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Mechanosensilla in the adult abdomen of *Drosophila*: *engrailed* and *slit* help to corral the peripheral sensory axons into segmental bundles

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SUMMARY

The abdomen of adult *Drosophila* bears mechanosensory bristles with axons that connect directly to the CNS, each hemisegment contributing a separate nerve bundle. Here, we alter the amount of Engrailed protein and manipulate the Hedgehog signalling pathway in clones of cells to study their effects on nerve pathfinding within the peripheral nervous system. We find that high levels of Engrailed make the epidermal cells inhospitable to bristle neurons; sensory axons that are too near these cells are either deflected or fail to extend properly or at all. We then searched for the *engrailed*-dependent agent responsible for these repellent properties. We found *slit* to be expressed in the P compartment and, using genetic mosaics, present evidence that Slit is the responsible molecule. Blocking the activity of the three Robo genes (putative receptors for Slit) with RNAi supported this hypothesis. We conclude that, during normal development, gradients of Slit protein repel axons away from compartment boundaries – in consequence, the bristles from each segment send their nerves to the CNS in separated sets.

KEY WORDS: *Drosophila*, Abdomen, *hedgehog*, *engrailed*, Compartments, Boundaries, Neurons, Axons, *slit*

INTRODUCTION

In the embryos of complex animals, including mammals and arthropods, the orderly deployment of selector genes determines cell identities and positions morphogen gradients. The result is a body plan that consists, in part, of segments and developmental compartments. Compartments are groups of cells, each with a unique genetic address; they do not intermingle with each other and the cells of each share distinct fates, identities and affinities (reviewed by García-Bellido et al., 1973; Lawrence, 1992). Although largely defined in the epidermis they also subdivide the nervous system. In vertebrates, compartments have been identified in the forebrain, midbrain and hindbrain (Keynes and Stern, 1988; Kiecker and Lumsden, 2005). The hindbrain is subdivided along its anteroposterior axis into lineage-based rhombomeres (Murphy et al., 1989; Fraser et al., 1990), which are also the units of neurogenesis, axonal guidance and axon bundling (Lumsden and Cohen, 1991). Rhombomeres are homologous to the parasegments of arthropods (Martínez-Arias and Lawrence, 1985; Lumsden and Cohen, 1991).

Since the meticulous studies of Lyonet (Lyonet, 1762), we have known that the peripheral sensory system of arthropods is segmented: neurons originate in sensilla in segmental groups in the epidermis and axons project from each of them to the corresponding segmental ganglion in the CNS (Bate, 1978). To achieve this, the nerves coming from each epidermal segment or compartment must not mix with nerves from neighbouring compartments (Hertweck, 1931; Wigglesworth, 1953). The epidermis of the fly and other arthropods is subdivided into a chain of anterior (A) and posterior (P) compartments, the P/A compartment boundary being the true segmental boundary (Blair and Ralston, 1997; Struhl et al., 1997b; Lawrence et al., 1999a; Lawrence et al., 2002). This segment boundary is recognised by neurons as they build the embryonic nervous system (Bate, 1976; Palka et al., 1981) and is not crossed by peripheral sensory neurons in later stages, as shown for example in *Rhyacophila* and *Galleria* (Hasenfuss, 1973) or *Oncopeltus* (Lawrence, 1975). However, little is known of the molecular mechanisms responsible for this process.

In the adult abdomen of *Drosophila*, the mechanoreceptive bristles are confined to a region of each A compartment (Fig. 1, left); they develop de novo as sensory organ precursor cells (Shirras and Couso, 1996; Fabre et al., 2008) that derive from the epidermal cells or 'histoblasts' that proliferate during the pupal stage (Madhavan and Madhavan, 1980). Sensory organ precursor cells divide asymmetrically to generate a bristle and their associated neurons and supporting cells (Gho et al., 1999); the neurons then extend axons towards the CNS in an orderly manner (Fabre et al., 2008). These axons remain within their compartments of origin because they are oriented with respect to the body axes: within each A compartment, the more anteriorly situated bristle axons grow backwards, while the posteriorly situated bristle axons grow forwards, and thus both sets of axons meet to form a segmental nerve bundle in the middle of the A compartment (Fig. 1, right) (Fabre et al., 2008).

A and P compartments differ fundamentally: all the P cells but not the A cells, except for a6 (Lawrence et al., 1999a), express *engrailed* (*en*). The *en* gene encodes a homeodomain-containing transcription factor that induces *hedgehog* (*hh*) expression in P cells. Hh is a secreted morphogen that spreads into the A compartment, forming a U-shaped gradient that patterns cell fate and determines cell affinity (Struhl et al., 1997b; Lawrence et al.,

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1999b). Only the epidermal cells of the A compartment produce Patched (Ptc) and Smoothened (Smo), proteins that act as receptors for Hh. Although the mechanosensory neurons are related to epidermal cells by lineage, it is not clear whether they retain all the compartmental properties of their origin.

Here, we ask how En- and Hh-dependent information positions the neuronal cell bodies, affects the dendrites and influences the pathways followed by axons. To investigate this, we alter cell identities by manipulating the relevant genes (*en*, *hh*, *ptc* and *smo*) within clones of cells and look for effects on the neurons. Strikingly, cells with P identity, but located within an A compartment, repel nearby neurons. We find that this neuronal repulsion is not directly mediated by En or Hh, but indirectly by activating the expression of *slit*, a molecule previously implicated in neuronal pathfinding (Brose and Tessier-Lavigne, 2000). Also, the response to Slit appears to be mediated by one or more of the Robo proteins. We propose that, during normal development, the secretion of Slit from P cells creates a Slit gradient in each A compartment that helps position neurons and orient axon outgrowth and thereby ensures segmental bundling of axons.

