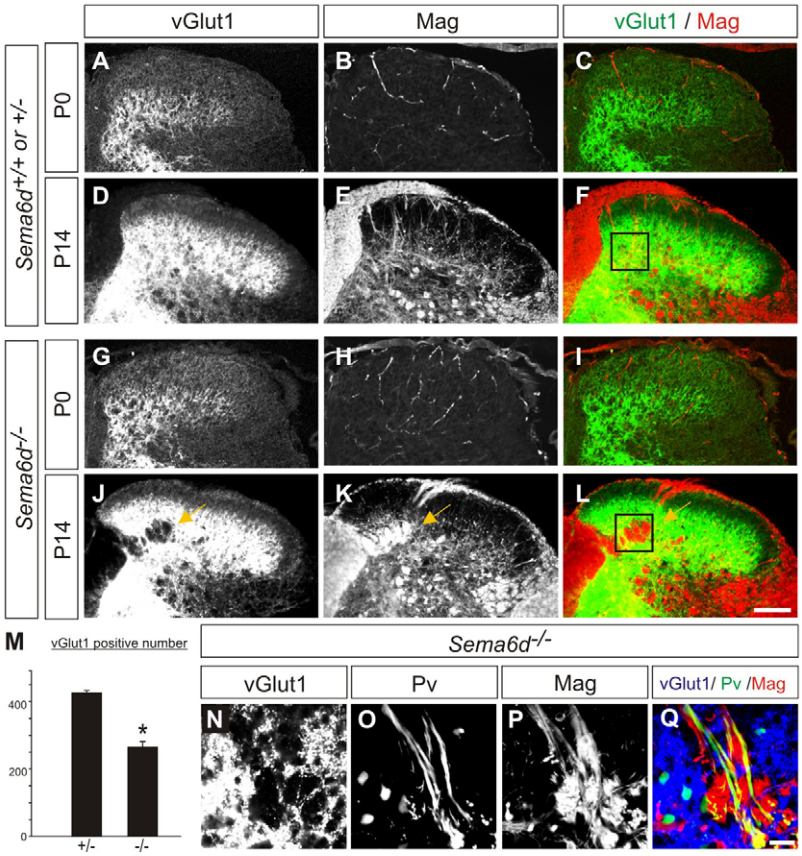


**Fig. 3. Organization of vGlut1-positive synaptic terminals in *Sema6d*$^{-/-}$ mice.** (**A-L**) vGlut1 (green) and Mag (red) expression in P0 and P14 *Sema6d*$^{+/+}$ or *Sema6d*$^{+/-}$ (A-F) and *Sema6d*$^{-/-}$ (G-L) mice. Arrows indicate vGlut1-negative areas (J-L). (**M**) Quantification of vGlut1-positive synaptic terminals in the boxed regions (100 μm × 100 μm) in F and L. *, $P<0.05$. Error bars indicate s.d. (**N-Q**) vGlut1 (blue), Pv (green) and Mag (red) expression in P14 *Sema6d*$^{-/-}$ mice. Ectopic proprioceptive axons with oligodendrocytes cause the disruption of vGlut1-positive synaptic terminals. Scale bars: 100 μm in L; 10 μm in Q.

sensory neurons in the ventral horn (Arber et al., 2000) and do not express vGlut1 in the ventral spinal cord (Hippenmeyer et al., 2005). Second, electron microscopy analysis has shown that vGlut1 labeling is present on putative synapses of proprioceptive sensory neurons and not in non-synaptic regions in the ventral spinal cord (Alvarez et al., 2004). Quantification of vGlut1-positive synaptic terminals in the ventral spinal cord did not show any significant difference between the *Sema6d* heterozygous and homozygous mutants at P14 (see Fig. S2A-E in the supplementary material). Thus, although proprioceptive axons in *Sema6d* mutants showed aberrant axonal trajectories in the dorsal spinal cord, they formed synapses in the ventral spinal cord.

To examine whether sensory-motor synapses are functional in *Sema6d* mutants, intracellular recordings of excitatory postsynaptic potentials (EPSPs) were obtained from motor neurons projecting in the sciatic nerve (see Fig. S2F-H in the supplementary material). The mean latency of the fastest EPSPs in wild-type mice was 3.8±0.4 mseconds ($n$=8 cells from five mice), whereas that in *Sema6d* mutants was 4.0±0.6 mseconds ($n$=7 cells from four mice) (see Fig. S2F,G in the supplementary material). Thus, proprioceptive axons can make monosynaptic synapses with motor neurons in the ventral spinal cord in *Sema6d* mutant mice. Furthermore, the mean amplitude of the fastest EPSPs in wild-type mice was 7.7±3.7 mV ($n$=8 cells from five mice), whereas that in *Sema6d* mutants was 8.1±3.0 mV ($n$=7 cells from four mice) (see Fig. S2F,H in the supplementary material), indicating that there were no obvious differences in the amplitude of monosynaptic EPSPs between wild-type and *Sema6d* mutant mice. Therefore, proprioceptive axons appear to make functional synapses with motor neurons in the ventral spinal cord in *Sema6d* mutant mice.

