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RESEARCH ARTICLE

Semaphorin 5B is a repellent cue for sensory afferents projecting into the developing spinal cord

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ABSTRACT

During vertebrate development, centrally projecting sensory axons of the dorsal root ganglia neurons first reach the embryonic spinal cord at the dorsolateral margin. Instead of immediately projecting into the grey matter, they bifurcate and extend rostrally and caudally to establish the longitudinal dorsal funiculus during a stereotyped waiting period of approximately 48 h. Collateral fibres then extend concurrently across multiple spinal segments and project to their appropriate targets within the grey matter. This rostrocaudal extension of sensory afferents is crucial for the intersegmental processing of information throughout the spinal cord. However, the precise cues that prevent premature entry during the waiting period remain to be identified. Here, we show that semaphorin 5B (Sema5B), a member of the semaphorin family of guidance molecules, is expressed in the chick spinal cord during this waiting period and dorsal funiculus formation. Sema5B expression is dynamic, with a reduction of expression apparent in the spinal cord concomitant with collateral extension. We show that Sema5B inhibits the growth of NGF-dependent sensory axons and that this effect is mediated in part through the cell adhesion molecule TAG-1. Knockdown of Sema5B in the spinal cord using RNA interference leads to the premature extension of cutaneous nociceptive axons into the dorsal horn grey matter. These premature projections predominantly occur at the site of dorsal root entry. Our results suggest that Sema5B contributes to a repulsive barrier for centrally projecting primary sensory axons, forcing them to turn and establish the dorsal funiculus.

KEY WORDS: Sema5B, Dorsal root entry zone, Repulsion, Chick

INTRODUCTION

The vertebrate dorsal root ganglion (DRG) contains a heterogeneous population of somatosensory neurons characterised by the sensory information they transmit (Eide and Glover, 1995). During spinal cord development, all afferent projections of DRG neurons initially respond to the same combination of cues that directs them into the dorsal root entry zone (DREZ), where their axial growth changes as they approach the dorsal horn grey matter (Davis et al., 1989; Masuda and Shiga, 2005). Here, they bifurcate and extend axons rostrally and caudally for numerous segments to form the dorsal funiculus and Lissauer's tract during what is known as the 'waiting period', before simultaneously projecting collateral fibres into the grey matter across multiple segments (Davis et al., 1989; Eide and Glover, 1996; Schmidt et al., 2007). Despite their common origin and initial pathfinding into the DREZ, sensory collaterals establish connections in different regions of the grey matter depending on their sensory modality (Eide and Glover, 1997; Mendelson et al., 1992). Whereas sensory axons involved in nociception terminate in the dorsalmost layers and synapse with interneurons of the pain pathway, proprioceptive afferents project to the motoneurons located in the ventral horns (Eide and Glover, 1997).

Sensory axons reach the DREZ as discrete roots at each segment along the length of the spinal cord (Mauti et al., 2007). Preventing sensory axons from entering the grey matter and forcing their bifurcation and rostrocaudal extension is crucial in establishing intersegmental sensory circuits along the length of the spinal cord. This permits appropriate perception and reflexes (Sprague, 1958). Although we have learned much about the cues that regulate laminar targeting of sensory axons (Messersmith et al., 1995; Shepherd et al., 1997), the precise combination of inhibitory guidance cues that acts as a barrier against premature entry into the grey matter is still unclear.

One of the first identified proteins suggested to restrict sensory axon growth into the spinal cord was semaphorin 3A (Sema3A) (Messersmith et al., 1995). In mouse and chick, Sema3A is expressed in the spinal cord at the approximate time of sensory axon growth into the spinal cord (Adams et al., 1996; Fu et al., 2000; Masuda et al., 2003; Shepherd et al., 1996), and the phenotypes observed in *Sema3a* and neuropilin 1 (*Nrp1*; the Sema3A co-receptor) knockout mice (Behar et al., 1996; Gu et al., 2003; Kitsukawa et al., 1997) suggest that Sema3A might function as a barrier to premature axon entry into the spinal cord. Indeed, increasing the levels of Sema3A in the dorsal horn at the time of axon entry can prevent the entry of TrkA (also known as Ntrk1) positive cutaneous axons (Fu et al., 2000). However, evidence derived from spinal cord and DRG co-culture experiments and analysis of knockout mice suggests that at least one additional repulsive cue is expressed in the spinal cord and functions through the cell adhesion molecule TAG-1 [also known as axonin 1 or contactin 2 (*Cntn2*)] (Law et al., 2008; Masuda et al., 2003; Zuellig et al., 1992). Here we show that the semaphorin Sema5B is expressed early and throughout the spinal cord when axons first enter the DREZ. Also, we show that Sema5B is inhibitory to sensory axons and that knockdown of Sema5B *in vivo* results in early entry of TAG-1-expressing sensory axons into the spinal cord grey matter. This suggests that Sema5B may be a key regulator of sensory axon entry into the developing spinal cord.

RESULTS

Sema5B is expressed in the developing spinal cord

Using *in situ* hybridization, we found chick Sema5B to be expressed in various tissues of the embryonic nervous system, including the spinal cord, dorsal root ganglia, retina, tectum and olfactory epithelium (supplementary material Fig. S1). Similar to observations in the mouse (Adams et al., 1996), expression of Sema5B in the chick spinal

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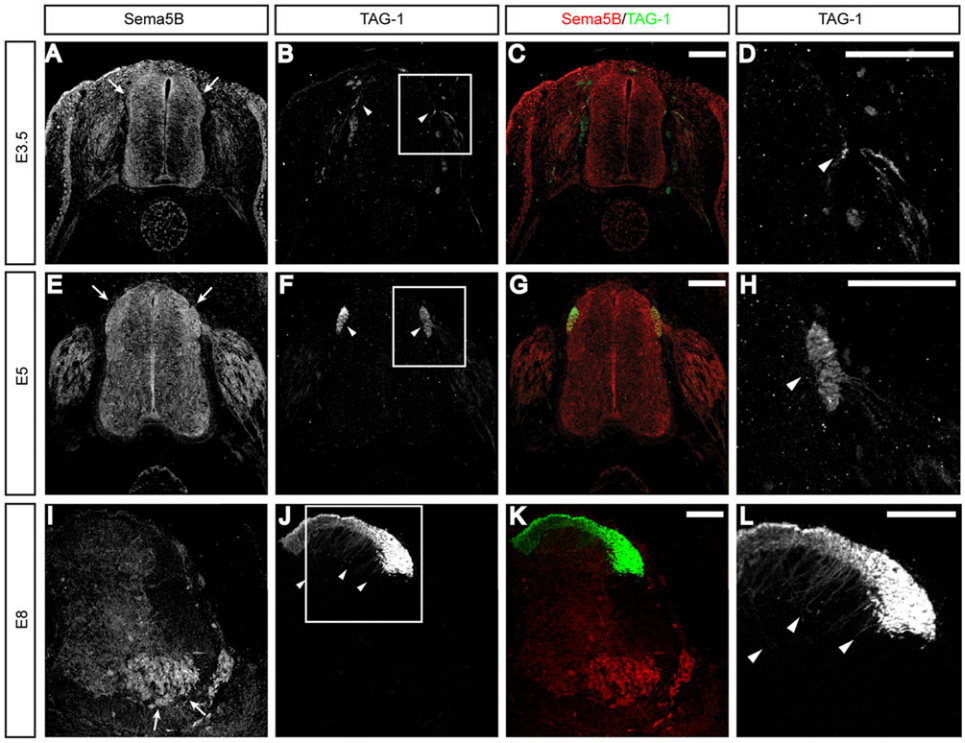

