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Supplemental Information

Comprehensive Monosynaptic Rabies Virus Mapping of Host Connectivity with Neural Progenitor Grafts after Spinal Cord Injury

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Supplemental Figures

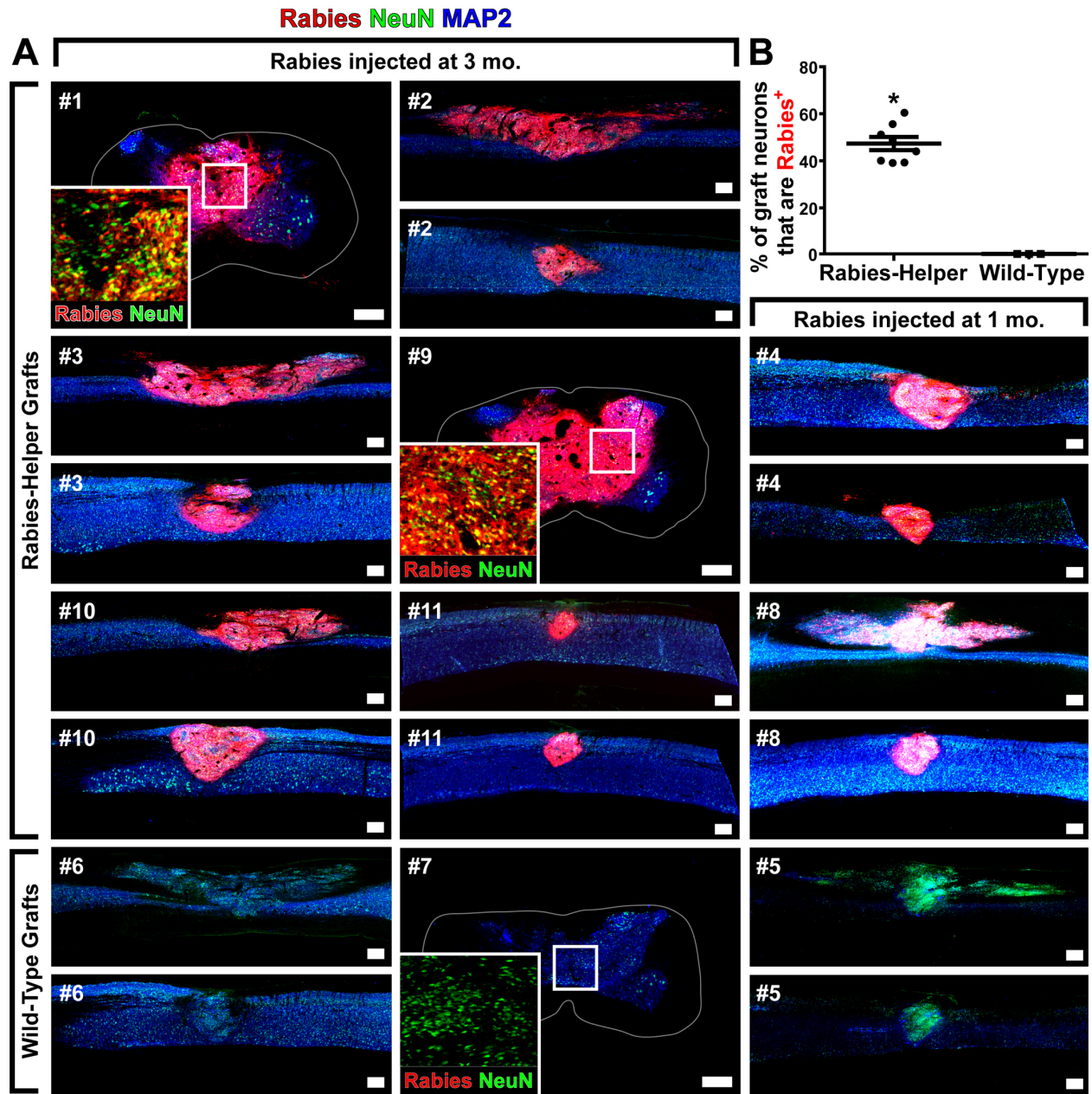


Figure S1. Graft sites of all animals used in these experiments, related to Figure 1. Host mouse identification numbers are indicated in the top left of panels. (A) Sagittal sections (with exception of transverse sections in #1, #7, #9), with rostral to the left, of all E12.5-E13.5 spinal NPC grafts in this study. When possible, a section near the central canal, as well as a more lateral section are provided here to illustrate the extent of the graft sites. All NPC grafts contained NeuN⁺MAP2⁺ neurons after either 1 or 3 months of maturation. Rabies-Helper grafts were infected and expressed EnvA-SADΔG-mCherry rabies virus throughout in all cases, whereas Wild-Type grafts were not infected. White lines denote the extent of white matter for transverse sections. Insets depict detail of boxed regions. Scale bars 250 μm, 30 μm-thick sections. (B) Quantification of the percentage of NeuN⁺ graft neurons that were also mCherry-Rabies⁺ at the end of the experiment (mean ± SEM, n = 8 Rabies-Helper grafts, n = 3 Wild-Type grafts). Asterisk denotes significance by Fisher's exact test of a 2×2 two-tailed contingency table comparing Rabies-Helper and Wild-Type NPC grafts, P < 0.01.

