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Specificity of Sensory-Motor Connections Encoded by Sema3e-PlexinD1 Recognition

Eline Pecho-Vrieseling¹, Markus Sigrist¹, Yutaka Yoshida^{2,3}, Thomas M. Jessell², and Silvia Arber¹

¹Biozentrum, Department of Cell Biology, University of Basel, and Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland ²Howard Hughes Medical Institute, Depts Neuroscience and Biochemistry and Molecular Biophysics, Kavli Institute for Brain Science, Columbia University, New York, USA ³Division of Developmental Biology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA

Abstract

Spinal reflexes are mediated by synaptic connections between sensory afferents and motor neurons^{1–3}. The organization of these circuits exhibits several levels of specificity. Only certain classes of proprioceptive sensory neurons make direct, monosynaptic, connections with motor neurons⁴. Those that do are bound by rules of motor pool specificity – they form strong connections with motor neurons supplying the same muscle, but avoid motor pools supplying antagonistic muscles^{1, 5–7}. This pattern of connectivity is initially accurate and is maintained in the absence of activity⁸, implying that wiring specificity relies on the matching of recognition molecules on the surface of sensory and motor neurons. But determinants of fine synaptic specificity here, as in most regions of the central nervous system (CNS), have yet to be defined. To address the origins of synaptic specificity in these reflex circuits we have used molecular genetic methods to manipulate recognition proteins expressed by subsets of sensory and motor neurons. We show here that a recognition system involving expression of Sema3e by selected motor neuron pools, and its high-affinity receptor PlexinD1 by proprioceptive sensory neurons, is a critical determinant of synaptic specificity in sensory-motor circuits. Changing the profile of Sema3e-PlexinD1 signaling in sensory or motor neurons results in functional and anatomical rewiring of monosynaptic connections, but does not alter motor pool specificity. Our findings indicate that patterns of monosynaptic connectivity in this prototypic CNS circuit are constructed through a recognition program based on repellent signaling.

Several observations led us to focus on the potential contribution of the class 3 semaphorin Sema3e and its receptors as mediators of sensory-motor synaptic specificity. Sema3e is expressed by a restricted set of brachial motor neurons and can serve as a bifunctional ligand, eliciting repellent responses through engagement of PlexinD1 and attractant responses through interactions with a Neuropilin-1/PlexinD1 receptor complex^{9–12}. Moreover, Sema3e expression is lost, and the pattern of monosynaptic sensory-motor connections altered, in mice mutant for *Pea3*, an ETS transcription factor expressed by several brachial motor neuron pools^{11, 13}.

Correspondence to S.A. (silvia.arber@unibas.ch) and T.M.J. (tmj1@columbia.edu).

Author contributions: E.P.V., M.S. and Y. Y. made critical primary contributions to this study. E.P.V. performed physiological analysis of sensory-motor connectivity and tracing experiments in wild-type and mutant mice. M.S. participated in Sema3e and PlexinD1 subpopulation assignment, anatomical analyses of sensory-motor organization, and generated *Sema3e^{nlz/nlz}* and *MN::Sema3e* mice. Y.Y. found that *PlexinD1* is expressed by subsets of proprioceptive sensory neurons and generated *PlexinD1^{cond}* mice. T.M.J. and S.A. initiated complementary aspects of this project, analyzed data, and wrote the manuscript.

We analyzed the role of *Sema3e* and its receptors in two sensory-motor reflex arcs. In one reflex arc that supplies the triceps (Tri) forelimb muscle, motor neurons receive monosynaptic input from Tri sensory afferents¹³. But in a second, atypical, reflex arc that controls the cutaneous maximus (Cm) muscle, motor neurons fail to receive monosynaptic input from Cm afferents¹³⁻¹⁵. Cm motor neurons also lack monosynaptic input from Tri proprioceptive neurons, or from any other proprioceptive afferents, and conversely, Tri motor neurons lack monosynaptic input from Cm proprioceptive afferents¹³. The contrasting circuitry of these two reflex arcs permitted us to examine monosynaptic connectivity between proprioceptive sensory and motor neurons, as well as pool specificity, revealed by the absence of connections between functionally unrelated sensory-motor pairs.