Fig. 1. Sema5B and TAG-1 expression in the developing chick spinal cord. (A,E,I) Immunohistochemical labelling of Sema5B in the spinal cord grey matter at the indicated stages. (B,F,J) Immunohistochemical labelling of TAG-1 in the spinal cord. Insets are magnified in D, H and L, respectively. (C,G,K) Merged images of Sema5B and TAG-1 labelling. Arrows in A and E indicate high expression of Sema5B throughout the grey matter at E3.5 and E5, respectively. Increasing numbers of sensory axons arrive at the DREZ at this time (arrowheads in B,D,F,H). At E8, Sema5B expression has decreased throughout the grey matter except within the ventral horn (arrows in I) and TAG-1-positive fibres are abundant in the dorsal horn (arrowheads in J, L). Scale bars: 100 µm.

cord is dynamic (Figs 1 and 2). At E3-3.5 (st21-23; Fig. 1A-D and Fig. 2A), Sema5B expression is observed broadly in the grey matter just as sensory axons are arriving at the DREZ. By E5 (st27), sensory axons have bifurcated and extended along the length of the spinal cord adjacent to the expression of Sema5B in the dorsal horn (Fig. 1E-H and Fig. 2B). At E6, Sema5B expression appears reduced along the periphery of the grey matter (Fig. 2C) and by E8 the expression of Sema5B has decreased throughout the grey matter, except in a population of cells in the ventral horn (Fig. 1I,K and Fig. 2D). It has been well described that sensory collaterals do not enter the grey matter until E6 (st29) and then project to specific laminae targets by E9 (st35) according to their sensory modality (Eide and Glover, 1997; Mendelson et al., 1992; Perrin et al., 2001). The correlation between these times and the dynamic expression of Sema5B suggests the possible involvement of Sema5B in the timing and targeting of sensory collateral axons.

Sema5B acts as a repellent for chick sensory neurons *in vitro*

Previously, we showed that Sema5B can act as a repellent guidance cue for different populations of neurons during development (Browne et al., 2012; Lett et al., 2009; O'Connor et al., 2009; To et al., 2007). To test whether Sema5B can affect axon outgrowth of sensory neurons, dissociated DRG were obtained from chicks ranging from E5 to E8 and cultured on a confluent monolayer of HEK293 cells stably expressing Sema5B or a control vector. The growth of nerve growth factor (NGF)-responsive and neurotrophin 3 (NT-3)-responsive populations of sensory neurons was selected by the addition of either neurotrophin as previously described (Chan et al., 2008; Law et al., 2008). In all cultures with Sema5B-expressing cells, the mean axon length of sensory neurons at all ages examined was significantly shorter (by 30-40%) than observed in control cultures (Fig. 3A-D). Although it is possible that the transfection of Sema5B into HEK293 cells might have resulted in the expression of an additional unknown inhibitory protein, we have previously shown that purified Sema5B can function as an inhibitory factor and collapse DRG growth cones (Browne et al., 2012). Thus, exogenous Sema5B inhibits axon outgrowth of different classes of sensory neurons *in vitro*.

Nociceptive sensory axons invade the grey matter prematurely following knockdown of Sema5B

Having determined that Sema5B can act as a repellent cue for sensory axons *in vitro*, we examined the function of Sema5B *in vivo* by knocking down its expression using RNA interference (RNAi). Two short hairpin RNA (shRNA) sequences were validated by their ability to reduce Sema5B expression when transfected into HEK293 cells stably expressing HA-tagged Sema5B (Fig. 4). GFP expression showed control (empty pLL vector) and shRNA-positive cells, and Sema5B expression was determined by HA labelling (Fig. 4A-I). Compared with the control (Fig. 4A-C), GFP-positive cells transfected with shRNA plasmids exhibited a substantial reduction in HA-Sema5B expression, confirming the effectiveness of the RNAi knockdown (arrows in Fig. 4D-I). Similarly, western analysis of lysates of shRNA-transfected HEK293 cells also showed a considerable reduction in HA-Sema5B expression compared with control transfected cells (Fig. 4J). Because the transfection efficiency with the shRNA plasmids was not 100%, some HA-Sema5B remained detectable in the cell lysates, but a significant reduction was observed for each of the shRNA constructs (Fig. 4J, compare lanes 2-4 with lane 1). Furthermore, the knockdown effect of the two shRNA constructs combined was as effective as when the two constructs were transfected individually (Fig. 4J, lane 4 compared with lanes 2 and 3); therefore, the two constructs were also used in combination (each at half the concentration of single transfection) for knockdown experiments *in vivo*. By contrast, the same shRNA vectors did not reduce the expression of HA-tagged mouse Sema5B (Fig. 4K, compare lanes 2-4 with lane 1), confirming their specificity to the chick homologue. Thus, the two shRNAs significantly knocked down chick Sema5B overexpression 24 h after transfection and they did not knockdown mouse Sema5B.

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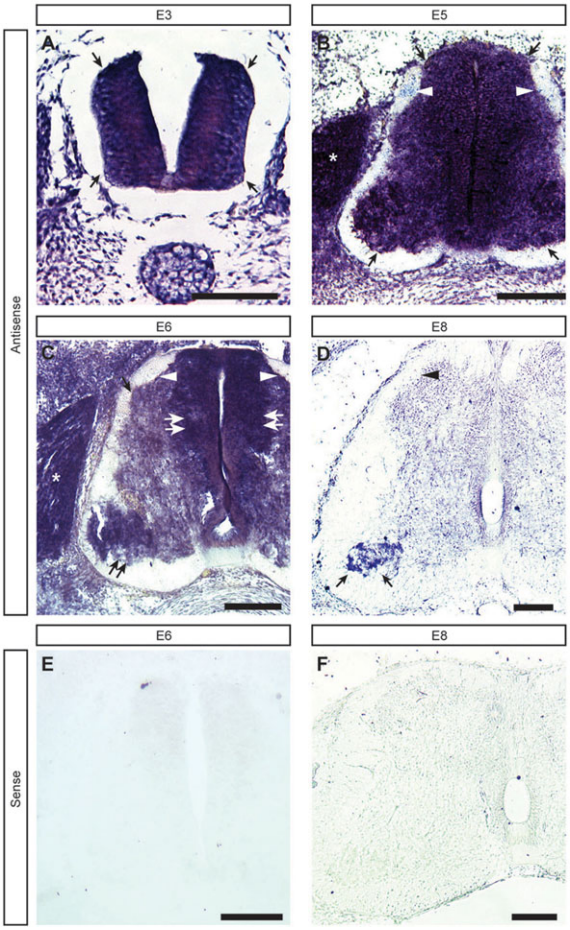

Fig. 2. The expression of *Sema5b* mRNA in the developing spinal cord is dynamic. (A-D) *In situ* hybridisation of *Sema5b* mRNA in the chick spinal cord at the indicated stages. (E,F) *In situ* hybridisation results for sense strand cRNA probe. *Sema5b* expression at E3 (arrows in A; note that the dorsal spinal cord tissue is disrupted during processing) and at E5 (arrows in B) is strong throughout the grey matter. Longitudinal axon tracts established by sensory axons arriving at the DREZ can be distinguished at older ages (arrowheads in B,C,D). At E6, *Sema5b* is highest along the ventricular zone and the ventral horn (double arrows in C) and decreases in the rest of the grey matter (arrow in C). *Sema5b* expression is only present at high levels in the ventral horn at E8 (arrows in D). Scale bars: 100 µm.

