Astrocyte-encoded positional cues maintain sensorimotor circuit integrity

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Astrocytes, the most abundant cells in the central nervous system, promote synapse formation and help to refine neural connectivity. Although they are allocated to spatially distinct regional domains during development, it is unknown whether region-restricted astrocytes are functionally heterogeneous. Here we show that postnatal spinal cord astrocytes express several region-specific genes, and that ventral astrocyte-encoded semaphorin 3a (Sema3a) is required for proper motor neuron and sensory neuron circuit organization. Loss of astrocyte-encoded Sema3a leads to dysregulated α-motor neuron axon initial segment orientation, markedly abnormal synaptic inputs, and selective death of α- but not of adjacent γ- motor neurons. In addition, a subset of TrkA+ sensory afferents projects to ectopic ventral positions. These findings demonstrate that stable maintenance of a positional cue by developing astrocytes influences multiple aspects of sensorimotor circuit formation. More generally, they suggest that regional astrocyte heterogeneity may help to coordinate postnatal neural circuit refinement.

Developing neural circuits must form and maintain appropriate regional connections in a rapidly expanding central nervous system (CNS). Although astrocytes (AS) are increasingly recognized as general regulators of synapse formation1, little is known about whether they encode heterogeneous positional signals involved in local neural circuit formation and/or maintenance. Recent studies indicate that AS develop and are regionally allocated in murine brain and spinal cord (SC) according to an embryonic segmental template1-11. AS derived from embryonic radial glia migrate in the trajectory of these fibres and proliferate locally12, yielding clonally related populations13-18 that retain spatial restriction into adulthood. Here we tested whether AS allocated to discrete dorsal–ventral (DV) SC domains might be functionally adapted to support specific neural circuits and neuronal subtypes. The SC sensorimotor circuit has well-defined organization in the DV axis (Fig. 1a). The ventral horn contains two types of motor neurons (MN), called α-MN and γ-MN, whose axons exit the ventral root to project to extrafusal (α) and intrafusal (γ) muscle fibres19. During development, afferent sensory fibres entering from the dorsal root ganglion (DRG) include type 1a proprioceptive afferents that synapse directly on ventral α-MN, and TrkA+ sensory axons that synapse in the dorsal grey matter20. Although programs that control MN diversification and connectivity are well established9,12, comparatively little is known about non-neuronal signals that influence local circuit formation11,13. We report that ventral AS-encoded semaphorin 3a (Sema3a), a secreted molecule that signals throughplexin A/neuropilin 1 receptor (Nrp1) complexes14-16, has critical roles in orienting MN axon initial segments (AIS), synapse regulation, MN subtype survival and normal patterning of a subset of TrkA+ sensory neurons. These findings establish a discrete molecular function for region-restricted AS.

Figure 1 | AS express region-specific genes. a, Concept of DV AS functional specialization. b, qPCR validation of differentially expressed genes in Aldh1l1–GFP+ dorsal or ventral SC AS (2 out of 4 samples had undetectable dorsal Sema3a). c, qPCR of Aldh1l1–GFP AS versus non-AS (purity >95%). d, Regional expression of Sema3a versus thrombospondin 2 (Thbs2), and glicpins 4/6 (Gpc4/Gpc6). D, dorsal; V, ventral. e, Sema3a proteins in ventral but not dorsal SC Aldh1l1–GFP+ protoplasmic AS (arrows, inset). ’Block’ denotes antibody blocking peptide. DAPI, 4',6-diamidino-2-phenylindole. VH, ventral horn. Scale bar, 20 μm. f, Graded expression of Sema3a protein as percentage of Sema3a+/Aldh1l1–GFP+ AS at E18.5. DH, dorsal horn; Med, medial; lat, lateral. All data are mean ± s.e.m. β-actin is used as a housekeeping gene in qPCR. n = 4 (b) or 3 (c-f) biological replicates per bar.

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AS express DV–restricted positional cues

To identify regionally distinct molecular differences we purified fibrous and protoplasmic AS from microdissected dorsal and ventral postnatal day 7 (P7) SC by flow cytometry using the Aldh111-GFP transgene reporter12,13,14 (Fig. 1a and Extended Data Fig. 1). Gene expression profiling and bioinformatic analysis identified 38 genes that were differentially expressed (Extended Data Fig. 1), and we validated these results by quantitative PCR. As shown (Fig. 1b), several genes encoded extracellular matrix molecules19 (Hapln1, Paml1, Enpep, Bgn) or factors with positional roles in brain development, including reelin (Reln)20 and Eph receptor A5 (Epha5; ref. 21). Of these, Sema3a was the most highly expressed ventral AS-specific gene, showing over threefold higher levels in radial glia and AS (versus non-AS) from embryonic day 13.5 (E13.5)–P7 (Fig. 1c), consistent with Sema3a expression in situ (Extended Data Fig. 2a–c)15. In contrast to Sema3a, genes for other AS-secreted molecules, including thrombospondin 2 (Tbss2) and glypican 4 and 6 (Gpc4, Gpc6), were expressed without positional distinction (Fig. 1d). Sema3b, -c, -d, -e and -f were low or undetectable in AS (data not shown).