MATERIALS AND METHODS

Fly genotypes

Unless stated otherwise, FlyBase (Tweedie et al., 2009) entries of the mutations and transgenes referred to in the text are as follows. *ptc.lacZ*: *Eco\lacZ$^{ptc\text{-}AT96}$*. *tub>y^{+}>en*: *en$^{Scer\FRT.Rnor\CD2.\alpha Tub84B}$*. *tub>y^{+}>hh*: *hh$^{Scer\FRT.Rnor\CD2.aTub84B}$*. *tub>Gal80,y^{+}>Gal4*: *Scer\GAL4$^{Scer\FRT.Gal80.\alpha Tub84B}$*. *UAS.en*: *en$^{Scer\UAS.cGa}$*. *ptc^{-}*: *ptc^{16}*. *en^{-}*: *Df(2R)enE*. *smo*: *smo^{3}*. *Fas2^{-}*: *Fas2^{eb112}*. *beatIa^{-}*: *beat-Ia^{3}*. *Fas3^{-}*: *Fas3^{E25}*. *Sema1a^{-}*: *Sema-1a^{k13702}*. *shg^{-}*: *shg^{k03401}*. *UAS.Dscam*: *Dscam$^{Scer\UAS.cWa}$*. *Dscam.lacZ*: *Dscam^{05518}*. *sca.Gal4*: *sca$^{109\text{-}68}$*. *18w.lacZ*: *18w^{k02701}*. *Sema-1a.lacZ*: *Sema-1a^{k13702}*. *elav.Gal4*: Scer\GAL4$^{elav\text{-}C155}$. *sli.lacZ*: *sli^{05248}*. *UAS.sli*: *sli$^{Scer\UAS.cBa}$*. *pnr.Gal4*: *Scer\GAL4$^{pnr\text{-}MD237}$*. *UAS.roboRNAi*: *robo$^{dsRNA.Scer\UAS}$*. *robo.Gal4*: *P{GawB}NP2099*. *UAS.robo2RNAi*: *lea$^{dsRNA.Scer\UAS}$*. *robo3.Gal4*: *P{GawB}NP4698*. *UAS.robo3RNAi*: *robo3$^{dsRNA.Scer\UAS}$*. *dock.lacZ*: *dock^{k13421}*. *CD2y^{+}*: *Rnor\CD2$^{hs.PJ.hs.FLP:\ Scer\FLP1hs.PS}$*. *Dpy^{+}*: *Dp(1;2)sc^{19}*. *Dpw^{+}*: *P{white-un1}30C*. *UAS.GFP*: *Mmus\Cd8a$^{Scer\UAS.T:Avic\GFP}$*. *hs.FLP*: *Scer\FLP1$^{hs.PS}$*. *FRT42*: *P{neoFRT}42D*. *FRT40*: *P{neoFRT}40A*. *FRT19*: *P{neoFRT}19A*.

Flies were cultured at 25°C on standard food. Clones were induced in flies with the following genotypes:

hh- or *en*-expressing clones, *y hs.FLP; tub>y^{+}>hh* or *y w hs.FLP; tub>y^{+}>en* or *y w hs.FLP; tub>Gal80,y^{+}>Gal4/+; UAS.en/+* flies;

ptc^{-} clones, *y w hs.FLP/ y hs.FLP; FRT42 CD2y^{+}/ FRT42 pwn ptc^{-}*;

ptc^{-} sli.lacZ clones, *y w hs.FLP/ y hs.FLP; FRT42 CD2y^{+}/ FRT42 pwn ptc^{-} sli.lacZ*;

UAS.en sli.lacZ clones, *y w hs.FLP; tub>Gal80,y^{+}>Gal4/ sli.lacZ; UAS.en/+*;

ptc^{-} en^{-} clones, *y hs.FLP/ y; FRT42; FRT42 CD2y^{+}/ pwn ptc^{-} en^{-}*;

smo^{-} en^{-} clones, *y hs.FLP/+; smo^{-} b FRT42 cn sha/FRT42 pwn en^{-}*;

Fas2^{-} clones, *Fas2^{-} sn FRT19/ w hs.FLP FRT19*;

beat1a^{-} Fas3^{-} clones, *y w hs.FLP; beat1a^{-} Fas3^{-} FRT40/ Dpy^{+} Dpw^{+} FRT40*;

Sema-1a^{-} clones, *y w hs.FLP; Sema-1a.lacZ FRT40/ Dpy^{+} Dpw^{+} FRT40; shg^{-}* clones, *y w hs.FLP; FRT42 pwn shg^{-}/ FRT42 CD2y^{+}*;

pnr.Gal4 UAS.sli flies, *pnr.Gal4/ UAS.sli*; and

elav.Gal4 UAS.#RNAi flies, *w elav.Gal4 UAS.GFP hs.FLP/ w; UAS.#RNAi/+* (where # is *robo*, *robo2* or *robo3*).

Clonal inductions and immunohistology

Clones were induced by heat shocking third-instar larvae for 1 hour at 34.5°C or 30 minutes at 37°C. Pupal and adult dissection and stainings were carried out as described previously (Fabre et al., 2008). For experiments with RNAi, flies were kept at 18°C until pupal stage P5 (Bainbridge and Bownes, 1981), when mechanosensory neurite extension is beginning (Fabre et al., 2008), and then shifted to 29°C. To control for this experiment, we used wild-type *elav.Gal4 UAS.GFP* or *elav.Gal4 UAS.#RNAi* flies kept during the whole development at 29°C or 18°C, respectively.

As primary antibodies we used: 22C10, anti-Fasciclin, anti-Fasciclin 2, anti-Fasciclin 3 and anti-Sema-2a mouse monoclonal supernatants (Developmental Studies Hybridoma Bank) at 1:20 to 1:50 dilutions; anti-Elav (rat serum) at 1:1000; anti-GFP (rabbit serum) at 1:500; and anti-β-galactosidase (rabbit serum) at 1:500 (Invitrogen, Paisley, UK). Samples were incubated with the primary antibodies for 2 hours, then incubated with FITC- or Texas Red-conjugated secondary antibodies at 1:200 (Stratech Scientific, Newmarket, UK), and kept overnight in Fluoromount-G medium (Southern Biotech, Birmingham, AL, USA). Abdomens were mounted flat on a slide with Fluoromount-G, with their bristles facing upwards, and kept for 2 days at room temperature in the dark for the Fluoromount to solidify. *lacZ* activity was visualised as described (Struhl et al., 1997b). Images were captured with Auto-Montage (Syncroscopy, Cambridge, UK) and processed with Adobe Photoshop (San José, CA, USA).

RESULTS

Background

In the abdominal segments, the U-shaped landscape of Hh concentration in each A compartment patterns the epidermis, creating distinguishable cuticle types (Fig. 1) (Struhl et al., 1997b). a1 and a6 cuticle are specified by the highest concentrations of Hh

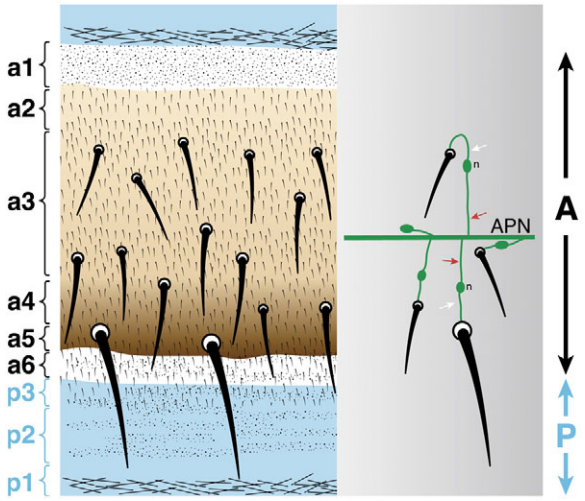

Fig. 1. A dorsal segment of the adult *Drosophila* abdomen showing mechanosensory receptors and associated neurons. (Left) In the dorsal cuticle of the anterior (A) compartment, the cuticles of types a3 to a5 contain oriented mechanosensory bristles, whereas a1, a2, a6 and the entire posterior (P) compartment do not. The cuticles of types a2 to a6 and the cells at the front of the P compartment (p3) display small trichomes. Cells of the P compartment express *en* (blue), which also contributes, late, to a6 cell identity. Cells of the P compartment produce Hh that spreads into and patterns the flanking A compartments. *ptc* is expressed in the A compartment at the front and back (see Struhl et al., 1997b). (Right) A right hemisegment showing the pattern of innervation of bristles and axonal pathways. The abdominal peripheral nerve (APN), within which mechanosensory axons ultimately bundle, forms in the medial area of the A compartment. In the anterior zone, the dendrites (white arrows) make U-shaped turns from the neuronal somata (n) to reach the bristle, and the axons (red arrows) extend posteriorly to meet the APN. In the posterior zone, both dendrites and axons extend anteriorly to join the nerve (Fabre et al., 2008). As with all subsequent figures, anterior is up and posterior is down.

