

Supplemental Information

**Dual Function of NRP1 in Axon Guidance
and Subcellular Target Recognition in Cerebellum**

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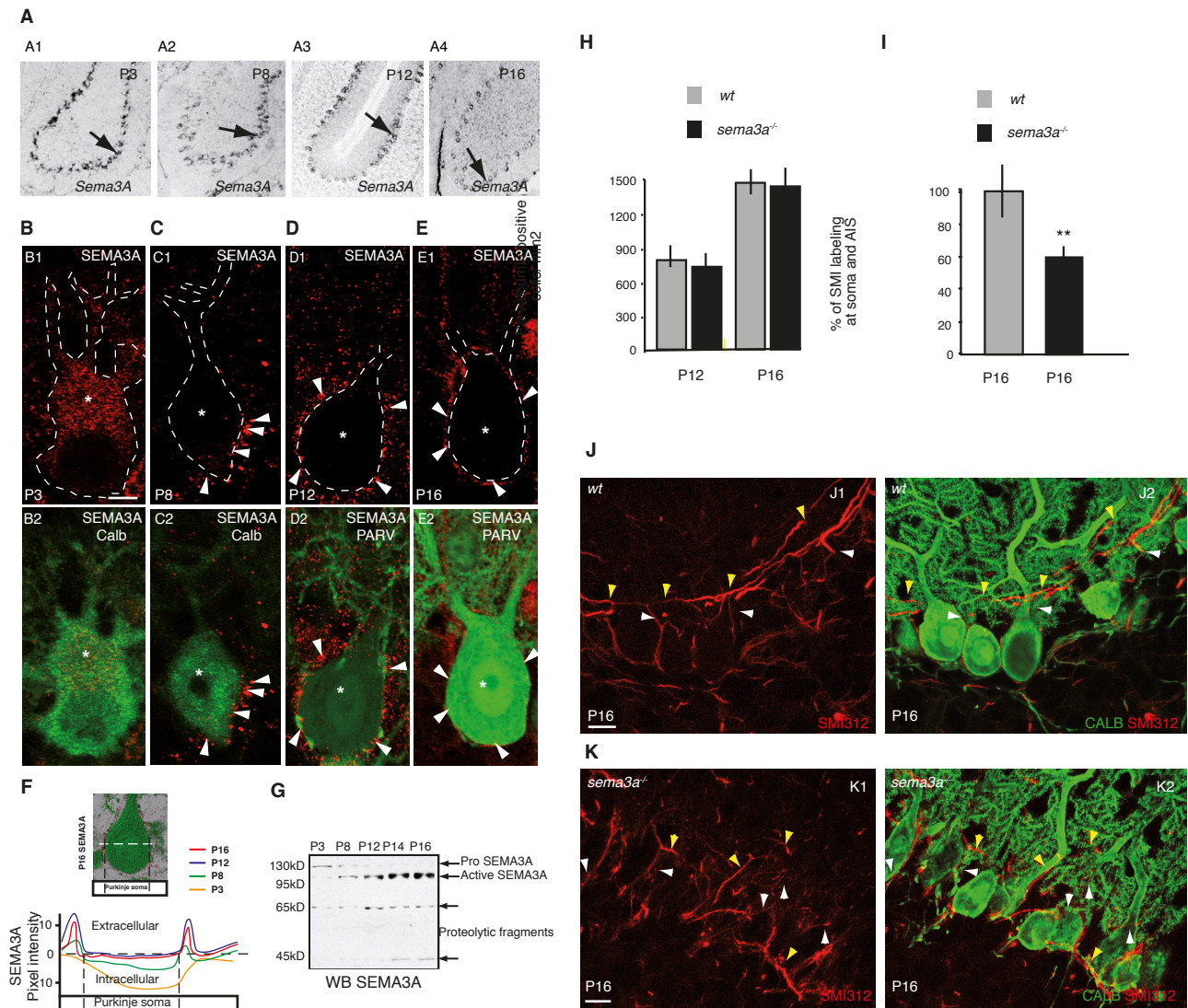


Figure S1, related to Figure 1. SEMA3A expression by Purkinje cells and GABAergic axon organization.

(A) In situ hybridization of *Sema3a* on cerebellar cortex at P3 (A1), P8 (A2), P12 (A3) and P16 (A4). Arrows point to PCL positive signals. Confocal images of endogenous SEMA3A (red) and PCs revealed with calbindin (Calb) or parvalbumin (Parv) antibodies (green) at P3 (B1-2), P8 (C1-2), P12 (D1-2) and P16 (E1-2). Note the expression of SEMA3A in PC soma (star) at P3 (B1). Arrowheads in C, D and E showed SEMA3A punctate accumulation around PC body. F1 is an example of a 3D rendering of PC (green) and SEMA3A (red) used for SEMA3A pixel intensity measurement along the dotted white line and quantified in F at P3, P8, P12 and P16. The horizontal dotted black line delineated intra and extracellular expression of SEMA3A. The vertical dotted lines delineated the PC soma. (G) Cerebellum at P3, P8, P12 and P16 were lysed and immunoblotted for SEMA3A protein expression. Four different forms of SEMA3A were dynamically observed during development, the ProSEMA3A isoform (125kD), the active isoform (92kD) and the proteolytic isoforms (65/45 kD). (H) Quantification of Parv positive cells in the ML at P12 and P16 of wt and *sema3a^{-/-}* mice. BCs axons labeled with SMI-312 (J, K) in wt (J) and *sema3a^{-/-}* deficient mice (K) at P16. PCs are labeled with calbindin (green, J2, K2). White arrowheads show BCs axons at soma. Yellow arrowheads show BCs axon main shafts. BCs axons at both soma and AIS were quantified by using SMI-312 labeling at P16 (I, **: n=14, P=0.0045, Student t-test). Scale bars represent 10 μ m.