AS Sema3a protein expression showed graded expression, with lowest numbers of Sema3a+ cells in the dorsal horn and highest numbers in the ventral horn. In ventral AS, Sema3a proteins appeared oriented towards MN soma (Fig. 1e, f). Although postnatally in near-normal numbers. Previous work indicates that Sema3a has roles in supporting dendrite versus axon identity17 and hippocampal neuron axon repulsion and dendrite growth in vitro18. To assess a potential role for AS-encoded Sema3a in orienting MN axons in vivo we used ankyrin G (AnkG)29 to define the direction of the AIS relative to the ventral root. By P7, large α-MN and smaller γ-MN normally exist in a 2:1 ratio in most MN pools16. To determine AIS orientation of both α-MN and γ-MN following loss of AS-encoded Sema3a, we used AnkG to identify the AIS and NeuN staining to distinguish α-MN (choline acetyltransferase (ChAT)+ NeuN+) from γ-MN (ChAT− NeuN+)10. This analysis was performed per lumbar and cervical levels and the results are plotted on a positional grid (Fig. 2a–c and Extended Data Fig. 4). In control animals, we found that α-MN AIS were oriented an average of 39°±33° (s.d.) from the ventral root. By contrast, we observed marked disorganization of α-MN AIS orientation in hGFAPcre:Sema3afl/fl animals, with increases in both mean angle and variability (62°±46° (s.d.); P<0.001) (Fig. 2d and Extended Data Table 1) at all topographic levels.
positions (Fig. 2c and Extended Data Fig. 4). Notably, we did not detect any differences in AIS orientation in γ-MN (control 52 ± 31° versus mutant 49 ± 29°; P = not significant; Fig. 2e). Furthermore, these prominent differences at P7 were no longer detectable by P30 (Fig. 2f).

To determine whether this AIS orientation defect reflected aberrant initial positioning or a failure to maintain MN orientation during later growth, we targeted radial glia and AS using Aldh11cre, which targets gliogenic radial glia at E12.5–13.5 (ref. 7). Aldh11cre fate maps most AS, some oligodendrocytes (that are Sema3a negative, Extended Data Fig. 2d), but excludes SC MN and interneurons (Extended Data Fig. 3b). Aldh11cre:Sema3afl/fl mice were perinatal lethal (28% expected survival at P1–P5, n = 112), but with normal numbers of Ankg+ MN, suggesting that axon specification is not affected in vivo (Ankg+ MN per confocal section: 42 ± 5% versus 46 ± 4%, P = not significant). At E14.5 (before the major period of AS expansion7), we found no Sema3a in MN, vGlut1+ presynaptic puncta on MN soma, which reflect type 1a propriospinal sensory afferents11. We observed a significant decrease in the number of vGlut1 puncta per MN (Fig. 4b; 4.82 ± 0.26 versus 2.91 ± 0.2; P < 0.0001) with unchanged input amplitudes of sEPSC and sIPSC were not changed (Extended Data Fig. 7). As shown (Fig. 4f–i), loss of AS-encoded Sema3a promoted MN survival in a dose-dependent manner that was abrogated by preincubating with an Nrp1-blocking antibody (Fig. 3g, h). Interestingly, the MN that survived in the absence of Sema3a typically had bipolar morphology (no add 86 ± 7% bipolar versus Sema3a 20 ± 11%, P < 0.01). Together, these findings indicate that AS-encoded Sema3a can directly promote MN survival (Fig. 3i) in a manner that is tightly linked to its tropic effects. Notably, abnormal MN AIS orientation was no longer evident in adult hGFAP-cre:Sema3afl/fl mice (Fig. 2f), suggesting that misoriented α-MN are lost by adulthood.

**AS Sema3a is needed for α-MN survival**

Given these findings, we investigated whether ventral AS-encoded Sema3a might also have later roles in maintenance of the sensorimotor circuit. At P7, numbers of both α- and γ-MN were normal in hGFAPcre:Sema3afl/fl mice (Fig. 3a, b) and added adenoviral Cre (AdE-Cre) to some wells to delete Sema3a at ventral AS (50 ± 9 μm versus 111 ± 16 μm, P < 0.001; Fig. 2j, k), suggesting a failure to properly repel the axon. Thus, AS-encoded Sema3a directly repels MN axons both in vivo and in vitro.