To examine whether Sema5B can function as a barrier in the grey matter to prevent the premature entry of sensory axons, we knocked down its expression by electroporating shRNAs into the spinal cord at E3.5 (st21), just after primary sensory axons first reach the DREZ (Eide and Glover, 1995; Mendelson et al., 1992; Perrin et al., 2001). Embryos were sacrificed at E6 (st29), when collaterals normally begin to invade the grey matter (Eide and Glover, 1995; Mendelson et al., 1992; Perrin et al., 2001). Sensory axons were labelled with anti-TAG-1 to examine the timing and extent of their entry into the grey matter (Law et al., 2008). Control transfected spinal cords showed no TAG-1-labelled sensory axons inside the grey matter on either side of the spinal cord (Fig. 5A-C,G). By contrast, when Sema5B expression was knocked down, TAG-1-positive afferents showed a striking change in their pathfinding pattern after reaching the DREZ, as a significant number of axons prematurely invaded the grey matter (Fig. 5D-G). Furthermore, these early penetrating nociceptive axons did not appear to pathfind correctly, as the majority extended beyond their normal sites of termination and reached the ventricular zone at the midline of the spinal cord (Fig. 5E,F).

A possible explanation of this phenotype is that, in animals with reduced Sema5B expression, sensory axons are not being forced to turn after reaching the spinal cord from the dorsal roots. If this is correct then it would be expected that the majority of early penetrating axons would be located at sites of dorsal root entry. To examine this, we analysed the position along the rostral-caudal axis at which the sensory axons prematurely entered into the grey matter of Sema5B knockdown animals. We compared the number of aberrant fibres found in sections in which the dorsal roots enter the spinal cord (root sections, Fig. 5H) with the number of fibres in sections between dorsal root entry sites (non-root sections, Fig. 5H). In all experimental spinal cords analysed, significantly more aberrant projections were found on root sections compared with those found on non-root sections (Fig. 5I). Furthermore, aberrant projections were observed in 88% of root sections, whereas only 15% of non-root sections showed a phenotype. To examine this further, we DiI labelled a subset of peripheral DRG axons and examined their central projections at the dorsal roots in the spinal cords of Sema5B knockdown animals. Although the majority of axons turned and extended along the dorsal funiculus (Fig. 5J, arrows), a significant number of axons had extended into the grey matter (Fig. 5J,K). We could not follow the path of a single aberrantly projecting axon in the DREZ due to the intense DiI labelling of axons, but many of the single axons that entered the grey matter appeared to extend straight into the grey matter (Fig. 5K, arrows). Collectively, these data suggest that when axons first enter the DREZ they are normally inhibited from growing into the spinal cord by Sema5B and instead extend collaterals along the rostral and caudal length of the spinal cord.

To control for possible off-target effects of the shRNAs, we co-transfected HA-tagged mouse Sema5B (m5B) with the shRNA constructs. Co-transfection of HA-m5B and shRNAs prevented the premature entry of sensory fibres, demonstrating that the effects observed following shRNA transfection were specific to the knockdown of Sema5B (Fig. 5G). However, no rescue effect was observed when the shRNA constructs to chick Sema5B (c5B-KD) were co-transfected with an empty pDisplay (pDis) vector (Fig. 5G). These data strongly support the finding that Sema5B regulates sensory axon entry into the spinal cord grey matter.

Proprioceptive axons do not invade the grey matter prematurely after knockdown of Sema5B

To examine whether Sema5B also acts as a barrier for other sensory afferents, we examined the projections of proprioceptive axons after Sema5B knockdown in the spinal cord of E3.5 embryos. At E6, axons were labelled with anti-TrkC (also known as Ntrk3) to examine the timing and extent of their entry into the grey matter. Control transfected spinal cords showed no TrkC-labelled sensory axons inside the grey matter on either side of the spinal cord (Fig. 6A,C). In addition, we did not observe premature entry of TrkC-labelled axons in the grey matter of Sema5B knockdown spinal cords (Fig. 6B,D). Indeed, we never observed premature axon entry or aberrant pathfinding of proprioceptive axons after Sema5B knockdown (n=10 animals, >100 sections examined).

Nociceptive afferents exhibit pathfinding errors following Sema5B knockdown

Next, we asked whether, in addition to acting as a barrier to early axon entry, Sema5B also regulates collateral fibre targeting of nociceptive axons once they have entered the spinal cord. We knocked down the level of Sema5B expression in the spinal cord at E5.5 (st27) by shRNA electroporation and examined the effect on nociceptive axon guidance

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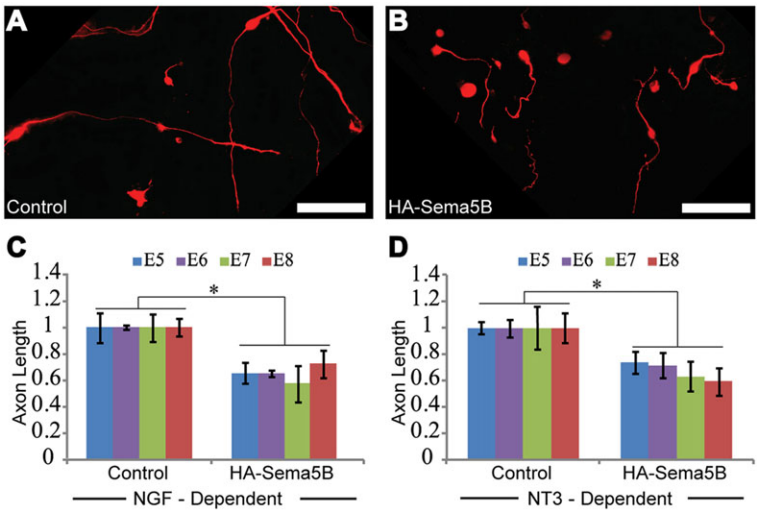

Fig. 3. Sema5B inhibits sensory axon outgrowth *in vitro*. (A,B) Sensory neurons (E6+1 day *in vitro* neurons shown here) were cultured overnight on a confluent monolayer of HEK293 cells expressing control (A) or HA-Sema5B (B) vectors in the presence of 40 ng/ml NGF (A-C) or 40 ng/ml NT3 (D). Compared with the control, cutaneous nociceptive axons (C) and proprioceptive axons (D) are both significantly shorter when grown on HA-Sema5B-expressing cells compared with the control at all time points tested (E5-E8). n=3 separate experiments with 66-228 neurons counted per treatment. Error bars represent s.e.m. Unpaired Student's t-test, *P<0.05 for all days tested.

at E8 (st33), when they normally establish contacts with their correct targets. In control spinal cords, TAG-1-positive collaterals extending from the dorsal funiculus remain confined to the dorsal lateral region of the dorsal horn (arrowheads, Fig. 7A-C). By contrast, after Sema5B knockdown, TAG-1-positive collaterals extend more ventromedially and target the ventricular zone surrounding the central canal (arrows, Fig. 7D-F), similar to our observations following Sema5B knockdown at earlier stages. We observed a significantly greater number of mistargeted nociceptive axons in Sema5B knockdown spinal cords (67±9 aberrant projections per 300 µm of spinal cord) compared with control spinal cords (20±3 aberrant projections per 300 µm; unpaired t-test, P<0.05; n=5 animals for each condition). These findings suggest that Sema5B within the grey matter might also be important for guiding the nociceptive collateral afferents to their correct targets in the dorsal horn laminae.

To investigate whether the early entry of axons or aberrant pathfinding was due to abnormal development of the neural tube following electroporation, the cellular patterning in the grey matter and the expression of various transcription factors were analysed in control and knockdown embryos that had been electroporated at E3.5 and fixed at E6 (Fig. 8). Upon comparing control (Fig. 8A,C,E,G,I) and Sema5B knockdown (Fig. 8B,D,F,H,J) spinal cords, no difference in cell density or patterning (Fig. 8C,D) was observed, nor between electroporated versus non-electroporated sides of the spinal cord. Similarly, no difference in the expression of Islet1 (Fig. 8E,F), Nkx2.2 (Fig. 8G,H) or Pax6 (Fig. 8I,J) was observed. These results indicate that the patterning of the neural tube is not affected by electroporation in general, nor by the knockdown of Sema5B specifically.