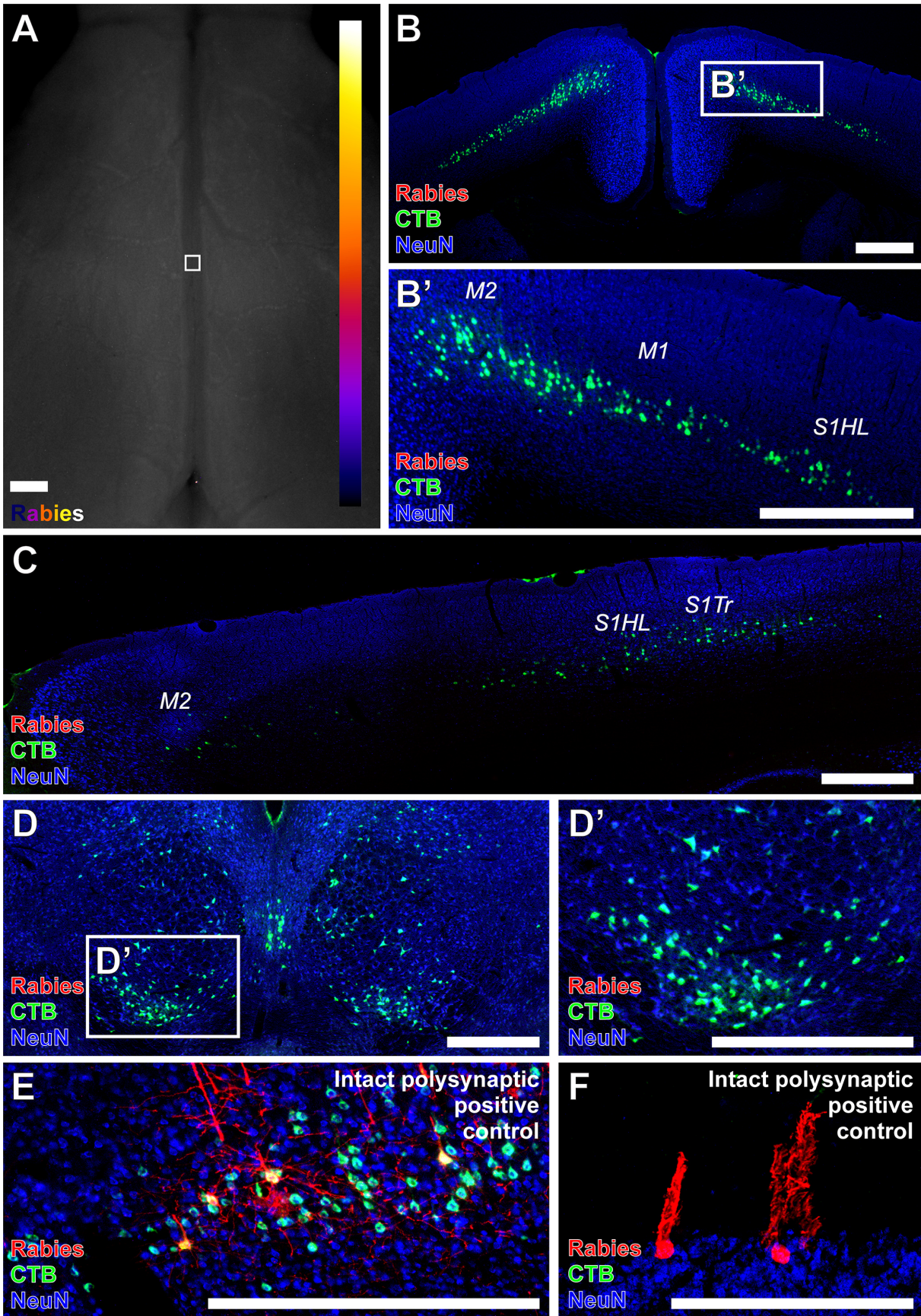


Figure S2. Negative Controls: Brains of hosts grafted with Wild-Type spinal NPCs were not labeled with EnvA-SADΔG-mCherry rabies virus, related to Figure 2. (A-D) Host brain regions in animals co-injected with CTB/EnvA-SADΔG-mCherry rabies virus following Wild-Type NPC grafting were retrogradely labeled with CTB in similar regions to animals receiving Rabies-Helper grafts, but were never labeled with rabies virus. Panels (A-B) and (C-D) correspond to Fig. 2A-B and 2D-E, respectively. Corresponding images were labeled, imaged, and processed identically. **Positive Controls: Brains of intact adult Rabies-Helper animals injected with EnvA-SADΔG-mCherry were extensively labeled with Rabies-mCherry, related to Figure 2.** (E) The M1 cortical section most densely labeled with polysynaptic Rabies-mCherry tracing among intact adult Rabies-Helper animals co-injected at C4 with CTB/EnvA-SADΔG-mCherry rabies virus. (F) Purkinje cells in cerebellar cortex were labeled with polysynaptic Rabies-mCherry tracing in intact adult Rabies-Helper animals, but never in Rabies-Helper grafted animals. Scale bars 500 μm, 30 μm-thick sections. (A) Horizontal, (B, D, E, F) coronal, (C) sagittal views. Abbreviations: M1 primary motor cortex; M2 secondary motor cortex; S1HL primary somatosensory cortex, hindlimb; S1Tr primary somatosensory cortex, trunk.