To define the brachial motor neurons that express *Sema3e*, we identified motor neuron pools by retrograde labeling in *Sema3e^{nlz}* mice in which nuclear LacZ is expressed from the *Sema3e* locus (Fig 1a, Supplementary Figs 1, 2a). In p0 *Sema3e^{nlz/+}* mice, all LacZ^{on} motor neurons were confined to the Cm pool and ~85% of all Cm motor neurons expressed LacZ (Fig 1a, b). In contrast, neither *Pea3* nor LacZ were expressed in Tri motor neurons (Fig 1a, Supplementary Fig 2b)¹³. We also determined the profile of expression of *Sema3e* receptors by sensory neurons. At e16.5, *Neuropilin-1* expression was excluded from TrkC^{on} proprioceptive neurons (Supplementary Fig 3a-d). In contrast, ~90% of *PlexinD1^{on}* DRG neurons co-expressed TrkC, although only ~50% of all TrkC^{on} DRG neurons expressed *PlexinD1* (Fig 1c, Supplementary Fig 3g). *PlexinD1* was expressed by ~80% of TrkC^{on} proprioceptive neurons labeled retrogradely from the Cm muscle nerve, by ~50% of TrkC^{on} Tri proprioceptors, but by only ~5% of TrkC^{on} Ulnaris proprioceptors (Fig 1b, d, data not shown). Thus within the Cm reflex arc, which lacks monosynaptic connectivity, most motor neurons express *Sema3e* and most proprioceptive sensory neurons express *PlexinD1*, whereas in the monosynaptically-connected Tri reflex arc the expression of *PlexinD1* by proprioceptors is not accompanied by motor neuron *Sema3e* expression (Fig 1b).

We used gene targeting to assess the contribution of *Sema3e* and *PlexinD1* to the formation and specificity of monosynaptic connections in the Cm and Tri reflex arcs. We analyzed homozygous null *Sema3e^{nlz/nlz}* mice (Supplementary Fig 1b-d) and conditional *PlexinD1^{flox}* mice in which *PlexinD1* function in DRG neurons was eliminated by intercrossing with *Wnt1^{Cre}* mice^{16, 17}. *Sema3e^{nlz/nlz}* and *PlexinD1^{flox/-}Wnt1^{Cre}* (*PlexinD1^{cond}*) mice are viable and fertile, permitting studies of sensory-motor connectivity at post-natal stages. Analysis of LacZ^{on}, f-dex labeled motor neurons in p1 *Sema3e^{nlz/+}* and *Sema3e^{nlz/nlz}* mice revealed no difference in the positions of the cell bodies and dendrites of Cm or Tri motor neurons (Supplementary Fig 4). *Pea3* mutant mice exhibit pronounced defects in Cm motor neuron positioning and dendrite patterning^{11, 13}, but our findings indicate that *Sema3e* does not mediate these aspects of *Pea3*'s influence on motor neuron differentiation.

To assess the contribution of *Sema3e* signaling to the functional specificity of monosynaptic connections in sensory-motor reflex arcs we stimulated, separately, the Cm and Tri muscle nerves in spinal cord preparations isolated from p5 to p7 mice and recorded intracellular responses from identified motor neurons (Supplementary Figs 5, 6)¹³. In wild-type mice, Cm motor neurons lacked monosynaptic input after stimulation of Cm afferents (Fig 2a)¹³. In contrast in *Sema3e^{nlz/nlz}* mice, 45% of Cm motor neurons received monosynaptic input after stimulation of Cm afferents (mean latency 2.9 ± 0.2ms; 21/47 cells in 10 mice) (Fig 2b). Cm motor neurons in *Sema3e^{nlz/nlz}* mice still lacked monosynaptic input from Tri afferents (mean latency 8.8±0.8ms; n=1/35 cells in 6 mice; Fig 2h). Moreover, the pattern of monosynaptic connectivity in the Tri arc was unaltered in *Sema3e^{nlz/nlz}* mice: Tri motor neurons received monosynaptic input from Tri but not Cm sensory afferents (Fig 2e, g, h). We conclude that the loss of *Sema3e* expression by Cm motor neurons permits monosynaptic input from Cm but not from Tri sensory afferents. The loss of *PlexinD1* expression from proprioceptive neurons

