Figure S1

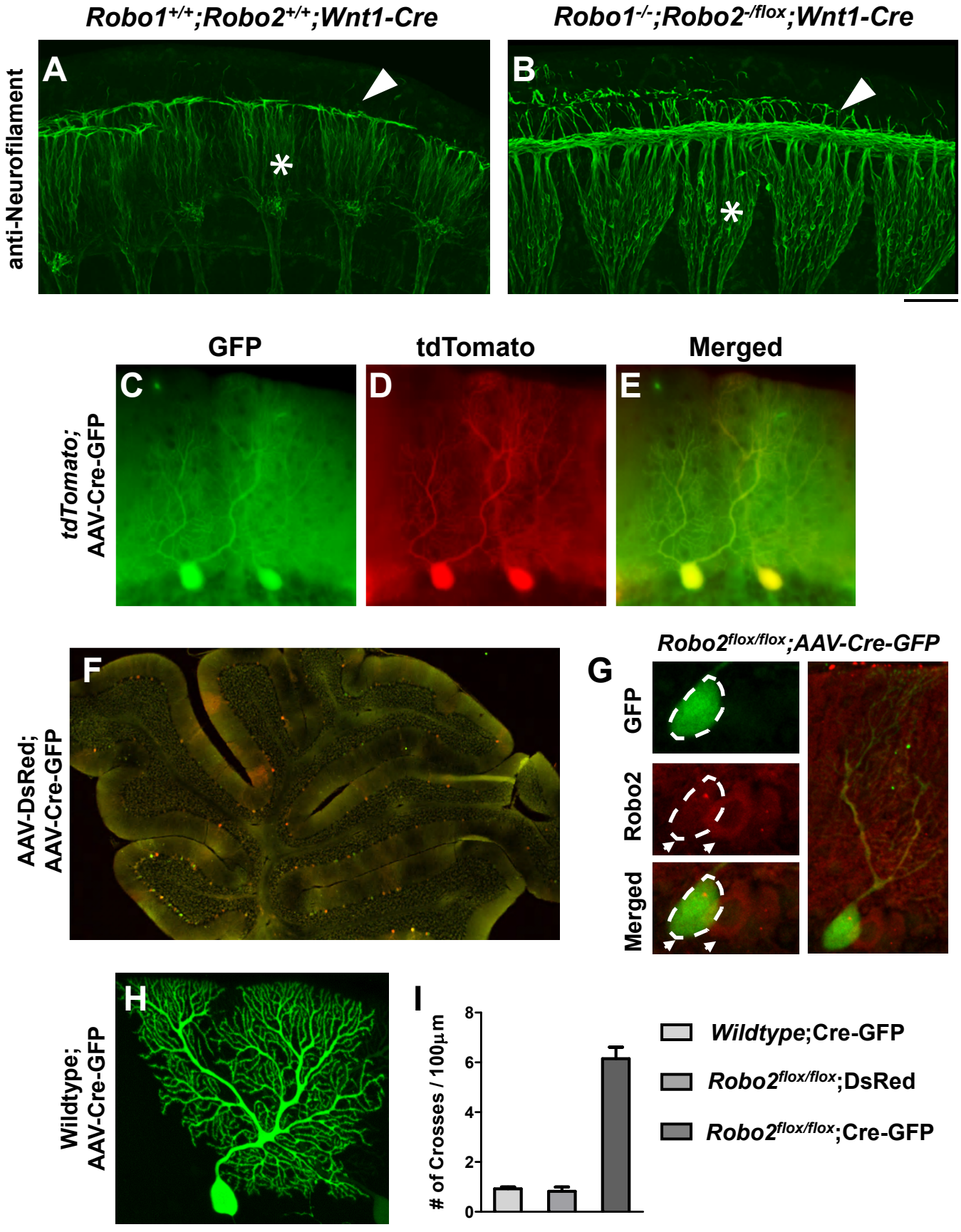

Robo1+/+;Robo2+/+;Wnt1-Cre
Robo1-/-;Robo2-/flox;Wnt1-Cre
anti-Neurofilament
A
B
GFP
tdTomato
Merged
C
D
E
tdTomato; AAV-Cre-GFP
AAV-DsRed; AAV-Cre-GFP
F
Robo2flox/flox;AAV-Cre-GFP
G
GFP
Robo2
Merged
Wildtype; AAV-Cre-GFP
H
I
# of Crosses / 100µm
0
2
4
6
8
Wildtype;Cre-GFP
Robo2flox/flox;DsRed
Robo2flox/flox;Cre-GFP

Figure S1. Validation of experimental tools. Related to Figure 1.

