Developmental Biology 430 (2017) 1–10

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Meninges-derived cues control axon guidance

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ARTICLE INFO

Keywords:
Meninges
Axon guidance
Motor neurons
Sensory neurons
Interneurons
Spinal cord

ABSTRACT

The axons of developing neurons travel long distances along stereotyped pathways under the direction of extracellular cues sensed by the axonal growth cone. Guidance cues are either secreted proteins that diffuse freely or bind the extracellular matrix, or membrane-anchored proteins. Different populations of axons express distinct sets of receptors for guidance cues, which results in differential responses to specific ligands. The full repertoire of axon guidance cues and receptors and the identity of the tissues producing these cues remain to be elucidated. The meninges are connective tissue layers enveloping the vertebrate brain and spinal cord that serve to protect the central nervous system (CNS). The meninges also instruct nervous system development by regulating the generation and migration of neural progenitors, but it has not been determined whether they help guide axons to their targets. Here, we investigate a possible role for the meninges in neuronal wiring. Using mouse neural tissue explants, we show that developing spinal cord meninges produce secreted attractive and repulsive cues that can guide multiple types of axons *in vitro*. We find that motor and sensory neurons, which project axons across the CNS-peripheral nervous system (PNS) boundary, are attracted by meninges. Conversely, axons of both ipsi- and contralaterally projecting dorsal spinal cord interneurons are repelled by meninges. The responses of these axonal populations to the meninges are consistent with their trajectories relative to meninges *in vivo*, suggesting that meningeal guidance factors contribute to nervous system wiring and control which axons are able to traverse the CNS-PNS boundary.

1. Introduction

During nervous system development, growing axons are guided to their targets by a combination of attractive and repulsive cues. These cues can be presented as gradients of diffusible factors or as cell surface- and extracellular matrix-attached molecules (Kolodkin and Tessier-Lavigne, 2011). In the developing embryo, axon guidance molecules are often produced by guidepost cells that are positioned at intermediate targets or boundaries of the axonal trajectory and serve as choice points (Chedotal and Richards, 2010).

One of the most fundamental decisions axons make is whether or not to cross the border between the CNS and PNS. While the vast majority of axons in the vertebrate nervous system do not traverse the CNS-PNS boundary, motor neurons in the spinal cord and hindbrain project axons into the periphery (Bonanomi and Pfaff, 2010). Conversely, sensory neurons in the trigeminal ganglion and the dorsal root ganglia (DRGs) send axon branches into the brain and spinal cord (Eide and Glover, 1995). The cellular and molecular mechanisms that prevent or allow axon growth across the CNS-PNS boundary remain largely unexplored.

The meninges are commonly characterized as a protective envelope for the mature CNS, but several studies over the last decade have demonstrated that the meninges contribute to nervous system development. The meninges regulate the survival and proliferation of radial glia in the forebrain, thereby contributing to cortical development (Radakovits et al., 2009; Siegenthaler et al., 2009). Furthermore, meninges-derived signals promote the migration of Cajal-Retzius cells (Borrell and Marín, 2006; Paredes et al., 2006) and other neural progenitors (Yang et al., 2013). The meninges have also been implicated as a negative regulator of corpus callosum formation but it remains unclear if this reflects a direct, *bona fide* axon guidance function (Choe et al., 2012). Because the meninges surround the entire CNS, they are in an ideal position to regulate axon behavior at the CNS-PNS interface. The spinal cord meninges originate from somitic mesoderm, which condenses around the neural tube shortly after neural tube closure at embryonic day 9 (E9) (McLone and Bondareff, 1975; Bagnall et al., 1989). Therefore, developing sensory and motor axons, which cross the CNS-PNS border starting at E9.5, must come into contact with meninges (Ozaki and Snider, 1997; Lieberam et al., 2005; Fraher et al., 2007). This places the meninges in the right

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http://dx.doi.org/10.1016/j.ydbio.2017.08.005
Received 7 March 2017; Received in revised form 3 August 2017; Accepted 3 August 2017
Available online 04 August 2017

location at the right time to regulate early axon guidance decisions at the CNS-PNS interface.

We hypothesized that the meninges contribute to nervous system wiring by producing axon guidance cues, and we tested this idea in a series of *in vitro* axon guidance assays. We report that the meninges surrounding the developing spinal cord produce a diffusible attractive guidance cue(s) for motor axons. The meninges also produce a secreted attractant(s) for DRG sensory axons and stimulate the growth of sensory axons in a contact-mediated manner. Furthermore, meninges transiently secrete a repellant for axons of both ipsi- and contralaterally projecting dorsal spinal cord interneurons. Our studies provide direct evidence that the meninges produce axon guidance molecules. These results suggest a meningeal function in neuronal wiring and raise the possibility that the meninges regulate axon entry and exit at the CNS-PNS border.

2. Materials and methods

2.1. Animals

HB9::GFP transgenic mice have been described before and were genotyped as originally reported (Wichterle et al., 2002). Mice were maintained on a CD-1 background. Explants were prepared from embryos of either sex.

2.2. Neuronal explant culture in collagen gels

For preparation of meninges explants, the meninges-covered brachial and thoracic spinal cord of E11.5, E12.5, or E18.5 embryos was exposed and forceps were laterally inserted between the meninges and spinal cord tissue about midway between the dorsal and ventral sides of the spinal cord. Incisions were made in the meninges along the length of both sides of the spinal cord, resulting in a dorsal and a ventral flap of meninges, which were removed from the embryo and trimmed to size. This produced 2–3 ventral and 2–3 dorsolateral pieces of meninges per E11.5/E12.5 embryo (more for E18.5), which were used for co-culture experiments. Dorsal spinal cord (DSC) explants from E11.5 mouse embryos were dissected and cultured in collagen gels as previously described (Serafini et al., 1994). Dorsolateral or ventral meninges from E11.5 or E18.5 embryos were either cultured alone or placed between one and two explant diameters away from DSC explants (Figs. 3 and 4A). Explants were grown in DSC medium (50% OptiMEM, 45% Ham's F-12, 1 x penicillin/streptomycin/glutamine (P/S/G) (all Gibco), 5% horse serum, 0.75% glucose), either for 20–23 h with 500 ng/ml Netrin-1 (R & D Systems) or 42–44 h without Netrin-1. Postcrossing explants from E11.5 embryos were dissected and cultured in collagen gels as previously described (Zou et al., 2000). Explants were grown in DSC medium for 22–23 h, either alone or with E11.5 ventral meninges explants. For preparation of ventral spinal cord (VSC) explants, open-book preparations of E11.5 *HB9::GFP* mouse brachial and cervical spinal cords were used to collect explants from the GFP-positive ventral horn. VSC explants were co-cultured in collagen gels with E11.5 ventral meninges in VSC medium (Neurobasal-A medium, 2% B-27 (both Gibco), 1 x P/S/G, 0.5% glucose) for 42–48 h (Fig. 1A). DRG explants were prepared from E12.5 mouse embryos by cutting individual DRGs into 4 equal pieces. Explants were co-cultured in collagen gels with either dorsolateral or ventral E12.5 meninges in DRG medium (VSC medium with Neurobasal instead of Neurobasal-A) containing 10 ng/ml Nerve Growth Factor (NGF; Promega), either for 24 h to measure axon growth/attraction or for 48 h to allow axons to contact the meninges (Fig. 2A).