**AS Sema3a regulates MN function**

To test whether AS-encoded Sema3a acts directly on MN without other cellular intermediates we co-cultured ventral SC AS from hGFAPcre:Sema3afl/fl animals at sub-confluent density with embryonic rat MN32 (Fig. 2i), and added adenoviral Cre (AdE-Cre) to some wells to delete Sema3a. We found that the length of proximal segment overlap in MN adjacent to AS was significantly increased after AdE-Cre-mediated deletion of Sema3a from ventral AS (50 ± 9 μm versus 111 ± 16 μm, P < 0.001; Fig. 2j, k), suggesting a failure to properly repel the axon. Thus, AS-encoded Sema3a directly repels MN axons both in vivo and in vitro. AS Sema3a is needed for α-MN survival

To test whether MN in hGFAPcre:Sema3afl/fl mutants integrated normally into local synaptic circuits (Fig. 4a), we first counted excitatory vGlut1+ presynaptic puncta on MN soma, which reflect type 1a propriospinal sensory afferents11. We observed a significant decrease in the number of vGlut1 puncta per MN (Fig. 4b; 4.82 ± 0.26 versus 2.91 ± 0.2; P < 0.0001) in adult mutant animals. These differences remained highly significant even when only large (>500 μm) putative α-MN were counted (5.59 ± 0.25 versus 4.13 ± 0.23; P < 0.001).

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**To test whether these synaptic changes correlated with changes in MN function, we performed whole-cell patch clamp electrophysiological recordings37 of lumbar α-MN at P13–14 from hGFAPcre:Sema3afl/fl, ChAT-GFP transgenic reporter mice and Cre-negative controls (Fig. 4c, d). As shown (Fig. 4f–i), loss of AS-encoded Sema3a conferred a large shift in the balance of excitation/inhibition with significantly decreased MN spontaneous excitatory postsynaptic current (sEPSC) frequency (control 3.19 ± 0.93 versus mutant 16.11 ± 0.88 P < 0.01) and increased spontaneous inhibitory postsynaptic current (sIPSC) frequency (control 0.38 ± 0.15 Hz versus mutant 1.21 ± 0.38 Hz; P < 0.01).
resistances (Extended Data Fig. 7), suggesting compensatory changes in MN function. Together, these findings demonstrate that loss of AS-encoded Sema3a leads to changes in MN excitatory and inhibitory synapse inputs and has global effects on MN firing properties.

**AS regulate sensory axon targeting**

Initial studies of Sema3a demonstrated selective chemorepellent activity for sensory axons expressing TrkA or calcitonin gene-related peptide (CGRP), which label overlapping sets of DRG neurons6. However, the cellular source of Sema3a in SC has never been defined. Sensory axon guidance takes place between E13.5 and E18.5 in the mouse SC (Fig. 1a), and Sema3a is expressed in the DRG15 (Extended Data Fig. 2b), and we observed normal numbers of both PV+ and TrkA+ cells in the DRG of Aldh1l1cre:Sema3afl/fl mice (Extended Data Fig. 8). DiI labelling of DRGs of Aldh1l1cre:Sema3afl/fl mutants at E18.5–P0 showed abnormal ventrally positioned axons in 7 out of 7 mutants examined versus 0 out of 7 controls (Fig. 5a).

We then investigated the sub-classes of DRG sensory afferents affected in Sema3a mutants. We observed normal DV positioning of PV+ type 1a proprioceptive afferents albeit with subtle fasciculation defects (Fig. 5b). By contrast, TrkA+ afferents showed numerous abnormal

![Figure 4](image)

**Figure 4 | AS-encoded Sema3a regulates MN synaptic function.** a, Schematic of MN synaptogenic puncta. b, Decreased sensorimotor excitatory puncta (vGlut1+), increased inhibitory puncta (VGAT+), and preserved vGlut2+ inputs in hGFAPcre:Sema3afl/fl animals. Scale bar, 10 μm. c, Electrophysiology schematic. d, e, Representative action potentials and mean rheobase value demonstrate hyperexcitability in hGFAPcre:Sema3afl/fl MN. F–I, Decreased sEPSC frequency (F, g) and increased sIPSC frequency (h, i) in hGFAPcre:Sema3afl/fl MN. Statistics: mean ± s.e.m. Data in b from cervical and lumbar levels of >4 per genotype and >200 MN per ea; data in d–h = 5–6 per genotype from lumbar slices. b, One-way mixed-effects ANOVA with Tukey’s comparison; vGlut2: student’s t-test. d–h, Student’s t-test. *P < 0.05, **P < 0.01, ****P < 0.0001.