(A-B) Whole-mount E10.5 mouse embryos viewed from the dorsolateral side of the body. Neurofilament immunostaining labels all sensory axon projections as they enter the dorsal root entry zone (DREZ). In the *Robo1*$^{+/+}$*;Robo2*$^{+/+}$*;Wnt1-Cre* (A) embryos the space between the left and right dorsal funiculi (arrowheads) contains no sensory axons. In the *Robo1*$^{-/-}$*;Robo2*$^{-/flox}$*;Wnt1-Cre* (B) embryos this space contains sensory axons that have overextended after growing out from the dorsal root ganglia (DRG) (asterisk), reproducing the defect found in the *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ mutant embryos (Ma and Tessier-Lavigne, 2007). Scale bars: A-B, 100μm.

(C-E) Demonstration of the coexpression of GFP (C) with Cre recombinase, which is assayed by tdTomato expression (D), from the AAV-Cre-GFP infected PCs carrying the *tdTomato* reporter. The merged image is shown in (E).

(F) Low magnification image of freshly cut cerebellar section demonstrates the density and specificity of co-infection with AAV-DsRed and AAV-Cre-GFP.

(G) Demonstration of Robo2-deletion following AAV-Cre-GFP infection in the mouse cerebellum carrying *Robo2*$^{flox/flox}$. The Robo2 signal (red) from antibody staining is present in GFP-negative PCs, but absent in a GFP-positive PC (green within the dashed white outline). A low magnification merged image is shown on the right.

(H-I) Infection of a wildtype PC with AAV-Cre-GFP (H) indicates that Cre recombinase has no effect on dendrite self-avoidance (I).

Figure S2

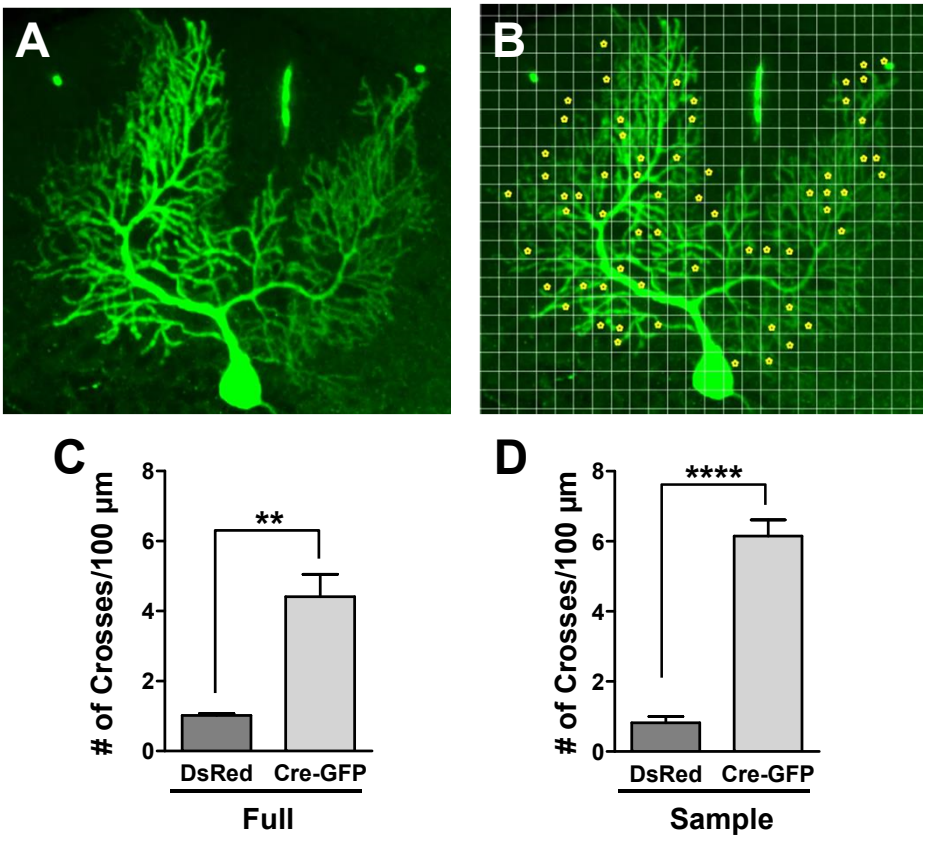

A
B
C
# of Crosses/100 µm
**
DsRed
Cre-GFP
Full
D
# of Crosses/100 µm
****
DsRed
Cre-GFP
Sample
Robo2flox/flox;DsRed
Robo1/2;DsRed
Robo1/2;Cre-GFP
E
# of Branches
F
Total dendrite length (µm)
G
Total Dendrite Area (µm²)
H
# of Crosses / 100µm
***
****
n.s.