and in consequence are located at the extreme front (anterior) and back (posterior) of the A compartment. Cells in the middle of the A compartment receive the lowest concentration of Hh and form either a2 or a3 cuticle (Struhl et al., 1997b). Late in development, *en* becomes necessary within a thin stripe at the back of the A compartment and helps specify a6 cuticle (Lawrence et al., 1999a). The P compartment is subdivided into three domains: p3-p1. We cannot easily distinguish a6 from p3 cuticle, and thus we sometimes have to describe cell identities as 'a6 or p3'. Mechanosensory axons originated at the front grow posteriorly and those originated at the back grow anteriorly to meet and join the abdominal peripheral nerve (APN) in the a3 region (Fig. 1) (Fabre et al., 2008). We now ask whether the growing nerves follow the Hh gradient itself or some other cue.

Can the mechanosensory neurons respond directly to Hh?

One possibility was that the neurons might simply grow down the slope of the Hh gradient produced by the P cells, which, in the innervated regions a3-a5, should decline consistently towards the anterior (Struhl et al., 1997b). But the two sets of bristle axons within this area grow in opposite directions, one up and one down the Hh gradient (Fabre et al., 2008). In addition, β-galactosidase immunostaining on *ptc.lacZ* flies did not show any expression of *ptc* in the mechanosensory neurons (see Fig. S1 in the supplementary material), arguing that mechanosensory neurons cannot 'see' Hh, at least via Ptc, a protein essential for Hh reception – any reception of the Hh signal should have caused an upregulation of *ptc.lacZ*, as occurs in the epidermis (Hooper and Scott, 1989; Nakano et al., 1989).

Are neurons sensitive to the amount of En protein and to the gradient of Hh in the epidermis?

UAS.en clones that make a6 or p3 cuticle, induced in A compartments, are avoided by neurons

It is possible that Hh might affect neurons indirectly, via effects on target genes in the epidermal cells, near which the mechanosensory neurons grow. We therefore made clones of cells that express *en* and, consequently, *hh* (Fig. 2). In the mid to back of the A compartment, these clones produced clear unpigmented cuticle (Fig. 2A), similar to the cuticle made by a6 or p3 cells (Lawrence et al., 1999a). They affected the wild-type axons near the clones (36 clones out of 40 were associated with defects; Fig. 2B,C); the axons avoided the clones, turning away from them, or they arrested within the clones. The orientation of dendrites and the relative positions of the cell bodies were also sometimes altered. For example, when a5 bristles were localised behind a clone, the soma was found lateral to the bristle – instead of in front, as is usual in this position (Fabre et al., 2008) – and the dendrite deviated from its normal path (Fig. 2B,C). Similar effects on neurons were seen when we induced *en*-expressing clones in the anterior part of A (bristle 1, Fig. 2). When clones were made in various parts of the A compartment that expressed *hh*, they also had effects on nearby axons (not shown).

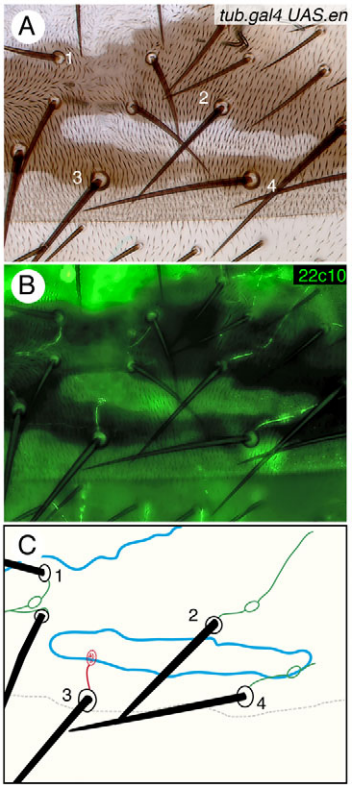

Fig. 2. Neurons avoid clones expressing *en*. (**A**) Two clones expressing *UAS.en* in the a3-a4 region. Mutant cuticle is unpigmented (a6 or p3). Pertinent bristles are marked 1-4. (**B**) The axons, dendrites and somata are labelled with 22C10 antibody. (**C**) Drawing to show innervation of the pertinent bristles. Clones are outlined in blue. The dendrite from bristle 1 does not show the usual U-shape and turns away from the clone. The axon from bristle 2 also deviates from its normal path and its soma is lateral (and not anterior as is usual). The axon from bristle 3 (red) enters the clone, expands and stops. The axon from bristle 4 turns right, avoiding the clone and the soma is also lateral to the bristle socket. The approximate position of the a5/a6 border (see Fig. 1) is indicated by a grey dashed line.

ptc^{-} clones that form a6 or p3 cuticle, induced ectopically amongst a5, a4 and a3 territories, are avoided by neurons

The loss of *ptc* activates the Hh transduction pathway (reviewed by Ingham, 2008) so that *ptc*$^{-}$ cells develop as if they were close to a source of Hh. When *ptc*$^{-}$ clones are made in the posterior part of A (amongst a5, a4 and a3), *en* is activated in these clones (we have no clue as to why), and their cells autonomously form unpigmented cuticle with hairs (a6 or p3) and also a5 bristles (Fig. 3) (Struhl et al., 1997a; Lawrence et al., 1999b). Wild-type neurons that arose near such *ptc*$^{-}$ clones were affected (38 clones out of 40 showed defects): the positions of the neuronal somata were altered and their processes either avoided the clone or even stopped growing when they came in contact with the clone (Fig. 3B,C,E,F). *ptc*$^{-}$ neurons that originated in these clones failed to develop or were defective (Fig. 3B,C,E,F). Some axons, outside the clones, turned away sharply (Fig. 3E,F). Thus, both *en*-expressing cells and *ptc* mutant cells – that activate *en* (Lawrence et al., 1999a) – have similar effects when ectopically located in the A compartment: they repel neurons. This result is consistent with the wild-type abdomen, in which neurons grow away from P cells and towards the central region of the A compartments.

ptc^{-} *en*$^{-}$ clones forming a5 cuticle, induced ectopically in the a4 or a3 territory, are crossed normally by neurons

We made clones in which both *ptc* and *en* were removed, the lack of *en* ensuring that all cells differentiate as A cells with a5 identity – the a5 state depends on a high level of Hh signalling