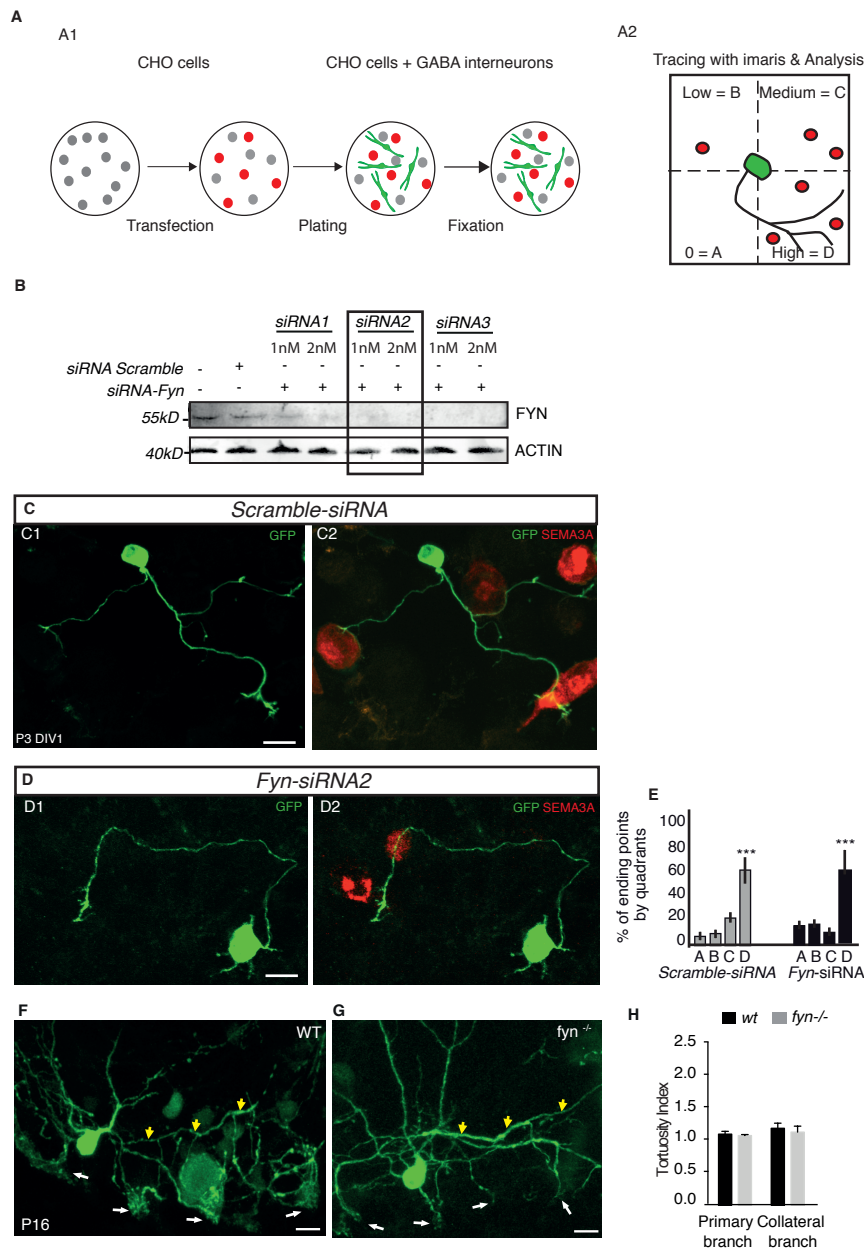
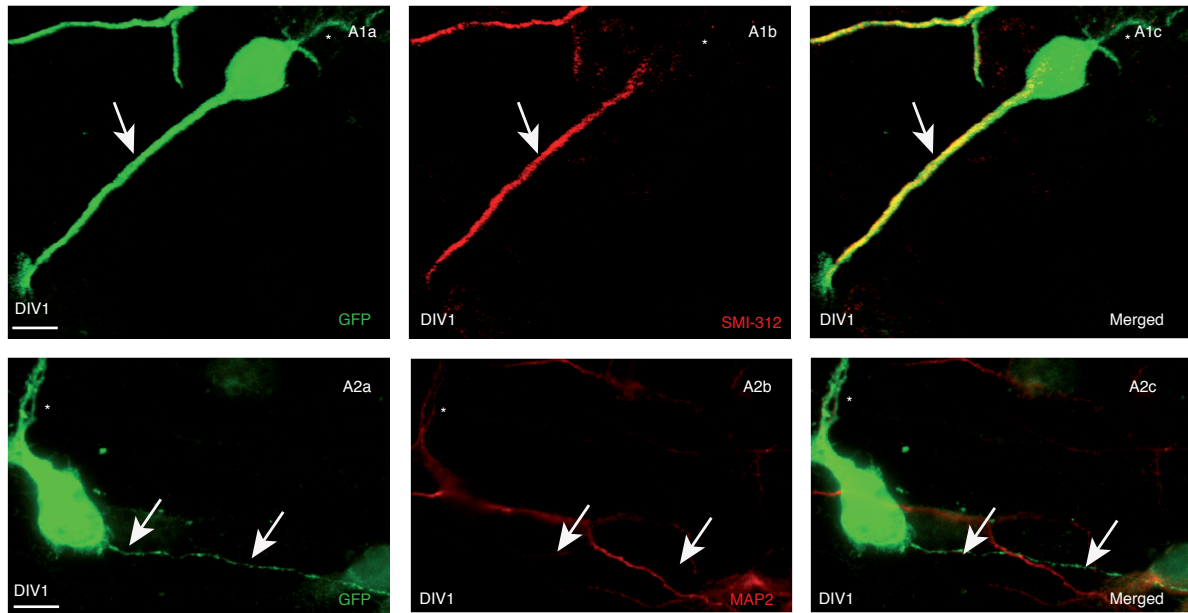