2.3. Heat inactivation of meninges and propidium iodide staining

Dorsal or ventral meninges were harvested as described above. Tissue was heat-killed via incubation at 55 °C for 30 min. Meninges were then co-cultured with VSC or DRG explants in collagen cushions as described above. Separately, pieces of heat-killed meninges were immediately placed in 1 μg/ml propidium iodide (Sigma) in phosphate buffered saline (PBS) for 30 min, counter-stained with Hoechst 33342 (Molecular Probes, 1:1000), mounted, and imaged to confirm cell death.

2.4. Meninges-conditioned media

E11.5 meninges from ventral spinal cord were dissected in L15 (Gibco), rinsed twice with Hank's Balanced Salt Solution (HBSS), and incubated for 20 min at 37 °C with 0.05% Trypsin/EDTA (Gibco) and HBSS (1:1). After two rinses with 5% horse serum in L15, meninges were triturated through fire-polished Pasteur pipettes in growth medium (DMEM (Gibco), 1 x P/S/G, 10% fetal bovine serum), passed through a 70 μm cell strainer, and cultured on plastic dishes. After 24 h, meningeal cultures were switched to collapse medium (Neurobasal-A, 2% B-27, 1 x P/S/G), and conditioned medium was harvested 72 h later. The proteolytic digest during tissue dissociation ensured that meninges-conditioned media only contain molecules actively produced and secreted by meninges, not factors that might be produced by other cell types and deposited into the meninges-associated basal lamina. For control experiments, E11.5 body wall fibroblasts were cultured under the same conditions as meninges (above) to produce fibroblast-conditioned medium.

2.5. Growth cone collapse assay

E11.5 DSC explants were grown in collapse medium on 8-chamber glass slides coated with poly-D-lysine and N-Cadherin. After 24 h, explants were incubated for 30 min with conditioned media at different dilutions and processed for immunohistochemistry. Unconditioned collapse medium served as a control.

2.6. Immunohistochemistry

Unless indicated otherwise, all incubations were performed at room temperature. Collagen-embedded explants were fixed in PBS containing 4% paraformaldehyde (PFA) overnight at 4 °C, washed three times 10 min in PBS, blocked in either 2.5% goat serum or 2.5% fetal bovine serum and 0.1% Triton X-100 in PBS for 2 h, and incubated with primary antibodies in blocking solution at 4 °C overnight. After six 1-h washes in 0.1% Triton X-100 in PBS, explants were incubated with secondary antibodies in blocking solution at 4 °C overnight. Explants were washed six times 1 h in 0.1% Triton X-100 in PBS and mounted on hanging drop slides using Fluoromount G. Collapse assay explants were fixed for 30 min by adding pre-warmed 8% PFA in PBS 1:1 to DSC cultures. After three 10-min washes in PBS, explants were incubated in blocking solution for 30 min and incubated with primary antibody in blocking solution at 4 °C overnight. After three 10-min washes in blocking solution, explants were incubated with secondary antibody in blocking solution for 2 h, washed three times in blocking solution (10 min each), and mounted under Fluoromount G. The primary antibodies used were rabbit polyclonal antibodies against TuJ1 (Covance, 1:1000) and Peripherin (Millipore, 1:200), goat polyclonal antibodies against SDF-1 (Santa Cruz, 1:500) and TAG-1 (R & D Systems, 1:200), a chick polyclonal antibody against GFP (Abcam, 1:200), and a mouse monoclonal antibody against NeuN (Millipore, 1:200). Secondary antibodies (from Invitrogen; 1:200) were Alexa488-conjugated goat anti-rabbit, Alexa488-conjugated donkey anti-rabbit, Alexa488-conjugated donkey anti-goat, Alexa594-conjugated donkey anti-rabbit, Alexa594-conjugated donkey anti-mouse, Alexa594-conjugated donkey anti-goat, Alexa647-conjugated rabbit anti-goat, and Alexa488-conjugated donkey anti-chick (Jackson Immuno; 1:200). Hoechst 33342 (1:1000) and Alexa594-conjugated Phalloidin (Molecular Probes, 1:100) were added with the secondary antibodies. All images were acquired on a Nikon Ti-E microscope.