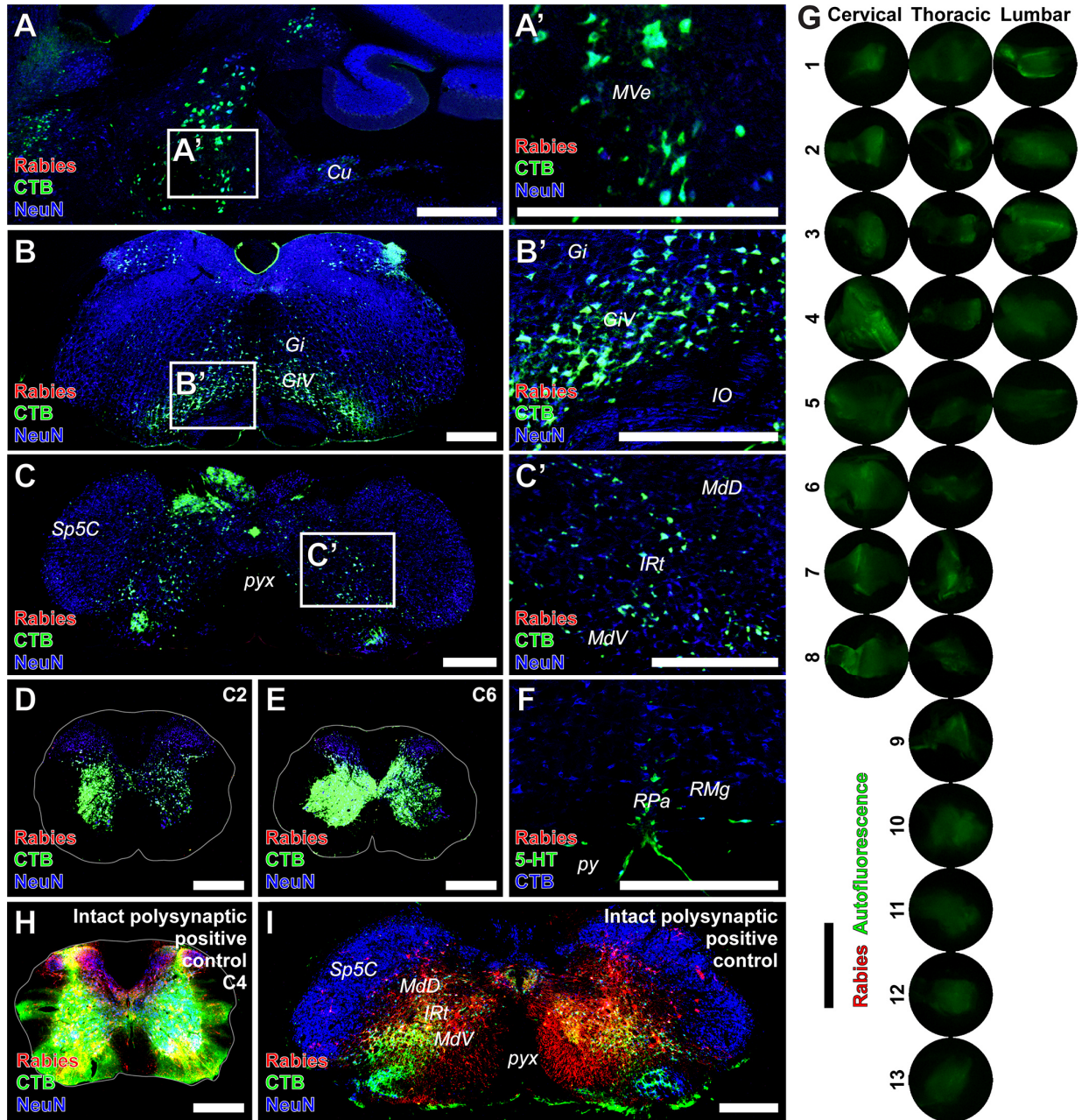


Figure S3. Negative Controls: Brainstems, spinal cords, and dorsal root ganglia of hosts grafted with Wild-Type spinal NPCs were not labeled with EnvA-SADΔG-mCherry rabies virus, related to Figures 3-4. Host brainstem, spinal cord, and dorsal root ganglia in animals co-injected with CTB/EnvA-SADΔG-mCherry rabies virus were retrogradely labeled with CTB in similar regions to animals receiving Rabies-Helper grafts, but were never infected with rabies virus. Panel (A) corresponds to Fig. 3J, (B) to Fig. 3A, (C) to Fig. 3D, (D-E) to Fig. 4A-B, (F) to Fig. 3G, and (G) to Fig. 4G. Corresponding images were stained, imaged, and processed identically to each other. **Positive Controls: (H) Injection sites and (I) brainstems of intact adult Rabies-Helper animals injected with EnvA-SADΔG-mCherry at C4 were extensively labeled with Rabies-mCherry.** Scale bars 500 μm, 30 μm-thick sections. (A) Sagittal view, (B-F, H-I) transverse views. Abbreviations: Gi gigantocellular reticular nu.; GiV gigantocellular reticular nu., ventral part; IO inferior olive, IRt intermediate reticular nu.; MdD medullary reticular nu., dorsal part; MdV medullary reticular nu., ventral part.; MVe medial vestibular nu.; py pyramids; pyx pyramidal decussation; RMg raphe magnus nu.; RPa raphe pallidus nu.; S1 primary somatosensory; Sp5C spinal trigeminal nu..