Sema5B functions through TAG-1

Considerable evidence from *in vitro* and *in vivo* experiments has demonstrated the presence of a spinal cord-derived chemorepellent that functions in part though the immunoglobulin superfamily cell adhesion molecule TAG-1 (Law et al., 2008; Masuda and Shiga, 2005; Perrin et al., 2001; Sharma and Frank, 1998). Thus, we tested the possibility that Sema5B may function through the TAG-1 protein. We employed the same dissociated DRG overlay assay used above but added a monoclonal mouse anti-TAG-1 antibody to some cultures to inhibit the function of TAG-1 on sensory axons as described previously (Law et al., 2008).

We first show that the outgrowth of E4 sensory neurons cultured in the presence of NGF or NT-3 was inhibited by Sema5B (Fig. 9A). At this stage sensory neurons are just beginning to discriminate between growth factors for survival (Lefcort et al., 1996), and all of their axons express TAG-1 (Perrin et al., 2001). Surprisingly, in the presence of TAG-1 antibodies only neurons cultured in the presence of NGF showed a reduction in inhibition by Sema5B (Fig. 9A). This suggests that a subpopulation of neurons might be differentiating into NGF-dependent neurons and that their sensitivity to Sema5B is mediated through TAG-1. At E6, Sema5B was inhibitory for both populations of neurons (Fig. 9B), although at this stage NT-3-dependent neurons no longer express TAG-1 (Perrin et al., 2001). This suggests that the inhibitory effects of Sema5B are not mediated via TAG-1 for proprioceptive NT-3-dependent neurons. Indeed, whereas the addition of a function-blocking anti-TAG-1 antibody to the cultures blocked the inhibitory effect of Sema5B on NGF-dependent neuron outgrowth, it did not effect NT-3-dependent axons (Fig. 9). It is also interesting to note that the effects of the TAG-1 antibody were more pronounced at E6 when NGF-dependent neurons are more differentiated and rely specifically on NGF for survival. To examine whether the TAG-1 antibody may be overcoming the Sema5B inhibition by directly stimulating neurite outgrowth, we also cultured E6 neurons on control cells in the presence of the antibody. The antibody had no effect on neurite outgrowth of either population of neurons relative to the control and to each other (Fig. 9B).

These findings demonstrate that acutely blocking TAG-1 function is sufficient to eliminate the inhibitory effect of Sema5B on TAG-1-positive nociceptive axons and support the hypothesis that Sema5B acts partly through TAG-1 to inhibit the outgrowth of these axons. By contrast, the repulsive effect of Sema5B on NT-3-dependent neurons was not inhibited by the addition of an anti-TAG-1 antibody, suggesting the presence of a non-TAG-1 receptor complex on these neurons that mediates the repulsive effect of Sema5B.

DISCUSSION

Neurons are guided by a combination of inhibitory and attractive cues as they establish precise and complex circuits. A goal of the present study was to identify inhibitory cues that prevent sensory axons from prematurely entering the spinal cord grey matter. As sensory axons extend into the spinal cord as fasciculated dorsal roots (Mauti et al., 2007), it is important that axons are forced to bifurcate and extend along the length of the spinal cord before entering the grey matter to form intersegmental neural circuits. It is most likely that this mechanism has evolved to maximise circuit formation along the entire length of the spinal cord, particularly

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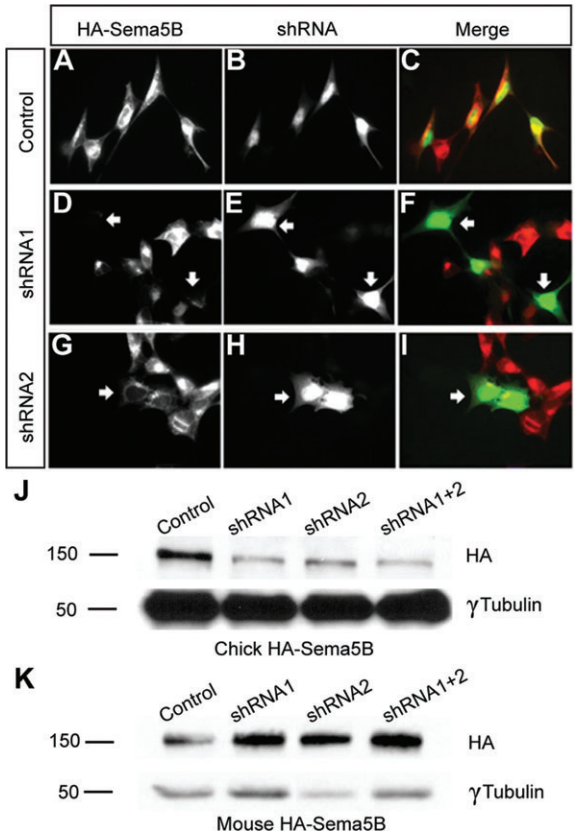

Fig. 4. shRNAs reduce the expression of chick Sema5B.
(A-I) Immunocytochemical labelling of HEK293 cells expressing HA-chick Sema5B after transfection with control and shRNA constructs. Compared with the control (A-C), HA-Sema5B (A,D,G, red in C,F,I) is reduced in shRNA-transfected cells (arrows in D-I). Transfection is identified by GFP expression (B,E,H, green in C,F,I). Images show a mixture of transfected and non-transfected cells. (J,K) shRNA constructs effectively reduce chick Sema5B but not mouse Sema5B protein levels. Sema5B in HEK293 cell lysates was analysed with antibodies against the HA tag (150 kDa bands). Both shRNA constructs effectively reduce the amount of chick Sema5B-HA in cell lysates when transfected individually or in combination (J), but did not reduce mouse Sema5B-HA levels (K). γ-tubulin provided a loading control (50 kDa bands).

between the regions where dorsal roots enter the spinal cord. We propose that Sema5B is a crucial contributor to this process.

In the present study we have shown that DRG neurons do not extend axons into the dorsal horn when Sema5B expression is high. *In vitro* assays show that sensory neuron outgrowth is inhibited by Sema5B over the embryonic time period (E5-E8) when sensory axons are extending into the spinal cord. Furthermore, functional analyses *in vivo* showed that Sema5B knockdown results in the premature entry of nociceptive TAG-1-expressing axons, particularly at the levels of dorsal root entry. Finally, we found that the adhesion molecule TAG-1 may play a role in the axonal responses to Sema5B. This is the first evidence of the involvement of Sema5B in sensory neuron circuit formation in the developing spinal cord and suggests that it plays a crucial barrier function that ensures uniform connectivity along the spinal cord (Fig. 10). It is important to note, however, that although proprioceptive axon outgrowth is inhibited by Sema5B, this inhibition does not appear to be mediated through TAG-1, and knockdown of Sema5B *in vivo* did not result in their premature entry into the spinal cord. This suggests that Sema5B might play a more restricted role in the regulation of proprioceptive axon entry into the spinal cord grey matter.

Sema5B is a functional barrier to sensory axons

We found that Sema5B is present in the chick spinal cord as early as E3, the developmental period when the first sensory axons are targeting the DREZ. Our *in vivo* analysis suggests that Sema5B acts as a barrier at the border of the dorsal grey matter to the DRG axons that have reached the DREZ. This repulsion must be significant as it forces the growth cones to turn ~90° in both rostral and caudal directions. At this time it is vital that sensory axons extend along the length of the spinal cord in order to form the nerve tracts in the dorsal white matter before extending collaterals into the grey matter. This facilitates the integration of sensory information across multiple segments along the rostrocaudal axis of the spinal cord. When the Sema5B barrier is removed or reduced, axons enter the grey matter prematurely at the dorsal roots and appear to extend straight into the grey matter. This barrier function of Sema5B is similar to its function in preventing corticofugal fibres from aberrantly projecting into the ventricular zone (Lett et al., 2009) as well as to its function in confining neurites of multiple neuron types to their appropriate lamina in the retina (Matsuoka et al., 2011).