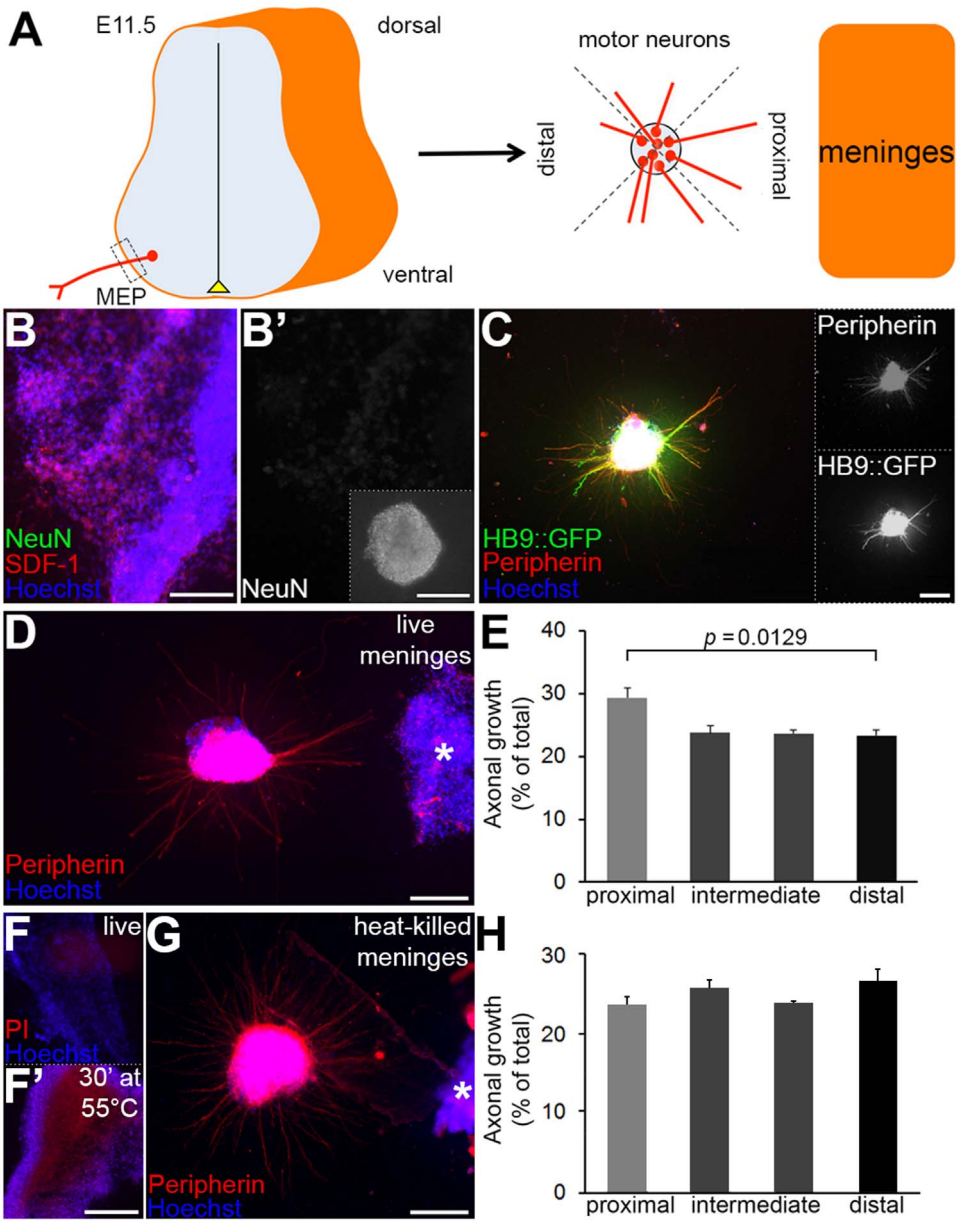


Fig. 1. Motor axons are attracted to meninges *in vitro*. (A) A schematic of motor axon projections out of the developing spinal cord and the *in vitro* assay for motor axon responses to meninges. (B, B') Meninges explants from E11.5 embryos were cultured and labeled with NeuN antibody to visualize neurons, SDF-1 to visualize meninges, and Hoechst stain for nuclei. Inset in B' shows a E11.5 VSC tissue explant with NeuN staining as positive control. (C) VSC explants from E11.5 *HB9::GFP* embryos were cultured and labeled with GFP and Peripherin antibodies to visualize motor axons and Hoechst stain. Insets show isolated Peripherin and GFP channels. (D, E) VSC explants and ventral meninges (*) from E11.5 mice were co-cultured and labeled with Peripherin antibody and Hoechst stain (D), and motor axon growth relative to meninges explants was quantified (E). Motor axon growth in the quadrant proximal to meninges explants is significantly higher than in the distal quadrant (n = 4 independent experiments). (F) Live (F) or heat-killed (F') ventral meninges explants from E11.5 embryos were treated with propidium iodide (PI) to visualize dead cells. (G, H) E11.5 VSC explants and heat-killed meninges were co-cultured and labeled with Peripherin antibody and Hoechst stain (G), and motor axon growth relative to meninges was quantified (H). Motor axon growth is similar in the proximal and distal quadrants relative to heat-killed meninges (n = 3). Scale bars: 125 μm in B; 250 μm in B' inset; 250 μm in C, D; 250 μm in C insets; 250 μm in F, F'; 250 μm in G. Error bars indicate SEM.

2.7. Quantification of axon growth and growth cone collapse

A minimum of 3 independent experiments (n, indicated in figure legends or Results section) were performed to quantify axonal growth responses. For each independent experiment (n = 1), between 6 and 39 TuJ1-, Peripherin-, or TuJ1/TAG-1-stained explants were quantified and averaged. The ImageJ plugin NeuronJ was used to measure the length of individual axons bundles emanating from tissue explants. Axons were measured from the point where they first emerged from the explant to their distal tip. Total axon growth was determined by measuring the summed length of all axon bundles from a given explant. To measure attraction or repulsion, quadrants were delineated by placing a right-angled crosshair at the center of each explant with the proximal quadrant directly facing the meninges (Figs. 1–4A), and explants with axon fascicles making contact with the meninges were excluded from analysis. Axon bundles were assigned to quadrants based on the location of the tip of the bundle. For DSC (with Netrin-1) and DRG explants, the total summed lengths of TuJ1-positive axons in the proximal, intermediate, and distal quadrants of all explants relative to the meninges were determined and divided by the total summed length from all quadrants of all explants in each experiment. For VSC explants, a similar analysis was performed using Peripherin as marker for motor axons. For DSC explants cultured without Netrin-1, commissural