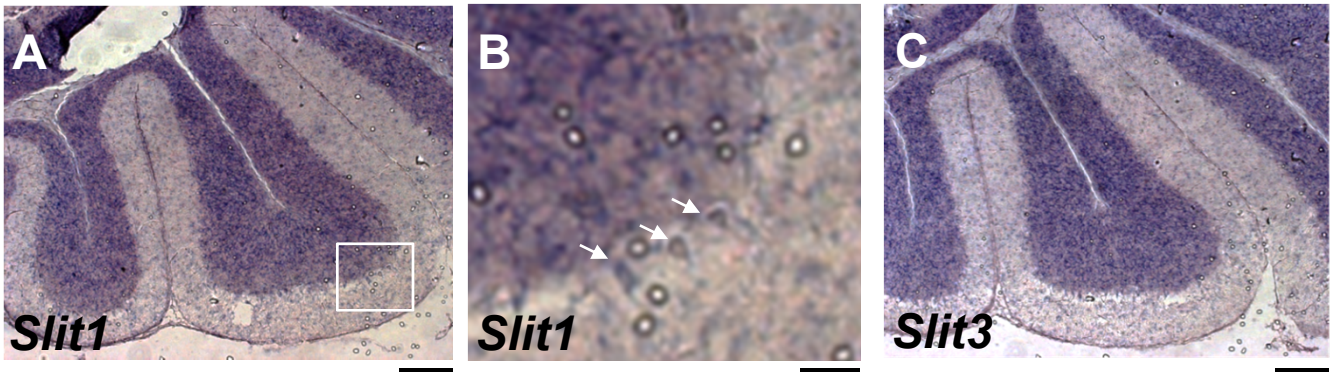
Figure S2. Validation the sampling method and analysis of Robo1 function in PC self-avoidance. Related to Figure 2.

(A-B) A maximum intensity *z*-projection of a PC (A) is created and a grid composed of 10μm x 10μm squares is overlaid on top of it (B). A random number generator is used to select a minimum of 20% of the grid squares for analysis (marked by yellow asterisks).

(C-D) Sampling method validation by quantitative comparisons of the frequency of self-crossing (#/100μm, mean ± SEM) measured in full reconstructions (C) or by sampled reconstructions (D) in *Robo2*$^{flox/flox}$ cells infected with either AAV-DsRed (dark bar) or AAV-Cre-GFP (light bar). Significant difference was found between DsRed^{+} control and GFP^{+} mutant cells by both methods (****p<0.0001 and **p<0.01, Student's t-test), whereas no significant difference was found for the GFP^{+} mutant PCs when analyzed by the two different methods (p =0.40).

(E-H) Quantification of dendritic arbor properties including total # of branches (E), total dendritic length (F), total dendritic area (G), and the frequency of self-crossing (H) in various *Robo* mutants. Note that only when *Robo2*^{flox} is deleted on top of the *Robo1* null background is there an increase in the frequency of self-crossing (***p<0.001**,** ****p<0.0001**,** n.s. not significant, Student's *t*-test). No other dendritic properties are changed by the deletion of *Robo1*. Values are expressed by mean ± SEM.

Figure S3



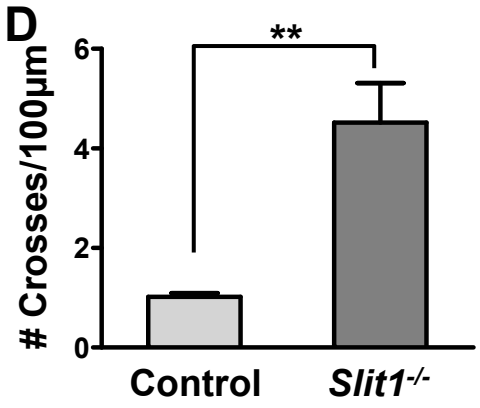


Figure S3. Expression of Slit1and *Slit3* mRNA in the cerebellum and analysis of Slit1 mutant PCs. Related to Figure 4.

