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The neural adhesion molecule TAG-1 modulates responses of sensory axons to diffusible guidance signals

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When the axons of primary sensory neurons project into the embryonic mammalian spinal cord, they bifurcate and extend rostrocaudally before sending collaterals to specific laminae according to neuronal subclass. The specificity of this innervation has been suggested to be the result both of differential sensitivity to chemorepellants expressed in the ventral spinal cord and of the function of Ig-like neural cell adhesion molecules in the dorsal horn. The relationship between these mechanisms has not been addressed. Focussing on the pathfinding of TrkA+ NGF-dependent axons, we demonstrate for the first time that their axons project prematurely into the dorsal horn of both L1 and TAG-1 knockout mice. We show that axons lacking TAG-1, similar to those lacking L1, are insensitive to wild-type ventral spinal cord (VSC)-derived chemorepellants, indicating that adhesion molecule function is required in the axons, and that this loss of response is explained in part by loss of response to Sema3A. We present evidence that TAG-1 affects sensitivity to Sema3A by binding to L1 and modulating the endocytosis of the L1/neuropilin 1 Sema3A receptor complex. However, TAG-1 appears to affect sensitivity to other VSC-derived chemorepellants via an L1-independent mechanism. We suggest that this dependence of chemorepellant sensitivity on the functions of combinations of adhesion molecules is important to ensure that axons project via specific pathways before extending to their final targets.

Key words: Axon guidance, Endocytosis, Neural cell-adhesion molecule, Semaphorin, Sensory neurons, Mouse, Cntn2

INTRODUCTION

Somatosensory neurons located in the mammalian dorsal root ganglion (DRG) comprise a number of different subclasses defined by the sensory information they transmit and the specific connections they make. NGF-dependent (TrkA+) afferents (including nociceptive axons) terminate in the dorsal horn of the spinal cord, whereas NT3-dependent (TrkC+) proprioceptive (Ia) afferents project to the ventral horn (Snider, 1994). Although these afferents arise from initially similar projections into the dorsal root entry zone (DREZ) followed by bifurcation and extension in the dorsal funiculus (DF), the subsequent branching of proprioceptive collaterals into the dorsal horn occurs earlier and more medially than TrkA+ collaterals (Mirnics and Koerber, 1995; Ozaki and Snider, 1997).

Differential responsiveness to spinal cord-derived chemorepellants, notably semaphorin 3A (Sema3A), may underlie the difference in the pathways taken. When proprioceptive collaterals are extending into the ventral spinal cord (Ozaki and Snider, 1997), Sema3A is expressed in the ventral horn and TrkA+ sensory collaterals remain restricted to the DF. Correspondingly, both ventral spinal cord and Sema3A repel NGF-dependent (TrkA+), but not NT3-dependent sensory axons in vitro (Fitzgerald et al., 1993; Messersmith et al., 1995; Pond et al., 2002; Puschel et al., 1996). Thus, entry into the dorsal horn was proposed to be determined by programmed sensitivity to Sema3A (Messersmith et al., 1995); initially all afferent axons are sensitive to Sema3A, but NT3-dependent axons lose this sensitivity coincident with collateral extension, owing to downregulation of a component of the Sema3A receptor, neuropilin 1 (NRP1) (Fu et al., 2000; Pond et al., 2002). Subsequent entry of TrkA+ fibres was suggested to be due to a general downregulation of Sema3A in the spinal cord (Messersmith et al., 1995).

However, although in chick Sema3A is expressed dorsally and then recedes followed closely by TrkA+ fibre entry (Fu et al., 2000), in rodents, Sema3A expression is restricted to the ventral horn from the earliest stages (Messersmith et al., 1995; Wright et al., 1995; Zou et al., 2000). A brief period of Sema3A expression in cells adjacent the dorsal funiculus (DF) may explain this in part (Wright et al., 1995), although mice lacking Sema3A or components of its receptor (NRP1, plexin A3/A4) have been reported to have relatively minor defects in the projections of NGF-dependent sensory afferents (Behar et al., 1996; Gu et al., 2003; Kitsukawa et al., 1997; Yaron et al., 2005). Together, this suggests that additional repulsive cues exist in the ventral spinal cord (Anderson et al., 2003; Masuda et al., 2003) or that local cues in the dorsal spinal cord play a role in modulating this process.

One class of molecules expressed on sensory afferents and known to mediate short-range interactions are the L1-like neural cell adhesion molecules (L1nCAMs) (Brummendorf and Rathjen, 1996). In chick, injection into the developing spinal cord of antibodies to axonin 1 or NgCAM (the orthologs of rodent TAG-1 and L1, respectively) disturbs pathfinding of nociceptive fibres (Perrin et al., 2001). However, although it is known that these molecules are binding partners, their ability to bind homo- and heterophilically (Brummendorf and Lemmon, 2001; Brummendorf and Rathjen, 1996), and their widespread expression in the developing spinal cord and on innervating sensory axons (Perrin et al., 2001), makes their precise role and site of action difficult to determine. The finding that L1 is required for cortical axon responses to Sema3A (Castellani et al., 2000) suggests these CAMs may be good candidates for short-range cues that could modulate responses to diffusible molecules in the spinal cord, particularly as L1 is known to bind both to NRP1 (Castellani et al., 2000; Castellani et al., 2002) and to TAG-1 (Cntn2 – Mouse Genome Informatics) (De Angelis et al., 2002; Kunz et al., 1998).

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Here, we show that TrkA+ afferents enter the dorsal horn prematurely in both L1 and TAG-1 mutant mice, and that NGF-dependent sensory axons require TAG-1 as well as L1 in order to be repelled by Sema3A. However, we show that, unlike L1, TAG-1 does not bind directly to NRP1 and suggest instead that TAG-1 modulates responses to Sema3A via its interactions with L1. Moreover, in the absence of TAG-1, there is a reduction in the disappearance of L1 and NRP1 from the growth cone surface that normally occurs in response to Sema3A, suggesting that TAG-1 may exert its effect by facilitating the endocytosis of the L1/NRP1 complex. Consistent with this, we find that, after Sema3A treatment, TAG-1 also disappears from the growth cone surface and can be found colocalised inside the cell with clathrin. The effect of loss of TAG-1 on TrkA+ afferent pathfinding is stronger than losing L1 and evidence is presented that TAG-1 is required for responses to other, as yet unidentified repulsive factors in the ventral spinal cord. These findings indicate a wider role for L1nCAMs in modulating responses to diffusible long-range inhibitory molecules.

MATERIALS AND METHODS

Animals

Production, characterisation and maintenance of L1 and TAG-1 null mutant mice was as reported (Cohen et al., 1998; Poliak et al., 2003). Mice were maintained on 129SvEv (L1 null; TAG-1 null) or C57/Bl6 (TAG-1 null; TAG-1^{a}) backgrounds. The phenotypes and data reported for TAG-1 nulls were the same on C57Bl6 or 129SvEv backgrounds.

TAG-1^{a} mutant allele was generated using a 1.65 kb fragment 5′ to *Eco*RI in exon 3 (13586-13730 in GenBank NC_000067) and 7.5 kb 3′ to *Eco*RI* site in exon 6 (14632-14841) cloned flanking MC1neopA and flanked by pMC1tk and PGKtk (Fig. 6). Homologous recombination in Bruce4 ES cells and germline chimaeras were created as described (Kontgen et al., 1993). Routine PCR genotyping was carried out using primers in exon 5 (5′-GGAGGAGAGAGACCCCGTGAAA-3′) and exon 6 (5′-ACACGAA-GTGACGCCCATCCGT-3′) plus neo-specific primers (Fig. 6C). Western analysis of postnatal cerebellum lysates was as described (Dodd et al., 1988). RT-PCR (Hybaid protocol) was carried out using postnatal cerebellum RNA using a primer in exon 2 (5′-TCTCAGTCTCCAGTTGACTCTCCTG-3′), with primers in exon 8 (5′-GAGTTCTCTGCCTCACATTCATAGG-3′), exon 9 (5′-CATCACAGCCCCAACGTAAGTT-3′) or exon 10 (5′-AATCGCAGGTCCCCAGCCAA-3′ (a-d respectively in Fig. 6E). Product from primers a and d was cloned for sequencing.

Noon of day when copulation plug found designated embryonic day 0.5 (E0.5). Animals generated and maintained with appropriate UK Home Office and Local Ethical Committee approval.