It is surprising that proprioceptive axons did not extend prematurely into the grey matter after Sema5B knockdown. These axons are responsive to Sema5B *in vitro* and show a similar reduction of neurite outgrowth at the same embryonic ages as the nociceptive fibres. Presumably, the proprioceptive fibres are inhibited *in vivo* by a combination of cues, including Sema5B, and the reduction of any one of these cues might not be sufficient to allow early entry into the spinal cord grey matter.

It has previously been suggested that other inhibitory cues are required for the correct pathfinding of sensory afferent axons. For example, Sema3A is expressed in the spinal cord and is a repellent cue to DRG axons (Messersmith et al., 1995; Shepherd et al., 1997). A number of reports have shown that Sema3A is expressed in the spinal cord at the time that sensory axons first reach the DREZ in chick and mouse (Adams et al., 1996; Fu et al., 2000; Masuda et al., 2003; Wright et al., 1995). In animals lacking Sema3A or Nrp1, only a few aberrant sensory projections into the central nervous system were observed (Kitsukawa et al., 1997; Taniguchi et al., 1997), although these reports were not examining early entry into the spinal cord specifically, and therefore might have underestimated this phenotype. Indeed, when the Sema-binding domain of Nrp1 was mutated, TrkA-positive fibres were observed to prematurely enter the spinal cord grey matter (Gu et al., 2003). Similarly, increasing the levels of Sema3A in the dorsal horn grey matter at the time of normal ingrowth can prevent the entry of TrkA-positive axons (Fu et al., 2000). Thus, these results suggest that additional semaphorins, in particular Sema3A, contribute to the barrier function.

Watanabe et al. (2006) have suggested that the brief appearance of netrin 1 in the mouse dorsal spinal cord (between E12.5 and E13.5) acts as an inhibitory cue to prevent axons from entering the mantle layer during the waiting period (Watanabe et al., 2006). However, as the authors pointed out, the upregulation of netrin 1 occurs midway through the waiting period, whereas the arrest of axial axon trajectory occurs from E10.5, when axons reach the DREZ. This means that there must be other molecules inhibiting the invasion of axons. Furthermore, netrin 1 expression is restricted to the floor plate in chick throughout sensory circuit development, which does not support its role as a barrier (Guan and Condic, 2003; Wang et al., 1999). Another molecule that has been proposed to function as a barrier between the central nervous system and the peripheral nervous system is Sema6A (Mauti et al., 2007). Mauti et al. (2007) showed that Sema6A is expressed by boundary cap cells near both the dorsal and ventral root entry sites in early

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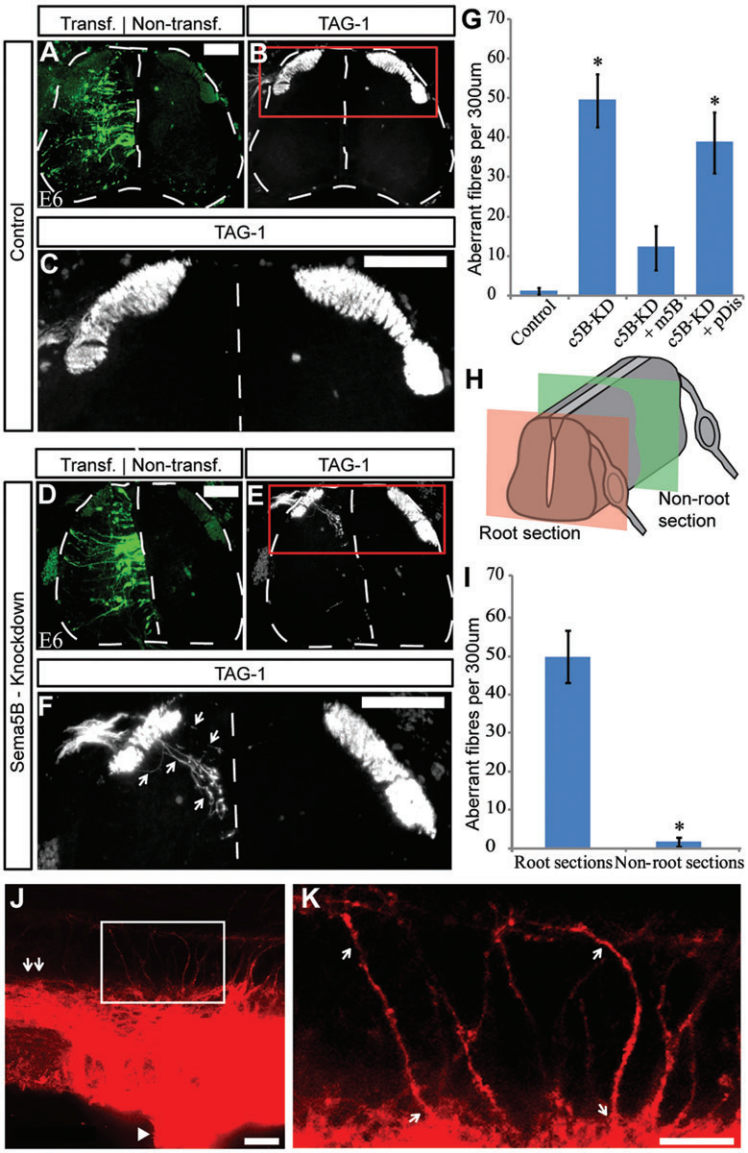

Fig. 5. Knockdown of Sema5B at E3.5 leads to premature entry of nociceptive afferents. (A,D) Positive unilateral transfection is illustrated by the expression of GFP in chick E6 spinal cord. (B,C,E,F) TAG-1 labelling shows the localisation of nociceptive axons. (A-C) Spinal cords transfected with control vectors at E3.5 show normal axon projection patterns at E6. (D-F) TAG-1-expressing nociceptive afferents prematurely enter the grey matter with the knockdown of Sema5B. Arrows in F highlight aberrant projections. Insets in B and E are enlarged in C and F. Dashed lines outline the spinal cord and the midline. (G) Significantly more cutaneous axons project prematurely into the dorsal grey matter where the expression of Sema5B has been reduced. The number of prematurely entering fibres returned to control levels when co-transfected with mouse *Sema5b* DNA construct (m5B). *n*=4-10 animals per treatment over more than three independent experiments. Error bars represent s.e.m. Unpaired *t*-test, **P*<0.005, *P*=0.66 between control and m5B rescue treatments. The knockdown phenotype was not rescued when co-transfected with an empty pDisplay plasmid (KD-pDis). (H,I) Significantly more aberrantly projecting fibres were found on root sections: 88% of root sections show the presence of aberrantly projecting fibres compared with 15% in non-root sections. *n*=5 animals. Error bars indicate s.e.m. Unpaired *t*-test, **P*<0.005. (J,K) Transverse view of DiI-labelled sensory afferents in a Sema5B knockdown spinal cord. Prematurely projecting fibres (box in J, as magnified in K) are observed at the level of the dorsal root (J, arrowhead). Collaterals extending along the dorsal funiculus are apparent (J, arrows). Higher magnification shows axons extending directly into the grey matter (K, arrows). Scale bars: 100 µm in A-F; 50 µm in J; 25 µm in K.

embryogenesis (E3) and the downregulation of Sema6A leads to the disorganisation of dorsal roots. The authors did not show, however, whether the subsequent timing or patterning of sensory afferents was changed; thus, further studies are required to fully elucidate the function of Sema6A in this light.