## Ectopic localization of myelinating oligodendrocytes in the superficial dorsal horn of *Sema6d* mutants

In *Plxna1* mutants, defects in proprioceptive axon positioning are detected before defects in oligodendrocyte positioning are observed (Yoshida et al., 2006). Therefore, aberrant proprioceptive axons appear to alter the localization of myelinating oligodendrocytes in the dorsal spinal cord in *Plxna1* mutants (Yoshida et al., 2006). To determine the temporal and spatial patterns of oligodendrocyte differentiation in the dorsal spinal cords of *Sema6d* heterozygous and homozygous mutant mice, we monitored the expression of two oligodendrocyte markers: myelin associated glycoprotein (*Mag*) and myelin basic protein (*Mbp*) (Mikoshiba et al., 1991) at P0 and P10 by in situ hybridization. At P0, there were a few *Mag*- and *Mbp*-positive oligodendrocytes in the spinal cords of *Sema6d* heterozygous and homozygous mutants (see Fig. S3A-D in the supplementary material). At P10 in the dorsal spinal cord, *Mag*- and *Mbp*-positive oligodendrocytes were present within the dorsolateral funiculus, dorsal columns and deep dorsal horn (laminae III to V) in *Sema6d* heterozygous mice, but very few were located in the superficial dorsal horn laminae I and II (Fig. 2A; see Fig. S3E,G in the supplementary material). By contrast, in *Sema6d* homozygous mutants, we observed a significant number of *Mag*- and *Mbp*-positive oligodendrocytes in the superficial dorsal horn (Fig. 2B; see Fig. S3F,H in the supplementary material).

Then, we quantified the total number of *Mag*-positive oligodendrocytes in the spinal cord at P10. There were no obvious differences in the number of oligodendrocytes in the dorsal horn or in the whole spinal cord between *Sema6d* heterozygous and homozygous mutant mice (Fig. 2C,D). There was also no obvious

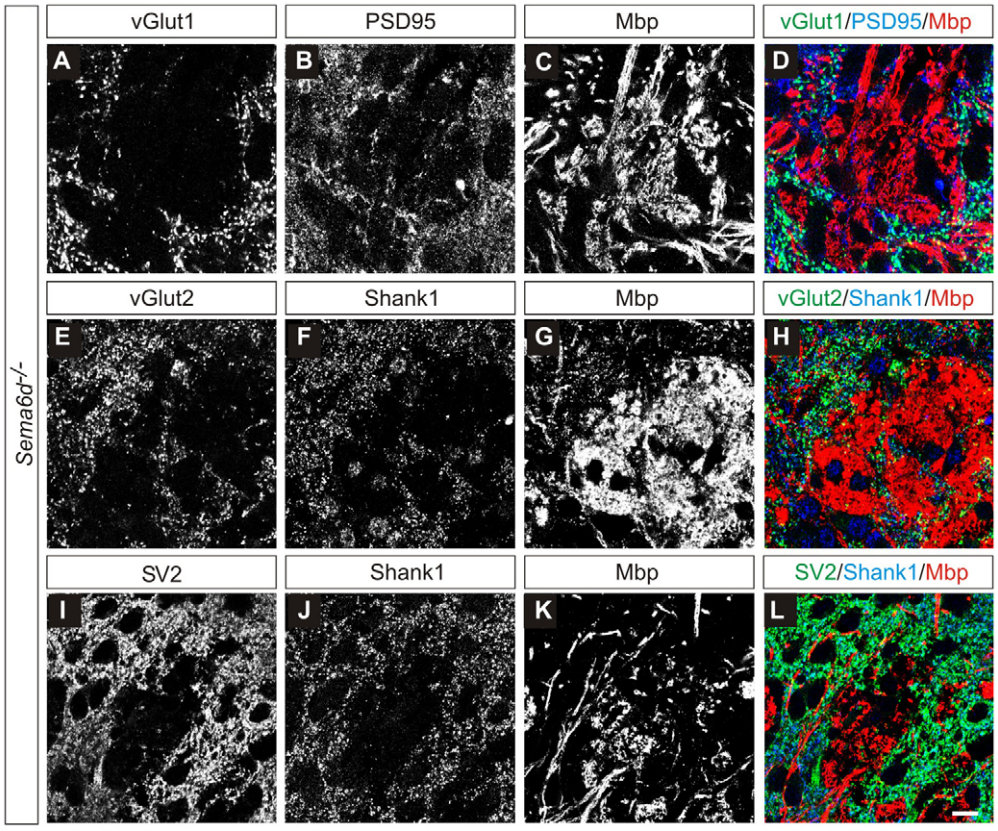


**Fig. 4. Various pre- and postsynaptic marker staining in *Sema6d*$^{-/-}$ mice.** (**A-L**) Immunofluorescence for the indicated markers (vGlut1 and SV2, presynaptic; Shank1 and PSD95, postsynaptic; Mbp, oligodendrocytes), with merges in the right-hand column, in *Sema6d*$^{-/-}$ mice at P14. Scale bar: 10 μm.

change in oligodendrocyte precursors stained by anti-Olig2 antibody between *Sema6d* heterozygous and homozygous mutant mice (see Fig. S3I-L in the supplementary material). Thus, the deficiency in *Sema6d* did not affect the proliferation of either oligodendrocyte precursors or oligodendrocytes. However, there was a significant increase in the number of *Mag*-positive oligodendrocytes located in the superficial laminae (Fig. 2E). These results suggest that aberrant proprioceptive axons in *Sema6d* mutants are likely to alter the localization of myelinating oligodendrocytes in the dorsal spinal cord. In fact, Pv-positive proprioceptive axons were associated with the Mag-positive oligodendrocytes in the dorsal spinal cord of *Sema6d* mutants (Fig. 3N-Q).