(A-C) *In situ* hybridization of *Slit1*(A-B) and *Slit3* (C) in P14 cerebellum. Both transcripts are primarily expressed in the GCL, however some PCs may weakly express *Slit1* (B, from boxed area in A, arrows indicate PC somas). Scale bars: A,C, 100µm; B, 25µm.

(D) Quantification of self-crossing frequency (mean ± SEM) in AAV-DsRed labeled control (wildtype or $Slit2^{flox/flox}$) or Slit1 mutant ($Slit1^{-/-}$) PCs. Significant crosses were found in mutant cells (**p<0.01, Student's *t*-test).

Figure S4

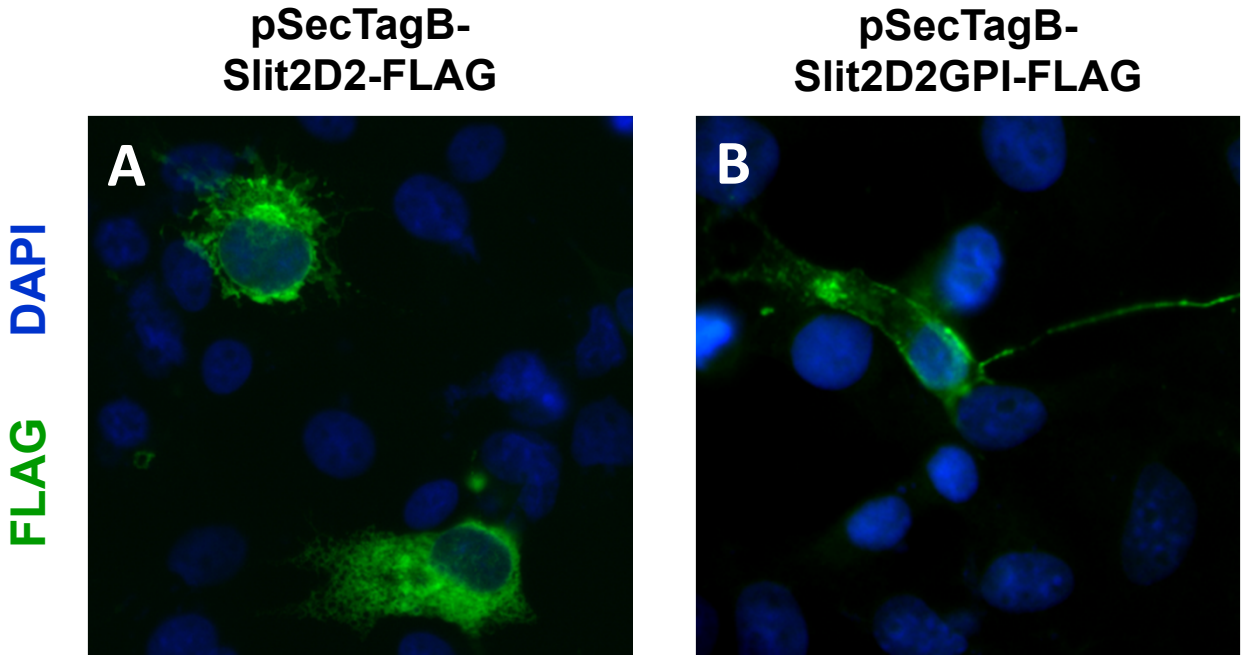

pSecTagB-
Slit2D2-FLAG
pSecTagB-
Slit2D2GPI-FLAG
A
B
DAPI
FLAG

Figure S4. Subcellular localization of Slit2D2 and Slit2D2GPI in COS cells. Related to Figure 6.

COS cells expressing Slit2D2 (A) or Slit2D2GPI (B) from pSecTagB vectors were stained with antibodies against a FLAG tag attached to the carboxyl terminal of the protein fragment (green) and counterstained with DAPI to show the nuclei (blue). Note the peri-nuclear staining for both proteins, whereas Slit2D2GPI also shows membrane association (B).