Sema5B functions through TAG-1

Cell adhesion molecules, including TAG-1, have long been known to function during axon guidance in processes such as fasciculation and outgrowth (Furley et al., 1990; Zuellig et al., 1992). Only recently have these molecules received attention as binding partners to mediate the responses to repulsive guidance cues in the nervous system (Law et al., 2008). TAG-1 is linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (Furley et al., 1990; Zuellig et al., 1992) and can bind homophillically to other TAG-1 molecules on adjacent cells (Freigang et al., 2000; Rader et al., 1993). Perrin et al. (2001) showed that TAG-1 is expressed by all cell bodies and axons of DRG neurons during early stages of development but its expression then becomes restricted to the NGF-dependent, TrkA-expressing nociceptive fibres after E6 (Perrin et al., 2001; Snider and Silos-Santiago, 1996). This temporal correlation provides support for TAG-1 as a component of the receptor complex for mediating the inhibitory effect of Sema5B on nociceptive sensory fibres.

Additional work by Perrin et al. (2001) has shown that TAG-1 is required for correct nociceptive cutaneous axon targeting in the dorsal region of the spinal cord (see Fig. 10). They found that, after injection of function-blocking antibodies against TAG-1 into the cerebral aqueduct of the developing spinal cord, nociceptive axons projected aberrantly into the dorsal horn (Perrin et al., 2001). Strikingly, the phenotype observed when TAG-1 function is perturbed is extremely similar to the phenotypes observed when Sema5B was knocked down in the developing spinal cord. Specifically, nociceptive axons projected prematurely into the dorsal horn grey matter (1 day before the normal time of collateral formation) and grew aberrantly toward the midline, dorsal to the central canal (the future lamina III region), instead of innervating laminae I and II as seen in normal animals. By contrast, the proprioceptive fibres were not affected by the function-blocking TAG-1 antibodies (Perrin et al., 2001). Law et al. (2008) examined the role of TAG-1 in regulating sensory axon responses to diffusible guidance cues in mice by following the pathways taken by sensory afferents in TAG-1 (*Cntn2*) null mice. Similar to observations in the chick, they first showed that TAG-1 is

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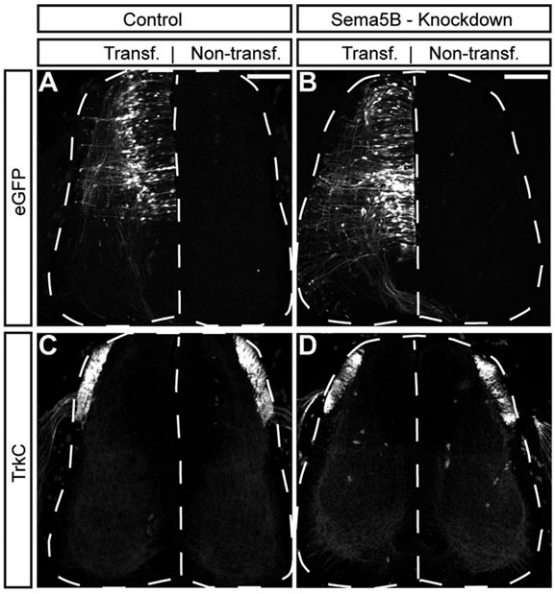

Fig. 6. Knockdown of Sema5B at E3.5 does not affect the entry of proprioceptive afferents. (A,B) GFP expression in chick E6 spinal cord shows positive unilateral transfection of control and Sema5B shRNA. (C,D) TrkC-labelled sections show no aberrant projections of proprioceptive afferents in either control (C) or Sema5B knockdown (D) spinal cords. *n*=10 animals with over 100 sections examined. Scale bars: 100 µm.

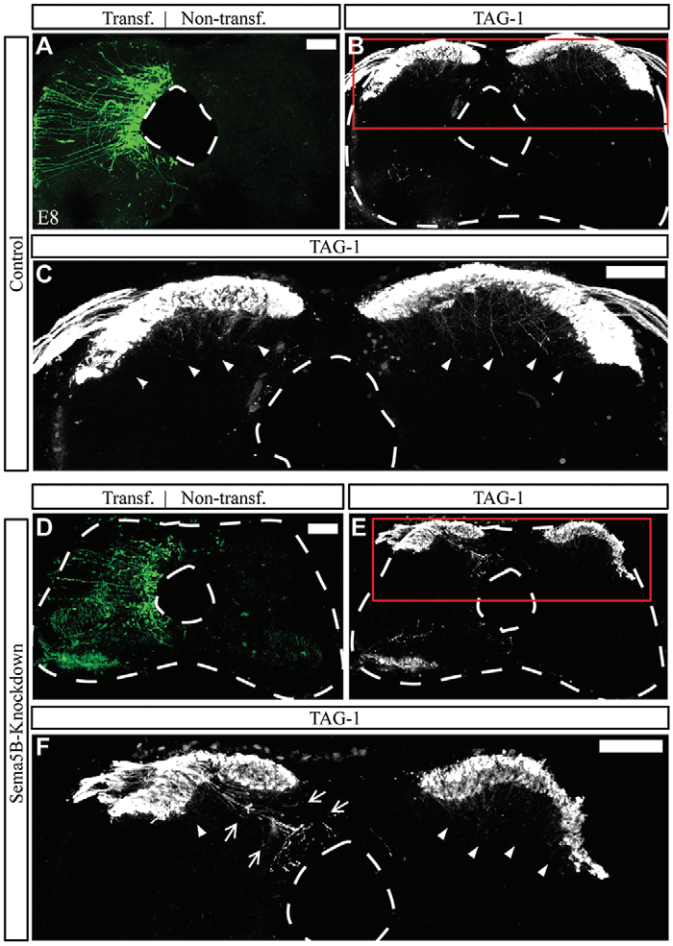

Fig. 7. Knockdown of Sema5B at E5.5-6 (St27) causes aberrant sensory fibre pathfinding. (A-C) In control electroporated chick spinal cords, normal short projections of nociceptive fibres were observed entering the dorsal horn grey matter on both control and transfected sides of the spinal cord (C, arrows). (D-F) When Sema5B expression was knocked down, axons aberrantly extended to the ventricular zone surrounding the central canal (F, arrowheads), whereas they extended normally on the non-transfected side (arrowheads). The spinal cord and central canal are outlined (*n*=5 animals). Scale bars: 100 µm.

expressed in all DRG neurons until the time of axon arrival at the DREZ (E10.5 in mice), but, by E12.5, TAG-1 expression became restricted to TrkA-expressing nociceptive fibres, 1 day before they extended collaterals into the dorsal horn. Law et al. (2008) also saw similar phenotypes in the TAG-1 null mice as were observed in our Sema5B knockdown experiments and which were also observed after TAG-1 antibody injections as discussed above (Perrin et al., 2001). They observed premature projections of cutaneous axons in TAG-1 null mice, particularly focused around points of dorsal root entry (Fig. 10). These authors argued that TAG-1 is required on sensory axons to mediate their response to a non-Sema3A diffusible repellent guidance cue(s) found in the spinal cord, although they had not identified the specific cue(s) (Law et al., 2008).

Presently, the mechanism of TAG-1 function in Sema5B signalling is unknown. Dang et al. (2012) have recently shown that TAG-1 regulates Sema3A signalling by differential endocytotic trafficking of components of the Sema3A receptor complex (Dang et al., 2012). Whether TAG-1 functions in a similar way for Sema5B signalling is not known. Additional potential receptors for Sema5B have also been described recently. Using a combination of *Sema5b* and plexin A1 and A3 null mouse lines, Matsuoaka et al. (2011) have shown that Sema5B signals in part through plexin A1 and/or A3 to regulate retinal lamination. Whether plexin A1 and A3 function with a co-receptor such as Nrp1/2 or TAG-1 is unknown.

MATERIALS AND METHODS

Animals

Fertilised White Leghorn chicken eggs were obtained from the University of Alberta and incubated at 38°C. Embryos were staged according to Hamburger and Hamilton (1951).