## Disruption of synapse formation in the dorsal spinal cord of *Sema6d* mutants

In *Plxna1* mutants (Yoshida et al., 2006), defects in the axonal terminals of different cutaneous sensory neurons are marked by the absence of vGlut1 (Todd et al., 2003) and by the lack of binding of isolectin IB4 (Molliver et al., 1997; Fang et al., 2006). IB4 and vGlut1 mark the termination of cutaneous sensory afferents that arborize, respectively, in laminae II and III-V (Molliver et al., 1997; Todd et al., 2003; Fang et al., 2006). We next addressed whether *Sema6d* mutants show a similar disruption of sensory terminals to *Plxna1* mutants. At P0, the pattern of vGlut1-positive sensory neurons in *Sema6d* mutants was similar to that of wild-type mice (Fig. 3A,G). However, in *Sema6d* mutants analyzed at P8, P14 and P30 we observed a local annulus of exclusion of vGlut1-positive axon terminals in laminae III and IV (Fig. 3D,J; data not shown). Quantification of the number of vGlut1-positive synaptic terminals in the boxed areas in Fig. 3F,L showed a significant decrease in *Sema6d* homozygous as compared with heterozygous mice (Fig. 3M). Similarly, we detected progressive disruptions of IB4-positive sensory terminals in lamina II, which were associated with aberrant proprioceptive axon positioning in *Sema6d* mutants at P14 and P30 (see Fig. S4 in the supplementary material; data not shown).

In *Plxna1* mutants, the ectopic positioning of oligodendrocytes is associated with the disruption of vGlut1- and IB4-positive sensory axon terminals (Yoshida et al., 2006). Consistent with this finding, there was a similar correlation between axon terminal disorganization and ectopic oligodendrocytes in *Sema6d* mutants (Fig. 3). At P0, when there was no obvious disruption of vGlut1-positive axon terminals in *Sema6d* mutants compared with wild-type mice, Mag protein was rarely detected in the spinal cord (Fig. 3A-C,G-I). However, in *Sema6d* mutants analyzed at P8, P14 and P30, the presence of Mag-positive oligodendrocytes correlated with the disruption of vGlut1- and IB4-positive axon terminals (Fig. 3D-F,J-L; see Fig. S4 in the supplementary material; data not shown).

We next examined whether defects in presynaptic terminals in *Sema6d* mutants are detected by other presynaptic markers and whether postsynaptic regions are also defective. For presynaptic markers, in addition to vGlut1 we used an anti-vesicular glutamate transporter 2 (vGlut2; Slc17a6 – Mouse Genome Informatics) antibody, which marks a subset of primary afferents and excitatory interneurons as well as descending fibers within the spinal cord (Todd et al., 2003), and an antibody to synaptic vesicle glycoprotein 2 (SV2), which is a general synaptic marker (Buckley and Kelly, 1985). As shown in Fig. 4, there were defects in both vGlut2- and SV2-positive terminals where ectopic oligodendrocytes were detected in *Sema6d* mutant mice. Furthermore, postsynaptic terminals positive for the postsynaptic

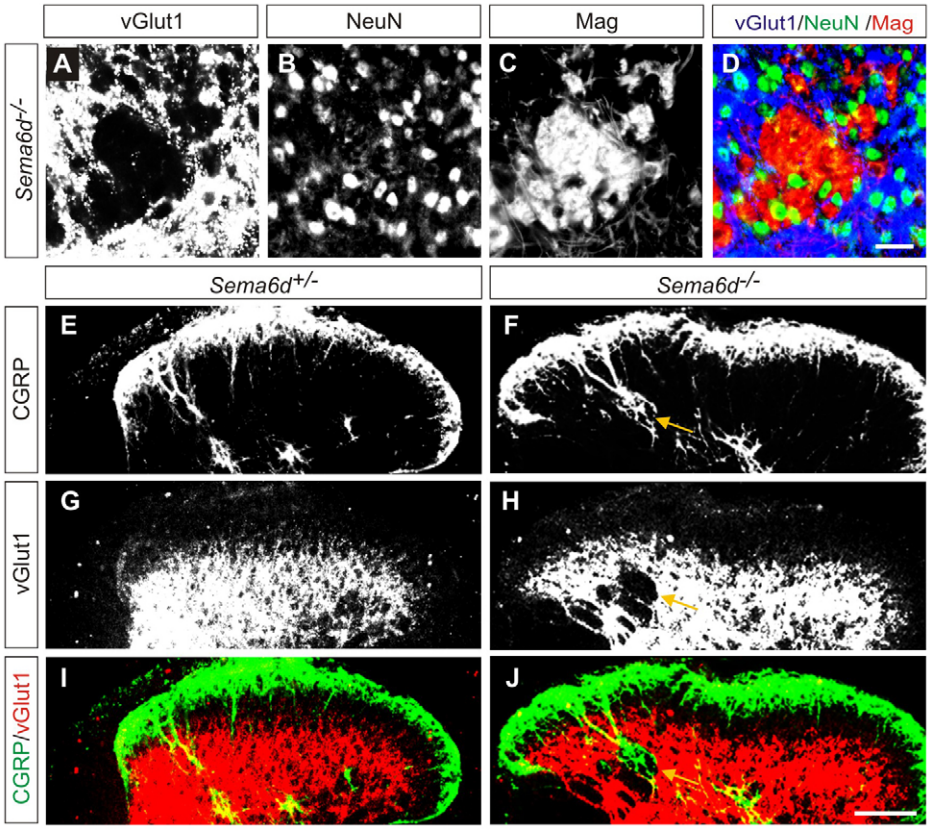


**Fig. 5. NeuN-positive neurons and CGRP-positive axons enter the vGlut1-negative regions in *Sema6d*$^{-/-}$ mice.** (**A-D**) NeuN-positive neurons enter the Mag-positive and vGlut1-negative regions in P14 *Sema6d*$^{-/-}$ mice. (**E-J**) CGRP-positive axons enter vGlut1-negative regions (arrows) in P14 *Sema6d*$^{-/-}$ mice. Scale bars: 50 μm in D; 100 μm in J.

markers Shank1 and PSD95 (Dlg4 – Mouse Genome Informatics) also showed defects in *Sema6d* mutant mice (Fig. 4). Taken together, *Sema6d* mutants showed synaptic disruption, using pre- or postsynaptic markers, for a variety of synaptic types in regions where ectopic oligodendrocytes were located.