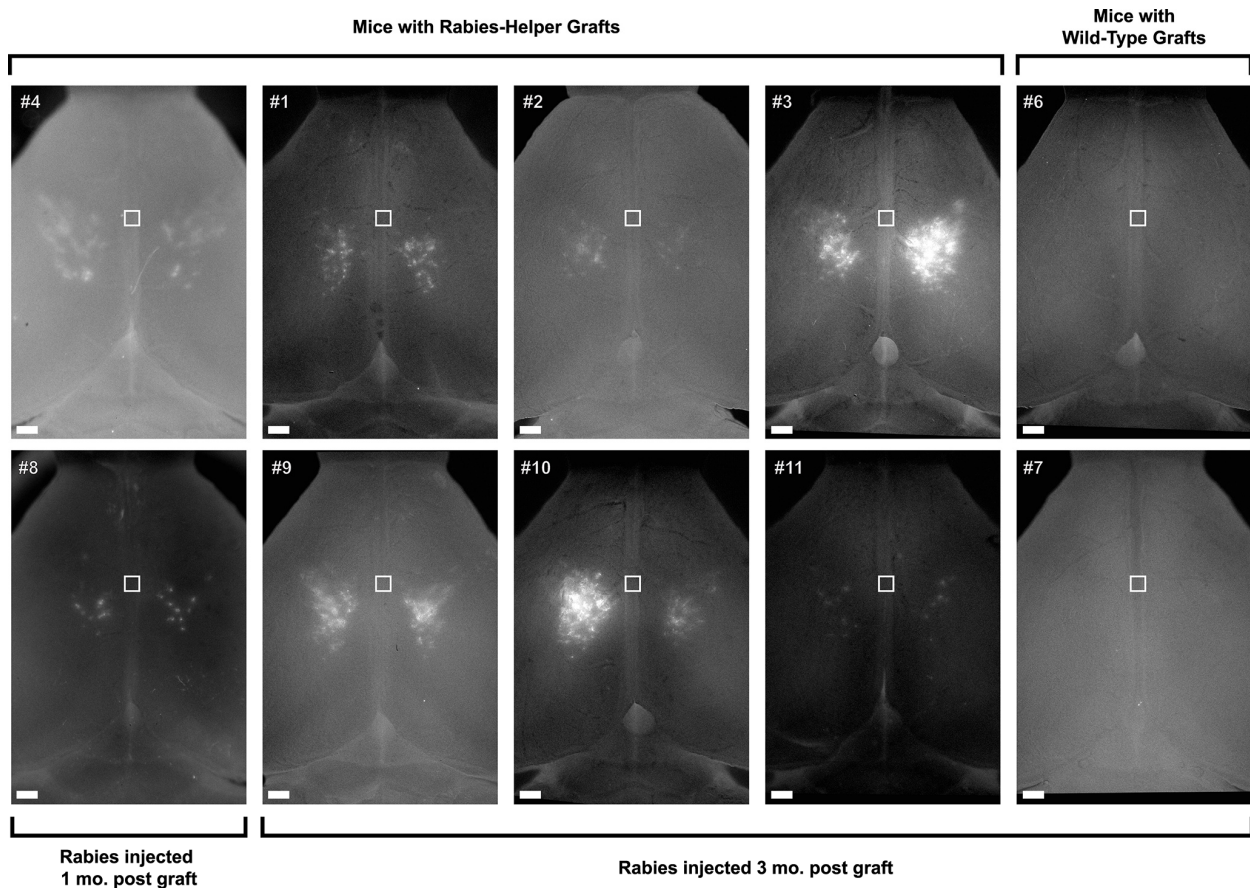


Figure S4. Cortical rabies labeling of individual animals, related to Figures 2A and S2A. Each individual image included in the production of Fig. 2A, and S2A. Corticospinal neurons of host animals receiving Rabies-Helper spinal NPC grafts were consistently transsynaptically labeled in similar regions of the cortex, whereas host animals receiving Wild-Type grafts were not labeled at all. Host mouse identification numbers are indicated in the top left of each panel. Mouse #5 is not depicted, because a gross cortical image was not collected before sectioning the brain; however, no Rabies-mCherry⁺ cells were present in animal #5 upon sectioning. Horizontal views, boxes denote bregma, with rostral at top of images. Scale bars 500 μ m.