![Figure 5](image)

**Figure 5 | AS-encoded Sema3a regulates DV positioning of sensory axons.** a, Dil labelling of upper thoracic SC demonstrates ectopic ventral fibres in Aldh1l1cre:Sema3afl/fl mice (inset, box, arrows) (n = 7 per group). Scale bar, 100 μm. b, No ectopic proprioceptive 1a afferents (white arrow) in Aldh1l1cre:Sema3afl/fl mice; n = 4 per group. Scale bar, 100 μm. c, Multiple ectopic ventral TrkA+ projections in Aldh1l1cre:Sema3afl/fl mice (box, insets). Scale bar, 100 μm. d, Overlay dot-plot of TrkA+ terminations in Cre-negative controls (black circles) and Aldh1l1cre:Sema3afl/fl (red stars); n ≥3 per genotype. e, Quantification of DV termination index** shows a significant increase in ventral terminations in Aldh1l1cre:Sema3afl/fl mice. f, Medial versus lateral terminations unchanged. g, Summary of sensory axon phenotypes. h, Diagram of AS-sensory neuron co-culture protocol. i, Representative TrkA+ DRG neuron grown on SC AS. Scale bar, 50 μm. j, Tracings of wild-type DRG neurons grown on dorsal/ventral SC AS from Sema3a−/− mice, under wild-type (no virus) or Sema3a−/− (+ Ade-Cre) conditions. Scale bar, 100 μm. k, Reduced total neurite length of neurons grown on ventral (red) vs dorsal (blue) AS significantly rescued on Sema3a−/− ventral AS. l, m, Sholl analysis shows significantly less branching on ventral AS (l), rescued with Sema3a deletion (m). Statistics: culture data from 4–6 independent experiments with >10 neurons per condition per experiment. Mean ± s.e.m.; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Student’s t-test.
ventral terminations in Aldh1l1cre:Sema3aΔ3b mutants (Fig. 5c–f). Thus, AS-encoded Sema3a regulates DV patterning of sensory axon projections in a subtype-specific manner in vivo (Fig. 5g), consistent with previous results and with the neuronal pattern of Nrp1 expression (Extended Data Fig. 2).

To test whether distinctions between regional SC AS exist independent of environmental positional cues, we co-cultured dorsal or ventral AS with dissociated DRG sensory neurons (Fig. 5h) using AS from Sema3aΔ3b animals and deleted Sema3a by adding Ade-Cre. Interestingly, AS cultures retained their distinct regional expression characteristics for many of the genes prospectively identified in vivo (see Fig. 1b and Extended Data Fig. 9), including higher ventral Sema3a levels.

We observed that both dorsal and ventral AS cultures preferentially supported the survival of TrkA-positive sensory neurons (>85% of neurons were TrkA⁺, none were PV⁺; Fig. 5i), with no significant survival differences between dorsal and ventral cultures. However, ventral SC AS cultures significantly (P < 0.01) inhibited neurite outgrowth and complexity relative to dorsal AS (Fig. 5j–m). Ade-Cre deletion of Sema3a in ventral AS with dissociated DRG sensory neurons (Fig. 5h) using AS (Extended Data Fig. 2).

Interestingly, AS cultures retained their distinct regional expression patterns, which might lead to deficient local supranuclear functions that determine structural and functional sensory-motor circuit integrity.

Our in vitro studies further suggest that AS positional identity is at least partly cell intrinsic, as cognate Sema3a-deleted regional AS properties were retained in co-cultures independent of local environmental cues. Such as, a testable prediction is that embryonic CNS patterning mechanisms might establish a template for generation of heterogeneous properties of AS. Furthermore, although maintenance of MN axonal orientation represents one tropic effect of AS-encoded Sema3a, further investigation is needed to assess other potential roles of this or other AS-encoded regional cues, such as promoting dendrite growth in vivo, maintenance of neuronal soma position, local synaptic strength and/or sensorimotor specificity.

Specialized local functions of AS in neural circuit formation may also have significance in human disease. For example, loss of ventral SC MN in amyotrophic lateral sclerosis (ALS) has been associated with mutant superoxide dismutase protein in ventral AS in animal models of the disease. Our findings suggest the possibility that the unique identity of ventral horn AS might lead to deficient local support for MN and disease progression in ALS. More generally, given that AS are regionally patterned throughout the CNS, the concept of regional AS function and dysfunction has implications for a variety of neurodevelopmental and psychiatric disorders.

METHODS SUMMARY

Animals were maintained in the University of California San Francisco animal facility. All protocols were IRB approved and in accordance with the Institutional Animal Care and Use Committee guidelines. Circular data and statistics were analysed using Oriana4 software (Kovach Computing Services). Astrocyte monolayers from microdissected P0 mouse spinal cord were cultured for 12–14 days before re-plating at sub-confluent density for MN co-cultures and high density for DRG co-cultures. Experiments with recombinant Sema3a (Peprotech) were on Matrigel in serum-free, growth-factor-free media. Nrp1 blockade with 10 μg/mL 85D antibody (R&D AF566) was added 30 min before Sema3a.

Online Content

Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions A.V.M. performed most experiments and data analysis. K.W.K performed electrophysiology under supervision of E.M.U. H.H.T. contributed to data analysis and experimental design. S.A.R. performed MN purification under supervision of J.R.C. S.M.C performed mouse genotyping. L.M. and S.E.B. performed bioinformatics data processing and analysis. A.V.M. and D.H.R. designed the experiments and wrote the manuscript.