### Aberrant proprioceptive axons and oligodendrocytes do not inhibit cutaneous axonal projections but inhibit synapse formation in *Sema6d* mutants

In *Sema6d* mutants, both aberrant proprioceptive axons and oligodendrocytes were detected in the dorsal spinal cord, and these proprioceptive axons and oligodendrocytes are likely to disrupt synapse formation (Figs 3 and 4). Interestingly, some neurons marked by the neuronal marker NeuN (Rbfox3 – Mouse Genome Informatics) were detected in the vGlut1-negative regions in *Sema6d* mutants (Fig. 5A-D). This suggests that ectopic proprioceptive axons and oligodendrocytes do not prevent neurons from entering these regions.

Then we investigated whether ectopic proprioceptive axons and oligodendrocytes inhibit axonal projections or alter synapse formation. If they inhibit cutaneous axonal projections, no axons would be detected in vGlut1-negative regions of *Sema6d* mutants. We examined CGRP-positive axons (Lawson, 2002) because some reached the intermediate region of the spinal cord in addition to lamina I and outer lamina II in both *Sema6d* heterozygous and homozygous mutant mice (Fig. 5E,F). Interestingly, these CGRP-positive axons entered vGlut1-negative areas in *Sema6d* mutants (Fig. 5F,H,J), indicating that at least some axons are unaffected by the presence of ectopic oligodendrocytes and proprioceptive axons.

Next, we used *Avil-hPLAP* mice, in which human placental alkaline phosphatase (hPLAP) is knocked into the *advillin* locus, because advillin is expressed by most, if not all, DRG sensory neurons but by neither spinal cord neurons nor oligodendrocytes (Hasegawa et al., 2007). DRG sensory axons can thus be detected in these mice by hPLAP activity. At P0, hPLAP activity was detected in both proprioceptive and cutaneous axons in *Sema6d* heterozygous and homozygous mutant mice (Fig. 6A,B). At P8 and P14, when disorganization of synaptic terminals in *Sema6d* mutants was detectable, there was no obvious disruption of hPLAP-positive DRG sensory axons in *Sema6d* mutant mice (Fig. 6C-F). Even regions very close to the proprioceptive axons and oligodendrocytes had clear hPLAP-positive sensory axons (Fig. 6D,F). Furthermore, the regions where there were defects in vGlut1-positive sensory terminals did not show any obvious disruption of hPLAP-positive axons in *Sema6d* mutants (see Fig. S5 in the supplementary material).

We also examined sensory axons by injecting fluorescein-Dextran (f-Dex) into the DRG of *Sema6d* mutant mice. f-Dex-positive sensory axons were uniformly distributed in the dorsal spinal cord regardless of the presence of Mbp-positive oligodendrocytes (Fig. 7A-G). Importantly, f-Dex-positive sensory axons appeared to have contacts in βIII-tubulin-positive postsynaptic neurons (Fig. 7H-K). To further examine the synapses in the dorsal spinal cord, we performed electron microscopy (EM) analysis (Fig. 7L,M). EM analysis showed that there were significant numbers of synapses in the dorsal spinal cord of *Sema6d* heterozygous mice (Fig. 7L). By contrast, few synapses were detected in the regions where ectopic myelins were observed in the dorsal spinal cord of *Sema6d* homozygous mutant mice (Fig. 7M). Thus, ectopic myelinating oligodendrocytes are likely to disrupt synapse formation in *Sema6d* mutant mice.

### Oligodendrocytes, but not proprioceptive axons, disrupt synapse formation in *Sema6d* mutants

Our findings suggest that either ectopic proprioceptive axons or oligodendrocytes in the dorsal spinal cord inhibit synapse formation in *Sema6d* mutants. To distinguish between these two possibilities, we investigated whether oligodendrocytes are required for the disruption of vGlut1-positive cutaneous sensory axon terminals in *Sema6d* mutant mice. Oligodendrocytes were genetically deleted in *Sema6d* mutant mice using oligodendrocyte-specific *Cnp-Cre* (Lappe-Siefke et al., 2003) together with *lox-stop-lox-fragment A of diphtheria toxin* (*DTA*) mice (Wu et al., 2006). In contrast to P10 wild-type or *Sema6d* mutant mice, in which strong Mag protein expression was observed in the spinal cords (Fig. 8A,B), Mag expression was very weak in the spinal cords of *Sema6d*$^{-/-}$; *Cnp-Cre*; *lox-stop-lox-DTA* mice (Fig. 8C). In P10 spinal cords of *Sema6d* mutants, there were disruptions in vGlut1-positive cutaneous axon terminals (Fig. 8H-K) compared with wild-type mice (Fig. 8D-G). Pv-positive proprioceptive axons as well as oligodendrocytes occupied the vGlut1-negative regions in *Sema6d* mutants (Fig. 8H-K). By contrast, we did not detect any disruptions of vGlut1-positive cutaneous axon terminals in *Sema6d*$^{-/-}$; *Cnp-Cre*; *lox-stop-lox-DTA* mice (Fig. 8L-O), although many Pv-positive proprioceptive axons were ectopically located in the dorsal spinal cord (Fig. 8M,O; see Fig. S6 in the supplementary material). Thus, ectopic myelinating oligodendrocytes, but not proprioceptive axons, are required for the disruption of vGlut1-positive cutaneous axon terminals in the dorsal spinal cords of *Sema6d* mutant mice.