Figure S5

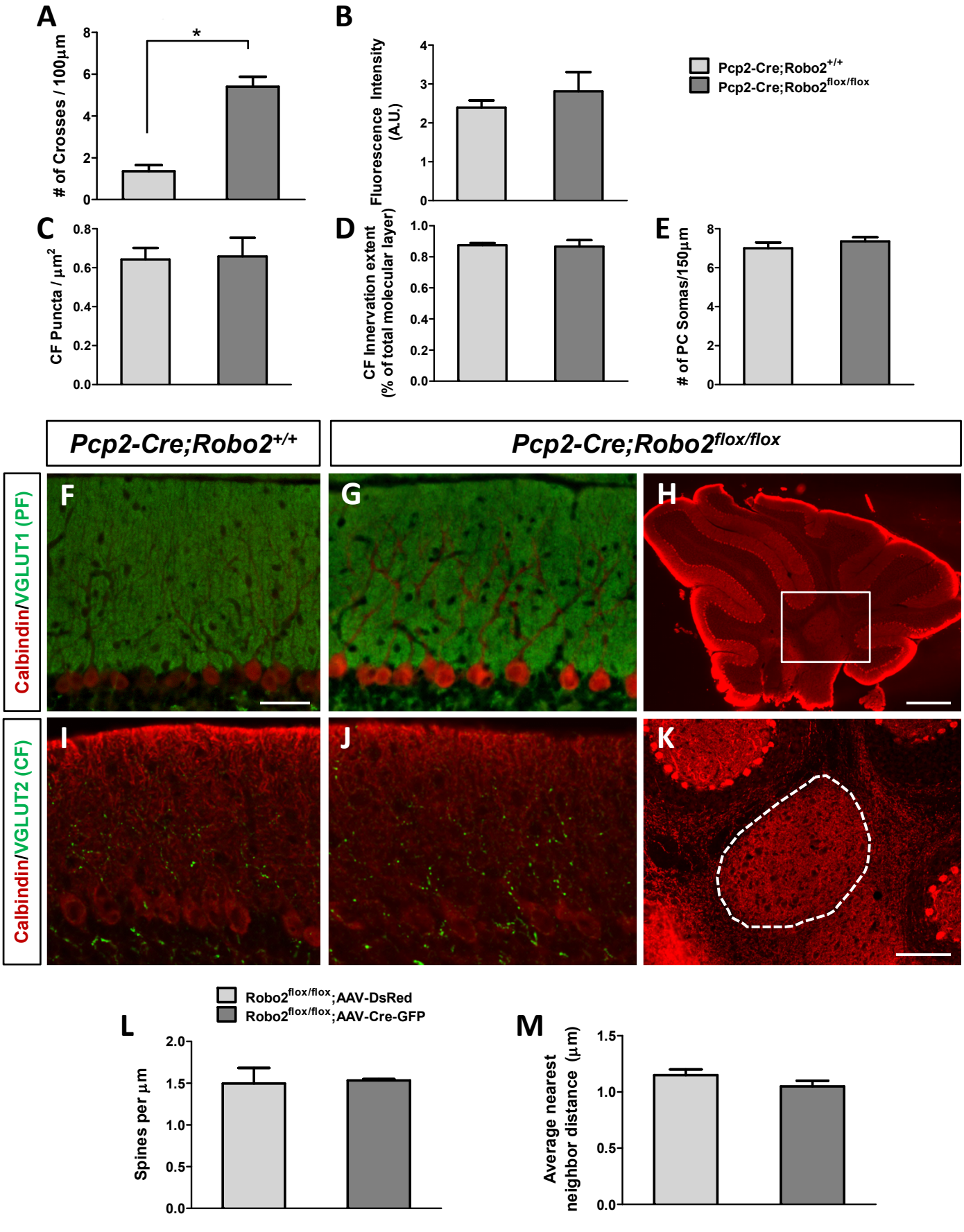

A
# of Crosses / 100µm
*
B
Fluorescence Intensity (A.U.)
Pcp2-Cre;Robo2+/+
Pcp2-Cre;Robo2flox/flox
C
CF Puncta / µm2
D
CF Innervation extent (% of total molecular layer)
E
# of PC Somas/150µm
Pcp2-Cre;Robo2+/+
Pcp2-Cre;Robo2flox/flox
Calbindin/VGLUT1 (PF)
Calbindin/VGLUT2 (CF)
F
G
H
I
J
K
L
Robo2flox/flox;AAV-DsRed
Robo2flox/flox;AAV-Cre-GFP
Spines per µm
M
Average nearest neighbor distance (µm)

Figure S5. Robo2 mutant PCs exhibit no changes in other neural development. Related to Figure 8.

(A) *Pcp2-Cre;Robo2*$^{flox/flox}$ PCs labeled with AAV-DsRed exhibit an increased frequency (mean ± SEM) of self-crossing relative to control *Pcp2-Cre;Robo2*$^{+/+}$ PCs (*$p<0.05$, Student's *t*-test).