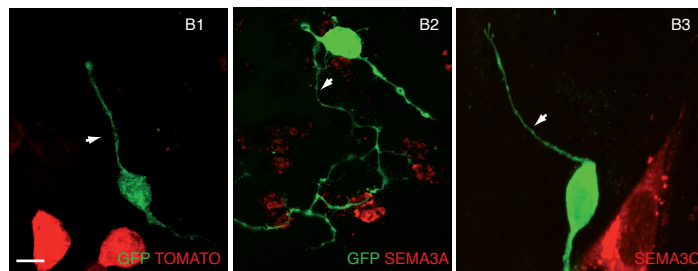
Figure S2, related to Figure 2. SEMA3A attracts BCs axon in SEMA3A/FYN deficient signaling cells.

(A) Schematic representation of the co-culture model. 24h after CHO cells transfection, GABAergic interneurons (green) from *gad67-gfp* mice were plated on the top of CHO cells. Red and grey dots are transfected and untransfected CHO cells respectively (A1). To quantify axons or axon branches preferential localization, images were separated in four equivalent quadrants centered on the neuron soma (A2). The number of branches was counted in each quadrant and scored against the pixel intensity of CHO cells. (B) Western-blot of FYN expression in the presence of *Scramble* and three different *Fyn* siRNA. (C) In this striking example of co-culture with SEMA3A, each BCs axon collateral follows a specific path towards cells expressing SEMA3A (red, C2). In *Fyn* deficient interneurons, SEMA3A failed to induce axon branches but the guidance is not affected. Note the change in axon directionality towards SEMA3A positive cells (D). (E) Quantification of BCs axon endings in both conditions revealed that quadrant with high SEMA3A expression level correlates with highest percentage of axonal endings (***: $n = 20$ cells per condition; $P = 0.0004$ (*Scramble*-siRNA) and 0.0009 (*Fyn*-siRNA), Two-way Anova). In WT (F) or *fyn* KO mice (G), both primary and collateral branches displayed the same tortuosity index as quantified in H. Scale bars represent $10 \mu\text{m}$.

A



B



C

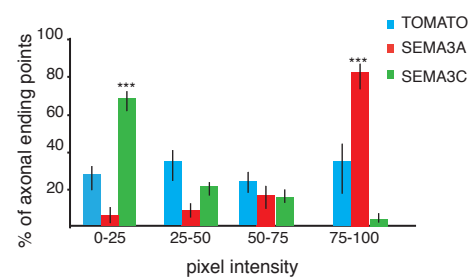


Figure S3, related to Figure 2. Specificity of SEMA3A effect in vitro. After one day in co-culture, GFP positive interneurons (green, A1a and A2a) are already differentiated with axons labeled with SMI-312 (red, A1b) and dendrites labeled with MAP2 (red, A2b). Merged images of A1 and A2 are presented in A1c and A2c respectively. White stars indicate dendritic domain and white arrows point to axons. CHO cells transfected with SEMA3A (red, B2) or SEMA3C (red, B3) attracted (C, ***: $n=18$; $P=0.0006$, Student t-test) or repelled (C, ***: $n=15$; $P=0.0009$, Student t-test) respectively GABAergic axons as compared to control CHO cells transfected with TOMATO (red, B1) and as quantified in (C). Scale bars represent 10 μm .