## DISCUSSION

This study shows that Sema6D in the dorsal spinal cord functions as a ligand for plexin A1 in proprioceptive axons, and that Sema6D-plexin A1 signaling regulates the proper axon positioning of proprioceptive sensory neurons. The defects in proprioceptive axon positioning precede defects in the migration of oligodendrocytes in the dorsal spinal cord of *Sema6d* mutants, suggesting that aberrant proprioceptive axons are likely to alter the localization of myelinating oligodendrocytes in the dorsal horn of *Sema6d* mutants. Since there are no obvious defects in the positioning of Olig2-positive oligodendrocyte precursors in *Sema6d* mutants, ectopic proprioceptive axons might induce nearby oligodendrocyte precursors to differentiate as myelinating oligodendrocytes. Proper axon positioning does not seem to be required for sensory-motor connectivity, but synapse formation in regions containing aberrant proprioceptive axons and ectopic oligodendrocytes was disrupted in *Sema6d* mutants. Deletion of oligodendrocytes using DTA in *Sema6d* mutants rescued the defects in synapse formation despite the aberrant positioning of proprioceptive axons. These findings suggest that the presence of aberrant myelinating oligodendrocytes disrupts synapse formation in the dorsal spinal cord in the absence of Sema6D signaling (Fig. 9).

### Sema6D appears to be a functional ligand for plexin A1

Two types of semaphorins, class 3 and class 6, have been identified as ligands for plexin A1 (Tran et al., 2007). Class 3 semaphorins indirectly bind to plexin A1 through neuropilin 1/2 (Tran et al., 2007). Plexin A1 interacts with the class 6 semaphorins Sema6C and Sema6D (Toyofuku et al., 2004; Yoshida et al., 2006), which may act as ligands for plexin A1 in the regulation of proprioceptive axon positioning (Yoshida et al., 2006). The ectopic expression of either Sema6C or Sema6D is sufficient to suppress proprioceptive axonal projections in chick spinal cords (Yoshida et al., 2006). However, we found that only *Sema6d* mutants exhibit defects in proprioceptive axon positioning similar to those seen in *Plxna1* mutants (Yoshida et al., 2006). This suggests that Sema6D is a functional ligand for plexin A1 in the control of proprioceptive axon positioning in the mouse spinal cord.

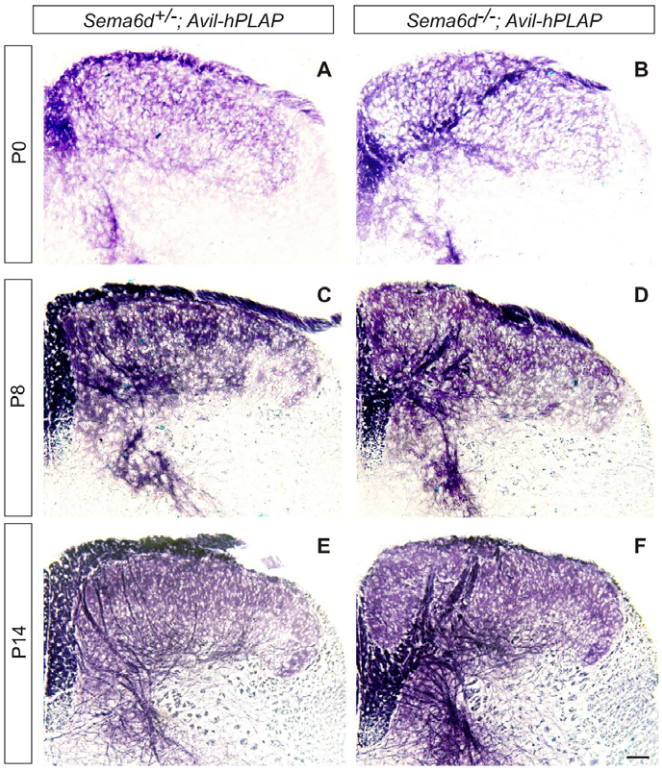


**Fig. 6. Ectopically located proprioceptive axons and oligodendrocytes in *Sema6d*$^{-/-}$ mice do not inhibit axonal projections.** (**A-F**) Both proprioceptive and cutaneous sensory fibers were detected by hPLAP activity in *Sema6d*$^{+/-}$; *Avil-hPLAP* (A,C,E) and *Sema6d*$^{-/-}$; *Avil-hPLAP* (B,D,F) mice at P0 (A,B), P8 (C,D) and P14 (E,F). Scale bar: 100 μm.

### Sema6D-plexin A1 signaling is not required for sensory-motor connectivity

Although proprioceptive axons were aberrantly located in the dorsal spinal cord in *Sema6d* mutants, these axons still reached the ventral spinal cord (Fig. 1D). Therefore, we addressed whether proprioceptive axons make functional synapses with motor neurons in the ventral spinal cord of *Sema6d* mutants. When we examined this possibility using anatomical and electrophysiological analyses (see Fig. S2 in the supplementary material), we did not see any differences in the numbers of synaptic terminals or in synaptic function between control and *Sema6d* mutant mice (see Fig. S2 in the supplementary material). Thus, correct axonal trajectories of proprioceptive sensory neurons in the dorsal spinal cord are not required for proper sensory-motor connectivity in the ventral horn. Recently, the interaction between Sema3E and plexin D1 has been shown to control synaptic connections between cutaneous maximus sensory and motoneurons (Pecho-Vrieseling et al., 2009). Whether Sema6D-plexin A1 signaling is similarly involved in synaptic specificity remains to be elucidated.