Immunodetection

Antibodies used were TAG-1 [monoclonal (mAb) 4D7, rabbit anti-TAG-1 (Dodd et al., 1988), TG3 polyclonal (Denaxa et al., 2001)]; L1 [mAb324; Chemicon (Lindner et al., 1983)]; neurofilament [mAb2H3 (Dodd et al., 1988)]; TrkA (rabbit anti-TrkA, a gift from L. Reichardt); neuropilin 1 (rabbit anti-neuropilin 1, a gift from A. Kolodkin); human immunoglobulin Fc domain (Goat anti-human Fc; Sigma); alkaline phosphatase (mouse IgM; Sigma). Immunodetection was carried out as described (Cohen et al., 1998).

DNA constructs, protein production and blocking reagents

DNA expression constructs gifts were as follows: Sema3A from A. Püschel (Puschel et al., 1996); full-length and soluble (AP and Fc fusions) neuropilin 1 (NRP1) and NRP2 from A. Kolodkin (Gu et al., 2003; Kolodkin et al., 1997); full-length rat TAG-1 in pcDNA-1 from R. Jia and T. Jessell (Furley et al., 1990); full-length ratL1 from D. Felsenfeld; huL1-Fc fusion from S. Kenwrick (De Angelis et al., 1999); full-length plexin A4 from A. Yaron and M. Tessier-Lavigne (Yaron et al., 2005); TAG-1-Fc fusion as described (Poliak et al., 2003). Cos7 cells were transiently transfected using lipofectamine (Invitrogen). Sema3A production was achieved using hanging-drop aggregates as described (Anderson et al., 2003); the amount of DNA transfected was titrated to produce repulsion of dorsal root ganglion (DRG) axons comparable with ventral spinal cord (VSC) explants co-incubated at a similar distance (cf. Anderson et al., 2003). Cell-binding assays with soluble ectodomain fusions as described (Anderson et al., 2003; Tamagnone et al., 1999). Growth cone collapse assays used Sema3A/mock-conditioned media from lipofectamine-transfected HEK293T cells (Kitsukawa et al., 1997).

TAG-1 function was blocked using monoclonal (4D7) and polyclonal (TG3) antibodies titrated for each repellant source to give maximal repulsion blockade. Glycosyl phosphatidylinositol (GPI) anchored membrane proteins were cleaved using phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringienis* (Stoeckli et al., 1996).

Axon repulsion and growth cone collapse assays

Collagen co-culture axon repulsion assays as described (Messersmith et al., 1995). DRG and VSC dissected from thoracic segments of E13.5 embryos. DRG explants were positioned 200-300 μm from VSC or mock-/Sema3A-transfected Cos7 cells and cultured with 50 ng/ml nerve growth factor (NGF) for 36 hours. Axons were counted in proximal (P; Fig. 3D) and distal (D) quadrants, then P/D ratio calculated. Data were analysed using Student's paired *t*-test (P versus D axons), Student's unpaired *t*-test (to compare mean P/D ratios between DRG from different genotypes) and ANOVA (to standardise separation distances). Similar results obtained when axon lengths were measured (not shown).

Growth cone collapse: E13.5 DRG were similarly cultured on PDL/laminin-coated coverslips. After ~18 hours of growth, Sema3A- or mock-conditioned media were applied for 30 minutes, before fixation with 4% PFA. The percentage of collapsed growth cones was calculated and differences analysed using Student's *t*-test.

Analysis of endocytosis

DRG were cultured as in the collapse assays; however, the Sema3A treatment lasted for 45 minutes. Cultures were stained on ice using anti-L1 and anti-NRP1 or anti-TAG-1 in L-15 for 20 minutes, fixed in 4% PFA and the secondary antibody [including FITC-Phalloidin (SIGMA), where appropriate] was applied. Lack of neurofilament staining confirmed inaccessibility of internal proteins. For experiments examining TAG-1/clathrin colocalisation, explants were treated with Sema3A for 10 minutes, fixed in 4% PFA, permeabilised, stained overnight in anti-clathrin heavy chain (X22, a gift from E. Smythe) and anti-TAG-1, then stained with appropriate secondary antibodies. Images were captured using a Volocity Grid Confocal (Improvision) on an Olympus BX61. Image analysis was performed using Volocity Classification. Briefly, average intensity of signal per unit area for the distalmost 50 μm of a growth cone was normalised to the average intensity of a 300 μm^2 region of cell-free background. *P*-values were calculated by use of either one-tailed (wild type versus wild type+Sema) or two-tailed (wild type+Sema versus null+Sema) Student's *t*-tests with Welch's correction (to account for differences in variance) being applied where appropriate. Values plotted (Fig. 8) are mean±s.e.m.

RESULTS

Co-expression of TAG-1 and L1 on TrkA+ spinal sensory afferents

The expression of TAG-1 and L1 on developing spinal sensory fibres has been detailed in chick (Perrin et al., 2001), but not in mouse. We compared expression of TAG-1 and L1 on TrkA+ afferents on embryonic days 12.5 to 15.5 (E12.5-E15.5; Fig. 1). For simplicity, the different fibre subsets will be referred to as TrkA+ and NGF dependent (to include both mechanoreceptive and nociceptive neurons) or TrkA– (proprioceptive, Ia, TrkC+ or NT3 dependent neurons).

In the thoracic spinal cord, afferents of early-born proprioceptive neurons arrive in the DREZ just before E10.5 and bifurcate to form the primordial dorsal funiculus [DF (Ozaki and Snider, 1997)]. New afferents continue to arrive and bifurcate over the next few days, taking progressively more lateral positions in the DF, the later-arriving and more lateral being strongly TrkA+

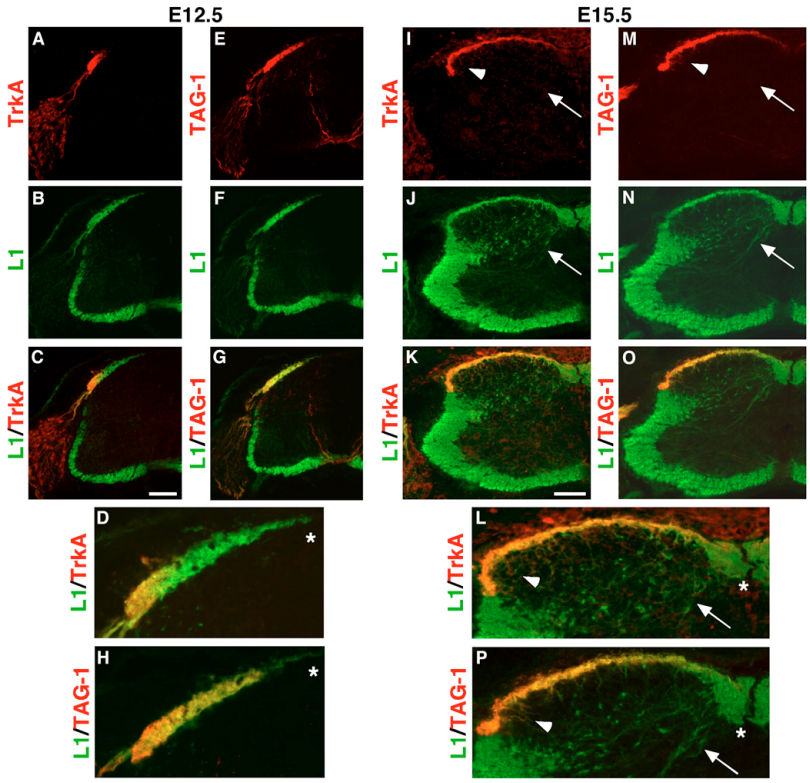

Fig. 1. Expression of TAG-1 and L1 on spinal sensory afferents. (**A-H**) Transverse sections of E12.5 spinal cord immunolabelled for TrkA and L1 (A-D) or L1 and TAG-1 (E-H). Magnified images of the dorsal funiculus (DF) from C and G shown in D and H, respectively. Asterisks indicate L1+, TAG-1/TrkA– region. (**I-P**) Transverse sections of E15.5 spinal cord with TrkA and L1 (I-L) or TAG-1 and L1 (M-P). Higher magnification of the DF from K and O is shown in L and P. Asterisks indicate L1+, TAG-1/TrkA– region. Arrows indicate position of TrkA– proprioceptive fibres emanating from the L1+, TAG-1/TrkA– region. Arrowheads indicate TrkA+ fibres. Scale bar: ~100 μm.