(B-K) Immunohistochemical analysis of animals used in the motor assays. No changes were detected between *Pcp2-Cre;Robo2*$^{+/+}$ and *Pcp2-Cre;Robo2*$^{flox/flox}$ cerebellum in the development of parallel fiber terminals as measured by relative fluorescence intensity (B, F, G), nor in the density (C) or innervation pattern (D) of climbing fibers (I, J). Furthermore, cerebella from both genotypes exhibit no difference in the number of PCs present (E, H) or in axonal projections to the deep cerebellar nuclei (H, inset; K, inset from H, dashed line indicates axonal target region). All data are expressed in mean ± SEM. Scale bars: F, G, I, J, 50µm; H, 500µm; K, 150µm.

(L, M) Comparison of DsRed^{+} control and Cre-GFP^{+} *Robo2* mutant PCs reveals no differences in dendritic spine density (L) or spine spacing (M). Values are expressed in mean ± SEM.

Supplemental Experimental Procedures

In situ hybridization

P14 cerebellar sections (16μm) were processed for in situ analysis following a standard procedure(Zhao and Ma, 2009) using digoxigenin (DIG) labeled specific antisense RNA probes for *Robo1, Robo2, Slit1, Slit2, and Slit3* (Brose et al., 1999). Sections were hybridized with probes overnight at 68°C followed by immunostaining with an anti-DIG antibody overnight at 4°C. Sections were developed in 1x NBT/BCIP for ~72 hours before the reaction was stopped. Images were obtained on a dissection microscope with AxioCamHMc (Zeiss).

Antibodies

Primary antibodies used: chick anti-GFP (Aves, 1:1000); rabbit anti-DsRed (Clontech, 1:1000); mouse monoclonal and rabbit anti-Calbindin-D28k (Sigma, 1:250, and Swant, 1:1000, respectively), guinea pig anti-VGLUT1 (Millipore, 1:250), guinea pig anti-VGLUT2 (Millipore, 1:250), rabbit anti-Robo2 (Abcam, 1:50), rabbit anti-Slit2 (ProteinTech, 1:1000, and rabbit-anti-α-tubulin (Abcam, 1:5000). Cy2- or Cy3-conjugated secondary antibodies were used for immunostaining (Jackson ImmunoResearch, 1:500).

DNA constructs

The D2 constructs covering the second leucine rich repeat of hSlit2 between residues 269 and 505 (SVLHC . . . FADLA) were constructed by PCR-based subcloning strategies. Sequences encoding the FLAG (DYKDDDDKAAA) were included right behind the D2 sequence in the primer for both Slit2D2 and Slit2D2GPI. The GPI linker was subcloned to the carboxyl terminal of Slit2D2GPI. Both constructs were first subcloned into the pSecTagB vector behind the IgK signal peptide. The entire coding region was then moved to the pAAV-GFP-cDNA6 vector (Vector Biolabs) for viral production.

Virus injections

All rAAVs except AAV-mCherry-Cre are based on serotype AAV8 that infects only Purkinje cells (PCs) in the cerebellum. AAV-Cre-mCherry is based on a synthetic serotype (AAV-DJ) that also infects PCs. They were all produced by Vector Biolabs (Philadelphia, PA) at the following stock titers (GC/ml): AAV-DsRed at 1×10^{13}, AAV-Cre-GFP at 1×10^{12}, AAV-Slit2D2-GFP at 5.5×10^{13}, and AAV-Slit2D2GPI-GFP at 2.2×10^{13}, and AAV-Cre-mCherry at 1×10^{13}. Viruses were diluted in sterile phosphate-buffered saline (PBS, pH = 7.4) before injection. rAAVs expressing *DsRed* (AAV-DsRed) was used to label control PCs in all mouse strains. rAAVs expressing both GFP and Cre recombinase under separate CMV promoters (AAV-Cre-GFP) was used to generate and label knockout PCs in conditional $Robo2^{flox}$, $Slit2^{flox}$, and $Pcdhg^{fcon3}$ mice, as well as $Pcdhg^{fcon3/fcon3}$;$Robo2^{flox/flox}$ mice. Rescue experiments were conducted in *Pcp2-Cre;*$Slit2^{flox/flox}$ mice using rAAVs expressing GFP and an active fragment of Slit2, either as a secreted form (AAV-Slit2D2-GFP) or as a GPI-linked membrane-bound form

(AAV-Slit2D2GPI-GFP); or in *Pcdhg*$^{fcon3/fcon3}$ mice co-injected with AAV-Slit2D2GPI-GFP and AAV-Cre-mCherry, which uses a single CMV promoter to express both Cre and mCherry that are linked by a 2A peptide.