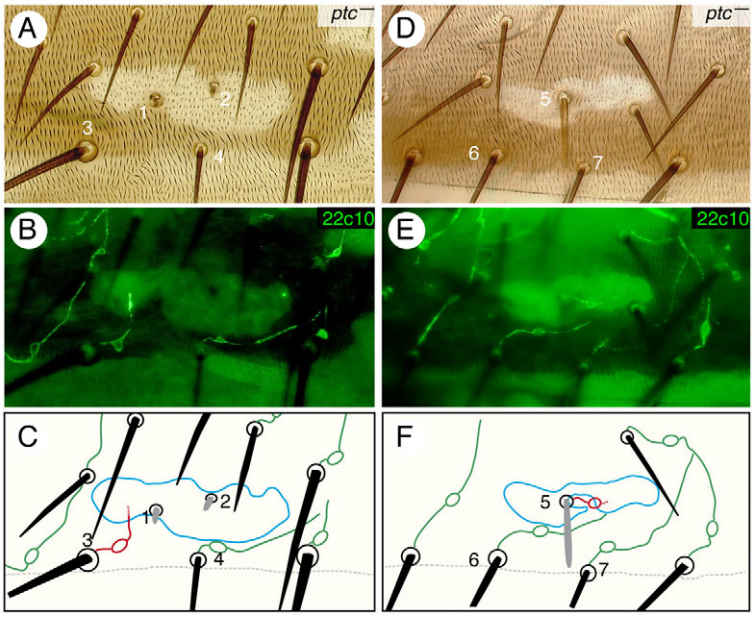

Fig. 3. Neurons avoid *ptc*⁻ clones. (**A**,**D**) Two clones in the posterior part of the A compartment that lack *ptc* are marked with *y* and *pwn*. They form a6/p3 cuticle and a5 bristles. (**B**,**E**) Staining of neurons with 22C10 antibody. (**C**,**F**) Pertinent bristles are numbered; mutant *ptc*⁻ bristles are shown in grey. The axons from bristles 3 and 5 (red) arrest within the clones, whereas those from bristles 4, 6 and 7 turn laterally to avoid the clones. The dendrite from one *ptc*⁻ bristle (5) appears to be confined within a small islet of wild-type cuticle. *ptc*⁻ bristles 1 and 2 are localised within the clone and have no detectable neurons, perhaps because they have degenerated within the clone.

but does not require *en* (Lawrence et al., 1999b). Accordingly, these clones make darkly pigmented cuticle and large bristles (a5) even when located in a4 and a3 territories (Lawrence et al., 1999b) (Fig. 4). Mutant mechanosensory axons arising within the clone grew normally towards the nerve. Also, wild-type axons regularly entered and crossed the clone to extend to the nerve (18 clones out of 21 were crossed normally by more than one neuron; Fig. 4A-C). As is usual (García-Bellido and Merriam, 1971), some bristles moved out of, or even into, clones during development. Such wild-type neurons, located within the clone and surrounded by mutant epidermis, grew anteriorly through the clone (Fig. 4A-C).

These results show that inducing a high Hh response in cells that lack *en* does not disturb the pathways of nearby neurons. Thus, it appears that the *ptc*⁻ clones have their effects on neurons via activation of *en*, and not through a high level of Hh signalling per se – more evidence that neurons do not respond directly to Hh and do not require the Ptc receptor to follow their

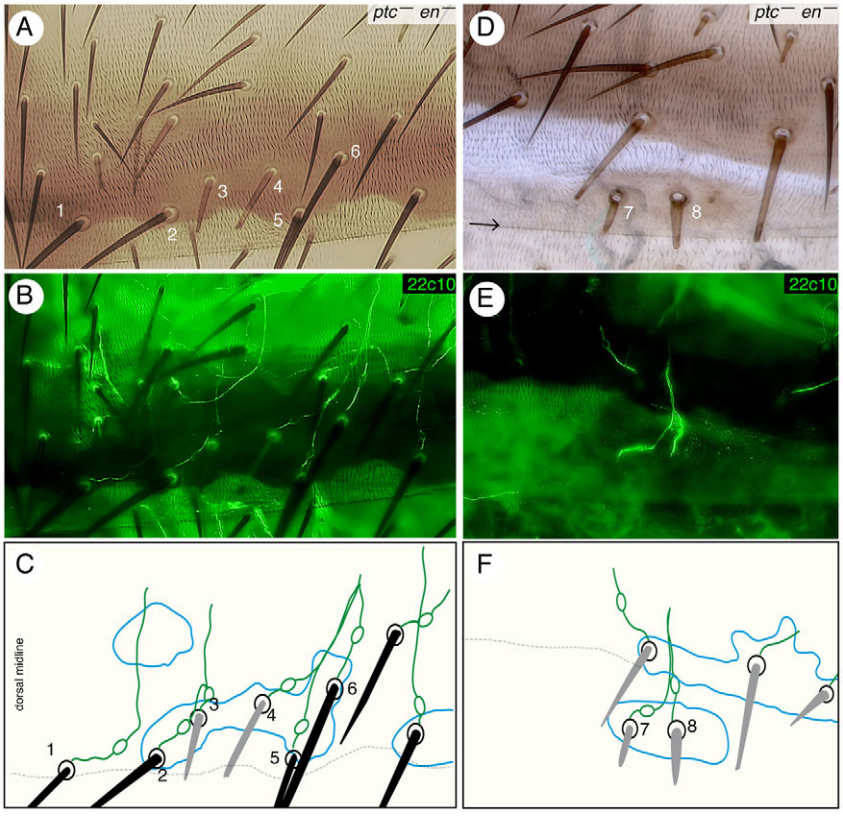

Fig. 4. Clones of *ptc*⁻ *en*⁻ cells do not affect neuronal pathfinding. (**A-C**) Clones that lack *ptc* and *en* (marked with *y* and *pwn*) were induced within the a3-a4 region of a right hemisegment, near the dorsal midline, as indicated. These clones make a5 cuticle and bristles (A). Pertinent bristles are numbered. The 22C10 antibody is used to reveal neurons (B); the top part of B is bleached by autofluorescence from adherent fat. Axons from wild-type bristles 1, 2, 5 and 6 (C) grow within and across the mutant clones in the normal fashion. Mutant bristles 3 and 4 behave similarly. Axons were not seen to arrest in any *ptc*⁻ *en*⁻ clones. (**D-F**) A *ptc*⁻ *en*⁻ clone was induced in the P compartment; the clone is marked with *pwn* and forms a5 cuticle and two bristles (7,8). The neurons are labelled with 22C10 antibody (E). Axons leave the two bristles, bundle together and extend anteriorly amongst the p3, a6 and a5 cuticle to join the APN in the segment ahead. Note that behind the clone (most easily seen in D) there is a deep fold (arrow), within which much of the P compartment is hidden.

paths towards the nerve. Rather, it seems that it is the presence of *en*-expressing cells that is inhospitable for mechanosensory neurons.