RABIES RAW CTB RAW	M1	M2	S1	S2	Red Nucleus	Reticular	Raphe	Vestibular	Other brainstem	TOTAL
Rabies-Helper Grafted #1	88 4688	32 3368	64 3352	8 448	16 1104	318 30228	0 1278	150 2028	210 10938	886 57432
Rabies-Helper Grafted #3	280 1176	136 2328	176 576	16 224	32 1664	438 5760	12 258	66 732	168 1890	1324 14608
Rabies-Helper Grafted #4	64	24	56	0	16	78	6	42	42	328
Rabies-Helper Grafted #8	64	8	16	0	0	48	12	0	42	190
Rabies-Helper Grafted #11	42 1232	12 3152	12 1240	0 912	0 1024	24 9568	6 480	6 1056	30 3168	132 21832
Wild-Type Grafted #5	0	0	0	0	0	0	0	0	0	0
Wild-Type Grafted #6	0 5280	0 7960	0 3104	0 864	0 1368	0 12780	0 576	0 996	0 5904	0 38832
Intact (Poly-synaptic) #1	88 1872	0 680	184 1928	0 128	192 616	3888 15600	408 912	516 1440	1800 9252	7076 32428
Intact (Poly-synaptic) #2	40 3136	0 1064	24 2640	0 240	272 2288	2532 31656	102 1140	288 2820	1422 19164	4680 64148
Intact (Poly-synaptic) #3	8 1264	0 560	8 1376	0 96	72 584	1104 15228	150 576	84 1320	558 6192	1984 27196

% ALL RABIES % ALL CTB	M1	M2	S1	S2	Red Nucleus	Reticular	Raphe	Vestibular	Other brainstem	TOTAL
Rabies-Helper Grafted #1	9.9 8	3.6 6	7.2 6	0.9 1	1.8 2	35.9 53	0.0 2	16.9 4	23.7 19	100.0 100
Rabies-Helper Grafted #3	21.1 8	10.3 16	13.3 4	1.2 2	2.4 11	33.1 39	0.9 2	5.0 5	12.7 13	100.0 100
Rabies-Helper Grafted #4	19.5	7.3	17.1	0.0	4.9	23.8	1.8	12.8	12.8	100.0
Rabies-Helper Grafted #8	33.7	4.2	8.4	0.0	0.0	25.3	6.3	0.0	22.1	100.0
Rabies-Helper Grafted #11	31.8 6	9.1 14	9.1 6	0.0 4	0.0 5	18.2 44	4.5 2	4.5 5	22.7 15	100.0 100
Wild-Type Grafted #5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Wild-Type Grafted #6	N/A 14	N/A 20	N/A 8	N/A 2	N/A 4	N/A 33	N/A 1	N/A 3	N/A 15	N/A 100
Intact (Poly-synaptic) #1	1.2 6	0.0 2	2.6 6	0.0 0	2.7 2	54.9 48	5.8 3	7.3 4	25.4 29	100.0 100
Intact (Poly-synaptic) #2	0.9 5	0.0 2	0.5 4	0.0 0	5.8 4	54.1 49	2.2 2	6.2 4	30.4 30	100.0 100
Intact (Poly-synaptic) #3	0.4 5	0.0 2	0.4 5	0.0 0	3.6 2	55.6 56	7.6 2	4.2 5	28.1 23	100.0 100
Rabies-Helper Grafted AVG	23.2 ± 4.3 8.9 ± 1.7	6.9 ± 1.3 14.2 ± 3.1	11.0 ± 1.8 5.9 ± 0.8	0.4 ± 0.3 2.2 ± 0.7	1.8 ± 0.9 5.4 ± 2.1	27.2 ± 3.2 42.2 ± 4.1	2.7 ± 1.2 1.9 ± 0.2	7.9 ± 3.1 4.0 ± 0.6	18.8 ± 2.5 15.4 ± 1.3	100.0 100.0
Intact (Poly-synaptic) AVG	0.8 ± 0.2 5.1 ± 0.3	0.0 ± 0.0 1.9 ± 0.1	1.2 ± 0.7 5.0 ± 0.5	0.0 ± 0.0 0.4 ± 0.0	4.1 ± 0.9 2.5 ± 0.5	54.9 ± 0.4 51.1 ± 2.4	5.2 ± 1.6 2.2 ± 0.3	5.9 ± 0.9 4.6 ± 0.1	28.0 ± 1.4 27.1 ± 2.2	100.0 100.0

Table S1. Individual raw counts and percentages relative to total of Rabies-mCherry⁺ neurons in all grafted and adult intact Rabies-Helper animals that were sectioned transversely, related to quantifications in Figures 2 and 3. CTB⁺ neuron counts and distributions are also provided for animals injected after 3 months of graft maturation (Grafted animals #1, #3, #6, #11), and for adult intact Rabies-Helper animals. (The idea to co-inject CTB had not yet been conceived at the time animals with 1 month old grafts were injected with rabies).