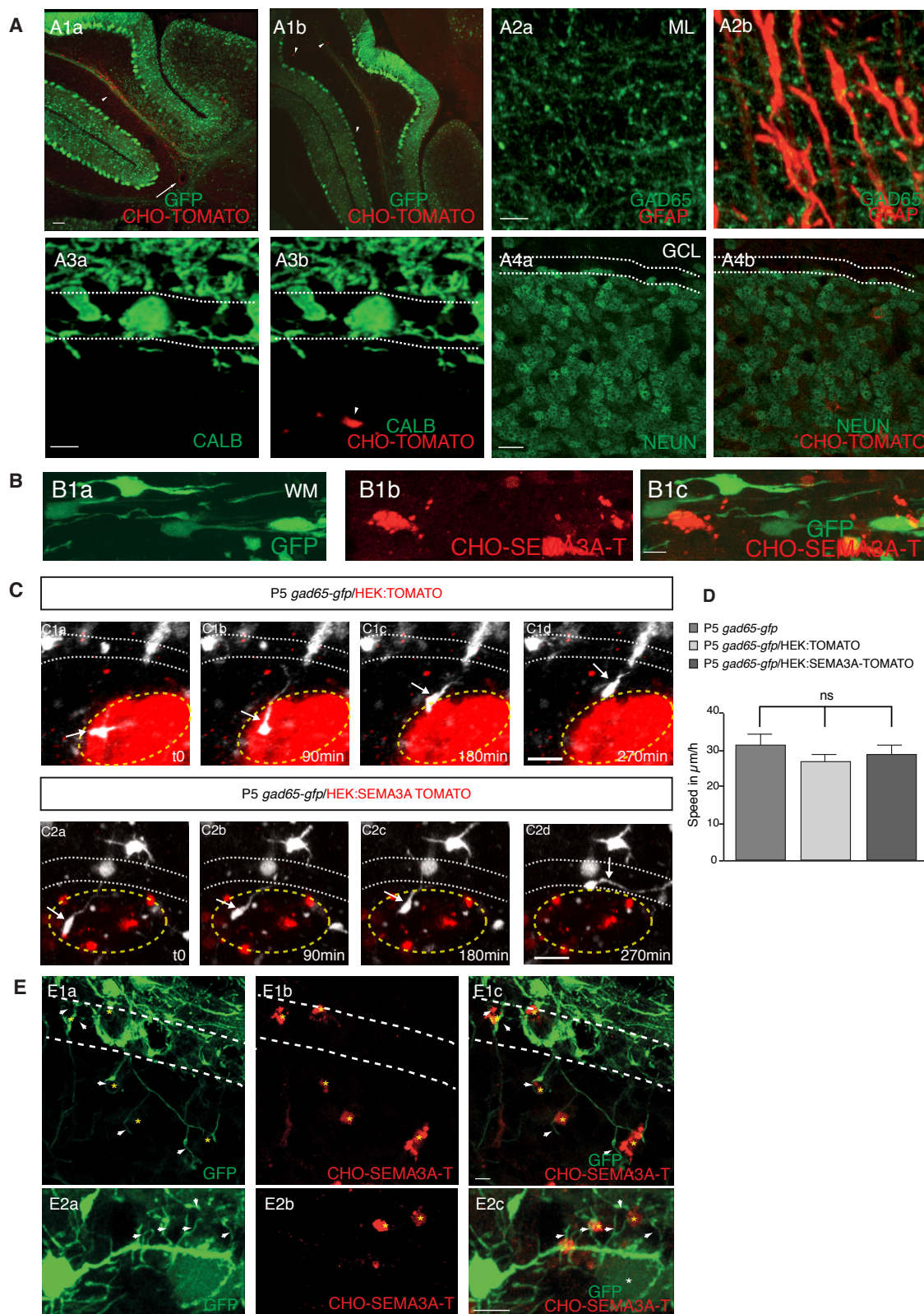


Figure S4, related to Figure 2

Normal cerebellar cytoarchitecture and GABAergic interneuron migration after heterologous cells injection *in vivo* but altered BCs axon organization in the presence of SEMA3A ectopic expression.

After 7 to 14 days post-injection, CHO cells are localized in different layers of the cerebellum (white arrowheads in A). The injection site is pointed with a white arrow (A1a). Cerebellum were cut into serial 100 μm -thick vibratome sagittal slices, (A1a-b). Note the diffusion of CHO cells away from the injection site in the same cerebellar folium. Injection of CHO cells that expressed TOMATO in cerebellar cortex did not alter major cellular organization in the ML (A1, A2), PCL (A1, A3), GCL (A1, A4) and WM (A1, A5). GAD65 punctates (green, A2) appeared normal and colocalized with GFAP in ML as previously described (Ango et al. 2008). Normal localization of granule cells labeled with NeuN is observed in the GCL (green, A4). Localization of CHO cells in the WM does not perturb interneurons migration that displayed normal morphology in fixed cerebellum (green, B1a-c). Time-lapse imaging performed in *gad65-gfp* mice injected with HEK cells expressing TOMATO (C1a-d) or SEMA3A-TOMATO (C2a-d) showed the same migration behavior with comparable migration speed (D). CHO grafts in ML (E1 and E2), PCL (E1 and E2) or GCL (E1) induced BCs axon reorganization. (E1-E2) White arrows point to BCs collaterals that grew either downward (E1a-c) or upward towards ML SEMA3A-expressing CHO cells (E2; yellow stars). Note that BCs axons grew towards SEMA3A-expressing CHO cells (yellow stars) localized in PCL or GCL (E1, E2). Dashed lines delineated the PCL. Scale bars represent 10 μm .