ptc⁻ *en*⁻ and *smo*⁻ *en*⁻ clones that form bristles in the P compartment produce neurons that grow towards their normal target

To see how neurons would respond to being surrounded by a6 or p3 cells, we made clones of cells with A identity within the P compartment. As described above, *ptc*⁻ *en*⁻ cells have A identity of a5 character. Similarly, because the loss of Smo blocks Hh transduction (Ingham, 2008), *smo*⁻ *en*⁻ cells also have A identity, but of a3 character (Struhl et al., 1997a; Lawrence et al., 1999b). We therefore made *ptc*⁻ *en*⁻ clones and *smo*⁻ *en*⁻ clones within the P compartment. Both types of clone formed ectopic bristles in the P compartment (Fig. 4D-F and see Fig. S2 in the supplementary material). We observed that axons arising from these bristles grew through the nearby p3 and/or a6 cells, and went straight anteriorly towards their normal target, the APN of the A compartment in front (10/10 *ptc*⁻ *en*⁻ clones and 10/12 *smo*⁻ *en*⁻ clones behaved this way; Fig. 4D-F and see Fig. S2 in the supplementary material). No axonal process arising from these clones grew backwards through the p2/p1 regions of the P compartment towards the A compartment located posteriorly to the clone.

These results confirm, yet again, that mechanosensory neurons are not repelled by a high level of Hh signalling in the epidermis; indeed, coming from the P compartment, where Hh signalling is absent, mechanosensory axons both approach and cross normal a6 territory where Hh signalling is maximal. The *smo*⁻ *en*⁻ clones show that the axons themselves do not need Smo, a protein required for Hh reception, in order to find their way to the peripheral nerve. This is consistent with the earlier finding that the neurons neither express nor require the other Hh receptor, Ptc, and are therefore unlikely to be able to detect the Hh signal.

The neurons that originate from the clones and grow forwards into the A compartment appear to cross the anterior region of the P compartment (p3) and certainly cross the rear of the A compartment (a6). Both p3 and a6 cell identities depend on En (Lawrence et al., 1999a). Thus, in this experimental situation, cells expressing *en* do not repel the neurons – so it cannot be En itself that repels. But, we also showed that neurons turned away from clones of cells that express *en* (*ptc*⁻ or *UAS.en* clones) when they

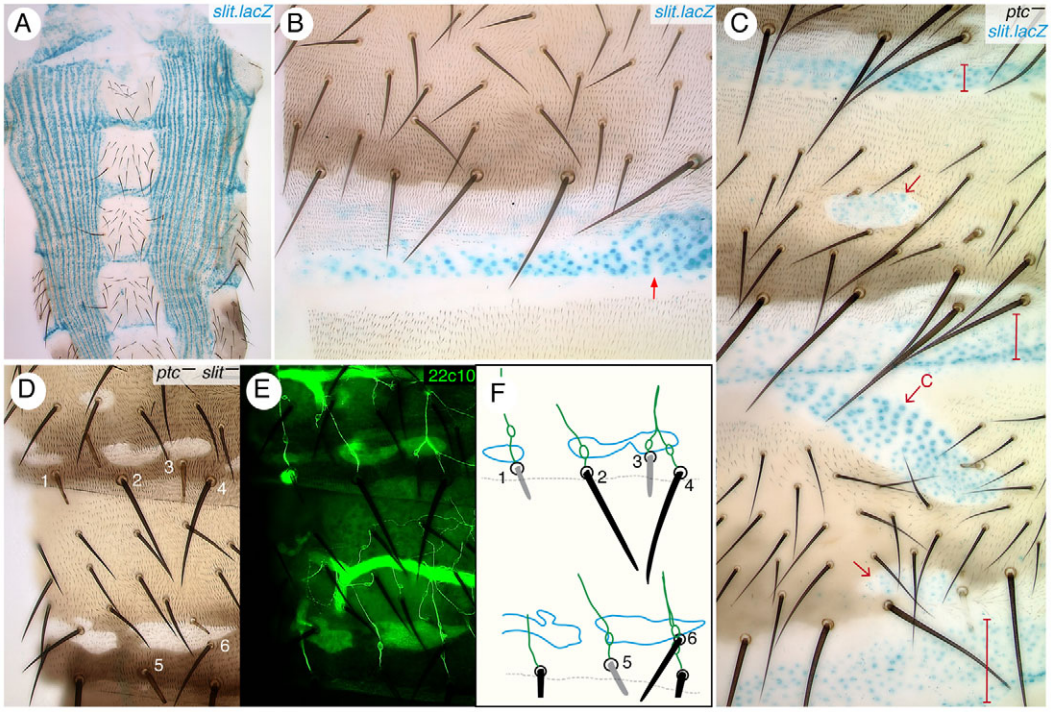

Fig. 5. *sli* expression in the abdomen. An enhancer trap drives expression of nuclear β-galactosidase (blue) in cells expressing *sli* (*sli.lacZ*). (**A**) Ventrally, *sli.lacZ* is expressed throughout the pleura but not in the sternites. *sli* expression extends across the midline in a region more or less coextensive with the P compartments. (**B**) Dorsally, *sli.lacZ* is expressed in most of the P compartments, strongly laterally (to the right in the image) and less strongly medially. The posterior boundary of the P compartment (the segment boundary, red arrow) coincides with a sharply demarcated boundary of expression. Anteriorly, the expression of *sli.lacZ* fades away and may not reach the front of the P compartment, even laterally. (**C**) *ptc*⁻ clones that carry *sli.lacZ* are shown in two segments. Clones (arrows) are marked with *pwn* and the abdomen is stained for β-galactosidase. Vertical bars indicate the normal zones of *sli* expression within the P compartments. In the anterior domain of the A compartments, clones are strongly transformed to P identity (Struhl et al., 1997b) and more posterior clones make a6 and/or p3 cuticle. All clones stained for β-galactosidase, the anterior ones most strongly. In the large clone (c), there is a gradient of *sli.lacZ*, which is stronger anteriorly and weaker posteriorly. This clone makes one *pwn* bristle. The β-galactosidase is entirely restricted to the cells of the clones. Note that the clones carry two doses of *sli.lacZ* and the background only one; this will tend to augment the blue staining in the clones relative to the endogenous stripes of expression in the P compartments. (**D-F**) Four *ptc*⁻ *sli*⁻ clones (marked with *y* and *pwn*) that carry a *sli.lacZ* transgene that knocks out the endogenous *sli* gene (Tayler et al., 2004) are shown in two segments. The neurons are stained with 22C10 (E). All the pertinent bristles, both wild-type and mutant (1-6), send their axons anteriorly and cross the clones normally; the somata are situated anterior to the bristles.

occur in a more anterior territory; perhaps these cells, in these abnormal positions, are now producing an effective repellent? We attempted to identify such a molecule.