elicited a similar change in the pattern of monosynaptic connectivity within the Cm and Tri reflex arcs. In *PlexinD1^{cond}* mice, 43% of Cm motor neurons received monosynaptic input from Cm sensory afferents (mean onset latency 3.3 ± 0.2 ms; 13/30 cells in 7 mice; Fig 2c, g), but they still lacked monosynaptic input from Tri sensory afferents (Fig 2h). Tri motor neurons received monosynaptic input from Tri but not Cm sensory afferents in *PlexinD1^{cond}* mice (Fig 2f-h). These findings provide functional evidence that *Sema3e* and *PlexinD1* interact during the formation of proprioceptive sensory-motor reflex circuits.

The functional changes in Cm connectivity observed in *Sema3e^{nlz/nlz}* and *PlexinD1^{cond}* mice were accompanied by a localized reorganization of proprioceptive sensory terminals, revealed by analysis of the pattern of expression of the vesicular glutamate transporter vGlut1^{on}. In wild-type mice, vGlut1^{on} proprioceptive terminals are distributed sparsely within the Cm domain (Fig 3a, Supplementary Figs 7, 8), and anatomically identified Cm motor neurons were contacted only by very few proprioceptive terminals (Fig 3c, d). In contrast, in the Cm domain of *Sema3e^{nlz/nlz}* and *PlexinD1^{cond}* mice we detected a ~5-fold increase in the density of vGlut1^{on} proprioceptive terminals within the Cm domain (Fig 3a, Supplementary Fig 8) and a ~3-fold increase in the number of proprioceptive synaptic contacts with identified Cm motor neurons (Fig 3d). Thus, monosynaptic sensory input to Cm motor neurons in the absence of *Sema3e*-*PlexinD1* signaling appears to result from the formation of new proprioceptive sensory contacts with Cm motor neurons.

We also examined whether *Sema3e* signaling by motor neurons is sufficient to repel proprioceptive synaptic inputs. To test this, we directed *Sema3e* expression to Tri (and all other spinal) motor neurons by crossing a Cre-inducible *Tau^{Sema3e.ires.nlz}* mouse line with a *ChAT^{cre}* line, to generate *MN::Sema3e* mice (Supplementary Fig 9). In *MN::Sema3e* mice, > 95% of brachial motor neurons now expressed *Sema3e* at levels equal or greater than that of endogenous *Sema3e* (Supplementary Fig 9b-d). In *MN::Sema3e* mice, only 23% of Tri motor neurons received monosynaptic input from Tri afferents, compared to >95% in wild-type (Fig 4a-c, Supplementary Fig 10). Yet, Tri and Cm motor neurons still lacked monosynaptic input from Cm afferents (Fig 4d, e). In addition, we detected a ~50% reduction in the number of vGlut1^{on} terminal contacts with the cell bodies and proximal dendrites of Tri motor neurons in *MN::Sema3e* mice, compared to wild-type (Fig 4f, g). The persistence of some vGlut1^{on} afferent terminal contacts with Tri motor neurons that express *Sema3e*, and the corresponding preservation of physiological inputs, may have its basis in the expression of *PlexinD1* expression by only about half of all Tri proprioceptive neurons, and could also reflect the existence of synaptic inputs from *PlexinD1^{off}* proprioceptive neurons that supply synergistic muscles. We conclude that *Sema3e* expression by Tri motor neurons is sufficient to reduce the incidence of monosynaptic input from Tri sensory afferents.