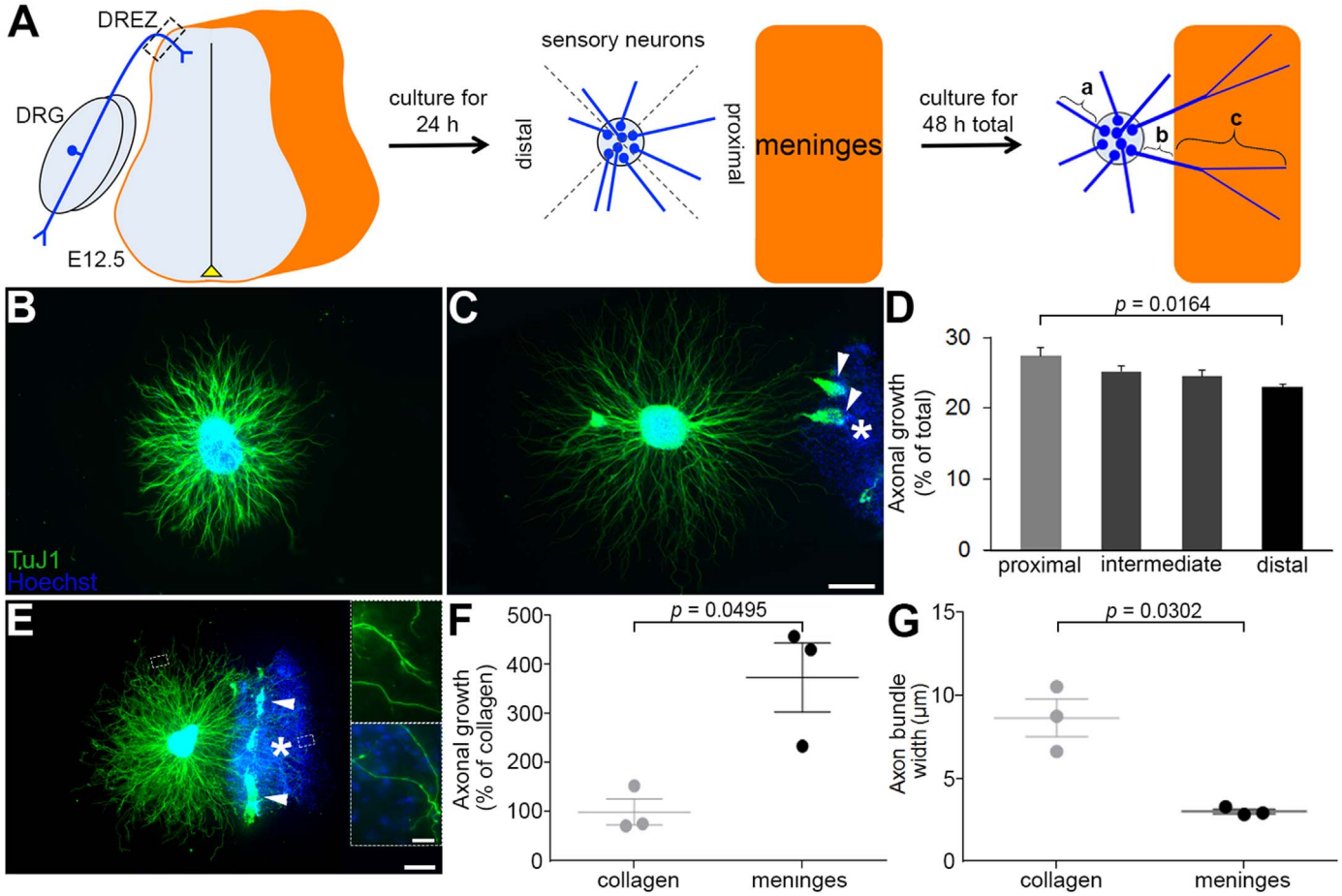
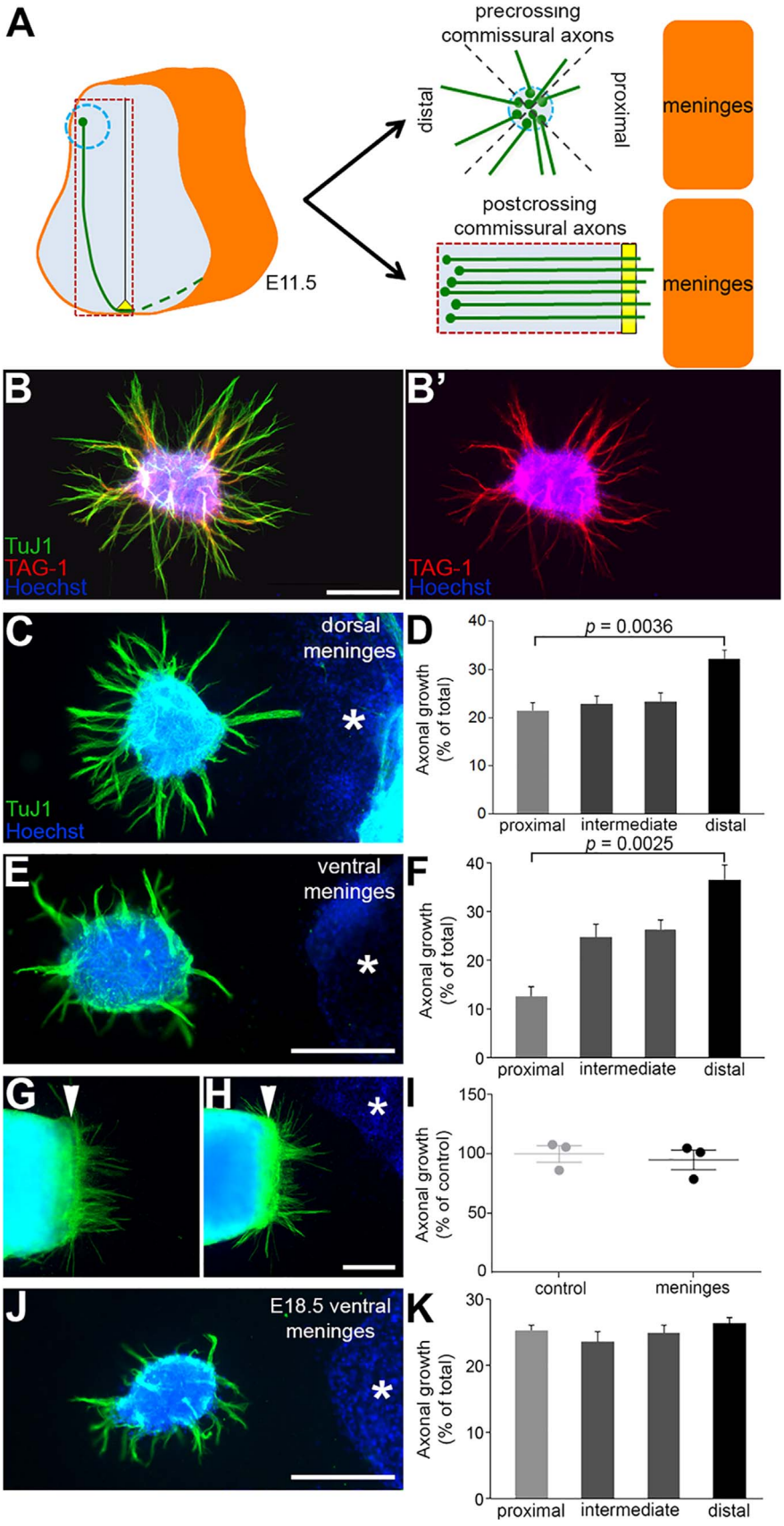


Fig. 2. Effects of meninges on sensory axon growth. (A) A schematic of sensory axon projections from DRGs into the developing spinal cord and the *in vitro* assay for sensory axon responses to meninges, as well as contact-dependent sensory axon responses to meninges. Brackets indicate measurements used in quantification of the contact-dependent growth effect (see Section 2). (B, C) DRG explants from E12.5 mice were cultured for 24 h, either alone (B) or in the presence of dorsal meninges (*) (C) and labeled with class III β–Tubulin (TuJ1) antibody to visualize axons and Hoechst stain. (D) Sensory axon growth relative to dorsal meninges explants was quantified. Sensory axon growth in the quadrant proximal to meninges explants is slightly but significantly higher than in the distal quadrant (n = 4). (E-G) When sensory axons are allowed to make direct contact with meninges after 48 h of co-culture, growth is significantly accelerated (E, quantified in F) and axon bundle diameter is significantly decreased (E, quantified in G) (n = 3). Insets in E depict axons growing through collagen (top) compared to axons that make contact with meninges (bottom). Arrowheads in C, E indicate remnants of DREZ that remained attached to dorsal meninges explants. Scale bars: 200 μm in B, C; 200 μm in E; 50 μm in E insets. Error bars indicate SEM.

(TuJ1-positive and TAG-1-positive) axons and ipsilateral (TuJ1-positive and TAG-1-negative) axons were analyzed separately. The means for normalized proximal and distal growth from several experiments were compared in an unpaired two-tailed *t*-test. Means for proximal growth of either motor or sensory axons in the presence of live versus heat-killed meninges were compared in an unpaired two-tailed *t*-test. Similarly, means for proximal growth of DSC explants with E11.5 ventral meninges and DSC explants with E18.5 ventral meninges were compared in an unpaired two-tailed *t*-test, and the means for growth in the proximal quadrant of DSC explants cultured adjacent to dorsal versus ventral E11.5 meninges were compared using an unpaired two-tailed *t*-test. For VSC and DSC (with Netrin-1) explants, total summed axon lengths, when cultured alone or co-cultured with age-matched ventral meninges, were determined. The normalized means for total growth from several experiments were compared in a paired two-tailed *t*-test. For post-crossing explants, the average summed length of axons per explant for each experimental condition was determined, and normalized means from multiple independent experiments were compared in a paired two-tailed *t*-test. For quantification of DRG axon growth after contact with meninges, a ratio of contact-mediated growth to non-contact mediated growth was determined for each explant. Three measurements were taken for each explant: first, the average length of all axons not contacting meninges (a_{avg}), second, the length of each meninges-contacting axon prior to meninges contact (b), and third, the length of each meninges-contacting axon after meninges contact (c) (Fig. 2A). Contact-mediated growth rate for each axon bundle was expressed as $x=\frac{c}{a(avg)-b}$. If meninges contact had no effect on growth rate ($b + c = a_{avg}$), this would yield x=1. Contact-mediated growth rates from all bundles belonging to an explant were averaged and multiplied by a_{avg} to generate an average expected bundle length if growth had been entirely in contact with meninges. The mean of these values was then calculated across all explants from the same experiment. Similarly, the mean of a_{avg} was calculated across explants. Normalized means of expected contact-mediated growth (expressed as "% of collagen" in Fig. 2F) and normalized means of a_{avg} (with the average defined as 100%) from multiple independent experiments were then compared in a paired two-tailed *t*-test. To quantify defasciculation of DRG axons upon contact with meninges, NeuronJ was used to measure the width of meninges-contacting and freely growing axon bundles at the most distal 10 μm of each bundle. The average diameter of all axon bundles for each condition was determined, and means from three independent experiments were compared in a paired two-tailed *t*-test.