In situ hybridisation

Chick embryos younger than E7 were fixed in 4% (v/v) paraformaldehyde (PFA; Sigma) in diethylpyrocarbonate (DEPC)-treated PBS at 4°C for 8 h followed by washing in PBS. Chicks at E7 or older were first fixed via pericardial infusion by injecting PBS into the heart for 2 min followed by 4% PFA for 10 min. Embryos were placed in 30% sucrose in DEPC-treated PBS overnight at 4°C for cryoprotection. RNA probes were prepared using a digoxigenin labelling kit and employed as described by the manufacturer (Roche Molecular Biochemicals). Antisense digoxigenin-labelled probes were generated from the C-terminus of Sema5B. Sense probes generated from the same region were used as a control.

Neurite outgrowth assay

DRG were dissected from E4, E5, E6, E7 and E8 chick embryos into cold DMEM (Invitrogen) and dissociated in 0.25% (v/v) trypsin-EDTA (Invitrogen) as previously described (Browne et al., 2012). Neurons (8×10^4 cells) were seeded on top of a confluent layer of stable HEK293 cells expressing either an empty pDisplay vector (control) or an HA-tagged chick Sema5B (chick HA-Sema5B), similar to as previously described (Matsuoka et al., 2011). To select for the growth of nociceptive neurons, culture medium was supplemented with 40 ng/ml 7S nerve growth factor (NGF; Invitrogen), and to select for the growth of proprioceptive neurons the same amount of neurotrophin 3 (NT-3, also known as Ntf3; PeproTech) was used (Chan et al., 2008; Law et al., 2008; Messersmith et al., 1995). Primary antibody incubations were performed with mouse anti-Tuj1 (also known as Tubb3; 1:500, Sigma, T8578) for visualisation of sensory neurons and

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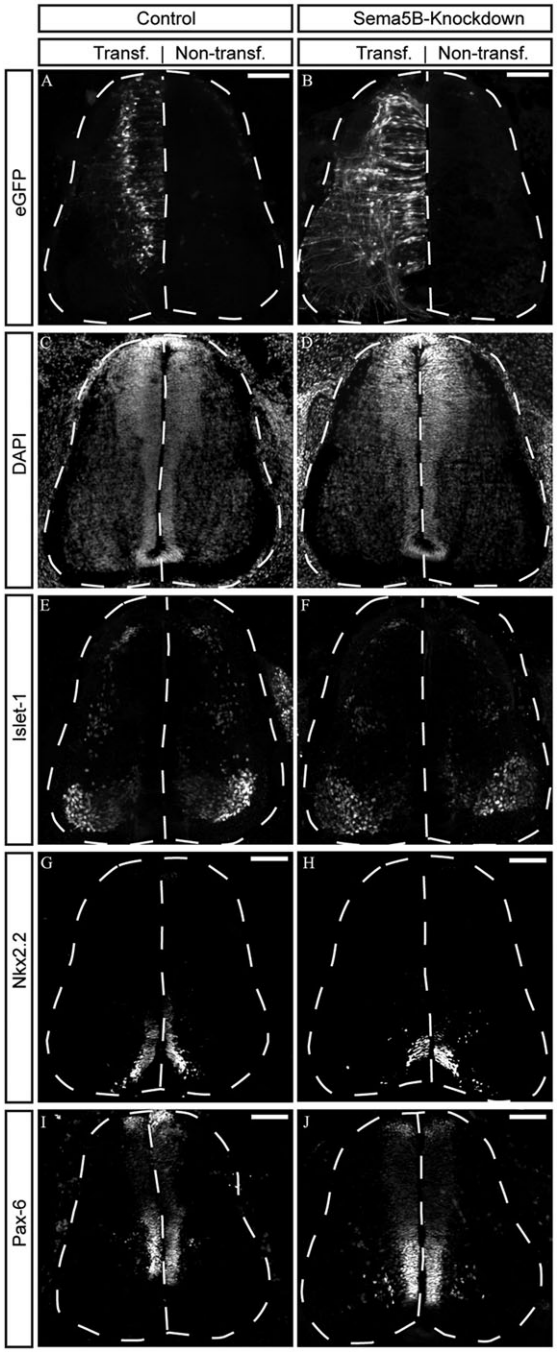

Fig. 8. Sema5B knockdown does not affect patterning and differentiation during development. (A,B) Electroporation of control and Sema5B shRNA in chick E6 spinal cords. In control (C) and Sema5B shRNA (D) transfected spinal cords, normal cell density and patterning were observed in both the transfected and the non-transfected side of the spinal cords. In addition, the expression of transcription factors Islet1 (E,F), Nkx2.2 (G,H) and Pax6 (I,J) were normal on both sides of control and Sema5B knockdown spinal cords. Scale bars: 100 µm.

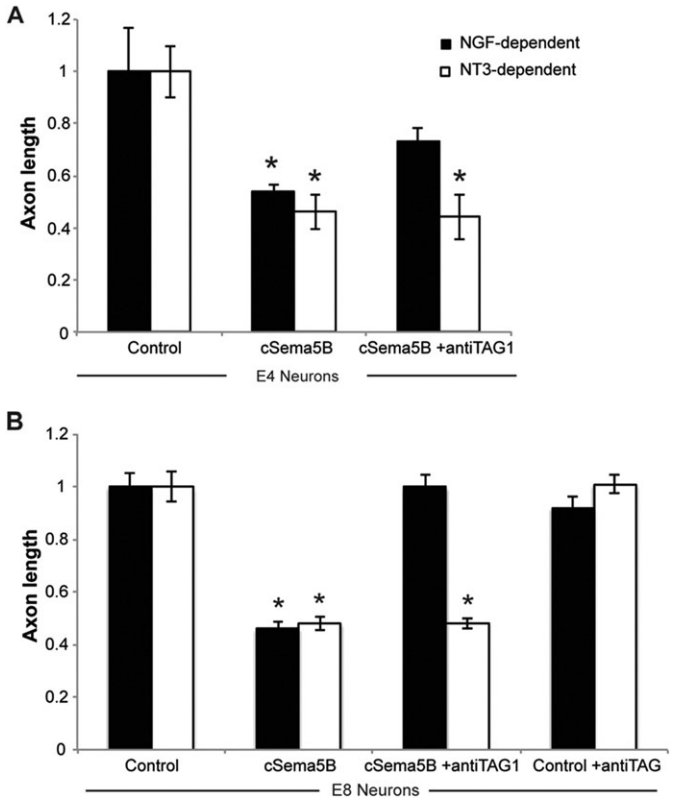

Fig. 9. Blocking TAG-1 function reduces the inhibitory effect of Sema5B. Inhibition of TAG-1 function reduces the inhibitory effect of Sema5B on NGF-dependent E4 (A) and E6 (B) neurons grown on Sema5B-expressing HEK293 cells. Blocking TAG-1 does not affect Sema5B inhibition of NT3-dependent E4 (A) or E6 (B) neuron outgrowth. In addition, TAG-1 function-blocking antibody did not affect the outgrowth of NGF-dependent or NT3-dependent neurites on control untransfected HEK293 cells (B). Unpaired Student's *t*-test, **P*<0.05. In a *t*-test between NGF-responsive neurons grown on control cells and Sema5B cells plus anti-TAG-1 antibody, *P*=0.138 for E4 neurons and *P*=0.475 for E6 neurons.

rabbit anti-HA (1:500, Cell Signaling, #3724) for visualisation of HEK293 cells expressing chick HA-Sema5B. Cultures were imaged and the length of the axons from each neuron was measured using ImageJ. For analysis of the function of TAG-1, E4 and E6 DRG were dissociated as above and incubated for 1 h at 37°C in either culture medium alone or in culture medium containing mouse anti-TAG-1 antibody (170 µg/ml, 23.4-5, Hybridoma Bank), and were then added to the appropriate cell culture wells and incubated overnight. The anti-TAG-1 antibody concentration was maintained in the cell cultures for the duration of the experiment.