Supplemental Experimental Procedures

Mouse breeding

Eighteen adult mice were used (Jackson Laboratory): wild-type (C57BL/6) n = 1, RΦGT (B6;129P2-Gt(ROSA)26Sor^{tm1(CAG-RABVgp4,-TVA)Arenk/J}) n = 2, athymic nude mice (NU/J) n = 12, Rabies-Helper (B6;129P2-Gt(ROSA)26Sor^{tm1(CAG-RABVgp4,-TVA)Arenk/J} × B6.Cg-Tg(Syn1-cre)671Jxm/J) n = 3. Twenty-seven embryonic mice were used: Wild-Type n = 9, Rabies-Helper n = 18. E12.5-E13.5 Rabies-Helper embryos were produced by timed mating of homozygous Syn-Cre sires with homozygous RΦGT dams, ensuring all progeny expressed the helper components.

Spinal cord injury and grafting

During surgery, mice were deeply anesthetized with a combination of acepromazine, xylazine, ketamine, and isoflurane. Laminectomies were performed on athymic nude mice at the fourth cervical vertebral level (C4). A wire knife (McHugh Milieux), which was selected to span from the center of each dorsal horn, was stereotactically inserted, such that the lowest point of the extended knife would be 1.1 mm down from the dorsal surface of the cord. The knife was then raised to lesion the central gray matter, the CST in its entirety, and to partially lesion the ascending sensory fibers, leaving a spared rim of dorsal white matter.

Spinal cords were dissected from E12.5-E13.5 Wild-Type or Rabies-Helper embryos on ice cold HBSS, dissociated with Accutase and gentle trituration, strained through a 70 μm cell strainer, centrifuged for 7 minutes at 300×g, resuspended in DPBS, counted, spun for another 7 minutes at 300×g, decanted, resuspended, and the final volume of concentrated cell suspension was measured with a micropipette. 1M viable spinal NPCs (Trypan Blue exclusion) were grafted immediately after lesion, into the center of lesion sites, in a volume of 2 μL DPBS, using a Picospritzer III (Parker Hannifin) and pulled glass pipettes. A fibrin matrix or growth factor cocktail was not necessary to enable graft survival in this enclosed lesion cavity. After surgery, animals were supported with heat, Neo-Predef, and banamine/ampicillin injections in Lactated Ringer's solution.

EnvA-pseudotyped-SADΔG rabies virus/CTB injections

Upon graft maturation, the grafting site was re-exposed, and fibrous scar overlying the dura was gently removed with a scalpel. Small pilot holes were punctured in dura with an insulin syringe, to allow stereotactic insertion of pulled and beveled glass micropipettes, containing 1×10^7 vg/mL EnvA-SADΔG-mCherry rabies virus (Salk Institute GT3 Core) in HBSS. Grafts at 3 months of maturation were co-injected with 1×10^7 vg/mL EnvA-SADΔG-mCherry rabies virus and 0.2% CTB (List Biological Laboratories) in HBSS. A total of 1.5 μL of viral suspension was injected with a Picospritzer evenly across 8 sites into each graft, at depths of 0.7, 0.5, 0.3 mm at each site, for an infusion of ~ 60 nL/injection, over a period of approximately one hour per animal. Picospritzer pressure and/or pulse duration was adjusted for each pipette to barely overcome capillary forces before insertion, enabling a gentle infusion of viral suspension. Injection sites were balanced as much as overlying vasculature would allow in the rostrocaudal and mediolateral directions for each graft. With exception of Rabies-Helper-grafted mouse #11, control and Rabies-Helper grafts were always injected in the same surgical session, drawing from the same tube of EnvA-SADΔG rabies virus or EnvA-SADΔG rabies virus/CTB mixture. For adult polysynaptic positive control Rabies-Helper mouse injections, a laminectomy was performed at C4, and a total of 1.5 μL of 1×10^7 vg/mL EnvA-SADΔG-mCherry rabies virus and 0.2% CTB suspension was co-injected bilaterally through the center of the dorsal horn, across 8 sites, at depths of 1.1, 0.7, 0.3 mm at each site, for an infusion of ~ 60 nL/injection, over a period of approximately one hour per animal. All animals were sacrificed 7 days after rabies virus injections.