Author Information Microarray data has been deposited to GEO under accession number GSE55054. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to D.H.R. (rowitchd@peds.ucsf.edu).
METHODS

Mice. All mouse strains were maintained in the University of California San Francisco (UCSF) specific pathogen-free animal facility, and all animal protocols were approved by and in accordance with the guidelines established by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Center. Mouse strains not otherwise referenced include CAG-GFP (MGL3849685) and ChAT-eGFP (MGL3694555). For embryonic tissues, plug date was considered as E0.5. Embryo age was confirmed by morphology and crown-rump length measurements at collection.

Astrocyte isolation by flow cytometry. Postnatal day 7 spinal cords were micro-dissected using an ‘open book’ preparation to separate dorsal and ventral halves. DRGs and meninges were removed then dissociated with papain (20 U ml⁻¹; Worthington) for 80 min at 33 °C as described previously. Aldh111-positive and -negative populations were as previously described on a BD Facs Aria II and gated on forward/side scatter, live/dead by DAPI exclusion, and GFP, using GFP-negative and DAPI-negative controls to set gates for each experiment. In some cases GFP-positive populations were re-selected using the same gates to >95% purity.

RNA isolation. RNA was isolated using TRIZOL reagent (Invitrogen) with glycogen added as carrier, DNase-digested to remove genomic DNA contamination, and further purified using the RNAeasy Kit (Qiagen). For microarray analysis, RNA samples were amplified using the Nugen Pico WT Ovation Kit and hybridized to Affymetrix Mouse Gene 1.0 ST arrays.

Bioinformatics. Microarray data were pre-processed in R using the Bioconductor suite of software packages. The ‘oligo’ package was used to background correct, normalize, and summarize the data (Welsh et al. 2001) on mouse gene 1.0 ST arrays via the robust multi-array analysis (RMA) algorithm. After non-specific filters were applied to remove low-intensity (<100 FU in at least 75% of the arrays), low-variance, and un-annotated probe sets, 7,799 probe sets remained. The Limma package was used to compare and assess differential expression between different groups of samples using the ‘treat’ algorithm. Dorsal astrocytes, ventral astrocytes, dorsal non-astrocytes, and ventral non-astrocytes were compared directly for the ‘dorsal versus ventral’ analysis. To generate an ‘asterocyte versus non-asterocyte’ data set, dorsal and ventral astrocyte probe sets were assigned equal weight and compared against dorsal and ventral non-astrocyte probe sets. With fold-change >1:2, false discovery rate = 0.15, we identified 5,158 genes differentially expressed between astrocytes and non-astrocytes, and 38 genes (39 transcripts) differentially expressed between dorsal and ventral astrocytes.

qPCR analysis. Complementary DNA was generated from purified RNA using Superscript III (Invitrogen) and random decamers. Primers were designed for amplions of 75–150 bp using Primer 3 and are available upon request. qPCR was done on a Roche Lightcycler 480 using Sybr Green Master Mix (Roche). Melting curves were analysed for each experiment to ensure primer specificity. In most cases both β-actin (Actb) and Gapdh were used as housekeeping genes for normalization with similar results, and Aldh111 was used as a housekeeping gene in vitro.

Immunohistochemistry/in situ hybridization. Most images were collected using a Leica SP5 confocal microscope and anti-Churchill Antibodies (CH), rabbit Sema3a (EMD biosciences), goat ChAT (Millipore), rabbit TrkA (gift of L. Reichardt, UCSF), chick GFP (Aveslabs), mouse Parvalbumin (Sigma), chick neurofilament (Enco), rabbit ankyrin G (Santa Cruz), mouse Err3 (PPMX), chick peripherin (Millipore), rabbit Map2 (Millipore) mouseIsl1/2 (DSHB), sheep Chx10 (Abcam) and rabbit calbindin (Swant). Rabbit FoxP1 and guinea pig Scip were provided by J. Dasen. In most cases staining was done overnight at 4 °C in 5% serum/0.4% Triton, following heat-mediated antigen retrieval for 2 min at 95 °C in 0.1 M citrate buffer, pH 6.0. For GFP labelling, antigen retrieval was 10 min at 70 °C. For TrkA staining, no retrieval was used, and slides were stained overnight at room temperature (20 °C) in 5% serum/1% Triton. All in situ hybridizations were performed using standard protocols. The probe for the full-length rat Sema3a transcript was provided by A. Kolodkin, the Wnt7a probe was from A. McMahon and Plp1 probe was from I. Griffiths.

Polarity analysis in vivo. Measurements of motor neuron orientation relative to the ventral root were calculated by measuring the angle between the following: (1) the vector from the MN nucleus to the axon hillock, marked by AChG staining of the proximal axon segment; and (2) the vector from the MN nucleus and the exit point of the ventral root from the grey matter. Angle measurements ranged from 0° (axon pointing towards the ventral root) to 180° (axon pointing directly away from ventral root). Circular data was analysed using Oriana4 software (Kovach Computing Services), and statistical analyses performed using a Watson’s U² test. Synapse and soma diameter counts. Counts of synaptic puncta on MN soma were performed at cortical (C4–5) and lumbar (L3–4) levels. Counts in the main figures represent pooled unbiased data from all levels with no size cutoff; however, all data were analysed in histogram format and sorted by MN soma area to determine whether results were likely to be biased by altered ratios of MN subtypes. For some subgroup analyses (Extended Data Table 1), putative α-MN and γ-MN were identified by size on the basis of histogram analyses of soma area.