(Fig. 1A,C,D) (Ozaki and Snider, 1997; Perrin et al., 2001). By E13.5, the first arriving TrkA– afferents begin to extend collaterals into the dorsal horn, reaching motoneuron targets by E15.5 (arrows in Fig. 1J,L,N,P). By contrast, the very first TrkA+ collaterals do not begin to appear in the dorsal horn until E14.5-E15.5 (Fig. 1I,K,L) and are not abundant until later stages (see below).

Expression of TAG-1, L1 and TrkA extensively overlaps on the dorsal roots and DF at E12.5 (Fig. 1A-H). However, whereas L1 is expressed on all axons within the DF (Fig. 1B-D,F-H), TrkA is restricted to the lateral region (Fig. 1A,C,D), indicating that L1 is expressed on both TrkA+ and TrkA– sensory axons. Although at E11.5 TAG-1 is present throughout the DF similar to L1 [not shown (Dodd et al., 1988)], by E12.5 its expression appears to be graded from high laterally to low or absent medially (Fig. 1E,G,H; asterisk indicates an L1+ TAG-1– region) and by E15.5 this difference becomes marked and the most medial fibres express only L1 (asterisks in Fig. 1H-N). These TrkA–, L1+ fibres (Fig. 1K,L) are almost certainly proprioceptive axons; their collaterals can be seen extending ventrally through the dorsal horn towards targets in the ventral spinal cord (Fig. 1I-P, arrows). By contrast, all TrkA+ fibres express TAG-1 and L1, including the few collaterals that have begun to extend (arrowheads in Fig. 1I,L,M,P). Therefore, TrkA+ afferents co-express TAG-1 and L1, whereas the TrkA– afferents continue to express L1, but downregulate TAG-1 at the time of collateral extension into the dorsal horn.

TrkA+ afferents project prematurely in TAG-null and L1-null mice

To determine whether loss of L1 or TAG-1 has an effect on TrkA+ sensory afferent guidance, we followed the path of these fibres in null mutant animals (Cohen et al., 1998; Poliak et al., 2003) (Fig. 2). Significant numbers of TrkA+ axons were found projecting into the dorsal horn from the DF at E12.5 and E13.5 in animals lacking either L1 or TAG-1, whereas few if any such projections are found in wild-type littermates (Fig. 2A-H,M). By E14.5, although a few TrkA+ collaterals were now present in wild-type animals, considerably more were found in the mutants (Fig. 2I,K-M). At this latter age, not only were aberrant projections more prevalent, but they were also significantly greater in length (Fig. 2N), sometimes projecting almost to the midline on both sides of the spinal cord in TAG-1 mutants (Fig. 2I,K). Aberrant projections also occurred all along the mediolateral extent of the TrkA+ region of the DF, with as many as 40% of projections located medially at E14.5 compared with only 5% in wild type (Fig. 2O). Even at E12.5 as many as 20% could be found in the medial half of the dorsal horn (not shown), suggesting that in mutants these projections are aberrant, rather than the result of accelerated development.

There was no obvious qualitative difference in the aberrant projections in TAG-1 nulls compared with L1 nulls – in both cases length and projection angle varied considerably – but quantitation of the number of such projections revealed significantly more in the TAG-1 mutants at all ages [as many as 70% more projections in TAG-1 versus L1 nulls (E13.5); *t*-test, $P<0.001$; Fig. 2M].

TAG-1 and L1 null sensory axons fail to respond to ventral spinal cord

Our observations indicate that TAG-1 and L1 are required to prevent premature entry of TrkA+ sensory afferents axons into the dorsal horn, in broad agreement with antibody perturbation experiments carried out in chick (Perrin et al., 2001). This could reflect a requirement for these molecules to mediate interactions with binding partners in the dorsal horn (Perrin et al., 2001). Alternatively, these molecules may be necessary for axonal responses to diffusible chemorepellants emanating from the ventral spinal cord [VSC

(Messersmith et al., 1995)], especially as cortical axons from neonatal mice lacking L1 are reported to be insensitive to Sema3A (Castellani et al., 2000).

To test whether embryonic sensory axons lacking L1 or TAG-1 are insensitive to diffusible cues from embryonic VSC, dorsal root ganglia (DRG) from wild-type or mutant embryos at E13.5 were co-cultured with wild-type VSC in collagen gels in the presence of NGF. As expected, wild-type DRG axons were robustly repelled away from the VSC explant (Fig. 3A). By contrast, DRG from L1-null embryos, or from TAG-1 null embryos, were repelled to a far lesser extent (Fig. 3B,C). Results were quantified by comparing the number of axons growing towards the explant in the proximal (P) quadrant with those growing away in the distal (D) explant (Fig. 3D). The ratio of these numbers (P/D) for wild-type DRG is low, indicating strong repulsion, whereas this ratio was ~0.70 for L1-null axons and ~1.00 for TAG-1 null axons, indicating, respectively, partial or complete loss of repulsion by VSC. The difference between these results is significant (P<0.01, unpaired t-test), suggesting that L1 null axons remain partially responsive to VSC-derived repellants (Fig. 3E). Similar results were obtained by blocking TAG-1 function using monoclonal (4D7) or polyclonal (TG3) anti-TAG-1 antibodies or phosphoinositol-specific phospholipase C (PI-PLC) to cleave TAG-1 from the cell surface (Fig. 3E; not shown), though in these cases the axons consistently remained partially responsive. Thus, acute loss of TAG-1 function, as well as long-term genetic ablation of the TAG-1 gene, leads to a loss of response to VSC. Therefore, NGF-dependent sensory axons require both L1 and TAG-1 to be fully responsive to VSC-derived chemorepellants.

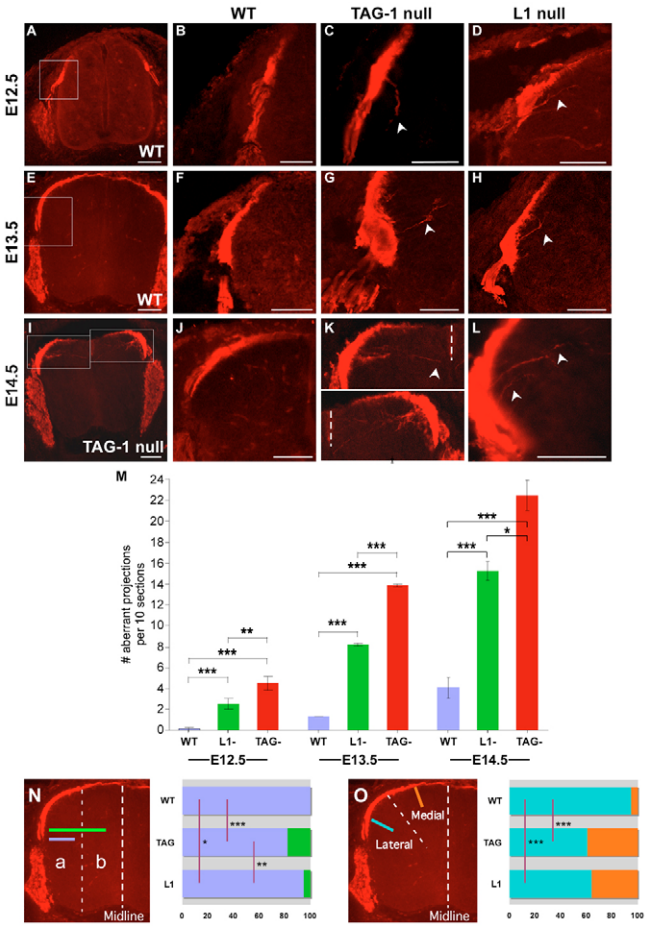

Fig. 2. Aberrant projections of sensory afferents. (**A-L**) Spinal cord sections immunolabelled for TrkA at E12.5 (A-D), E13.5 (E-H) and E14.5 (I-L) in wild-type (WT; A,B,E,F,J), TAG-1-null (C,G,I,K) and L1 null (D,H,L) mice. Boxes in A and E indicate typical regions shown in B-D and F-H, respectively, at higher power; boxes in I are magnified in K; broken lines indicate the dorsal midline. Arrowheads highlight aberrant projections. Scale bars: ~100 μm. (**M**) Mean number of aberrant projections calculated at E12.5, E13.5 and E14.5 in wild-type (n=8, 4 and 6 embryos, respectively), L1-null (n=6, 3 and 5) and TAG-1-null mice (n=7, 5 and 6). Horizontal bars indicate significant differences (unpaired t-test, ***P<0.001, **P<0.01, *P<0.05). Error bars throughout are s.e.m. (**N**) Proportion of axons projecting into medial (green) or lateral (blue) regions. Red lines indicate significant differences (Chi-squared test; significance as above). (**O**) Proportion of axons projecting from medial (orange) or lateral (cyan) regions of the DF. Red lines indicate significant differences (Chi-squared test; significance as above).