Immunofluorochemistry

Animals were deeply anesthetized with a mixture of ketamine and xylazine, and transcardially perfused with ice-cold phosphate-buffered saline (PBS), then 4% PFA in phosphate-buffered saline. Spinal columns were removed, post-fixed for 24 hours at 4 °C in 4% PFA, then cryoprotected for a minimum of 24 hours in 30% sucrose in PB. The CNS and DRGs were then dissected, and cryoprotected for an additional 24 hours in 30% sucrose in PB. Brains and spinal cords were blocked, frozen, and sectioned at a thickness of 30 µm with a sliding microtome. DRGs were embedded in OCT, and sectioned at a thickness of 20 µm on a cryostat.

For free-floating section immunofluorescence, sections were blocked for 1 hr at room temperature (RT) in blocking buffer (0.2% Triton-X and 10% donkey serum in Tris-buffered saline). Primary antibodies were incubated with sections in blocking buffer overnight at 4 °C, washed, and then incubated with streptavidin or AlexaFluor 488-, 594-, or 647-conjugated secondary antibodies raised in donkey. DRG sections were direct-mounted to subbed slides, and subjected to 30 min of heat-induced antigen retrieval at 80°C in pH 9.0 Tris-HCl buffer before staining on-slide. Primary antibodies used included: Ch α mCherry (EnCor Biotechnology CPCA-mCherry, 1:1000), Ch α MAP2 (EnCor Biotechnology CPCA-MAP2, 1:1000), Gt α mCherry (SICGEN AB0040-200, 1:1000), Gt α CTB (List Labs #703, 1:2500), Rb α NeuN (Biosensis R-3770-100, 1:1000), Rb α 5-HT (Immunostar 20080, 1:2500), Rb α TH (Millipore AB152, 1:1000), Gt α ChAT (Genetex GTX82725, 1:500), Sh α CHX10 (Abcam ab16141, 1:200), Gt α SATB1 (Santa Cruz sc-5989, 1:500), Ch α CALRETININ (EnCor Biotechnology CPCA-CALRETININ, 1:1000), Ch α NF200 (Millipore AB5539, 1:500), Gt α CGRP (Abcam ab36001, 1:1000), biotin-conjugated IB4 (Sigma L2140, 1:500).

Image Analysis

Sections were mounted in Mowiol, and imaged with Olympus AX70 (widefield) or FV-1000 (confocal) fluorescence microscopes. Image window/level were adjusted with ImageJ (FIJI), and long exposures in the far red channel were processed with the “despeckle” command. Comparable regions imaged between Figs. 2-4 and S2-S3 (indicated in caption) were imaged together, with identical acquisition settings and processing in ImageJ. For gross anatomical imaging, autofluorescent background images were captured in the 488 channel, and subtracted from mCherry signal in the 594 channel to produce Figs. 1E, 1J, 2A, 4G, and S2A, S3G, and S4; this background subtraction was performed equivalently for all samples. For quantification of Rabies-mCherry⁺ neurons in Figs. 2-3, all neurons in every 6th (8th) section through brainstem (cortex) were manually counted for all animals cut transversely (#1, 3, 4, 8, 11 Rabies-Helper, and #5, 6 Wild-Type) from olfactory bulb to the pyramidal decussation. No significant differences in the ratio of host labeling were detectable between animals injected with rabies at 1 or 3 months following injury and engraftment, so their counts were pooled. Counter was blinded to experimental conditions for the determination of whether any Rabies-mCherry⁺ cells were observed in control animals during quantifications performed for Figs. 2-4 (none were observed). ImageJ macros were used to count polysynaptic mCherry⁺ neurons, CTB⁺ neurons, and mCherry⁺NeuN⁺ graft neurons (Fig. S1B) in an automated fashion. ImageJ macros set a local threshold of a defined radius around each pixel, excluded masked objects that were too large or small to be neurons, and then counted the remainder within ROIs defined with the aid of an atlas of the mouse brain.