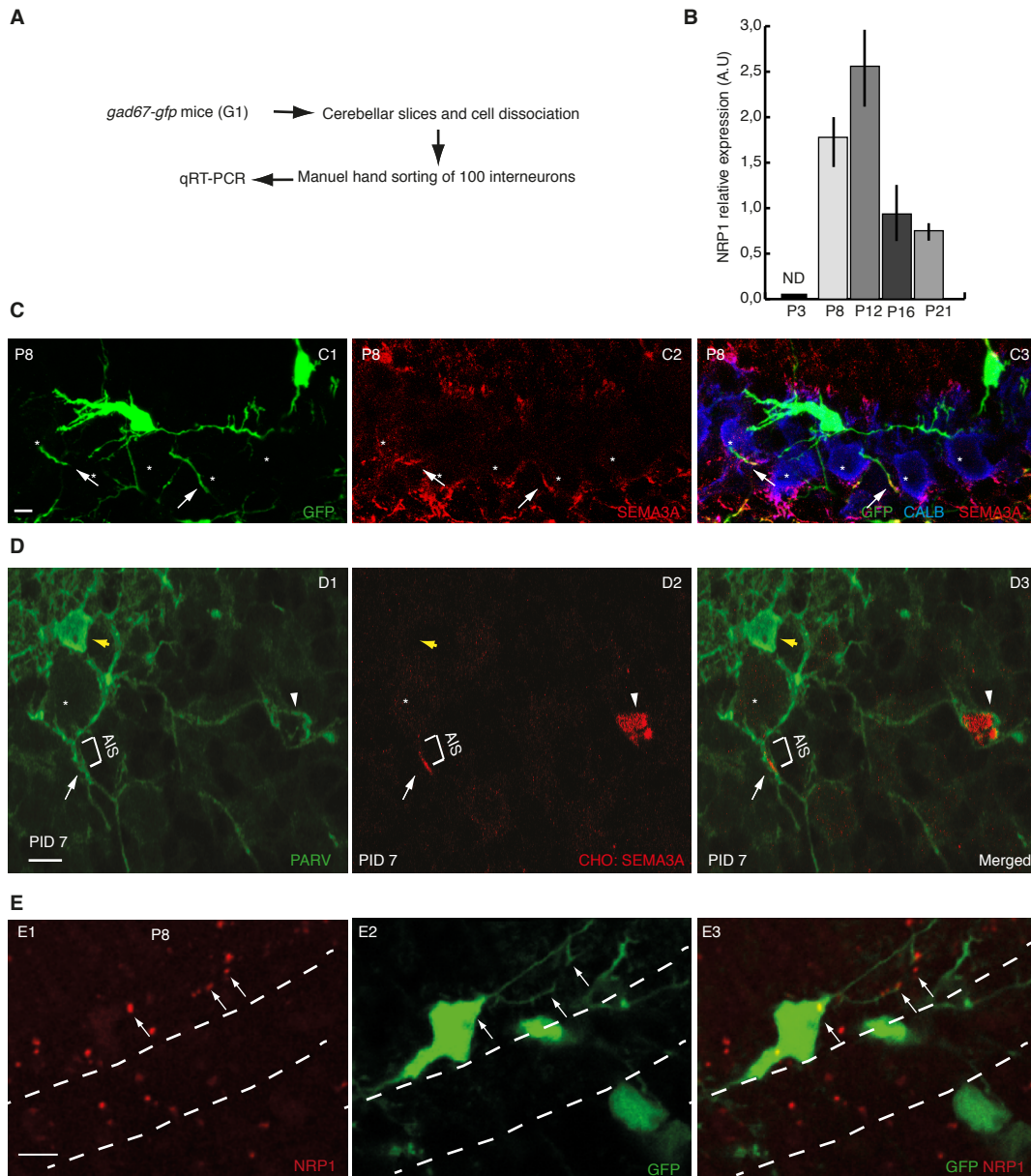


Figure S5, related to Figure 3. SEMA3A receptor is expressed by BCs and preferentially localized in pinceau synapse. (A) Rapid description of the experiment to analyze the expression of *Nrp1* mRNA in BCs using qRT-PCR. (B) *Nrp1* mRNA relative expression at P3, P8, P12, P16 and P21. (C) P8 *gad67:gfp* (C1) immunohistochemistry of SEMA3A (C2) shows local stabilization of SEMA3A around PCs labeled with CALB (C3, *). Arrows show SEMA3A accumulation on BCs axon endings. (D) In vivo localization of SEMA3A-TOMATO secreted by grafted CHO cells accumulated at pinceau, assuming the presence of a receptor for SEMA3A. White stars: PC soma, white arrowheads: CHO cells, white arrows: SEMA3A-TOMATO accumulation at AIS, yellow arrow: BC soma. (E) PLA immunohistochemistry of NRP1 at P8 in young BCs. White arrows point to NRP1 expressed in both BCs soma and axons. Scale bars represent 10 μ m.