NGF-dependent sensory axons lacking TAG-1 fail to respond to Sema3A and other chemorepellants

The repulsion of NGF-responsive sensory afferents by VSC has been shown to be mediated in part by Sema3A (Fu et al., 2000; Messersmith et al., 1995; Shepherd et al., 1997). However, the differential loss of response to VSC explants exhibited by L1 compared with TAG-1 null DRG (Fig. 3E) suggested the possibility that other chemorepellants were acting in our assay, in accordance

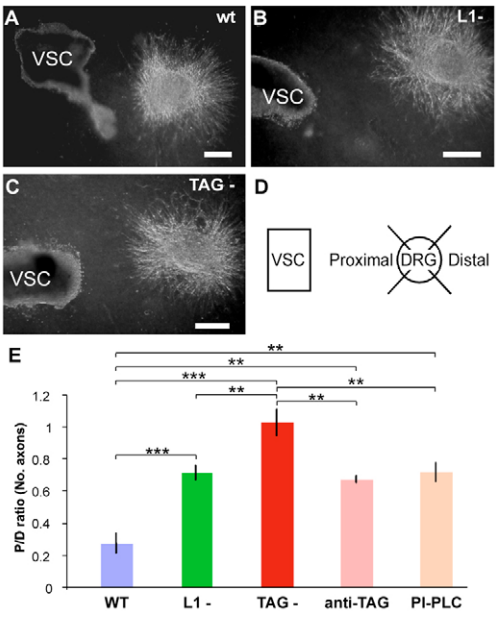

Fig. 3. Mutant sensory axons fail to respond to ventral spinal cord. (**A-C**) Co-cultures of wild-type E13.5 ventral spinal cord (VSC) with DRG from E13.5 wild-type (A), L1-null (B) or TAG-1-null (C) embryos in collagen gels. (**D**) Quantitation of axon growth: DRG were divided into quadrants and axons in each counted to give proximal:distal (P/D) ratio. (**E**) Graph showing the P/D ratios in wild type (WT; mean=0.269, s.e.m.=0.06, n=27), L1 null (mean=0.713, s.e.m.=0.043, n=15), TAG null (mean=1.03, s.e.m.=0.08, n=15), wild type plus polyclonal anti-TAG-1 (mean=0.67, s.e.m.=0.02, n=7) and wild type plus PI-PLC (mean=0.72, s.e.m.=0.06, n=7). ***P<0.001, **P<0.01, *P<0.05; unpaired t-test. Similar results were obtained counting axon length (not shown). Scale bars: 100 μm.

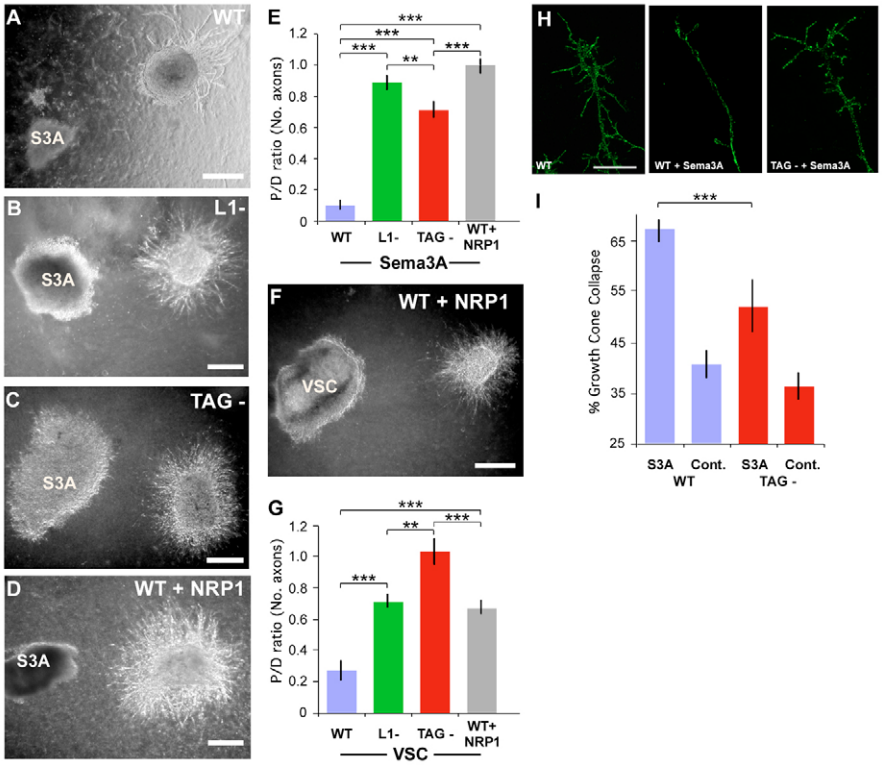

Fig. 4. Mutant sensory axons fail to respond to Sema3A chemorepulsion. (**A-C**) Co-cultures of Sema3A-transfected Cos7 cell aggregates (S3A) with E13.5 DRG from wild-type (A), L1-null (B) or TAG-1-null (C) embryos. (**D**) Wild-type DRG co-cultured with Sema3A-transfected Cos7 cells in the presence of soluble NRP1-AP. (**E**) P/D ratios of axons numbers in wild type (WT; mean=0.096, s.e.m.=0.03, *n*=25), L1 null (L1–; mean=0.88, s.e.m.=0.047, *n*=20), TAG-1 null (TAG–; mean=0.71, s.e.m.=0.05, *n*=15) and wild type plus soluble NRP1-AP (WT+NRP1; mean=1.0, s.e.m.=0.04, *n*=10). (**F**,**G**) Wild-type DRG and VSC cultured together with soluble NRP1-AP (F) and plotted with data from Fig. 3E for comparison (G). There was no significant difference ($P>0.05$) between L1– and WT+NRP1 in either E or G. Scale bars: 100 μm. (**H**) NGF-dependent growth cones from wild type (WT) or TAG-1 null (TAG–) with (+Sema3A) or without Sema3A. Growth cones are immunolabelled with anti-L1. Scale bar: 20 μm. (**I**) Percentage of collapsed growth cones from wild-type or TAG-1-null DRG with (S3A) or without (cont.; supernatant from mock-transfected Cos7 cells) Sema3A. Over 600 growth cones were assessed for each DRG; each datapoint is the mean of data from *n*=18 (WT+S3A), *n*=17 (WT cont.), *n*=7 (TAG– +S3A) and *n*=5 (TAG– cont.) DRG. ***$P<0.001$, **$P<0.01$; unpaired *t*-test.

with other studies (Masuda et al., 2003) and the fact that this region expresses multiple secreted inhibitory molecules (Zou et al., 2000). To test whether the altered responses of sensory neurons from L1 and TAG-1 mutant mice could be attributed to changes in response to Sema3A, we assayed their response to Sema3A expressed in Cos7 cell aggregates.

As reported for postnatal sensory neurons (Castellani et al., 2000), E13.5 L1-null sensory axons were completely refractory to Sema3A-mediated repulsion (Fig. 4B,E), even at levels of Sema3A expression that repel wild-type axons (Fig. 4A) to the same or to a greater extent than VSC under the same conditions [P/D ratio of 0.096 (Fig. 4E), compare with 0.269 (for VSC versus wild-type axons; Fig. 4G)]. Indeed, the P/D ratio obtained with L1-null, NGF-dependent sensory axons was not significantly different from that found when Sema3A activity was blocked by the addition of soluble NRP1 ectodomain-alkaline phosphatase fusion protein [NRP1-AP (Chen et al., 1998)] or when DRG were cultured alone (Fig. 4B,D,E; not shown). The response of E13.5 TAG-1 null, NGF-dependent sensory axons was also significantly reduced, though to a lesser degree than L1-null axons (P/D=0.71; Fig. 4C,E). To demonstrate that this is a direct effect on growth cone response to Sema3A, we compared TAG-1 mutant growth cones with wild-type in a growth cone collapse assay. As has been shown previously for L1-null growth cones (Castellani et al., 2000), the proportion of growth cones collapsing in response to Sema3A was significantly reduced in TAG-1-null mutants (Fig. 4H,I). Thus, NGF-dependent embryonic sensory axons require both TAG-1 and L1 to be fully responsive to Sema3A.