For growth cone collapse assays, an average of about 100 axon terminals per experimental condition were scored for collapse, and means from three independent experiments were compared in a paired two-tailed *t*-test.


A
precrossing commissural axons
distal
proximal
meninges
postcrossing commissural axons
meninges
E11.5
B
TuJ1
TAG-1
Hoechst
B'
TAG-1
Hoechst
C
dorsal meninges
TuJ1
Hoechst
D
p = 0.0036
Axonal growth (% of total)
proximal
intermediate
distal
E
ventral meninges
F
p = 0.0025
Axonal growth (% of total)
proximal
intermediate
distal
G
H
I
Axonal growth (% of control)
control
meninges
J
E18.5 ventral meninges
K
Axonal growth (% of total)
proximal
intermediate
distal

3. Results

3.1. Spinal cord meninges produce an attractant cue(s) for motor axons

Motor axons must leave the developing neural tube ventrally through motor exit points (MEPs) to project toward peripheral targets (Bonanomi and Pfaff, 2010) (Fig. 1A). This raises the possibility that the meninges surrounding the ventral spinal cord contribute to guiding motor axons out of the CNS. To test this idea and study the response of motor axons to meninges, we established a collagen gel co-culture assay for E11.5 VSC explants, which contain motor neuron cell bodies, and ventral spinal cord meninges (Fig. 1A,D). Using immunohistochemistry, we confirmed that meninges explants express the meningeal marker SDF-1 (Lu et al., 2002; Borrell and Marín, 2006) and do not contain neuronal cell bodies, as marked by NeuN (Fig. 1B). Furthermore, we established the peripheral axon cytoskeletal protein Peripherin (Escurat et al., 1990) as an *in vitro* marker for motor axons by staining VSC explants from E11.5 *HB9::GFP* embryos, which express GFP specifically in motor axons (Wichterle et al., 2002), with a Peripherin antibody (Fig. 1C). We found that motor axon growth from VSC explants was radially symmetric when these explants were cultured in isolation (range of average summed axonal length per quadrant as fraction of total (per experiment): 22.6–26.8%) (Fig. 1C). When we co-cultured VSC explants and ventral meninges, we found that motor axons preferentially grow towards the meninges explants (Fig. 1D). Quantification of this effect (Fig. 1E) showed that growth in the meninges-proximal quadrant of explants was significantly increased compared to growth in the distal quadrant (summed axonal length as fraction of total: proximal, 29.3 ± 1.4%; distal, 23.3 ± 0.8%; p = 0.0129). We compared total motor axon growth between VSC explants that were cultured alone and VSC explants co-cultured with ventral meninges and observed a significant reduction in total axonal growth in the presence of meninges (normalized summed axon length per explant: VSC, 100.0 ± 22.0%; VSC + meninges: 73.9 ± 17.6%; n = 3 independent experiments; p = 0.0157). This indicates that the ventral meninges attract motor axons without stimulating axon growth per se. To ascertain the specificity of this effect, we sought to abolish it by heat-killing the meninges. We confirmed meningeal cell death after heat treatment using propidium iodide staining (Fig. 1F). We found that motor axon growth from VSC explants that were co-cultured with heat-killed ventral meninges was radially symmetric (proximal growth, 23.7 ± 0.9%; distal, 26.7 ± 1.4%; p = 0.1593) (Fig. 1G,H) and that growth proximal to heat-killed meninges was significantly reduced compared to proximal growth in the presence of live meninges (p = 0.0331) (see Fig. 1E). Thus, the meninges produce one or more diffusible attractant(s) for motor axons, supporting the idea that meningeal guidance factors can guide motor axons towards their spinal cord exit points.

3.2. Meninges attract sensory axons and serve as a growth-promoting substrate

Sensory neuron cell bodies are located within the DRG, and the central branches of sensory axons cross the CNS-PNS boundary at the dorsal root entry zone (DREZ) to innervate various CNS targets (Eide and Glover, 1995) (Fig. 2A). To investigate the possibility that the dorsal meninges regulate CNS entry of sensory axons, we confronted sensory axons with meningeal tissue *in vitro*. We cultured DRG explants from E12.5 mouse embryos either alone or in the presence of pieces of dorsolateral meninges from age-matched spinal cord in collagen gels (Fig. 2A-C). These explants were grown in the presence of NGF, which promotes outgrowth of TrkA-positive sensory axons (Reichardt, 2006). We found that axons from DRG explants cultured alone exhibited largely radially symmetric growth (range of growth per quadrant: 24.5–25.4%) (Fig. 2B). When we quantified sensory axon growth in co-culture with dorsolateral meninges (Fig. 2C,D), we found that growth in the meninges-proximal quadrant of explants was slightly but significantly increased (27.4 ± 1.2%) compared to growth in the distal quadrant (22.9 ± 0.5%; p = 0.0164). Similar results were obtained when DRG explants were grown in the presence of NT-3 (data not shown), which promotes outgrowth of TrkB- and TrkC-positive axons (Reichardt, 2006). Additionally, DRG explants co-cultured with heat-killed dorsal meninges showed a significant reduction of axonal growth in the proximal quadrant (23.4 ± 0.5%) when compared to either the distal quadrant (28.1 ± 0.5%; n = 4; p = 0.0012) or the quadrant proximal to live meninges (p = 0.0499), confirming the specificity of the attractive effect that live meninges have on DRG axons.

In separate experiments, sensory axons were allowed to make direct contact with dorsal meninges explants by extending the culture duration (Fig. 2A,E). We found that axon bundles in contact with meninges grow significantly longer than axons that do not make contact with meninges (Fig. 2E,F) (axon length after meninges contact normalized to meninges-free growth: non-contact growth, 100.0 ± 26.6%; contact-mediated growth, 374.0 ± 70.5%; p = 0.0495; see Materials and Methods for details of quantification). Additionally, we observed that sensory axons become less fasciculated once they contact meninges compared to axon bundles extending into meninges-free collagen (mean axon bundle width: no meninges contact, 8.63 ± 1.13 μm; meninges contact, 3.00 ± 0.14 μm; p = 0.0302) (Fig. 2E,G). Moreover, axons that make contact with meninges tend to stay closely associated with meninges and do not re-enter the collagen substrate (Fig. 2E). Together, these results indicate that meninges exert a weak long-range attractive effect on sensory axons and possess a short-range or contact-mediated growth-promoting activity, consistent with the idea that the meninges can positively regulate sensory axon growth at or near the spinal cord surface.