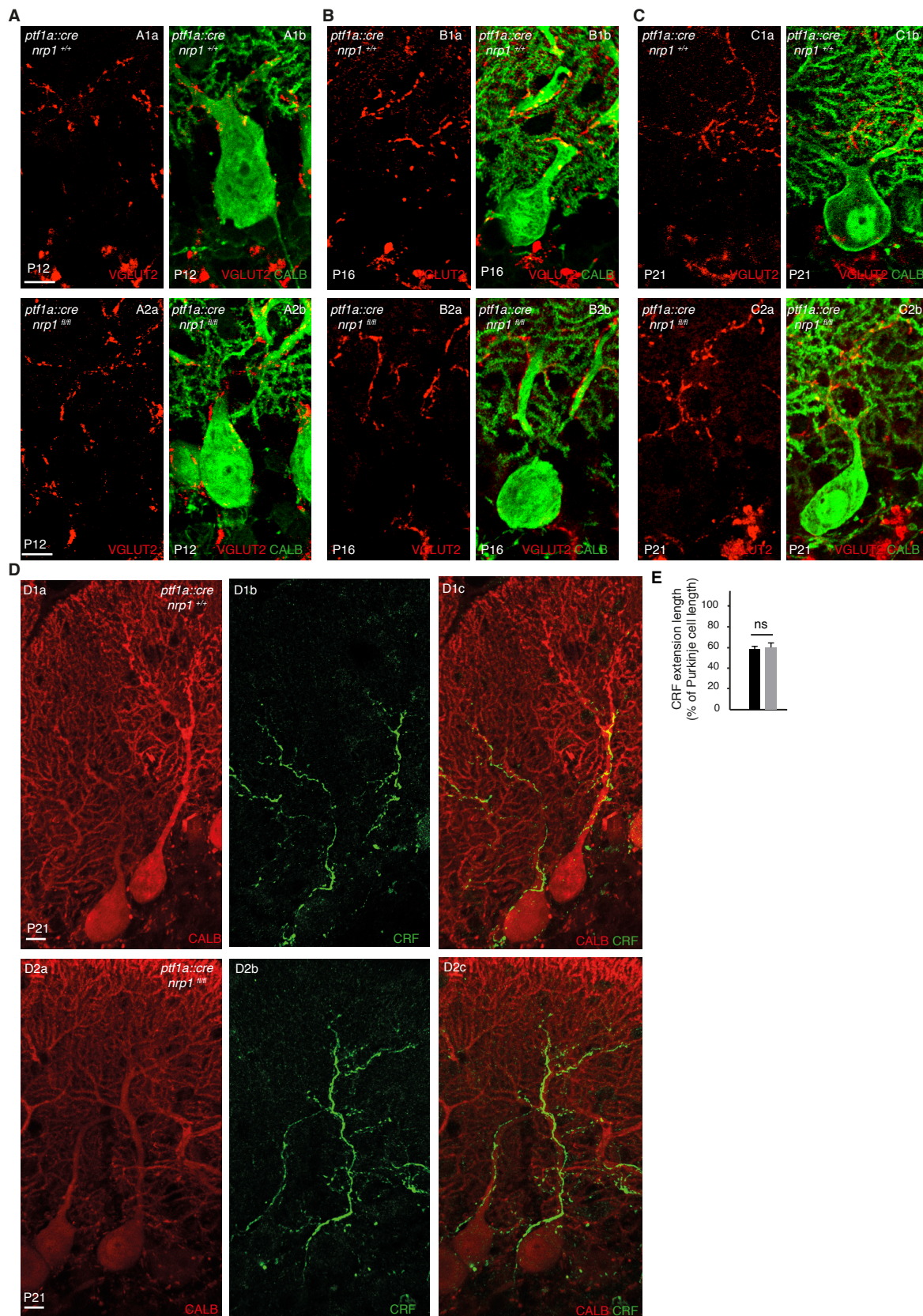


Figure S6, related to Figure 4. Climbing fiber synapses on PCs are not affected on *ptf1a::cre* ; *nrp1*^{-/-} mice. (A-C) Climbing fiber terminals are labeled with VGLUT2 (red, A-C), PCs labeled with CALB (green, A-C) at P12 (A) P16 (B) and P21 (C) in *ptf1a::cre* ; *nrp1*^{fl/fl} (A1, B1, C1) and *ptf1a::cre* ; *nrp1*^{+/+} (A2, B2, C2). (D) Climbing fibers labeled with corticotropin-releasing factor (CRF, green) show similar extension in the ML in both wt (D1) and *ptf1a::cre* ; *nrp1*^{fl/fl} (D2) mice and as quantified in E. Scale bars represent 10 μ m.