A candidate approach to find molecules expressed in the abdominal epidermis that might guide or repel neurons

Families of proteins implicated in neuronal guidance were tested. We report on five candidates, all of which showed a suggestive pattern of expression in the abdomen (see Table S1 and Fig. S5 in the supplementary material): *Fasciclin 2* (*Fas2*), *Fasciclin 3* (*Fas3*), *18 wheeler* (*18w*), *slit* (*sli*) and *Down syndrome cell adhesion molecule* (*Dscam*). We perturbed their expression and looked for effects on the mechanosensory neurons (see Table S1 and Figs S6 and S7 in the supplementary material). We decided to concentrate on *sli*.

The pattern of expression of *sli* suggests that it might repel neurons in the abdominal epithelium

Expression of *sli.lacZ* in a wild-type background

We found that *sli.lacZ*, a transgene that signals *sli* expression, is expressed in all or part of the P compartments (Fig. 5A,B). Dorsally, the expression is broad in the lateral parts but confined within the P compartment, whereas medially it is more narrowly expressed at the back of the compartment. There is a sharp boundary at the rear, at the segment border (Fig. 5B, arrow). Thus Sli, a secreted protein (Rothberg et al., 1988) and an axonal repellent in other systems (Brose and Tessier-Lavigne, 2000), is expressed in a pattern that could produce a gradient of Sli protein that peaks at the back of the P compartments and declines both forwards and backwards. In this way, Sli could force mechanosensory neurites to grow away from the P compartments. Even the higher expression laterally fits with this hypothesis, as axons arising from lateral bristles tend to turn medially, towards the dorsal midline (Fabre et al., 2008). We test this hypothesis further below.

ptc⁻ clones express *sli*

We generated *ptc*⁻ clones that carry *sli.lacZ*. This same transgene is associated with a strong mutation for the endogenous *sli* gene (Tayler et al., 2004). In the anterior domain of the A compartment, these *ptc*⁻ *sli.lacZ* clones had the typical phenotype of *ptc*⁻ clones: they were clearly transformed to P character, affected the identity of the nearby A cells outside the clone and reversed polarity behind the clone (Fig. 5C) (Struhl et al., 1997a; Lawrence et al., 1999b). The clones stained for β-galactosidase, indicating that *sli* is upregulated in *ptc*⁻ cells and consistent with our observations that *ptc*⁻ clones repel neurons. Careful examination of the *pwn* marker showed that the β-galactosidase is expressed only and precisely in the cells of the clone. This pattern of *sli* expression might argue that *ptc*⁻ cells are autonomously transformed towards P, as *sli* is normally expressed only by P cells. Anteriorly located clones exhibited *sli.lacZ* staining most strongly; more posterior cells expressed *sli.lacZ* clearly, but less strongly (Fig. 5C). This suggests that the transformation towards P is less extreme in the posteriorly located clones, but is still sufficient for them to repel neurons.

ptc⁻ clones that lack *sli* do not repel neurons

We tested the hypothesis that the neurons are repelled by Sli. Whereas *ptc*⁻ clones repel axons, *ptc*⁻ *sli.lacZ* clones lack *sli* function and should not. Indeed this is the case: axons entered and left the marked clones freely, behaving normally and showing no indication of any response to the mutant tissue (Fig. 5D-F). This clear result argues strongly that normal *ptc*⁻ clones cause axonal avoidance by producing Sli protein. It also supports the earlier conclusion that Hh itself does not guide axons as these *ptc*⁻ *sli.lacZ* clones are completely or partially transformed to make P cuticle and should therefore produce Hh (yet they have no effect on the axons).

en-expressing clones express *sli*

en-expressing clones transform cells towards P identity and also repel neurons (see above). As would be expected under the current hypothesis, these clones also expressed *sli.lacZ* (Fig. 6). As with *ptc*⁻ clones, these clones expressed *sli.lacZ* more strongly in the anterior region of the A compartment (where they appeared to transform towards posterior P, p1,p2) than in the posterior region (where they transform towards anterior P, p3). This fits with the wild-type expression of *sli* being strongest in the posterior region of the P compartment.

sli-expressing clones disturb axon pathfinding

Clones were made that express *sli* under a strong *Gal4* driver. We found that they caused a generalised disruption of the axonal pathways, sometimes up to a segment's width away from the clones; they did not produce definable local effects (not shown). The level of Sli produced by these clones is likely to be high and, given the presumed long-range action of the molecule, perhaps the results are not surprising.

A change in the gradient of Sli can turn the sensory axons

pannier is expressed in a broad stripe mid-dorsally in the adult epidermis (Calleja et al., 2000). *sli* was driven by *pannier* in order to produce a change in the Sli gradient, towards peaking in the dorsal midline and declining laterally. As a consequence, the paths followed by peripheral axons were meandrous and longer than normal, presumably because they were repelled by the ectopic peak of the Sli gradient (Fig. 7A,B).

Mechanosensory neurons may express *robo* and *dock*

In order for mechanosensory neurons to respond to Sli, they should express molecules involved in the reception of Sli. Three receptors, Roundabout (Robo), Leak (Robo2) and Robo3, have been

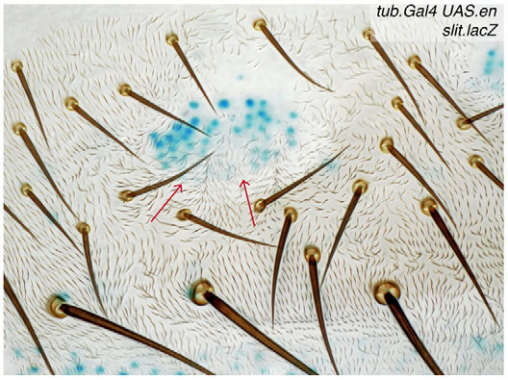

Fig. 6. *sli* expression is downstream of *en*. Overexpression of *en* in clones of A compartment cells endows posterior character and causes ectopic *sli* expression, most strongly in the anterior of the A compartment. This clone is near the middle of the segment, is associated with reversed polarity (red arrows) as well as hairless areas of cuticle, and expresses *sli.lacZ*.