All injections were performed on newborn mouse pups on the day of birth, designated postnatal age P0, and performed as previously described (Gibson and Ma, 2011). Briefly, mouse pups were subjected to cold anesthesia and 1μl of diluted rAAVs (~1 x 10^{11} GC/ml) was injected through the skull and into the cerebellar midline using a digital pressure pump and a Hamilton syringe with a beveled 34G stainless steel needle; pups were then warmed and rolled in bedding before being returned to their home cage.

Analysis of PC dendrites

Mice (P14-P60) were anesthetized by intraperitoneal administration of a lethal overdose of ketamine/xylazine and sacrificed by transcardial perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. The cerebellum was removed and post-fixed in 4% PFA overnight at 4°C and processed into either thick sections or thin cryosections.

Thick sections (100μm) were prepared using a vibratome (Leica) and processed as floating sections. Sections were washed with 1% Triton X-100 in PBS, incubated for 1 hour at room temperature in blocking buffer (1% Triton X-100, 10% normal goat serum in PBS), and then incubated for 2-3 days at 4°C in blocking buffer containing primary antibodies. Sections were then washed, blocked, and incubated with secondary antibodies in blocking buffer for 2 hours at room temperature, followed by excess washes in PBS. Sections were then mounted onto glass slides with homemade antifade solution for imaging.

Cryosections were cut at 16μm on a cryostat (Leica CM3050S) and mounted on SuperFrost Plus slides. Sections were washed with 0.1% Triton X-100 in PBS, incubated in blocking buffer (0.1% Triton X-100, 10% goat serum) for 15 minutes at room temperature, and incubated with primary antibodies overnight at 4°C. Sections were then washed, blocked, and incubated with secondary antibodies for 1 hour at room temperature, then coverslipped with antifade solution for imaging. Confocal stacks were collected on a Zeiss LSM5 laser scanning confocal microscope; non-confocal images were collected on an upright epifluorsecent microscope (AxioImager, Zeiss) equipped with a digital camera (AxioCamMRm, Zeiss).

3D *z*-stacks of labeled PCs were collected on a Zeiss LSM5 laser scanning confocal microscope from anatomical positions throughout the cerebellum. GFP^+ or $DsRed^+$ PC dendrites were manually reconstructed in 3D using Neurolucida (MBF Bioscience) to generate a skeletonized 3D reconstruction of the entire arbor or selected arbor regions. Neurolucida Explorer was subsequently used to analyze the reconstruction data. Self-crossings were counted manually and marked in flattened *z*-projections of skeletonized reconstructions; any two branches crossing over each other were classified as a self-crossing. Total dendrite area was calculated using the 2D convex hull method; total number of dendritic branches, total dendrite length, and analysis of *z*-separation between crossing branches were calculated from 3D

reconstruction data. Coexpression of GFP^+ or $DsRed^+$ in the same PC showed identical labeling and reconstruction.

Western blots

Postnatal mouse cerebellum tissue was collected from mice euthanized at postnatal age P14. Whole cerebellum tissue was further cut under a dissection microscope into separate GCL and ML+PCL samples. Tissue was homogenized using a tight fitting glass-glass homogenizer in RIPA buffer (20mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EGTA, 1mM EDTA, 20mM NaF, 1% Triton X-100, 0.1% SDS, 0.5% SDC) plus PhosStop (Roche) and a protease inhibitor cocktail at 4°C, followed by mechanical trituration using successively finer syringe needles. The homogenate was then incubated on ice for 30 minutes followed by a 13,000RPM centrifugation for 10 minutes at 4°C. An equal volume of 2x SDS sample buffer (100mM Tris-Cl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200mM β-mercaptoethanol) was added to the supernatant and the samples were boiled for 5 minutes before electrophoresis.

Proteins were separated by SDS-PAGE (8%) and then transferred to nitrocellulose membranes (0.2 µm pore size, Whatman). Membrane blots were blocked for 1 hour in blocking buffer (PBS-T plus 4% nonfat dry milk) at room temperature and then probed overnight on a rocker with primary antibodies diluted in blocking buffer at 4°C. Blots were washed in PBS-T and incubated with appropriate HRP-conjugated secondary antibodies for 1 hour at room temperature. After wash with PBS-T, blots were developed using SuperSignal West Dura Chemiluminescent Substrate (Thermo) and imaged on a UVP BioSpectrum 500 Imaging System with VisionWorksLS software (UVP). Densitometry was performed using ImageJ analysis software (NIH).