Preparation and validation of shRNA vectors

Sequences for RNAi targeting were analysed using pSico Oligomaker v1.5 software (the Jacks Lab, Massachusetts Institute of Technology, USA) and the oligoduplex palindromes designed for hairpin loop formation were generated by Invitrogen. Two shRNA sequences were generated to target sequences unique to chick *Sema5b* mRNA: shRNA1 (1203), 5′-GAAATCCCTTTCTATTATA; and shRNA2 (3442), 5′-GGAGTTCAAG-ACACTTTAA. Oligoduplex palindromes were cloned into the *Xho*I/*Hpa*I restriction sites of the Lentilox 3.7 (pLL3.7) expression vector, which contains an enhanced green fluorescent protein (eGFP) sequence driven by a CMV promoter located downstream of the cloning site (Reynolds et al., 2004). shRNA plasmids were transfected into HEK293 cell lines expressing HA-tagged full-length chick Sema5B or mouse Sema5B using polyethylenimine (Polysciences) as described previously (Browne et al., 2012). The specificity of the shRNA plasmids was verified by its ability to knock down chick Sema5B expression and the lack of knockdown effect on mouse Sema5B expression. This was confirmed by western blot analyses of cell lysates as described previously (Browne et al., 2012).

In ovo electroporation

In ovo unilateral electroporation of developing chick spinal cords was performed as previously described (Nakamura and Funahashi, 2001). At the time of electroporation (E3.5/st21 and E5.5/st27), 1 µl purified plasmid DNA

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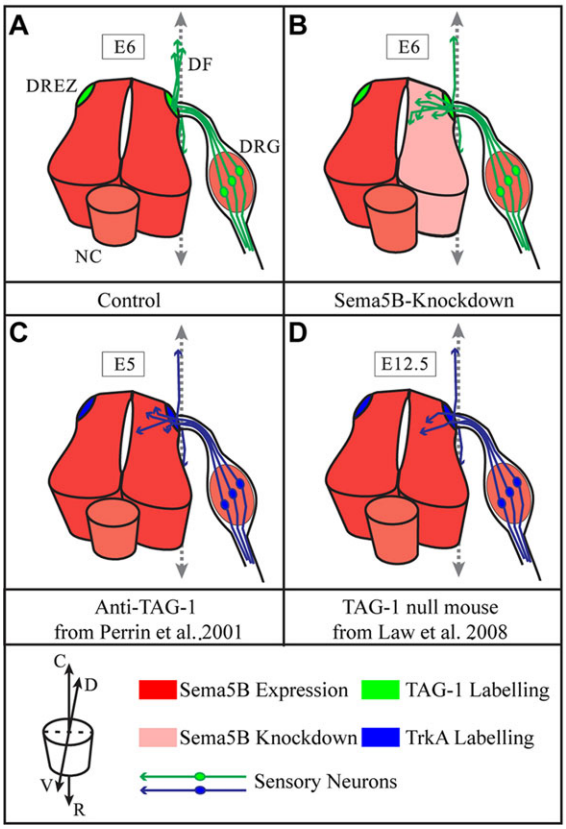

Fig. 10. Sema5B functions as a barrier to sensory axons. (A) Sema5B acts as a repulsive barrier in the grey matter, forcing axons to turn in both rostral and caudal directions to form the longitudinal tracts by E6. (B) Knockdown of Sema5B at E3.5 (right side) is sufficient to cause cutaneous afferents expressing TAG-1 to enter the dorsal horn prematurely before E6. (C) Summary of findings presented by Perrin et al. (2001). The acute loss of TAG-1 causes TrkA-positive axons to prematurely extend into the dorsal horn by E5. (D) Summary of findings presented by Law et al. (2008). Cutaneous axons expressing TrkA project prematurely into the dorsal horn in TAG-1 (*Cntn2*) null mice by E12.5. DREZ, dorsal root entry zone; DRG, dorsal root ganglion; DF, dorsal funiculus; NC, notochord; D, dorsal; V, ventral; C, caudal; R, rostral.

(4 µg/µl) was mixed with Fast Green (Sigma) at a ratio of 25:1 (by volume) and injected into the developing chick neural tube using a glass micropipette. A few drops of Hank's Balanced Salt Solution with calcium and magnesium (Invitrogen) were added on top of the embryo to facilitate electric field formation. Paddle electrodes (CUY650-P3 platinum plate tweezers electrode, Protech International) were placed on either side of the neural tube such that the DNA will migrate toward the positive electrode into one side of the developing spinal cord. At st21, electroporation was performed with five 50 ms pulses of 26 V at 1 s intervals. At st27, electroporation was performed at 45 V. The electrodes are moved along the length of the lumbosacral spinal cord to ensure sufficient electroporation of the entire region. For rescue experiments, equal amounts of the shRNA plasmids and full-length HA-tagged mouse *Sema5b* DNA constructs were co-transfected (c5B-KD+m5B in Fig. 5). As an additional control for the rescue experiments to ensure that the lack of phenotype under the rescue treatment was not due to the dilution of the shRNA plasmids injected, shRNA plasmids and an equal volume of a control pDisplay plasmid were co-transfected (c5B-KD+pDis in Fig. 5). After electroporation, the openings of the eggs were sealed and the embryos were allowed to grow further at 38°C until the desired stage for analysis.

Axon-tracing analysis

To visualise the extension of afferent axons into the grey matter of the spinal cord as well as their longitudinal extension, whole mounts of chick spinal cord were labelled with the lipophilic tracer DiI as in Schmidt et al. (2007). Control and shRNA-electroporated spinal cords were dissected with attached, intact DRG, and fixed in 4% PFA in PBS overnight. Small DiI crystals were placed against the DRG or against large peripheral nerve trunks. Spinal cords were left in PBS for 3-4 days before being imaged as whole mounts with a Leica DM6000CS confocal microscope.

Immunohistochemistry

At the desired stage, the embryonic spinal cords were dissected out into cold PBS and fixed overnight at 4°C in 4% PFA. On the next day, the spinal cords were washed in PBS and immersed in 15% (v/v) followed by 30% (v/v) sucrose solutions for cryoprotection. Spinal cords were embedded in O.C.T. (Sakura Finetek) and cross-sections of 16-30 µm were collected.

The following dilutions were used for labelling: rabbit anti-chick Sema5B (1:500) (O'Connor et al., 2009) was used to examine Sema5B expression; mouse anti-TAG-1 (as above; 1:500); rabbit anti-TrkC (1:1500, Cell Signaling, #3376); rabbit anti-HA (as above; 1:500); rabbit anti-Pax6 (1:500, Hybridoma Bank), mouse anti-Islet1 (1:500, Hybridoma Bank) and mouse anti-Nkx2.2 (1:500, Hybridoma Bank). Immunolabelling was visualised with a Leica confocal microscope. Aberrantly projecting axons were counted per section and 5-15 sections were quantified per animal. The average number of aberrant collaterals per section was calculated and averaged across the number of animals (n=4-10 chicks per treatment).

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Competing interests

The authors declare no competing financial interests

Author contributions

R.Q.L. performed the majority of experiments, analysed the data and prepared the manuscript. W.W. prepared the shRNA constructs and confirmed their knockdown of Sema5B in heterologous cells and contributed to manuscript preparation. A.L. performed *in situ* hybridisation and immunocytochemistry on developing spinal cords. J.A. generated *in situ* hybridisation probes. T.P.O. developed the experimental concepts, supervised experiments, wrote and edited the manuscript.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.103630/-/DC1

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