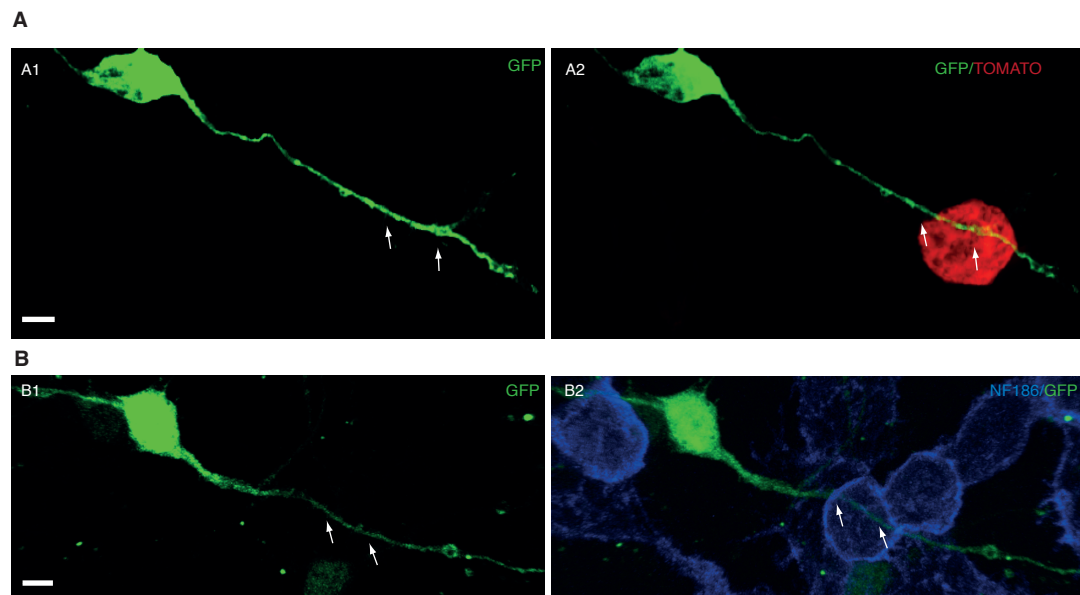


Figure S7, related to Figure 7. NF186 alone is not sufficient to induce target innervation. (A-C) Co-culture of GFP expressing interneurons mixed with CHO cells transfected with control TOMATO (red, A2) or NF186 (blue, B2). Note that interneuron axons (white arrows) show similar interaction with CHO cells expressing NF186 or TOMATO. Scale bars represent 10 μm .

Supplemental method:

Animals and surgical procedures

The experimental plan was designed according to the European Communities Council Directive and the French law for care and use of experimental animals with authorization number: B 34-309 and approved protocol number: CEEA-LR-11031. For mixed coculture assay we used *gad67-gfp* BAC transgenic mice as previously described (Ango et al., 2004). For injection procedure, we used Swiss Webster (CFW®) mice. *Sema3a*^{-/-} mice were obtained from Riken Bioresource Center previously generated by (Taniguchi et al, 1997) MGI: 2158944. For *ptfla:cre;nrp1^{fl/fl}*, animals were handled and housed according to the federal guidelines for the use and care of laboratory animals, approved by the Helmholtz Zentrum München Institutional Animal Care and Use Committee and the Regierung von Oberbayern. The *nrp1^{fl/fl}* line is from (Gu et al., 2003), MGI identification number is MGI:3512101. The *ptfla:cre* mice are from (Nakhai et al., 2007), MGI identification number is MGI:3701996. The day of birth in this study is designated as postnatal day (P). All surgical procedures were performed under deep general anesthesia obtained by intraperitoneal administration of Ketamine (0,56mg/g of body weight) supplemented by Xylazine (0,03mg/g of body weight).

Plasmids.

Tomato-SEMA3A was generated by replacing EGFP in EGFP-SEMA3A (gift from Joost Verhaagen) by a fragment of 5'NHE1-TdTomato-BAMH13' amplified with primer :
AAAAAAAAAGCTAGCGCCACCATGGTGAGCAAG and
AAAAAAAAAGGATCCCTTGTACAGCTCGTCCATG on the sequence of PcmvTdTomato (Addgene).

In Situ Hybridization

The pBluescriptII-KS-semaIII (clone#52 – gift from Alain Chédotal) was linearized with Not1 (NEB-Biolabs) and used as a template for Digoxigenin-labelled antisens riboprobe synthesis with SP6 RNA polymerase (Promega) according to the supplier's instructions (Roche). Mouse brains were fixed as for immunohistochemistry and cryoprotected in saccharose 30% prior to cryosectioning. Free-floating cryostat sagittal sections (30µm thick) were processed for hybridization in 50% formamide, overnight at 65°C. Hybridization was detected using an alkaline phosphatase-coupled anti-Digoxigenin antibody (Roche 1:2000). Alkaline phosphatase staining was developed with NBT/BCIP (Roche) as a substrate.