Astrocyte cell culture. Dorsal and ventral spinal cords from P0–1 mice were isolated and dissociated as above. Cells were plated at a density of >1 x 10⁶ per 25 cm² flask in DMEM+hi-glucose with 10% FCS/10 μM hydrocortisone, 5 μg ml⁻¹ N-acetylcyesteine, 2 μg ml⁻¹ insulin and 20 μg ml⁻¹ EGF. Six days after plating cells, flasks were shaken to remove oligodendrocyte contamination. At 8 days, ArCt was added to kill rapidly proliferating cells. For DRG co-cultures, 10–12 days after initial plating, cells were re-plated into assay containers to 80% confluence and retained for 8–10 days. Glass chamber slides (BD) coated with poly-1-lysine and recombinant human fibronectin (Biomedical Technologies) to promote astrocyte adhesion, plating 30,000 cells per well. In most cases, cultures were established using Semasa3a mice, and adenosine Cr recombinase (Vector Biolabs) was added to some wells 2–4 h after re-plating. For MN co-cultures, astrocytes were re-plated at 2,000 cells per well onto a reduced GF Matrigel substrate (BD) diluted 1:25 in DMEM. Astrocyte monolayers were then cultured for 2–3 days before adding neurons.

DRG isolation and co-culture. DRGs from E13.5–14.5 mice were isolated and dissociated for 45 min in 0.25% trypsin/EDTA (Invitrogen). Five hundred cells per well were plated onto astrocyte monolayers in minimal neural growth media containing DMEM:F12, 10% FCS, N2 and B27 supplements (Invitrogen), and cultured for 48 h before fixation in 4% paraformaldehyde and immunolabelling.

Motor neuron isolation and co-culture. Spinal cord neurons were isolated from embryonic rat spinal cords on the basis of previous protocols. In brief, spinal cords were dissected from E15 rat embryos, dissociated in 0.25% trypsin (Gibco) for 15 min and triturated to form a single-cell suspension in 1–15% plus 10% FBS media (Gibco). The suspension was immunopanned in a series of negative selection plates against rat neural antigen 2 (Ran2) and galactocerebroside, and then motor neurons were positively selected for on a final p75NTR panning plate. Adherent cells were released from the plate with a brief application of 0.05% trypsin (Gibco) and re-suspended in growth media (DMEM, B27, N2, Pen-Strep (Gibco)) before culturing. At re-plating (onto Matrigel-plated sub-ventricle astrocytes for co-cultures, or Matrigel-coated wells for recombinant Semasa3a experiments), fresh media was added consisting of DMEM hi-glucose supplemented with N2 and B27 supplements 5 μg ml⁻¹ N-acetylcyesteine, 5 μg ml⁻¹ insulin and 5 μM forskolin.

Recombinant Semasa3a addition and Nrp1 blockade in vitro. MN were plated at 1,000 cells per well as above. 12 h after plating, they were assessed by light microscopy, at which point most could be seen to have budded polar processes. 24 h after plating, recombinant human Semasa3a was added (Peprotech) at indicated concentrations. For Nrp1 blockade, an Nrp1-blocking antibody validated for this purpose (R&D Systems, AF566) was added at a concentration of 10 μg ml⁻¹ and incubated with MN for 30 min before Semasa3a addition. Goat IgG was added separately to control for non-specific effects (not shown). Cells were cultured for an additional 36 h before fixation and immunolabelling.

Culture image analysis. For DRG co-cultures, neurons were identified with NF-H (Enco), in some cases TrkA, and PV immunostaining was used to identify sensory neuron subtypes. MN were identified with peripherin and traced with an overlay of peripherin and Map2 (to label distal dendrites). Spatially distinct neurons were photographed and analysed using Neuron plugin/ImageJ to trace total neurite length for each neuron. Sholl analyses were performed using the Sholl analysis plugin after thresholding each neuron using ImageJ (parameters: start 25 μM, step size 10 μM). More than ten neurons per condition per experiment were analysed. For axon overlaid in vitro, only MN directly abutting an astrocyte were included in the analysis, and overlap of proximal axon (defined as the axon segment proximal to the first branch point) was quantified using ImageJ. For neurite length, statistical analyses were performed on means of each experiment, and in Sholl and axon overlap analyses, t-tests were performed on pooled data.