3.3. Meninges repel spinal commissural axons

Contrary to sensory and motor axons that cross the CNS-PNS boundary, the axons of spinal commissural interneurons remain within the CNS. Commissural axons initially grow through the dorsal neuroepithelium towards the ventral midline of the spinal cord and enter the developing fiber tracts adjacent to the ventrolateral meninges after crossing the midline (Dickson and Zou, 2010) (Fig. 3A). To determine whether commissural axons respond to meningeal guidance cues, we confronted them with either dorsal or ventral spinal cord meninges *in vitro*. Collagen-embedded DSC explants from E11.5 mouse embryos were cultured in the presence of Netrin-1, which promotes radially symmetric growth of commissural axon fascicles (Serafini et al., 1994) that express the cell surface molecule TAG-1 (Yamamoto et al., 1986)

Fig. 3. Meninges repel precrossing commissural axons *in vitro*. (A) A schematic of commissural axon projections in the developing spinal cord and the *in vitro* assays for commissural axon responses to meninges. (B, B') DSC explants from E11.5 mice were cultured in the presence of Netrin-1 and labeled with TAG-1 antibody to visualize commissural axons, TuJ1 antibody, and Hoechst stain. (C-F) DSC explants and meninges (*) from dorsal (C) or ventral (E) spinal cord of E11.5 mice were co-cultured in the presence of Netrin-1 and labeled with an antibody against TuJ1 and Hoechst stain, and commissural axon growth relative to meninges explants was quantified (D, F). Commissural axon growth in the quadrant proximal to meninges explants is significantly lower than in the distal quadrant, both for dorsal meninges (D, n = 4) and for ventral meninges (F, n = 3). (G, H) Half spinal cord explants from E11.5 mice were cultured either alone (G) or with ventral meninges explants (*) (H) and labeled with TuJ1 antibody and Hoechst stain. Commissural axons leave spinal cord explants through the floor plate (arrowheads) under both conditions. (I) Postcrossing axon growth was quantified and is not affected by the presence of meninges (n = 3). (J, K) E11.5 DSC explants and E18.5 ventral meninges (*) were co-cultured in the presence of Netrin-1 and labeled with TuJ1 antibody and Hoechst stain (J), and commissural axon growth relative to meninges explants was quantified (K). Commissural axon growth is similar in the proximal and distal quadrants relative to E18.5 meninges (n = 3). Scale bars: 200 μm in B; 200 μm in C, E; 200 μm in G, H; 200 μm in J. Error bars indicate SEM.

(Fig. 3B). When we co-cultured DSC explants with pieces of dorsal E11.5 meninges (Fig. 3A,C), we found that commissural axons preferentially grow away from meningeal explants (Fig. 3C). We quantified this effect (Fig. 3D) and found that axon growth in the meninges-proximal quadrant of DSC explants is reduced when compared to growth in the meninges-distal quadrant (proximal growth, 21.5 ± 1.5%; distal, 32.2 ± 1.7%; p = 0.0036), indicating that dorsal meninges produce a diffusible repellant(s) for commissural axons. When we co-cultured DSC explants from E11.5 mouse embryos with age-matched ventral meninges, including meninges covering the floor plate, commissural axons again exhibited reduced growth in the meninges-proximal quadrant (12.6 ± 2.0%) when compared to growth in the distal quadrant (36.5 ± 2.9%; p = 0.0025) (Fig. 3E,F). Interestingly, we found that repulsion from the ventral meninges is