PC explant co-cultures

Co-cultures of cerebellar foliar explants and COS cell aggregates were prepared as follows: Mouse pups were sacrificed at postnatal age P7 and the cerebellum was removed into ice-cold low sodium ACSF (1mM $CaCl_2$, 10mM D-Glucose, 4mM KCl, 5mM $MgCl_2$, 26mM $NaHCO_3$, 246mM sucrose). The cerebellum was cut in half along the rostral-caudal axis at the midline, embedded in 4% low-melt agarose, and sectioned on a vibratome (Leica) at 400µm. After sectioning the agarose was removed from around the sections and individual foliar explants were cut off of the section. Foliar explants were transferred to 6-well plates containing cell-culture inserts with a 0.4µm pore size (Millipore) floating on top of culture media (75% MEM, 25% horse serum, 25mM HEPES, 1mM glutamine, 5mg/ml glucose, 1% pen/strep) that was preconditioned by a 2 hour incubation at 37°C and 5% CO_2. Culture media was exchanged with fresh media by 50% every 3 days.

COS cell aggregates were prepared via the hanging drop method (Kennedy et al., 1994). The day after transfection, DMEM drops containing cells were inverted and incubated overnight at 37°C and 5% CO_2 to form aggregates. The next day the aggregates were squared off, cut into small pieces, and then placed adjacent to the foliar explants that had been growing for 10 days *in*

vitro (DIV), the time at which PC dendrites begin to grow out of the explant. Explant/aggregate co-cultures were grown together for an additional 3DIV.

After growing for an additional 3DIV, cocultures were fixed with 4% PFA in PBS, washed with PT buffer (0.5% Triton X-100 in PBS) overnight at 4°C, then blocked with blocking buffer (20% goat serum in PT) overnight at 4°C. Co-cultures were labeled with rabbit anti-Calbindin-D28k (Swant, 1:1000) overnight at 4°C to label PCs and their dendrites followed by 3-hour incubation at room temperature with Cy3-conjugated anti-rabbit secondary antibody. The membrane insert to which co-cultures were attached was mounted on a glass slide and imaged on a Zeiss LSM5 laser scanning confocal microscope to obtain both fluorescent and DIC images. Images were analyzed in ImageJ (NIH).

Behavioral Assays

Gait analysis was performed using the footprint test (Crawley, 2007; Crawley, 2008). Briefly, a runway was constructed out of acrylic glass to dimensions of 55 cm long and 6 cm wide, with walls 5 cm in height. White paper was used to line the runway during testing. The feet of the mice were painted with nontoxic paint (fore-paws red, hind-paws blue). Each animal was given several (3-5) trial runs with painted paws before testing in order to acclimate them to the procedure. Footprints were analyzed for print separation (distance between corresponding fore- and hind-paw prints), base width (medial-lateral distance between left and right fore- and hind-paws), stride length (rostral-caudal distance between two prints from the same paw), and maximum stride difference (difference between shortest and longest stride lengths; a measure of stride variability). Footprint images were thresholded and analyzed in ImageJ. Measurements were made by comparing the centroid coordinates of each paw print; three sets of prints were used for each measurement to calculate the mean.

The dowel rod test was performed using a square wooden dowel rod 0.9 cm in diameter and 55 cm long; at the starting end was a flat platform and a darkened goal box was placed at the opposite end. The entire setup was elevated so that animals could not step or jump off of the dowel. Test subjects were put through 5 training runs per day for 3 days prior to the day of testing. On each training day and the day of testing animals were acclimated to the testing room in the dark for 1 hour prior to the assay; the testing was performed in the dark with a bright light positioned behind the animals to provide a motivating stimulus to cross the dowel. Mice were place on the flat platform and allowed up to 60 seconds to cross the dowel rod into the goal box. The latency to cross was measured from the time the animal first stepped onto the beam to the time the last foot was lifted off of the beam. The number of hind foot slips was also counted.

For both tests, animals were normally housed in a standard colony room on 12:12 light:dark cycle where food and water were freely available. The tests were done at the same time of day. Prior to testing, mice were transferred to the behavioral testing room for 60min to allow them to acclimate to the room conditions.

Supplemental References

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