Whole-cell patch clamp recordings. Acute fresh lumbar (L3–4) spinal cord slices were prepared from KGAP re-express Semasa3a ChAT-GFP mice and Cre-negative littermates aged 2–14 using a modified protocol36. In brief, transverse slices (350-μm thick) were cut with a vibratome (Leica Micro-systems) in a chamber filled with ice-cold sucrose cutting solution followed by a brief (60 s) incubation in polyethylen glycol (Mn = 1,900–2,200). The slices were then incubated in cutting solution at 35 °C for 30 min followed by 2 min in artificial cerebrospinal fluid then equilibrated to room temperature. Whole-cell recordings were made using patch clamp amplifiers (Multiclamp 700B) under an infrared-differential interference contrast microscope. Data acquisition and analysis were performed using digitizers (DigitiData 1440A) and analysis software pClamp 10 (Molecular Devices). Signals were filtered at 6 kHz and sampled at 20 kHz. pSCSs were recorded from motor neurons in the whole-cell patch clamp mode where the chloride reversal potential was 0 mV. sIPSCs were recorded at −55 mV where chloride currents were positive deflections in voltage clamp. Glass pipettes with a resistance of 2.5–4 MΩ were filled with a K-methanesulphonate internal solution. To ensure currents measured were sEPSCs and sIPSCs, control
recordings were performed using standard pharmacology (Extended Data Fig. 7). Series resistance (15–25 MΩ) was monitored throughout the whole-cell recording and data were discarded if the change in series resistance was >20% during the course of the experiment.

**Dil labelling of sensory afferents.** Spinal cords from E18.5–P0 mice were fixed for at least 48 h in 4% paraformaldehyde, then dissected from spinal column with DRGs intact. The ventral root was severed and a crystal of Dil (Molecular probes) was applied to the DRG with a needle. Cords were incubated for 12 days at 37 °C, then 300-μm thick sections were cut by vibratome, mounted in PBS, and imaged by collecting confocal z-stacks at 2 μM spacing.

**Sensory neuron termination analysis.** Confocal sections of cervical spinal cords were overlaid with a uniform grid using Adobe Illustrator and terminations per image were labelled. When more than one break in a process was visible, only the distal-most termination was labelled. Data from all sections and animals were overlaid on a normalized grid and the number of terminations per quadrant calculated.

**Statistical analysis.** Consultation obtained from the UCSF department of Biostatistics. Coefficient of variance (standard error as a percentage of the mean) was used to calculate minimum sample sizes. In most cases, sample sizes were well in excess. Student’s t-tests were two-tailed and based on Gaussian distributions. In all cases, replicates refer to biological rather than technical replicates. Blinding during analysis was used whenever possible for all in vivo studies. Most statistics analysed using GraphPad Prism software. Circular data in Fig. 2 were analysed using Oriana4 software (Kovach Computing Services).
Extended Data Figure 1 | Flow cytometry gating strategy and microarray.

a, Schematic indicating microdissection of Aldh1l1–GFP-positive P7 spinal cord and isolation by flow cytometry using scatter gates, doublet exclusion (not shown) and sorting for GFP-positive cells with live/dead exclusion by DAPI staining. Percentage of Aldh1l1–GFP cells was not significantly different between dorsal and ventral (not shown).
b, Summary of differentially expressed genes in astrocytes (AS), whole cord, or both using the analysis parameters indicated.
c, Heatmap of all 39 genes differentially expressed between dorsal and ventral cord, highlighting astrocyte-enriched genes with known roles in neural circuit development (red) or extracellular matrix (blue).
Extended Data Figure 2 | Coordinate expression of Sema3a and Nrp1 in astrocytes and neurons. a–c, Sema3a mRNA is expressed in radial glia (RG) and in protoplasmic cells that are NeuN negative throughout the embryonic and early postnatal period. Sema3a was not detected in DRG or in SC white matter (b). d, Sema3a is segregated from Plp-positive oligodendrocytes. e, MN Sema3a expression is detected in α-MN but not γ-MN in cervical SC. f, g, High levels of Nrp1 expression in TrkA+ fibres and cell bodies (white arrowhead) and in MN, but not in PV-positive fibres and cell bodies (yellow arrows). h, Quantification of percentage of Nrp1+ neurons per condition.
Extended Data Figure 3 | Fate map of conditional astrocyte deletion lines used in this study. **a**, hGFAPcre fate map labels fibrous and a subset of protoplasmic AS but not MN or interneurons in P10 SC. **b**, Aldh1l1cre fate maps to astrocytes but not to neurons in P10 SC, including α-MN (purple), γ-MN (blue) and interneurons (red).
Extended Data Figure 4 | Motor neuron AIS orientation defects in cervical spinal cord. a, Representative images of cervical SC confocal sections stained to distinguish α- and γ-MN and identify their proximal axon segment (asterisk denotes ventral root). b, Inset shows high-magnification view of representative MN with identifiable AIS and a schematic of their location with respect to the ventral root. c, Overlay of all cervical α-MN angles measured to generate data summarized in Fig. 2c, with positional information preserved, demonstrates that misoriented AIS can be seen at all DV positions.
No evidence of abnormal MN cell body positioning with loss of astrocyte-encoded Sema3a. a, Representative FoxP1 Islet1/2 co-labelling at three rostrocaudal levels in control and mutant animals shows no differences between control and mutant. b, Similar stainings using Scip (a PMC and LMC marker. c, d, No obvious differences in DV or mediolateral boundaries of ChAT MN at comparable cervical or lumbar levels at P0 (using Aldh1l1cre to delete Sema3a) and P7 (with hGFAPcre), both time periods where misorientation of AIS is clearly evident.
Extended Data Figure 6 | Quantification of ventral interneuron populations after loss of astrocyte-encoded Sema3a. a, Chx10 staining at E18 and quantification. b, Calbindin staining of Renshaw interneurons at P30 and quantification demonstrates a significant increase at this age. Data are mean ± s.e.m., student’s t-test. Data in a from n = 2 per group, 4 sections per animal; data in b from 4 per group, 4 sections per animal.
Extended Data Figure 7 | Additional data and controls for MN electrophysiology.  