Attraction analysis

Isolated interneurons with at least two transfected CHO cells in immediate proximity ($\leq 150 \mu\text{m}$) were chosen for analysis. Images of isolated interneurons were divided into four quadrants centered on the interneuron soma. For all quadrants, the relative fluorescence intensity of the transfected CHO was measured with ImageJ and normalized to the highest pixel value being 100%. The pixel intensity values were classified into 4 groups A (0-25%), B (25%-50%), C (50%-75%) and D (75-100%). The same analysis was done with interneurons whose number of ending points in each quadrant was quantified after 3D reconstruction using Neurolucida software. The number of ending points was then scored against the pixel value intensity of the transfected protein. If our molecule of interest has an attractive function, a significant increase in the number of ending points is expected towards the quadrant with highest pixel intensity value (D), as compared to quadrants A, B and C. By contrast, if our molecule has a repulsive effect, the number of ending points should increase in quadrant (A) with the lowest protein expression.

Western blotting and Co-immunoprecipitation

HEK293 cells or mouse cerebellar tissues were lysed with solubilization buffer (HEPES 20 mM, NaCl 150 mM, 1% NP40, 10% glycerol, 4 mg/ml dodecylmaltoside, protease and phosphatase inhibitors) during 1h at 4°C under agitation. Then, samples were centrifuged and the supernatants were collected. For co-immunoprecipitation, lysates (HEK293 :0,5 mg ; Brain :1 mg) were incubated with NF186 specific antibody (rabbit polyclonal antibody, 2 $\mu\text{g/ml}$, Santa Cruz Biotech) for overnight at 4°C, and protein A Sepharose (GE Healthcare) or protein AG (protein Bio-Adembeads PAG, Ademtech) was added for 1h at 4°C. Proteins extracts were separated with 8% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C, Amersham Biosciences). Proteins were detected with primary antibodies overnight at 4°C, then washed and incubated with secondary antibodies for 1h at room temperature. The following antibodies were used: anti-pan-Actin (mouse monoclonal antibody, 1:2000, Cayman), anti-NF186 (rabbit polyclonal antibody, 1:1000, Abcam), anti-phosphotyrosine 4G10 (mouse monoclonal antibody, 1:500, Millipore), anti-Neuropilin1 (goat polyclonal antibody, 1:2000, R&D Systems). Immunoreactivity was detected using ECL Plus Western blotting Detection System, Amersham.

Time-lapse imaging.

P5 *gad65-gfp* mice brains were removed in ACSF (119 mM CaCl₂, 2,5 mM KCl, 2 mM MgCl₂, 2,5 mM CaCl₂, 1 mM Na₂HPO₄, 26,2 mM NaHCO₃, 20 mM glucose) and then cut in 300µm-thick slices with vibratome in frozen and oxygenated ACSF. After transfection of HEK cells with TOMATO or SEMA3A-TOMATO, inverted drops were prepared in order to form a cell layer. These drops were then cut into small pieces and inserted into the granule cell layer in the prepared slices, using a tungsten filament. The slices were then cultured with fresh Neurobasal medium supplemented with Glutamax (GIBCO), B27 (GIBCO) and a mix Penicillin/Streptomycin for recovery during 6 hours in the incubator. Finally, slices were imaged overnight with a confocal microscope (Nikon A1R, 20x, 37°C and 5% CO₂). Analyzes were done using the ImageJ MTrackJ plugin.

Microscopy and image processing.

All pictures were taken with a Zeiss confocal LSM780. Imaris software was used to adjust image contrast and assemble the final plates. Most quantitative and morphometric analysis were made using ImageJ (Research Service Branch, National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>) or Neurolucida (MWF Biosciences) which is used particularly to quantify and trace axons. For GAD65, Parv and SMI-312 local expression quantification, we measured the mean pixel intensity value in the PC layer using ImageJ and as previously described (Ango et al; 2004).

Axons were traced using Neurolucida software (Microbrightfield, Williston, Vermont, United States). From these tracings, we could easily derive data regarding axon tortuosity. The tortuosity index is the ratio of the true contour length of the axon segment (actual length) divided by the end-to-end distance (straight length) (Portera-Cailliau et al., 2005). The primary axon was identified as the main axonal branch attached to the soma. The secondary branches corresponded to axon collaterals issued by the primary axon.

List of antibodies used: Parvalbumin (1:1000; Chemicon), Calbindin (1:1000; Swant), GAD65 (1:300; Chemicon), GFAP (1:500; Dako), NeuN (1:200; Chemicon), VSV (1:500; Sigma), HA (1:300; Aves Lab), NF186 (1:1000 ; gift from Van Bennett), SEMA3A (1:100; Santa Cruz and 1:100; ECM Biosciences), MAP 2 (1:1000; Abcam), SMI-312 (1:200; Abcam), GFP (1:1500; Aves Labs), KV1.1 (1:100; Neuromab), HCN1 (1:100; Neuromab), NRP1 (1:300; R&D systems), VGLUT2 (1/2000; Millipore). Sections were incubated with either Alexa546-conjugated goat anti-mouse or anti-rabbit IgG and Alexa488-conjugated goat anti-rabbit, anti-chicken or anti-mouse IgG (1:500; Molecular Probes) 633-conjugated goat

anti-chicken (1:500; Millipore) and 546-conjugated rabbit anti-goat IgG (1:500; Molecular probes) and mounted.