Together, our findings show that the specificity of monosynaptic sensory-motor connections in the mammalian spinal cord depends on a recognition system in which the matching expression of a Sema ligand and its Plexin receptor prevents synapse formation. Yet even for these relatively simple reflex circuits, an additional layer of specificity is evident (Fig 5a). Elimination of *Sema3e*-*PlexinD1* signaling uncovers a latent propensity of Cm sensory afferents to form monosynaptic connections with Cm motor neurons, but Tri motor neurons remain off-limits (Fig 5a). Similarly, Tri sensory afferents stripped of *PlexinD1* still ignore Cm motor neurons. These observations argue that a recognition system independent of *Sema3e*-*PlexinD1* signaling underlies motor pool specificity (Fig 5a). Other Sema and Plexin proteins are expressed by motor and sensory neurons^{12, 16} and thus pool specificity could involve a more elaborate matrix of Sema-Plexin recognition. More generally, our findings extend the influence of repellant Sema signaling from the guidance and long-range pruning of axons^{19, 20} (see Supplementary Material) to fine synaptic specificity.

The recruitment of a repellent recognition system to the cause of synaptic specificity in spinal sensory-motor circuits provides an intriguing contrast with recent studies of connectivity in the nematode motor system and vertebrate retina, where specificity appears to depend on adhesive interactions between Ig superfamily proteins expressed by pre- and post-synaptic partners^{21, 22}. Whether the intricate patterns of sensory-motor connectivity in the mammalian spinal cord emerge solely through layers of repellent filtering or also involve adhesive recognition remains to be determined. Nevertheless, a prominent role for repellent recognition in synaptic specificity seems likely, given recent evidence that Wnt-mediated repellent signaling controls the position and selectivity of synaptic contacts in invertebrate neural circuits^{23, 24}.

Methods Summary

Sema3e^{nlz} mice were generated using a targeting strategy similar to that described¹⁰ but by the integration of a *CreERT2-IRES-Nls-LacZ-pA* cassette into the endogenous start codon of the *Sema3e* locus. For the generation of *Tau*^{*Sema3e*} mice, a *lox-STOP-lox-Sema3e-IRES-Nls-LacZ-pA* targeting cassette was integrated into exon 2 of the *Tau* locus^{25, 26}. *PlexinD1*^{fllox} mice were generated by insertion of *LoxP* sites flanking the first coding exon of the *PlexinD1* locus as described¹⁷. Electrophysiological recordings from p5 to p7 animals and analysis were carried out as described¹³. Retrograde tracing experiments from Cm and Tri muscles were carried out by f-dex injection at p12 and followed by analysis of vGlut1 input to f-dex labeled motor neurons at p14. Cryostat sections were processed for immunohistochemistry by sequential application of primary antibodies and fluorophore-conjugated secondary antibodies (Invitrogen and Jackson Laboratories)²⁷. For *in situ* hybridization experiments on cryostat sections, *PlexinD1* (Accession number BC19530) and *Sema3e*¹¹ plasmids were used to generate probes. Images were collected on an Olympus confocal or dissection microscope. For quantitative analysis of synaptic input to Cm and Tri motor neurons, IMARIS colocalization tool (version 6.1.5.) was used to assess proprioceptive input contacting motor neurons retrogradely labeled by f-dex, using images acquired at 0.2µm confocal steps and 150× magnification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *Sema3e* marks Cm motor neurons and *PlexinD1* proprioceptive neurons
 (a) *Sema3e* directed LacZ in p0 *Sema3e*^{nlz} spinal cords after retrograde f-dextran tracing from Cm or Tri muscle nerves and summary of Cm, Ld, Tri motor pool position.
 (b) *PlexinD1* expression in *TrkC*^{on} proprioceptive afferents (top) and *Sema3e* expression in motor neurons (bottom) projecting to Cm, Tri or Uln muscles.
 (c) *PlexinD1* expression in e16.5 c7 DRG, matched with TrkC and TrkA.
 (d) *PlexinD1* (left) and TrkC (right) expression in e16.5 DRG neurons after retrograde f-dextran tracing from Cm or Tri muscle nerves.