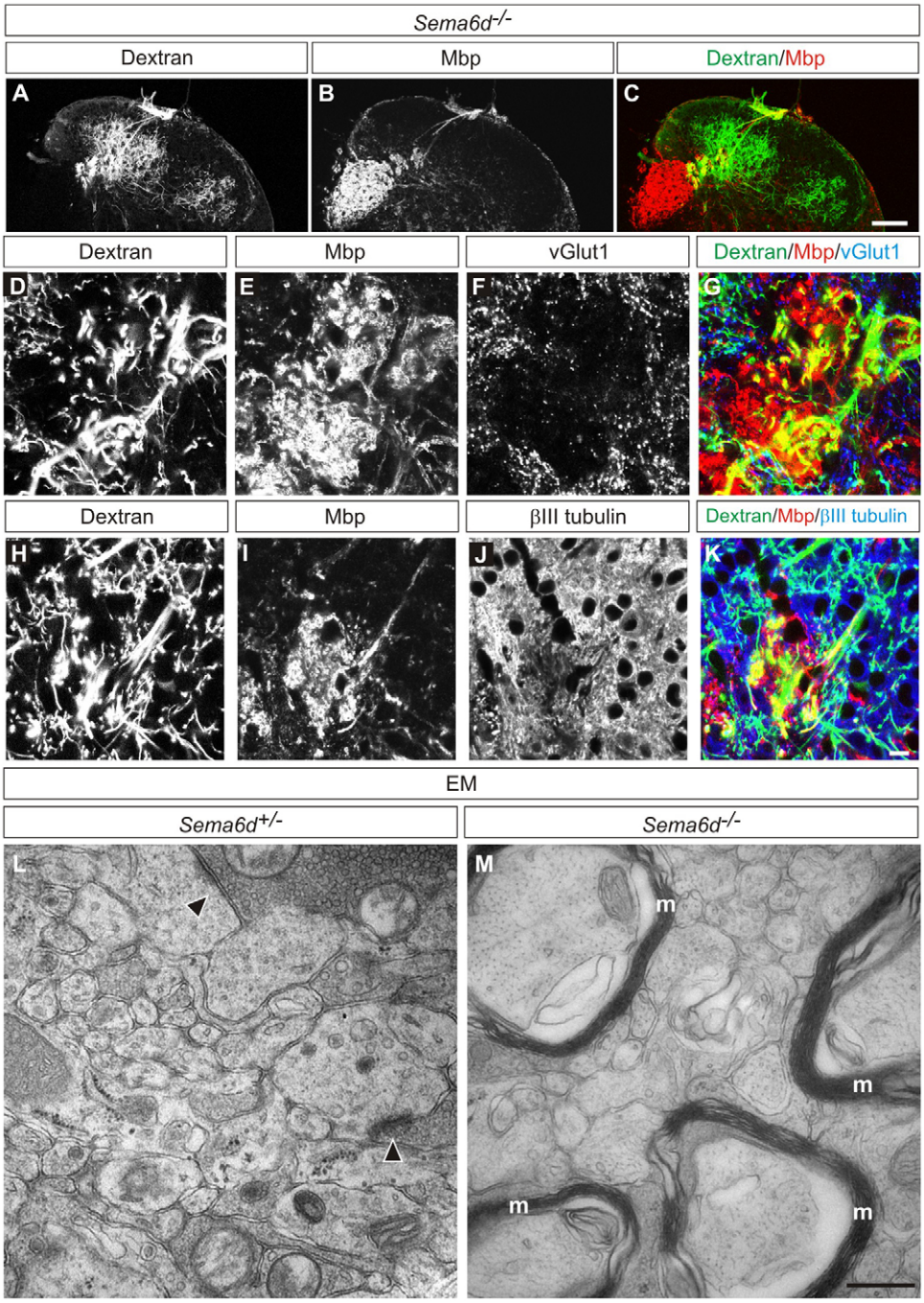


**Fig. 7. Aberrant proprioceptive axons and oligodendrocytes do not inhibit cutaneous axonal projections.** (**A-K**) Dorsal root ganglia (DRG) neurons were labeled with f-Dex (Dextran) by injection from the dorsal nerve in *Sema6d*$^{-/-}$ mice at P7. (A-C) Confocal images of dorsal horn showing f-Dex (A) and Mbp immunostaining (B), with merge (C). (D-K) Confocal images of Mbp-positive and vGlut1-negative areas with f-Dex (D,H), Mbp (E,I), vGlut1 (F) and βIII tubulin (J), with merges (G,K). (**L**,**M**) Electron microscopy images from *Sema6d*$^{+/-}$ (L) and *Sema6d*$^{-/-}$ (M) mice. Synapses were observed in *Sema6d*$^{+/-}$ mice (arrowhead in L). Myelinated axons (m) and non-myelinated processes, but few synapses, were observed in *Sema6d*$^{-/-}$ mice (M). Scale bars: 100 μm in C; 10 μm in K; 500 nm in M.