Because the contribution of Sema3A to VSC repulsion of DRG axons is reported to vary according to embryonic age (Masuda et al., 2003), we established its specific contribution in our assay. The repulsion of wild-type axons by E13.5 VSC was substantially reduced in the presence of soluble NRP1-AP (Fig. 4F,G), although significant repulsion remained even at concentrations of NRP1-AP that completely blocked the strong repulsion by Sema3A-transfected Cos7 cells noted above (compare Fig. 4E,G), suggesting that other repellants are active in our assay. The amount of repulsion remaining after blockade of Sema3A was similar to that when L1 function was lost (Fig. 4G). Together with our data and previous data (Castellani et al., 2000) showing that L1 is required for responses to Sema3A, this suggests that the loss of response to VSC seen with embryonic L1-null sensory axons is due to loss of sensitivity to the Sema3A synthesised in these tissues.

Whereas the loss of response of L1-null axons to VSC was partial, TAG-1 null axons showed no response at all (P/D ~1.0), a significantly greater loss than when L1 was lost or when Sema3A

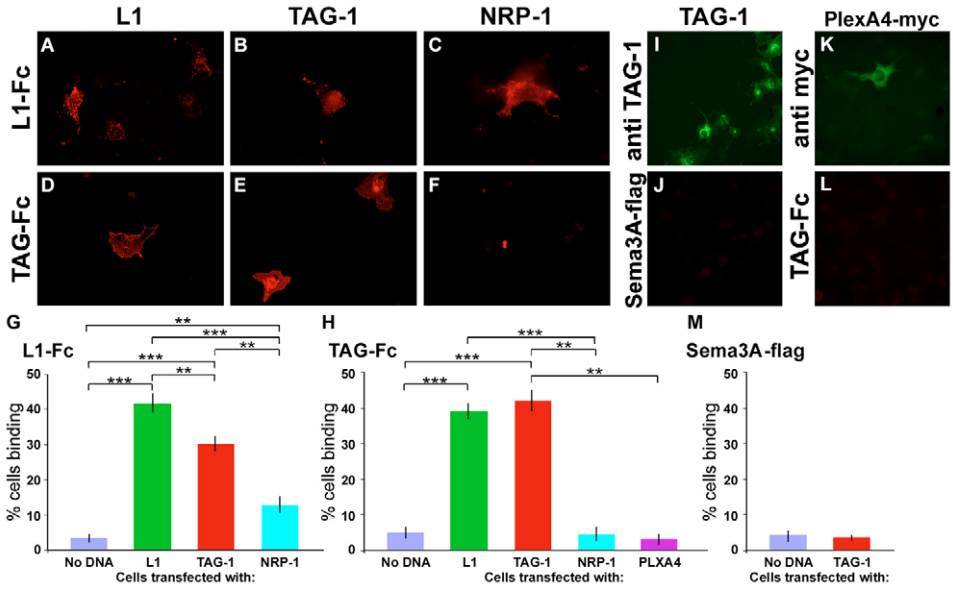

Fig. 5. TAG-1 does not bind neuropilin 1, plexin A4 or Sema3A. Cos7 cells transfected with full-length L1 (A,D), TAG-1 (B,E,I,J), NRP1 (C,F) or plexin A4 (K,L) and probed with L1-Fc (**A-C**), TAG-1-Fc protein (**D-F**,**K**,**L**) or Sema3A-Flag (**I**,**J**). Quantification of the percentage of cells binding the probe is presented in **G** (L1-Fc), **H** (TAG-1-Fc) and **M** (Sema3A-Flag). ***P<0.001, **P<0.01; unpaired t-test. The transfection efficiency (as assessed by immunolabelling; shown for anti-TAG-1 (I) and anti-Myc (detects plexin A4-myc; K) was similar in each case.

was blocked (Fig. 4G). This indicates both that TAG-1 is required for responses to Sema3A expressed in embryonic VSC and that it is probably required for responses to other VSC-derived chemorepellants. The identity of these latter factors remains unknown, but they are unlikely to be other Sema3 class molecules as addition of soluble NRP2-Fc had no effect on VSC repulsion (not shown).

Together, these data indicate that both L1 and TAG-1 are required for responses of NGF-dependent embryonic sensory axons to Sema3A and that TAG-1 is probably involved in responses to other unidentified repellants in VSC.

TAG-1 does not bind directly to Sema3A, neuropilin 1 or plexin A4

L1 has been shown to bind directly to NRP1 and mediate its Sema3A-induced endocytosis (Castellani et al., 2000; Castellani et al., 2002; Castellani et al., 2004). To establish whether TAG-1 also binds components of the Sema3A receptor expressed by sensory axons (Yaron et al., 2005), we assayed the binding of soluble protein to full-length proteins expressed on the surface of Cos7 cells (Fig. 5). As expected, this assay demonstrated the homophilic binding of both L1 and TAG-1, and heterophilic binding between the proteins (Fig. 5A,B,D,E). We were also able to demonstrate binding of soluble L1-Fc to membrane-bound NRP1 (Fig. 5C). By contrast, soluble TAG-1-Fc did not bind either NRP1- or plexin A4-expressing cells (Fig. 5F,L), even though the same protein bound L1- and TAG-1-expressing cells at relatively high frequency (Fig. 5G,H). Similarly, whereas flag-tagged Sema3A collapsed growth cones, it did not bind to cells expressing TAG-1 (Fig. 5J,M). Thus, although we were able to confirm the binding of L1 to NRP1 (Castellani et al., 2000; Castellani et al., 2002), our data indicate that TAG-1 binds neither NRP1, plexin A4 or Sema3A.

Deletion of L1-binding domains from TAG-1 is sufficient to disrupt response to Sema3A

TAG-1 might be involved in responses to Sema3A through its known cis interactions with L1 (Bizzoca et al., 2003; Buchstaller et al., 1996; Malhotra et al., 1998; Rader et al., 1996). Binding of TAG-1 to L1 is mediated by the first four Ig domains of TAG-1 and deletion of any of these disrupts binding (De Angelis et al., 1999; Rader et al., 1996). Therefore, we made use of a hypomorphic targeted TAG-1 allele which disrupts these domains. This allele, which we call TAG-1^{a}, was created through deletion of part of the TAG-1 locus between exons 3 and 6 that encodes the first three Ig domains (Fig. 6A). Aberrant splicing of exon 2, which encodes the start codon and leader sequence, to exons 7 or 9 in these mutants (Fig. 6E-H) results in the production of truncated anti-TAG-1 immunoreactive proteins, lacking the first two (minor product) or three Ig domains (major product) but retaining the leader sequence (Fig. 6H,J) at ~50% of the wild-type level (Fig. 6D; data not shown). We confirmed that these truncated protein products reach the cell surface by staining live cells in conditions in which intracellular proteins are not detectable (Fig. 6I). Thus, TAG-1^{a} homozygous mice (TAG-1$^{a/a}$) make no detectable wild-type TAG-1 protein (Fig. 6D), but instead produce truncated proteins lacking the domains necessary for binding to L1. As reported for the TAG-1-null allele (Poliak et al., 2003), TAG-1^{a} homozygotes are viable and without overt phenotype as adults.

We tested whether deletion of the L1-binding domains of TAG-1 is sufficient to mimic complete loss of TAG-1 protein in responses to Sema3A. In co-cultures with Sema3A-expressing Cos7 cells, NGF-dependent sensory axons from TAG-1$^{a/a}$ embryos displayed a significant decrease in response, comparable with that seen with TAG-1 null axons and likewise significantly less than the loss seen with L1-null axons (Fig. 7A,B). This indicates that loss of the L1-binding domains of TAG-1 indeed has an effect on axonal Sema3A responses equivalent to complete loss of TAG-1 protein.