Figure 2. Loss of *Sema3e* or *PlexinD1* function perturbs monosynaptic specificity (a-f) Cm and Tri onset latency histograms upon homonymous muscle nerve stimulation (0-8ms in 0.15ms bins). Blue box: monosynaptic latency. Right of two lines: # neurons with onset latencies >8ms. Right of each histogram: Summary diagram # neurons with monosynaptic input/ #cells assayed. *Sema3e* or *PlexinD1* mutation lead to monosynaptic Cm-Cm connections (light green), absent in wild-type (no presynaptic terminal). (g) Mean onset latencies (ms $\pm$ SEM) of homonymous pairs. Cm: data within (green) and outside (white) monosynaptic window. Significant differences: Cm; Tri in wild-type, Cm(no mono); Tri and Cm(no mono); Cm(mono) in *Sema3e*^{-/-} and *PlexinD1*^{cond} ($p \leq 0.001$; Student's t-test).

(h) Summary of heteronymous recordings (Tri–Cm and Cm–Tri).

Figure 3. Increase in vGlut1 sensory contacts with Cm motor neurons in *Sema3e* mutant mice
 (a) Digitalized vGlut1^{on} proprioceptive terminals overlaid with ChAT^{on} motor neuron cell bodies in wild-type (c5 and c8), *Sema3e*^{-/-} (c8) and *PlexinD1*^{cond} (c8) mice.
 (b-d) vGlut1 apposition with f-dex labeled Tri (b) or Cm (c) motor neuron in wild-type (b, c) and *Sema3e*^{-/-} (c) mice. Single confocal planes (b) or 3-dimensional view (c). White indicates contact site. (d) Frequency histograms of vGlut1 input to Cm motor neurons. (Bottom) average vGlut1 number in contact with Cm motor neurons ($\pm$ SEM; $p \leq 0.001$; Student's t-test). Scale bar = (a): 30 μ m; (c): 10 μ m.

Figure 4. Ectopic expression of *Sema3e* in motor neurons prevents monosynaptic connectivity
(a, b) Tri onset latency histograms upon Tri muscle nerve stimulation (0-8ms in 0.15ms bins). Blue box: monosynaptic latency. Right of each histogram: Summary diagram #neurons with monosynaptic input/ #cells assayed. Light grey color of presynaptic terminal (b): reduction in Tri motor neurons with monosynaptic Tri input.
(c) Mean onset latencies (ms $\pm$ SEM) measured from homonymous pairs. Tri: data within (dark grey) and outside (light grey) monosynaptic window. Significant differences: Cm;Tri in wild-type mice ($p \leq 0.001$; Student's t-test) and Tri(mono);Tri(no mono) ($p \leq 0.01$; Student's t-test) and Tri(mono);Cm ($p \leq 0.001$; Student's t-test) in *MN::Sema3e* mice.
(d, e) Summary of Cm-Cm, Tri-Cm and Cm-Tri recordings.
(f, g) Analysis of vGlut1 apposition with f-dex labeled Tri motor neurons (contact sites: white). (g) (top rows) values from individual motor neurons; (bottom) mean of analyzed cells ($\pm$ SEM). Significant differences: wild-type; *MN::Sema3e* and *Sema3e*^{-/-}; *MN::Sema3e* ($p \leq 0.001$; Student's t-test). Scale bar=10 μ m.

Figure 5. Regulation of synaptic specificity by repellant Sema3e-PlexinD1 signaling

(a) Connectivity patterns in Cm and Tri reflex arcs in wild-type mice (top) and with altered Sema3e-PlexinD1 signaling (bottom). Top: In wild-type, Tri but not Cm motor neurons receive direct homonymous proprioceptive inputs. Bottom left: changing the profile of Sema3e-PlexinD1 expression rewires homonymous connectivity. Loss of Sema3e-PlexinD1 signaling results in monosynaptic connections between Cm afferents and Cm motor neurons; Ectopic Sema3e expression reduces monosynaptic connections between Tri afferents and motor neurons. Bottom right: changing Sema3e-PlexinD1 signaling does not erode pool-specificity: no aberrant heteronymous sensory-motor connections are observed (red cross).

(b) Genetic program for Sema3e expression in Cm motor neurons. Hox transcription factors program Cm motor pool identity²⁸, conferring Cm motor neuron sensitivity to the retrograde inductive influence of Gdnf²⁹. Pea3 expression by Cm motor neurons controls cell position, dendrite pattern and the selectivity of proprioceptive inputs through distinct downstream pathways^{11, 13, 30}.