## Oligodendrocytes, but not proprioceptive axons, inhibit synapse formation in the dorsal horn of *Sema6d* mutants

Although it has been shown that ectopic proprioceptive axons and oligodendrocytes in the dorsal horn of the spinal cord alter the distribution of synaptic terminals of cutaneous sensory neurons in *Plxna1* mutants (Yoshida et al., 2006), two important questions have not been resolved. First, do the ectopic proprioceptive axons and oligodendrocytes inhibit cutaneous axonal projections or do they alter synapse formation? Second, which agents inhibit synaptic terminals: proprioceptive axons or oligodendrocytes? We addressed these two questions using *Sema6d* mutant mice.

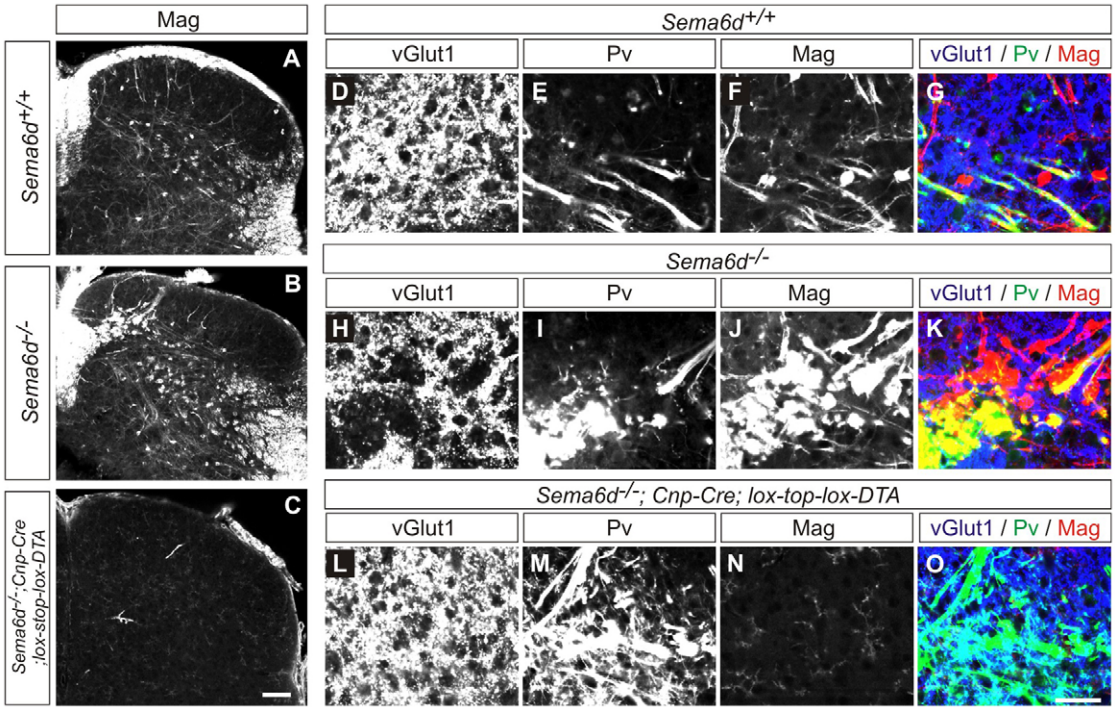


**Fig. 8. Genetic deletion of oligodendrocytes from *Sema6d*$^{-/-}$ mice.** (**A-C**) Dramatic reduction of Mag protein expression in the dorsal spinal cord of *Sema6d*$^{-/-}$; *Cnp-Cre*; *lox-stop-lox-DTA* mice at P10. (**D-O**) vGlut1 (blue), Pv (green) and Mag (red) expression at P10. Defects in vGlut1-positive terminals exhibited by *Sema6d*$^{-/-}$ mice were not detected in *Sema6d*$^{-/-}$; *Cnp-Cre*; *lox-stop-lox-DTA* mice. Scale bars: 100 μm in C; 10 μm in O.

To determine whether ectopic proprioceptive axons and oligodendrocytes could inhibit axonal projections of cutaneous sensory neurons in *Sema6d* mutants, we crossed *Sema6d* mutants with *Avil-hPLAP* mice that express hPLAP in all, or most, DRG sensory neurons (Hasegawa et al., 2007). We did not detect any disruptions in hPLAP activity in the dorsal spinal cords of *Sema6d*$^{-/-}$; *Avil-hPLAP* mice (Fig. 6), indicating that axonal projections of DRG sensory neurons were not inhibited by ectopic proprioceptive axons or oligodendrocytes. Furthermore, to examine the sensory axons in detail, we injected f-Dex into DRG in *Sema6d* mutant mice (Fig. 7). Similar to *Sema6d*$^{-/-}$; *Avil-hPLAP* mice, f-Dex-positive sensory axons were detected in Mbp-positive and vGlut1-negative areas in *Sema6d* mutant mice (Fig. 7). Taken together, oligodendrocytes in ectopic regions in *Sema6d* mutants are unlikely to inhibit axonal projections, although we cannot exclude the possibility of defects in axon branching of sensory neurons in *Sema6d* mutant mice.