TAG-1 mediates responses to non-Sema3A repellants via domains other than those that bind L1

Experiments above suggested that Sema3A was only partially responsible for the repellant activity of VSC and that TAG-1, but not L1, was important for responses to both the Sema3A and the non-Sema3A components of the repulsion derived from this tissue. If loss of the L1-binding domains of TAG-1 was simply equivalent to loss of all TAG-1 protein, TAG-1$^{a/a}$ sensory axons should also display a

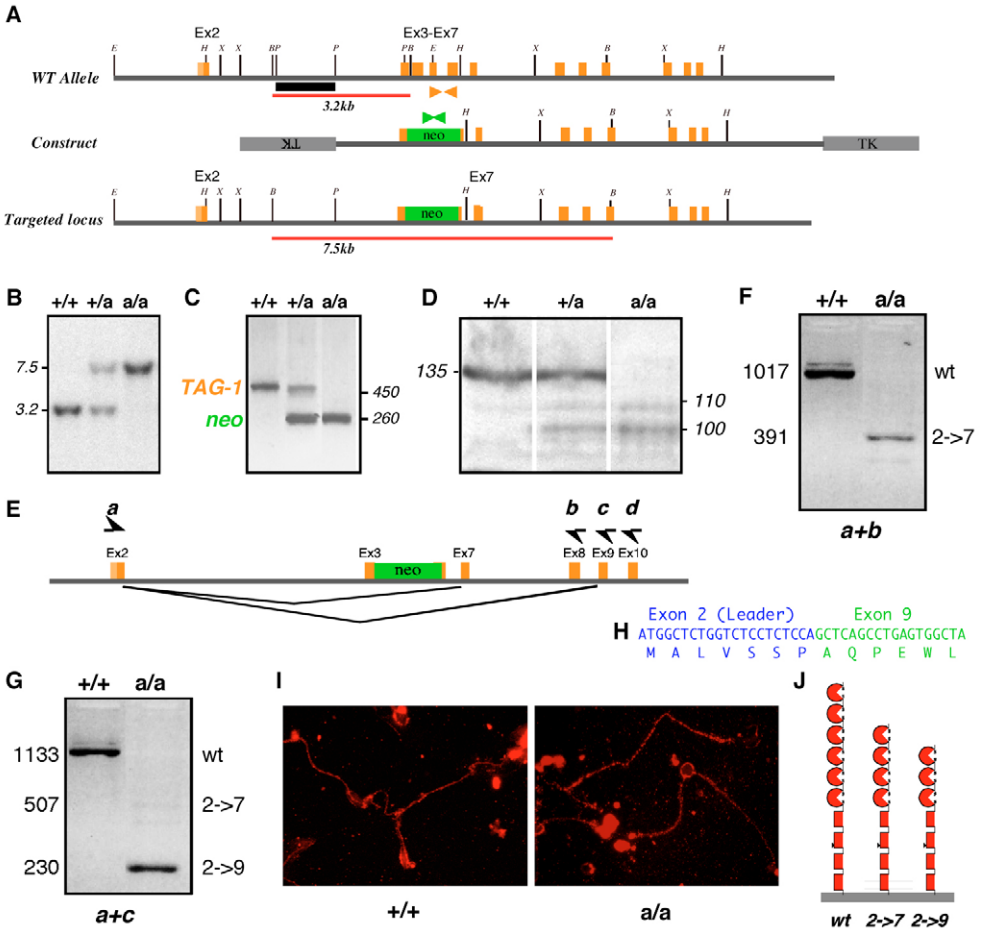

Fig. 6. Construction and analysis of a TAG-1 mutant allele that encodes N-terminally truncated TAG-1 protein. (**A**) Recombination strategy showing part of TAG-1 gene locus, including exon 2 (ATG and leader) and exons 3-13 (Ig domains 1-5), targeting construct and targeted TAG-1^{a} locus. Red lines indicate diagnostic *Bam*HI fragments; black box, probe PP; arrowheads, polymerase chain reaction (PCR) primers. (**B**) Southern analysis of targeted ES cells with *Bam*HI digest and probe PP. (**C**) PCR detection of wild-type (+/+) allele (orange primers in A) ~450 bp product; TAG-1^{a} allele (green primers in A) ~260 bp product. +/a, heterozygote; a/a, homozygote. (**D**) A major 135 kDa product detected by anti-TAG-1 in western analysis of +/+ and +/a mice, but not in a/a. Novel bands at ~100 kDa and 110 kDa present only in +/a and a/a (see J). (**E**) Aberrant splicing of mRNA from TAG-1^{a} allele and primers (arrows) used for reverse-transcription (RT) PCR in F and G. (**F**) RT-PCR detects aberrant exon 2-7 splice using a and b primers (see E); 391 bp in a/a animals instead of normal 1017 bp product (+/+). (**G**) RT-PCR using a and c primers detects exon 2-9 splice (230 bp) in a/a instead of 1133 bp product (+/+). Faint band at 507 bp=exon 2-7 splice. (**H**) Cloning and sequencing of exon 2 to 9 product (a and d primers in E) confirms splice is in-frame. (**I**) Surface immunolabelling of wild-type and TAG-1$^{a/a}$ sensory neurons with anti-TAG-1 (see Materials and methods). (**J**) Predicted proteins. Full-length TAG-1 mRNA encodes a 1041 aa protein, calculated MW 113 kDa (compare with observed MW of 135 kDa) (D). Exon 2-7 splice: 831 aa; calculated MW 89.9kDa; observed 110 kDa. Exon 2-9 splice: 740 aa; calculated MW 79.7 kDa; observed 100 kDa.

total loss of response to VSC. However, if TAG-1 is involved in chemorepellant responses independent of L1 and Sema3A, deletion of the L1-binding domains may not affect this function. To test this possibility we assayed responses of TAG-1$^{a/a}$ sensory axons to VSC and compared these with our previous results. Although TAG-1$^{a/a}$ axons showed a significant decrease in response, this decrease was significantly less than that seen with TAG-1 null axons and similar to that seen with L1-null axons (Fig. 7C,D). Thus, in response to VSC, TAG-1$^{a/a}$ axons behave comparably with L1-null axons and with wild-type axons when Sema3A activity is blocked (Fig. 4G), but retain a residual response that is completely lost in TAG-1-null axons. This is consistent with TAG-1 being necessary for responses to Sema3A via its L1-binding domains, but also being required for responses to other, non-Sema3A chemorepellants through an L1-independent mechanism.

Loss of TAG-1 alters the internalisation of L1 with neuropilin 1 on sensory axons

Whereas sensory axons lacking L1 are completely refractory to Sema3A repulsion, TAG-1 null axons remain partially responsive. This suggested that TAG-1 in some way enhances L1 function in Sema3A responses. By analogy with the role of TAG-1-like molecules in stabilising or localising CASPR molecules at the node of Ranvier (Boyle et al., 2001; Faivre-Sarrailh et al., 2000; Poliak et al., 2003), we considered the possibility that TAG-1 might affect levels of L1 protein expression. However, we were unable to detect any obvious change in L1 expression in TAG-1 mutants on cryostat sections or by western blotting (not shown). We were also unable to see any change in the amount of L1 protein reaching the plasma membrane of cultured TAG-1-null sensory axons labelled in conditions in which only extracellular

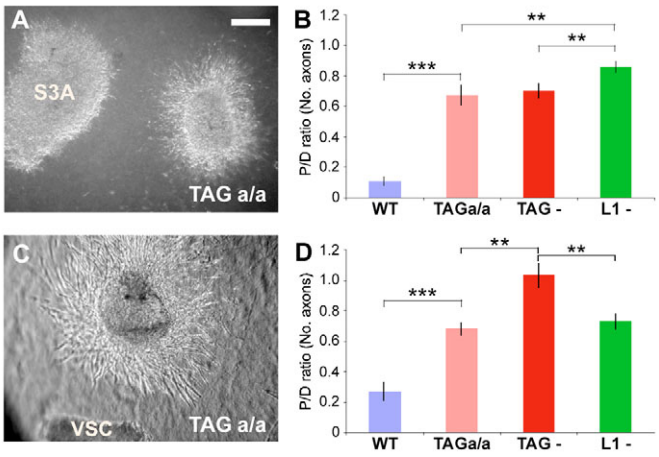

Fig. 7. Deletion of L1-binding domains from TAG-1 disrupts responses to Sema3A and ventral spinal cord. (**A**) E13.5 TAG-1$^{a/a}$ DRG co-cultured with Sema3A-expressing Cos7 cells. (**B**) P/D ratio of number of axons from TAG-1$^{a/a}$ DRG cultured with Sema3A (mean=0.677, s.e.m.=0.068, n=8) compared with wild type, TAG-1-null and L1-null results (from Fig. 4E). (**C**) E13.5 TAG-1$^{a/a}$ DRG co-cultured with VSC. (**D**) P/D ratio of number of axons from TAG-1$^{a/a}$ DRG cultured with ventral spinal cord (mean=0.68, s.e.m.=0.042, n=15) compared as in B. ***P<0.001, **P<0.01; unpaired t-test. Scale bar: 100 μm.

proteins were detectable (Fig. 8A,B), which we confirmed quantitatively by structured illumination (GRID) confocal microscopy (Fig. 8I; see Materials and methods). Thus, loss of TAG-1 does not appear to affect levels of L1 at the cell surface of normally growing axons.