**a**, 2 μM strychnine and 20 μM bicuculline block postsynaptic currents (at $-55 \text{ mV}$) in a ChAT–GFP$^+$ lumbar MN.  

**b**, 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 50 μM (2R)-amino-5-phosphonovaleric acid (AP5) block postsynaptic currents (at $-75 \text{ mV}$) in a ChAT–GFP$^+$ lumbar MN.  

**c**, No difference in input resistance, sIPSC amplitude or sEPSC amplitude between control (Cre-) and hGFAPcre:Sema3a$^{+/+}$ (fl/fl) MN.  

$n = 5$ each; mean ± s.e.m., Student’s $t$-test.
Extended Data Figure 8 | Normal dorsal root ganglia in *Aldh1L1cre:Sema3a*−/− mice. a, No difference in the number of subtype-specific neurons per DRG in control or *Aldh1L1cre:Sema3a*−/− mice (n = 3 from 4–5 sections per animal; mean ± s.e.m.; Student’s t-test).
Extended Data Figure 9 | Differential expression of regionally heterogeneous astrocyte genes is partly preserved in vitro. qPCR quantification demonstrates that many regionally heterogeneous microarray genes prospectively identified in vivo remain differentially expressed in vitro after 17 days in culture, including ventral Sema3a. Mean ± s.e.m., n = 3 independent experiments.
Extended Data Table 1 | Subgroup analyses of motor neuron data presented in Figs 2 and 3.

|                          | Control (cre-) | hGFAPcre: Sema3a<sup>fl/fl</sup> | p-value |
|--------------------------|----------------|---------------------------------|---------|
| **MN axon orientation, P7** |                |                                 |         |
| All MN                   | 42±33°(SD)     | 58±42°(SD)                      | **      |
| Cervical MN              | 42±34°(SD)     | 62±49°(SD)                      |         |
| Lumbar MN                | 42±32°(SD)     | 55±42°(SD)                      | *       |
| α-MN (NeuN+)             | 39±33°(SD)     | 62±46°(SD)                      | ***     |
| γ-MN (NeuN-)             | 52±31°(SD)     | 49±29°(SD)                      | ns      |
| **MN soma size, P30**    |                |                                 |         |
| All MN                   | 664±14         | 483±10                          | ****    |
| Cervical MN              | 599±24         | 497±16                          | ***     |
| Lumbar MN                | 687±17         | 469±14                          | ****    |
| **#vGlut1/MN**           |                |                                 |         |
| All MN                   | 4.8±0.26       | 2.9±0.20                        | ****    |
| Cervical MN              | 4.3±0.29       | 2.9±0.19                        | ***     |
| Lumbar MN                | 5.1±0.33       | 3.3±0.33                        | ***     |
| α-MN (>500 μM)           | 5.6±0.25       | 4.1±0.24                        | ***     |
| γ-MN (<500 μM)           | 2.27±0.28      | 1.95±0.19                       | ns      |
| **#VGAT/MN**             |                |                                 |         |
| All MN                   | 21±0.74        | 27±0.96                         | ****    |
| Cervical MN              | 19.8±1.1       | 24.5±1.5                        |         |
| Lumbar MN                | 21.8±1.0       | 27.8±1.4                        | **      |
| α-MN (>500 μM)           | 25.7±1.1       | 37.5±1.3                        | ***     |
| γ-MN (<500 μM)           | 11.4±0.82      | 22.7±1.0                        | ***     |
| **#vGlut2/MN**           |                |                                 |         |
| All MN                   | 18.29±0.93     | 16.11±0.88                      | ns      |
| Cervical MN              | 15.0±0.82      | 13.0±0.68                       | ns      |
| Lumbar MN                | 23.5±1.4       | 21.7±1.4                        | ns      |
| α-MN (>500 μM)           | 22.98±1.0      | 21.21±1.2                       | ns      |
| γ-MN (<500 μM)           | 12.18±0.81     | 11.7±0.57                       | ns      |