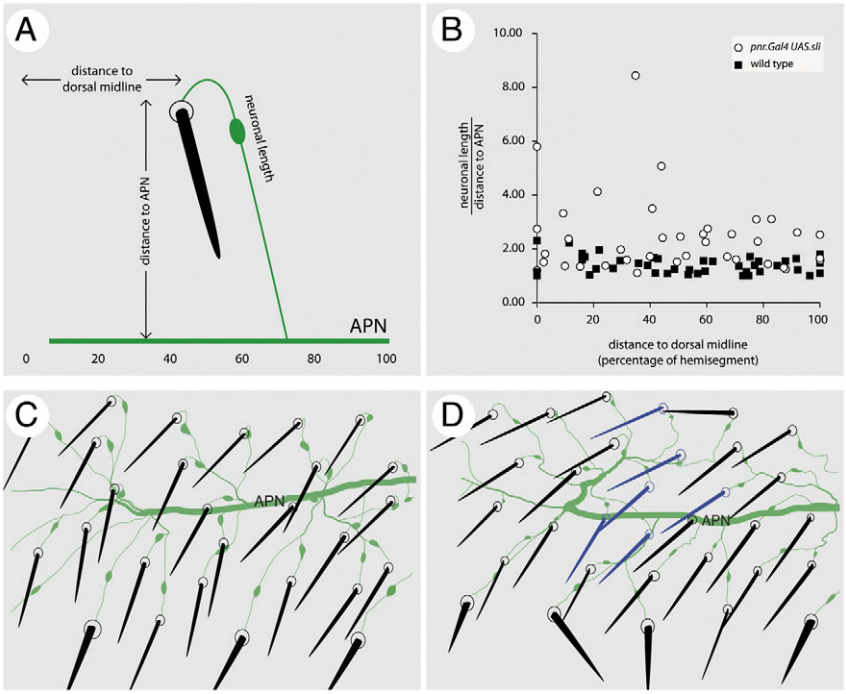

Fig. 7. Effects of disturbing *sli* signalling on axon pathfinding. (**A**,**B**) Effects of ectopic *sli* expression. When the Sli gradient is altered (by driving the gene with *pnr.Gal4*) axons follow a less direct path to the APN. How this is measured is shown in A. A beeline to the nerve is measured and compared with the actual course of the nerve. The ratio between these measures is plotted in B and shows a strong effect on many axonal pathways when four wild-type (black squares,) and four mutant (white circles) A3 hemisegments are compared. (**C**,**D**) RNAi against the *robo2* gene affects axon patterning. For clarity, the mechanosensory neurons have been drawn to scale from the microscope; for corresponding images of the neuronal staining, see Fig. S3C,D in the supplementary material. (C) Wild-type mechanosensory neurons of a right dorsal hemisegment (Fabre et al., 2008). The anteriorly located axons turn right and then extend posteriorly to join the nerve (APN). In the posterior part, the axons extend directly anteriorly to the nerve. A maximum of four axons bundle together before they reach the nerve. (D) The effect of expressing an RNAi that targets *robo2*. Many abnormalities can be seen; bristles with axons that bundle abnormally or take unusual paths are shown in blue. At least 12 axons bundle together to make an aberrant nerve.

described in *Drosophila*, all of which can mediate axon repulsion in response to secreted Sli protein. Also, the adaptor protein Dreadlocks (Dock) functions during Robo repulsion by binding to the cytoplasmic domain of Robo upon stimulation by Sli (Fan et al., 2003; Dickson and Gilestro, 2006). Unfortunately, the abdominal cuticle interferes with staining by some antibodies, which was the case for those against Robo, Robo2, Robo3 and Dock. We therefore used reporter assays under the control of the *robo* and *dock* promoters. To investigate *robo* expression, we used a putative *robo.Gal4* driver in combination with a GFP reporter. We found that both the mechanosensory neurons and the nerve show strong GFP expression, arguing that these cells may normally express *robo* (see Fig. S3A in the supplementary material). We were not able to test for *robo2* expression. To investigate *dock*, we expressed *lacZ* under the control of the *dock* promoter. Antibodies against β-galactosidase strongly stained the neuronal cell bodies of the mechanosensory bristles, together with large parts of the abdominal epithelium, arguing that the neurons also express *dock* (see Fig. S3B in the supplementary material).

Evidence from RNAi implicates the Robo genes in neuron guidance

We built flies carrying the neuronal driver *elav.Gal4* and *UAS.RNAi* constructs targeting each of the Robo genes (*robo*, *robo2* and *robo3*). These flies were grown at 18°C until early pupal stages, when the sensory neurites begin to extend (Fabre et al., 2008); they were then transferred to 29°C to produce the RNAi. Each of the three RNAi constructs caused defects in the pathways followed by mechanosensory neurons, particularly in the anterior part of the segment, but varied in their efficacy. RNAi for *robo2* caused the strongest phenotype, with many misrouted axons and some unusual bundling; some axons even grew away from the peripheral nerve instead of towards it (Fig. 7D). Flies of the same genotype raised at 18°C throughout development, or *elav.Gal4 UAS.GFP* flies raised at 29°C, had normal patterns (Fig. 7C).

Finally, we show evidence which suggests that *robo3* is expressed exclusively in at least some of the multidentritic neurons (Grueber et al., 2007) (see Fig. S4 in the supplementary material). Perhaps these neurons respond to Sli via Robo3? Note that multidentritic neurons normally cofasciculate with mechanosensory neurons, and this might explain the abnormal fasciculation caused by RNAi against *robo3* (not shown).

Together, our results argue that both *ptc*$^{-}$ and *en*-expressing clones repel neurons because they produce Sli. Moreover, the mechanosensory neurons appear to require *robo2*. There is evidence that they might also express *dock* and *robo*; all these three proteins are involved in receiving the Sli signal. We therefore propose that the mechanosensory neurons are, at least in part, guided by a Sli signal emanating from cells within P compartments that acts to repel the neurons away from the compartmental borders.

DISCUSSION

In *Drosophila*, a region of each A compartment bears mechanosensory bristles that send axons centrally, but, between these regions, there are bare strips of integument that contain no bristles. As nascent axons leave the bristles en route to the CNS they orient away from these bare strips so that the nerves from each segment form separate bundles. The result is an orderly and somatotopic display of incoming neurons in the segmental ganglia of the CNS. The oriented paths followed by the axons are in no way influenced by the planar cell polarity of the epidermal cells nearby (Fabre et al., 2008), so we sought to identify what does orient them. Here, we present evidence that Sli, a secreted ligand for the transmembrane receptors Robo, Robo2 and Robo3, helps position the neurons and orients the outgrowing axons.

In the wild-type fly (and perhaps therefore also in many other invertebrates), we find that Sli is normally made in the P compartments, spreading forwards and backwards to repel neurons at the back and the front of the A compartments. As a consequence, the axons meet in the middle of the A compartments. Thus, En regulates *sli* expression to form a Sli gradient, the axons growing away from the source of Sli and down that gradient (Fig. 8). Sli may also drive oriented nucleokinesis of the mechanosensory cell bodies away from the compartmental boundaries (Fabre et al., 2008).

Hh is not involved directly in mechanosensory neuronal pathfinding

Gradients of morphogens, such as Wingless (Wg), Hh and Decapentaplegic, can act at short or long range to specify cell identity and have also been implicated in axon pathfinding. Numerous studies have concluded that Hh can act as an axonal repellent or attractant, and that axons can respond directly to the gradient of Hh (reviewed by Charron and Tessier-Lavigne, 2005). Surprisingly, in the abdomen we have presented evidence that Hh does not guide the mechanosensory neurons. We see no dependence on the Hh receptors Ptc or Smo (Fig. 4D,E,F, Fig. 5D,E,F and see Figs S1 and S2 in the supplementary material). This raises the possibility that some of the previously described effects of Hh (Charron and Tessier-Lavigne, 2005) might also be indirect. Indeed, in the zebrafish forebrain, Hh acts to guide commissural and retinal axons indirectly by regulating *sli* expression (Barresi et al., 2005).