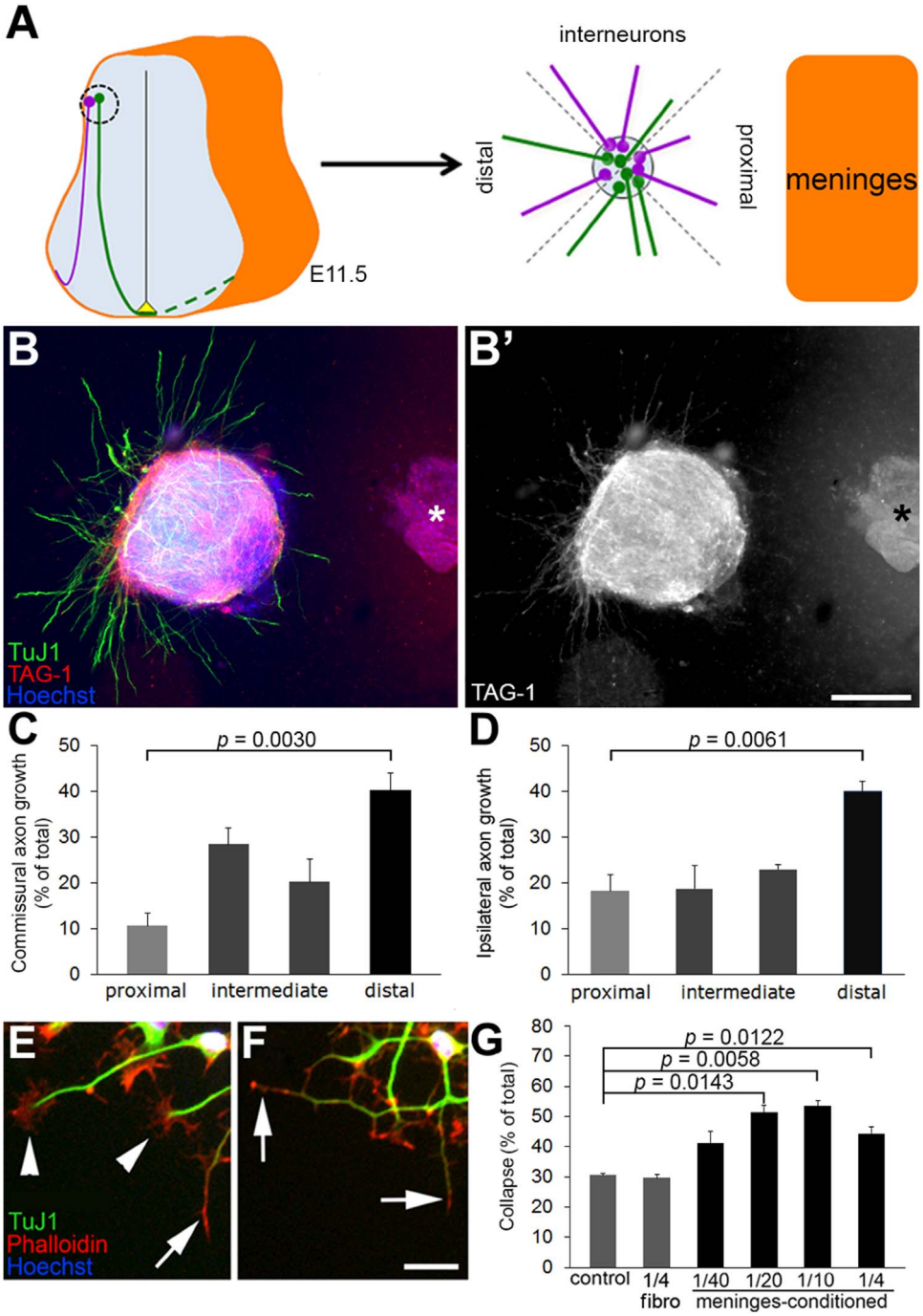


Fig. 4. Meninges repel multiple populations of spinal interneuron axons. (A) A schematic of the *in vitro* assay for commissural and ipsilateral axon responses to meninges. (B-D) DSC explants and ventral meninges (*) from E11.5 mice were co-cultured and labeled with antibodies against TuJ1 and TAG-1 to identify commissural axons (TAG-1-positive) and ipsilateral axons (TAG-1-negative), as well as Hoechst stain (B, B'), and axon growth relative to meninges was quantified (C, D). In the absence of Netrin-1, commissural axon growth in the quadrant proximal to meninges explants is significantly lower than in the distal quadrant (C, n = 3). Similarly, ipsilaterally projecting axons display significant growth reduction in the quadrant proximal to meninges compared to the distal quadrant (D, n = 3). (E-G) DSC interneurons from E11.5 mice were acutely exposed to control medium or meninges-conditioned medium and stained with TuJ1 antibody, Hoechst stain, and fluorescent Phalloidin to label growth cone actin. Under control conditions (E), most axons are tipped by fan-like actin-rich growth cones with numerous filopodia (arrowheads). When exposed to meninges-conditioned medium (F), many growth cones are collapsed (arrows). Growth cone collapse under different experimental conditions was quantified (G), and meninges-conditioned medium significantly increases the number of collapsed growth cones at 20-fold, 10-fold, and 4-fold dilutions when compared to unconditioned or fibroblast-conditioned ("fibro") control media (n = 3). Scale bars: 200 μm in B; 20 μm in E, F. Error bars indicate SEM.

stronger than the repulsive effect of dorsal meninges (comparison of proximal growth, ventral versus dorsal meninges: $p = 0.0007$), suggesting that meningeal repellants are less abundant dorsally. Total axon growth from DSC explants cultured in the presence of ventral meninges is not significantly different than growth from DSC explants that were cultured without meninges (DSC, 100.0 ± 13.5%; DSC + ventral meninges, 112.8 ± 7.5%; n = 4; $p = 0.1871$). Thus, meningeal cues repel commissural axons without inhibiting their growth.

Commissural axons undergo a switch from floor plate attraction to repulsion after crossing the spinal cord midline, as they transition from circumferential to longitudinal growth (Shirasaki et al., 1998; Zou et al., 2000). We reasoned that, similarly, commissural axons might lose their responsiveness to meningeal repellants when they extend in close proximity to the meninges during and after floor plate crossing. To address this possibility, we used a postcrossing explant assay and studied the response of axons that have crossed the floor plate to meningeal tissue (Fig. 3A). In this assay, repulsive cues elicit a reduction in total axon growth (Zou et al., 2000). We cultured postcrossing explants from E11.5 embryos either in isolation (Fig. 3G) or in the presence of ventral meninges (Fig. 3H) and quantified axonal growth under both conditions (Fig. 3I). We found that meninges do not affect axon growth from postcrossing explants (normalized summed axon length per explant: control, 100.0 ± 6.9%; meninges, 95.1 ± 8.2%; $p = 0.7388$), and we observed no deflection of postcrossing axons from the meninges when presented at an angle (Fig. 3H). These data indicate that commissural axons become unresponsive to meningeal repellants after crossing the floor plate.

To determine if expression of meninges-derived repulsive cues is transient or persists at later developmental stages, we co-cultured E11.5 DSC explants (with Netrin-1) and ventral meninges from E18.5 embryos (Fig. 3J). We quantified axon growth (Fig. 3K) and observed similar levels of growth proximal (25.2 ± 0.8%) and distal to the meninges (26.3 ± 0.8%; $p = 0.4005$). Proximal growth in these experiments was significantly higher when compared to results obtained with E11.5 ventral meninges ($p = 0.0040$) (see Fig. 3E,F). Thus, the meninges lose their repulsive activity for commissural axons between E11.5 and E18.5.

3.4. Meninges produce a secreted repulsive cue(s) for axons of multiple spinal cord interneuron populations

Netrin-1 selectively promotes the growth of commissural axons from DSC explants (Serafini et al., 1994). To determine if other interneuron populations, including those that project their axons ipsilaterally within the spinal cord, respond to meningeal cues, we co-cultured DSC explants and age-matched meninges in the absence of Netrin-1 (Fig. 4A,B). Under these conditions, explants exhibit concurrent growth of both ipsilaterally and contralaterally projecting (i.e. commissural) axons, as shown by immunohistochemistry for the cell adhesion molecule TAG-1 (Fig. 4B), which is expressed on commissural but not ipsilateral axons (Dodd et al., 1988). We found that TAG-1-positive commissural axons cultured in the absence of Netrin-1 are strongly repelled by meninges (proximal growth, 10.9 ± 2.8%; distal, 40.3 ± 3.6%; $p = 0.0030$) (Fig. 4C). This response is comparable to the observed repulsion of these axons when cultured with Netrin-1 and confronted with ventral meninges (Fig. 3E,F), suggesting that commissural axon repulsion from meninges in the latter assay is not the result of Netrin-1 antagonism. Quantification of ipsilateral (TAG-1-negative) axon growth (Fig. 4D) also showed substantial axon repulsion from the meninges (proximal growth, 18.2 ± 3.6%; distal, 40.3 ± 2.1%; $p = 0.0061$), indicating that the meninges produce a repellant cue(s) for axons of both contra- and ipsilaterally projecting DSC interneurons.

To examine the acute effects of secreted meningeal cues on the axons of DSC interneurons, we cultured dissociated E11.5 meningeal cells and harvested conditioned media from these cultures. We then grew DSC explants on a two-dimensional substrate, exposed axons to meninges-conditioned media, and examined growth cone morphology. Under control conditions, most axons are tipped by fan-like, actin-rich growth cones with numerous filopodia (Fig. 4E), while the addition of meninges-conditioned medium caused many growth cones to collapse (Fig. 4F), which is a hallmark activity of many repulsive axon guidance cues. We quantified growth cone collapse in response to different concentrations of conditioned medium (Fig. 4G) and found that 20-fold, 10-fold, and 4-fold dilutions of meninges-conditioned medium cause significant collapse when compared to control medium (fraction of collapsed growth cones: control, 30.5 ± 0.6%; 40-fold dilution, 41.0 ± 4.0%, $p = 0.1028$; 20-fold, 51.3 ± 2.4%, $p = 0.0143$; 10-fold, 53.4 ± 2.0%, $p = 0.0058$; 4-fold, 44.3 ± 2.1%, $p = 0.0122$), while a 4-fold dilution of fibroblast-conditioned medium did not cause any collapse (29.6 ± 1.1%; $p = 0.6365$). Thus, meninges secrete one or several cues that collapse growth cones of DSC interneuron axons. Taken together, our results indicate that the meninges produce a diffusible repellant(s) for axons of DSC interneurons and suggest that meningeal guidance cues help shape axonal trajectories within the spinal cord.