Because both NRP1 and TAG-1 are known to associate with L1 on the cell surface (Buchstaller et al., 1996; Castellani et al., 2004), and because Sema3A binding to NRP1 leads to co-internalisation with L1 in Cos7 cells (Castellani et al., 2004), we considered whether loss of TAG-1 might affect this internalisation. We first established that, as for L1, levels of NRP1 at the cell surface in TAG-1 mutants were similar to those in wild-type growth cones (Fig. 8C,D,J). We next determined whether the internalisation of L1 and NRP1 in response to Sema3A was affected by loss of TAG-1. We showed first that our system of quantitation could detect the disappearance of L1 and NRP1 from the cell surface; the relative intensity of both L1 and NRP1 fluorescence dropped significantly after Sema3A treatment (Fig. 8E,G,I,J), presumably reflecting internalisation. By contrast, no such decrease occurred when growth cones lacking TAG-1 were treated (Fig. 8F,H,I,J). Together, this suggests that TAG-1 modulates L1-mediated responses to Sema3A by facilitating the internalisation of L1 and NRP1. Consistent with this reflecting a decrease in the levels of endocytosis normally seen after Sema3A treatment (Fournier et al., 2000), we also saw a decrease in the internalisation of FITC-labelled dextran in Sema3A-treated growth cones from TAG-1-null mice (Fig. 8K). Similar to L1 and NRP1, TAG-1 substantially disappears from the cell surface of Sema3A-treated wild-type growth cones (Fig. 8L) and, moreover, can be found colocalised with clathrin heavy chain in the cytoplasm of these collapsed structures (Fig. 8M-Q). Together, these data suggest that TAG-1 controls responses to Sema3A in NGF-dependent sensory growth cones by modulating the endocytosis of L1 and NRP1, and is itself endocytosed in these responses.

DISCUSSION

Accumulating evidence indicates that growth cone responses to diffusible guidance signals change according to context through integration with contact adhesive signals (Castellani et al., 2000; Hopker et al., 1999; Shewan et al., 2002). Previous studies have begun to uncover the intracellular signalling mechanisms involved in this integration (Castellani et al., 2002; Hopker et al., 1999; Shewan et al., 2002). Here, we focused on mechanisms that operate at the plasma membrane to modulate guidance responses. We have shown that responses of NGF-dependent sensory axons to diffusible chemorepellants present in the spinal cord are dependent on both L1 and TAG-1, and that loss of either leads to premature innervation of the dorsal horn. We confirmed that a key component of VSC repulsion is Sema3A and that L1, which associates with the Sema3A receptor NRP1 (Castellani et al., 2000), is required for sensory axon repulsion by Sema3A. We showed for the first time that TAG-1 is also required for these responses but that it does not to bind directly to Sema3A, NRP1 or plexin A4. Instead, our evidence suggests that the known cis interaction of TAG-1 with L1 (Buchstaller et al., 1996; De Angelis et al., 2002) regulates the co-internalisation of L1 with NRP1 from the cell surface. Our evidence also suggests that TAG-1 may be involved in responses to other, as yet unidentified, chemorepellants.

TAG-1 and L1 are required for timely dorsal horn innervation

Experiments using antibodies to axonin 1 (TAG-1) or NgCAM (L1) on chick embryonic spinal cord in ovo (Perrin et al., 2001) or in vitro (Shiga et al., 1997) indicated a role for these molecules in TrkA+ sensory axon pathfinding. Our findings are consistent with these studies in indicating that these L1nCAMs are required to prevent the premature entry of TrkA+ axons into the dorsal horn; injection of axonin 1 antibodies into chick embryos resulted in premature projections comparable in number with those observed in TAG-1 null mice [40 premature projections per 600 μm of spinal cord at E6.5 in chick (Perrin et al., 2001) compared with at least 90 per 600 μm at E14.5 in mice] and focused around points of dorsal root entry. However, whereas these studies suggested that TAG-1 and L1 are involved in providing ‘positive’ guidance cues in order to retain axons in the dorsal funiculus, most probably through selective fasciculation (Perrin et al., 2001; Shiga et al., 1997), our in vitro explant culture evidence indicates that these molecules are required on sensory axons for responses to diffusible ‘negative’ cues present in the spinal cord; NGF-dependent axons lacking L1 or TAG-1 are partially or completely unresponsive to ventral spinal cord-derived chemorepellants, respectively.

At least part of this loss of response is due to loss of response to Sema3A that, consistent with previous studies (Messersmith et al., 1995; Puschel et al., 1996), we find to account for a substantial proportion of the VSC repulsive activity at this stage in rodents. For axons lacking L1, the proportion of response to VSC lost correlates exactly with the proportion due to Sema3A repulsion, as judged by blockade with soluble NRP1 ectodomain. Such axons also no longer respond to soluble Sema3A. This agrees with previous studies demonstrating a requirement for L1 in responses to Sema3A in postnatal cortical and sensory axons, mediated by direct binding of L1 to NRP1 (Castellani et al., 2000; Castellani et al., 2002).

In the case of NGF-dependent sensory axons lacking TAG-1, the loss of response to VSC repulsion is complete and significantly greater than that seen with axons lacking L1. Correspondingly, the