By deleting oligodendrocytes using DTA from *Sema6d* mutants, we were able to identify oligodendrocytes as the agents that inhibit synapse formation. Although similar defects in proprioceptive axon positioning were observed in *Sema6d* mutants regardless of the presence or absence of oligodendrocytes, only the mutants lacking oligodendrocytes displayed wild-type patterns of vGlut1-positive cutaneous synaptic terminals in the dorsal spinal cord (Fig. 8). This might represent one of the few in vivo descriptions of such a relationship between oligodendrocytes and synapse formation. How, then, do ectopic oligodendrocytes inhibit synapse formation in *Sema6d* mutants? Although cutaneous axons can project or pass through regions of ectopic proprioceptive axons and oligodendrocytes, the oligodendrocytes might inhibit synapse formation in the dorsal horn. Another possibility is that synapses are initially formed, but the subsequent presence of oligodendrocytes causes the elimination or disassembly of those synapses. The molecular mechanisms of this process are still unclear. Several inhibitors for DRG axonal growth are expressed by oligodendrocytes, including Mag, Nogo-A (Rtn4 – Mouse Genome Informatics) and OMgp (Omg) (He and Koprivica, 2004). It will be interesting to determine whether oligodendrocytes and such oligodendrocyte inhibitors can inhibit synapse formation in vitro. Such in vitro experiments might provide new insights into the mechanisms of how oligodendrocytes inhibit synapse formation. We still cannot exclude the possibility that oligodendrocytes create a physical barrier between pre- and postsynaptic regions, although our EM analysis did not provide any evidence of this. However, regardless of whether oligodendrocytes prevent synapse formation by releasing inhibitors or by creating a physical barrier, our findings provide evidence that the precise positioning of myelinating oligodendrocytes is important for appropriate synapse formation in the dorsal spinal cord.

## Oligodendrocytes and synapse formation

There were defects in not only vGlut1-positive presynaptic terminals but also vGlut2- and SV2-positive presynaptic terminals in the dorsal spinal cord of *Sema6d* mutant mice (Fig. 4). In addition, there were defects in postsynaptic markers, such as PSD95 and Shank1, in the dorsal spinal cord of *Sema6d* mutant mice (Fig. 4). Thus ectopic oligodendrocytes are likely to inhibit synapse formation of both sensory neurons and spinal cord neurons in the dorsal horn of *Sema6d* mutant mice.

Although many studies have shown that oligodendrocytes inhibit axonal growth in vitro (Yiu and He, 2006), our studies demonstrate that ectopic oligodendrocytes in the dorsal spinal cord are likely to inhibit only synapse formation in vivo, as axonal growth was not altered in *Sema6d* mutants. This might

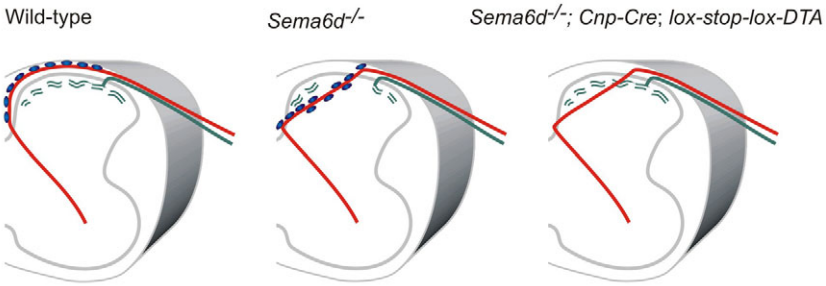


**Fig. 9. Dorsal spinal cords of wild-type and *Sema6d* mutant mice used in this study.** Transverse sections illustrate proprioceptive axons (red lines), oligodendrocytes (blue ovals), cutaneous axons (green lines) and cutaneous synapses (green wavy lines). Cutaneous synapses are disrupted when oligodendrocytes enter the dorsal horn of the *Sema6d*$^{-/-}$ spinal cord.

highlight a major difference between in vitro and in vivo systems. In vitro, oligodendrocytes are likely to repel the growth cones of axons, whereas in *Sema6d* mutants cutaneous axons have already reached their target regions when the myelination events occur. Therefore, it is possible that oligodendrocytes could inhibit axonal growth if we were able to ectopically 'express' them in the dorsal spinal cord before cutaneous axons reached the dorsal horn, although such an experiment would be technically challenging.

Oligodendrocytes are present everywhere in the spinal cord except in lamina II, which is also known as the substantia gelatinosa. Why, then, is it that oligodendrocytes do not prevent synapse formation in oligodendrocyte-positive regions in the spinal cord? The temporal and spatial patterns of oligodendrocyte migration might be important for precise synapse formation in the spinal cord. The axons of proprioceptive and cutaneous sensory neurons invade the spinal cord at embryonic day (E) 13.5 and E14.5, respectively (Ozaki and Snider, 1997). The proprioceptive sensory axons start making synapses with motoneurons at E17 (Mears and Frank, 1997), and some cutaneous sensory neurons make synapses with spinal cord neurons at early postnatal stages (Pignatelli et al., 1989). Since there are few oligodendrocytes at P0 (see Fig. S3 in the supplementary material), sensory axonal growth into the spinal cord and synapse formation precede mature oligodendrocyte migration into the spinal cord. This temporal order might be important for proper synapse formation. Furthermore, if proteins derived from oligodendrocytes inhibit synapse formation, the types of neurons that are affected might be dependent on the expression of the receptors for the inhibitors.

Is the exclusion of oligodendrocytes that are associated with heavily myelinated proprioceptive axons in the dorsal spinal cord instrumental for further maturation of the neural circuits in *Sema6d* mutants? Cutaneous axons supplying discrete regions of the limb project to sagittally oriented columns of target interneurons in the dorsal spinal cord (Schouenborg, 2004). Thus, there is a somatotopic organization of cutaneous inputs in the dorsal spinal cord (Schouenborg, 2004). As such, defects in synapse formation in the dorsal spinal cord in *Sema6d*$^{-/-}$ mice might result in sensory deficits upon cutaneous stimulation of the peripheral receptive field, although this remains to be tested.

In summary, our studies in the *Sema6d* null mouse reveal that ectopic myelinating oligodendrocytes in the dorsal spinal cord can inhibit synapse formation and might provide insights into problems of nervous system repair.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at
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