4. Discussion

Our results establish the developing spinal cord meninges as a source of attractive and repulsive cues that selectively guide several types of axons. We show that motor and sensory axons are attracted to meninges via one or several secreted attractive cues produced during development. Additionally, the axons of spinal interneurons are repelled by secreted meninges-derived cues. We provide the first direct evidence that meningeal cues can guide developing axons, raising the possibility that the meninges act as a choice point during neuronal wiring.

4.1. Meninges-derived attractive cue(s) guide sensory and motor axons

Motor and sensory axons must cross the border between the CNS and PNS. We hypothesized that attractive or permissive cues expressed by the developing meninges at the CNS-PNS interface facilitate axon crossing of this boundary. Here, we show that motor axons are attracted to the ventral meninges at a distance *in vitro*. Perhaps surprisingly, total motor axon growth is reduced (by about 26%) in the presence of meninges. This indicates that the observed motor axon attraction is not the result of a meningeal growth-promoting factor and that meninges instead secrete an attractive guidance cue(s) for motor axons, possibly in conjunction with growth-inhibitory factors. Our data suggest that the meninges might assist in guiding motor axons out of the spinal cord and into the periphery *in vivo*.

We also observed a weak attractive effect of dorsal meninges on E12.5 sensory axons at a distance, although we cannot fully exclude the possibility that our results reflect a growth-promoting effect. *In vivo*, sensory axons have a peripheral and a central branch, and it is unclear if sensory neurons grown *in vitro* elaborate axons that reflect a PNS or CNS fate (or both). While the central branch needs to enter the dorsal spinal cord and might therefore be attracted to meninges, the peripheral branch might ignore meningeal cues or exhibit repulsion from meninges. Hence, it is possible that a potentially stronger attractive effect on the central branch of sensory axons *in vitro* is partly obscured by peripheral branch growth. Additionally, we found that sensory axon growth rate is stimulated (approximately 4-fold) by direct contact with meninges, indicating that the meninges are a favorable substrate for these axons. Our finding that sensory axons defasciculate upon contact with meninges further suggests that adhesive axon-meninges interactions dominate over axon-axon adhesion forces. Meninges produce extracellular matrix molecules that could potentially mediate this observed effect (Sievers et al., 1985). Together, our results support the idea that diffusible meninges-derived cues aid in guiding sensory axons toward the spinal cord while strong contact-

mediated interactions between sensory axons and meninges can stimulate axon growth after spinal cord entry.

4.2. Meningeal cues(s) repel the axons of spinal cord interneurons

Unlike sensory and motor axons, spinal commissural axons remain within the CNS. Commissural axon guidance is largely orchestrated by floor plate-derived molecules (Dickson and Zou, 2010), but additional repellants expressed within the spinal cord, such as NELL2, help create a permissive corridor for commissural axons *en route* to the floor plate (Jaworski et al., 2015). We considered the possibility that the meninges further help restrict commissural axon growth to the CNS and found that both ventral and dorsal meninges repel commissural axons at a distance. Ventral meninges produce a stronger repulsive effect than dorsal meninges, suggesting that the meningeal repellant(s) are expressed in a gradient that increases from dorsal to ventral. Meninges do not affect total commissural axon growth *in vitro*, arguing against production of a growth-inhibitory molecule. Moreover, commissural axons are repelled by meninges irrespective of the presence of Netrin-1 in the culture media, indicating that the meninges do not exert their repulsive effect via a Netrin-1 antagonist. Because Netrin-1 itself is a growth-stimulatory and attractive cue for commissural axons, it is unlikely that meninges-derived Netrin-1 mediates axon repulsion in our assay. Together, our results support the idea that commissural axons respond to *bona fide*, as yet unidentified, repulsive guidance cues secreted by meninges.

The repulsive activity of meninges is specific for commissural axons that have not crossed the floor plate, as postcrossing axons do not respond to meninges. Hence, commissural axons down-regulate their responsiveness to meningeal repellants after midline crossing, similar to the down-regulation of sensitivity to floor plate-derived Netrin-1 in postcrossing axons (Shirasaki et al., 1998). We found that production of the meningeal repellant(s) is transient and coincides with commissural axon growth in the spinal cord, as meninges from later developmental stages (E18.5) do not repel commissural axons. Furthermore, the meninges also repel the axons of ipsilaterally projecting spinal cord interneurons, and meninges-conditioned media collapse DSC growth cones. Together, our data indicate that the meninges are transiently producing one or several diffusible repellant cue(s) for multiple populations of spinal interneuron axons, which could help shape their trajectories within the spinal cord and prevent their exit from the neural tube.

4.3. A possible contribution of meninges to shaping axonal trajectories in the developing spinal cord

The *in vitro* axon guidance activities of meninges for different classes of neurons are consistent with the respective axonal projection patterns *in vivo*, supporting the idea that meningeal guidance cues help sculpt axonal trajectories in the developing nervous system. Motor axons must project through the meninges at MEPs in order to innervate the periphery. Initial motor axon orientation towards the MEPs is thought to be facilitated by floor plate derived repellants and mesenchyme-derived attractive cues (Guthrie and Pini, 1995; Lieberam et al., 2005). Our results support the idea that meningeal attractants further help guide motor axons towards the CNS-PNS border.

The central branch of sensory axons crosses the dorsal meninges to enter the spinal cord and then travels along the rostral-caudal axis directly adjacent to the meninges in the dorsal funiculus. Our findings indicate that meninges-derived long-range attractive cues might contribute to guiding the central sensory axon branch to the spinal cord, while short-range growth-promoting and adhesive molecules could facilitate sensory axon extension in the dorsal funiculus.

Ipsilateral and precrossing commissural axons project through the grey matter within the spinal cord and do not cross the meninges. Our results support the idea that precrossing commissural axon repulsion by dorsal meninges might prevent these axons from projecting laterally and help guide them towards the midline in collaboration with other sources of guidance cues. Ventral meninges, on the other hand, could prevent commissural axons from overshooting and leaving the spinal cord as they approach the strongly attractive floor plate, which is in immediate vicinity to the CNS-PNS border. Postcrossing commissural axons project in the ventrolateral funiculus in very close proximity to the meninges, which may be facilitated by attenuating responsiveness to meningeal repellants. It is likely that other factors keep postcrossing commissural axons contained within the spinal cord – these could be attractive cues produced within the CNS or repulsive cues originating from non-meningeal tissues surrounding the spinal cord.

In summary, while the identities of meningeal axon guidance cues and their precise functions *in vivo* remain elusive, our results show that these cues could help shape the trajectories of multiple axonal populations during nervous system wiring. Because the meninges envelop the entire CNS, our results suggest that the meninges might serve as a "gatekeeper" in the sense that meninges-derived guidance factors can control which axons are able to traverse the CNS-PNS boundary.

Author contributions

TS, ZD, and AJ performed research, analyzed data and prepared the manuscript. AJ also designed research.

Conflict of interest

Authors report no conflict of interest.

Funding source

This work was supported by Brown University and the NIH Training Grant T32GM077995.

Acknowledgments

We thank Diane Lipscombe, Gilad Barnea, and members of the Jaworski lab for their thoughtful comments on the manuscript.

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