En indirectly repels neurons away from the compartment borders via the induction of *sli* expression in the P compartment

In vertebrates, En affects axon routing (Saueressig et al., 1999; Wenner et al., 2000). In invertebrates, En modifies axon morphology via the expression of cell adhesion molecules such as Connectin and Neuroglian (Siegler and Jia, 1999) or the cell adhesion receptor Frazzled (Joly et al., 2007). In the cockroach cercus, En is essential for axonal pathfinding, perhaps acting directly on genes needed for guidance and synaptic recognition (Marie and Blagburn, 2003). There is a hypothesis that En acts directly: En protein has structural domains that could regulate nuclear export, secretion and cell-internalisation, processes also needed for axon pathfinding and target recognition (Wenner et al., 2000; Brunet et al., 2005; Wizenmann et al., 2009). However, our experiments in the fly abdomen point to a different conclusion. When *smo*$^{-}$ *en*$^{-}$ and *ptc*$^{-}$ *en*$^{-}$ (Fig. 4D-F and see Fig. S2 in the supplementary material) clones were produced in the P compartment, mechanosensory axons traversed anterior cells of the P compartment and a6 cells in which *en* is expressed. Thus, we judge it unlikely that En itself repels axons in the abdomen of *Drosophila*. Our evidence suggests instead that En drives the expression of *sli* autonomously (Fig. 6), the effects of En on pathfinding being due to local gradients of Sli concentration. The behaviour of axons emanating from *smo*$^{-}$ *en*$^{-}$ and *ptc*$^{-}$ *en*$^{-}$ clones in the P compartment can be understood in this context: the clones are small and even though they do not themselves secrete Sli (because they are transformed into A cells), they nevertheless find themselves in a Sli gradient, high behind and lower in front. Axons leaving such clones behave as expected and grow down that gradient.

The mode of action of Sli in neuronal and axonal repulsion has been studied in numerous systems (for reviews see Guthrie, 1999; Brose and Tessier-Lavigne, 2000; Wong et al., 2002; Piper and Little, 2003). In vertebrates, a gradient of one or more of the three Slit genes can induce the arrest of growth cones (Jia et al., 2005; Piper et al., 2006), similar to that observed here with *ptc*$^{-}$ and *en*-expressing clones, which are ectopic sources of Sli (Figs 3, 5 and 6).

Reception of Sli

In other systems, Sli is received by one or more Robo receptors acting with Dock (Fan et al., 2003; Dickson and Gilestro, 2006). In *Drosophila*, the three Robo genes are typically expressed in distinct but overlapping regions, but only Robo binds to Dock (Fan et al., 2003; Dickson and Gilestro, 2006). We present evidence that Robo and the co-receptor Dock are expressed in the mechanosensory neurons (see Fig. S3 in the supplementary material) and also that *robo3* is expressed in the multidendritic neurons (see Fig. S4 in the supplementary material). Results with

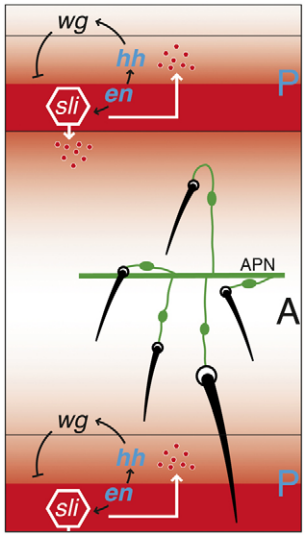

Fig. 8. A model of *sli* regulation. The *en* gene drives *sli* expression mainly in the back (posterior) of the P compartment, and secreted Sli (red) forms a U-shaped gradient in the A compartment. The Sli gradient repels axons away from both flanking P compartments and towards the middle of the A compartment, where they bundle with the APN. *sli* expression is subject to inhibition by Wg that spreads posteriorly into P from the more anteriorly adjacent A compartment. As a result, *sli* is more strongly expressed at the back of the P compartment. Wg is expressed at the back of the A compartment because the *wg* gene is activated by Hh at the rear, but not the front (anterior), of the A compartment (Struhl et al., 1997a).

RNAi suggest that all three Robo genes are required for the normal fasciculation of the mechanosensory axons; the strongest effect was found with RNAi for *robo2* (Fig. 7). Note that knockdown of any one of the Robo genes is unlikely to produce a very clear phenotype as they can partially substitute for each other (Spitzweck et al., 2010).

p3 and/or a6 normal territories are not avoided by neurons, but p3 and/or a6 in clones can repel axons: an inhibitory role for Wg on the *Sli* signal?

Flies carrying *sli.lacZ* suggest that *sli* is normally strongly expressed only at the back of the P compartments (Fig. 5), raising the question of how its expression is controlled in the wild type. In the adult tergites, *wg* is expressed at the rear of each A compartment (Shirras and Couso, 1996) and Wg protein is thought to cross over the A/P border to form a gradient that patterns the P compartment (Lawrence et al., 2002). If so, and if a high concentration of Wg were to inhibit *sli* expression, then *sli* expression might be blocked in the anterior part of P (p3), but allowed in the posterior part of P (p1) (Fig. 8). There are two other arguments supporting this hypothesis. First, *wg* is not (or is weakly) expressed in the most lateral tergite, which could explain why the band of *sli* expression is broader laterally and fills, or almost fills, the P compartment there. Second, *wg* is not expressed in the pleura (Lawrence et al., 2002), where *sli.lacZ* expression is ubiquitous (Fig. 5A). By contrast, in the sternites, *wg* is expressed and there *sli.lacZ* is confined to the P compartments (Fig. 5). It could therefore be that ptc^- as well as the *en*-expressing clones that are transformed towards P identity would not express *wg* themselves. Thus, when located far from the endogenous source of Wg they should escape repression and transform into p1, which is of extreme posterior P identity, and become sources of Sli, as observed (Fig. 5C).

Other cues?

Sli might work with other guidance cues in the fly abdomen. In the *Drosophila* eye, disruption of the Sli/Robo mechanism disturbs the boundary between the lamina and the distal cell neurons (Tayler et al., 2004). Tayler and colleagues suggest that the Fasciclin adhesion molecules also support the boundary: *Fas3* is expressed in the region where distal cell neurons are found, and *Fas2* is expressed by the photoreceptor axons that carry Hh to the lamina. We suspect that Fas2 and Fas3 might contribute to corralling neurons inside of the A compartment by promoting axonal bundling to the APN (see Fig. S5 and Table S1 in the supplementary material).

Marc Tessier-Lavigne wrote that “an individual axon might be pushed from behind by a chemorepellent, pulled from afar by a chemoattractant, and hemmed in by attractive and repulsive local cues” (Tessier-Lavigne and Goodman, 1996). These signals constitute what Ramón y Cajal proposed to be an ‘intelligent force’ guiding axons (Ramón y Cajal, 1890). It is not easy to dissect out these various signals, but we have found one repulsive signal, Sli, that hems in neurons and helps bundle segmental sets of sensory neurons in an arthropod.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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