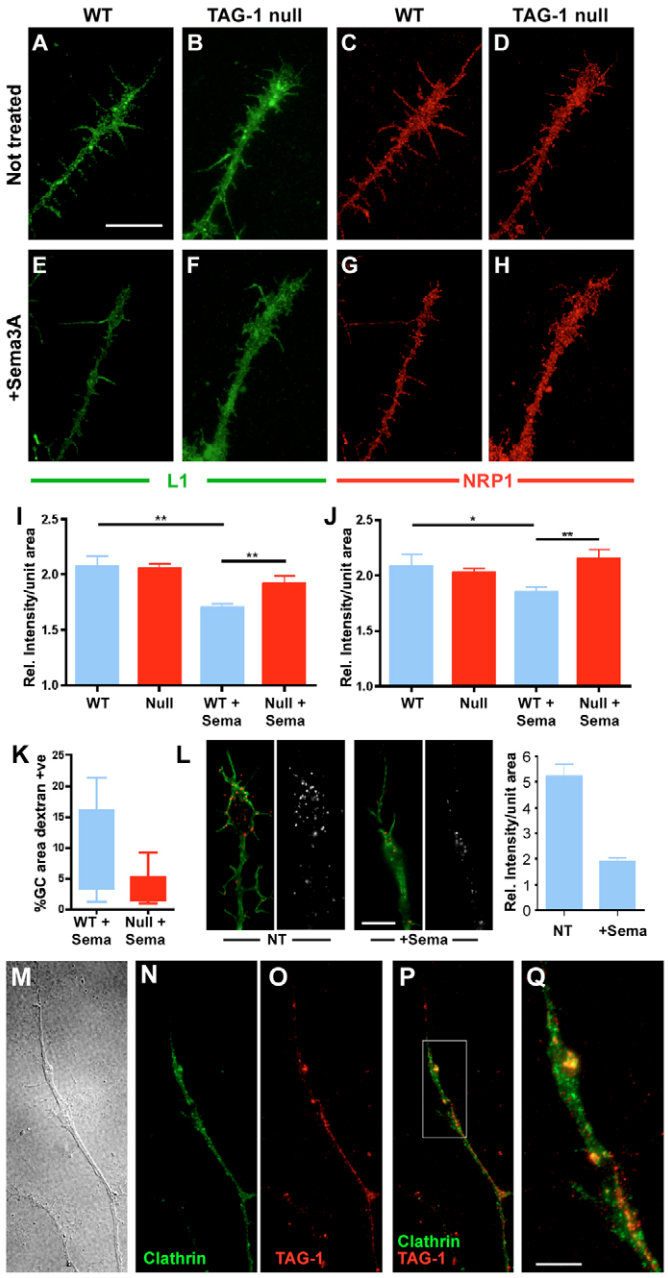

Fig. 8. Internalisation of L1 and NRP1 is affected in TAG-1 mutants. Immunolabelling of growth cones from wild-type (A,C,E,G; WT) and TAG-1-null (B,D,F,H; null) sensory neurons shows that L1 (**A**,**B**) and NRP1 (**C**,**D**) reach the surface of TAG-1-null growth cones at normal levels, as quantitated in I (L1) and J (NRP1); *n*=16 for wild type, *n*=21 for null (see Materials and methods). Quantitation of surface immunolabelling after Sema3A treatment of wild-type and TAG-1-null growth cones shows that surface levels of L1 (**E**,**F**,**I**) and NRP1 (**G**,**H**,**J**) are significantly reduced in wild type (E,G; WT + Sema) but not TAG-1-null (F,H; Null + Sema) growth cones (***P*<0.01, **P*<0.05; unpaired *t*-test). Results from both collapsed and extended growth cones are pooled for each genotype in both conditions (and are present in each population in the proportions indicated in Fig. 4I) but results are expressed as average intensity per unit area to normalise for differences in growth cone size [similar results were obtained when comparing growth cones from the different genotypes according to morphology (i.e. extended versus collapsed; not shown)]. (**K**) FITC-labelled dextran is taken up to a lesser extent by TAG-1-null growth cones (null + Sema) than by wild-type growth cones (WT + Sema) after Sema3A treatment. (**L**) Cell-surface TAG-1 levels (red) in wild-type growth cones (counterlabelled with phalloidin; green) fall significantly (*P*<0.0001) after Sema3A treatment (+Sema; *n*=13) compared with no treatment (NT; *n*=12). Panels on the right in each pair show TAG-1 only. Quantitation as for L1 and NRP1 above. (**M-Q**) Colocalisation of TAG-1 and clathrin heavy chain in wild-type growth cones after Sema3A treatment. Area outlined by white box in P shown in Q. Scale bars: 10 μm in A-H,Q; 20 μm in L.

loss of response cannot be accounted for simply by loss of response to Sema3A; as indicated above, soluble NRP1 was insufficient to block VSC-derived repulsion completely, even at concentrations of NRP1 that completely block the stronger repulsion seen with Sema3A-transfected Cos7 cells. Moreover, although axons lacking TAG-1 are less responsive to Sema3A-transfected Cos7 cells, the loss of response is less than that seen with L1 mutant axons under the same assay conditions, the reverse of the situation seen when VSC is the source of repellant. This indicates that Sema3A, or indeed other NRP1-binding semaphorins cannot account for all VSC repulsion at this age, and that TAG-1 is required for responses to an unidentified repulsive activity as well as to VSC-derived Sema3A. The greater loss of response to VSC repulsion in TAG-1 nulls compared with L1 nulls is consistent with the greater number of TrkA+ axons prematurely entering the dorsal horn in TAG-1 nulls.

That TAG-1 is required for responses to chemorepellants other than Sema3A is in agreement with observations in chick (Masuda et al., 2003). In this study, as with ours, antibodies to axonin-1/SC2 diminished responses to VSC repulsion. However, in contrast to our results, these authors argue against a role for TAG-1 in mediating responses to Sema3A, in part because antibodies to axonin 1/SC2 did not significantly block responses to Sema3A-transfected Cos7 cells, and because responses of DRG to VSC were not affected by genetic loss of NRP1 or Sema3A. Two key differences between the studies may account for the apparent discrepancy. First, our study tested E13.5 VSC with DRG of the same age and species, whereas the earlier study mixed both ages and species. This is important because there are significant differences in the expression profiles of Sema3A and NRP1 both between the species [compare Zou et al. with Fu et al., for example (Fu et al., 2000; Zou et al., 2000)] and at different ages in the same species (Pond et al., 2002; Puschel et al., 1995; Puschel et al., 1996; Wright et al., 1995; Zou et al., 2000). In particular, Masuda et al. make the argument that because E5 chick DRG are still repelled by E11.5 VSC from Sema3A-null mice, Sema3A has no role in VSC repulsion at this time and that therefore, because axonin 1/SC2 antibodies do block repulsion by E11.5 VSC, axonin 1/SC2 may have no role in Sema3A responses. However, Sema3A is not expressed at high levels in mouse VSC until E12.5 or later (Puschel et al., 1995; Zou et al., 2000); therefore, it is not possible to draw conclusions about the role of TAG-1 in responses to Sema3A from these data. Second, in our study we focussed on NGF-dependent (TrkA+) sensory axons known to respond to Sema3A (Messersmith et al., 1995), whereas Masuda et al. included both NGF and NT3 in their cultures, and it is known that NT3-dependent axons lose their response to Sema3A with age (Pond et al., 2002).

TAG-1 affects responses to Sema3A by modulating the internalisation of the L1/NRP1 complex

Disruption of TAG-1 function by gene knockout is not as effective in blocking the Sema3A response as is genetic removal of L1 function. This indicates that, although TAG-1 is required for the full Sema3A response, some residual activity remains in its absence. Together with our observation that TAG-1 does not appear to bind directly to Sema3A, NRP1 or plexin A4, this suggests that TAG-1 influences responses to Sema3A indirectly via its ability to bind L1 on the same membrane (i.e. in cis) (Brummendorf and Rathjen, 1996; De Angelis et al., 1999). Consistent with this, disruption of the domains known to be important for L1 binding (De Angelis et al., 1999; Rader et al., 1996) in our TAG-1^{a} mutant reduces responses to Sema3A as effectively as complete loss of TAG-1.

How might TAG-1 binding to L1 affect Sema3A responses? One possibility is that TAG-1 is required for the trafficking of L1 to the cell surface, by analogy with the role of F3/contactin in the trafficking of CASPR/paranodin (Faivre-Sarrailh et al., 2000). However, we could find no significant change in the levels of cell surface L1 in TAG-1 mutants. However, it has been reported that L1 is co-internalised with NRP1 in response to Sema3A (Castellani et al., 2004) and that Sema3A treatment induces endocytosis locally (Fournier et al., 2000). Consistent with this, we found that cell surface levels of both L1 and NRP1 were significantly reduced after Sema3A treatment of wild-type sensory growth cones, but this reduction did not occur in growth cones lacking TAG-1. Moreover, the proportion of growth cones taking up FITC-dextran after Sema3A treatment was reduced in the absence of TAG-1. This suggests that the binding of TAG-1 to L1 on the neuronal surface may facilitate endocytosis of the Sema3A receptor complex. Whether TAG-1 directly associates with the L1-NRP1-plexin A4 complex or modulates the participation of L1 in the complex indirectly is unclear, although our data do indicate that TAG-1 itself is endocytosed and becomes associated with clathrin after Sema3A treatment. Interestingly, because of its known association with lipid rafts (Kasahara et al., 2000), one possibility is that TAG-1, through its binding to L1, may recruit components of the Sema3A receptor into these membrane microdomains, which have been suggested to be portals for endocytosis (Parton and Richards, 2003) and which are known to be required for Sema3A-mediated growth cone repulsion (Guirland et al., 2004). Further analysis will be necessary to determine the exact mechanism by which TAG-1 exerts its effect.

The role of L1nCAMs in axon pathfinding in vivo

Despite our evidence that TAG-1 and L1 expression is required to prevent premature entry of TrkA+ sensory afferents into the dorsal horn, their exact role in controlling the pathfinding of these axons remains unclear. Of particular interest is why molecules known to mediate fasciculation modulate responses to soluble chemorepellants. One possibility is that this may render axonal responses context dependent, as suggested by the inhibition of growth cone collapse by Sema3A by soluble L1 (Castellani et al., 2000; Castellani et al., 2002). Thus, TrkA+ growth cones fasciculating on L1-expressing proprioceptive axons as they enter and bifurcate in the DREZ may be insensitive to local Sema3A (Fu et al., 2000; Wright et al., 1995), so long as they maintain their TAG-1/L1-mediated fasciculation. Accordingly, the aberrant TrkA+ projections seen in TAG-1 and L1 mutant mice were most evident at the entry point (Fig. 2) (Perrin et al., 2001), suggesting that these axons fail to bifurcate. Subsequent downregulation of Sema3A (Wright et al., 1995) normally would release this restraint and allow collateral formation (Messersmith et al., 1995). Together with recent evidence from zebrafish that class 3 semaphorins may regulate L1 expression (Wolman et al., 2007), this suggests that the mechanisms by which semaphorins and adhesion molecules regulate fasciculation are more intricate than a simple balance of repulsion and adhesion (Yu et al., 2000).

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