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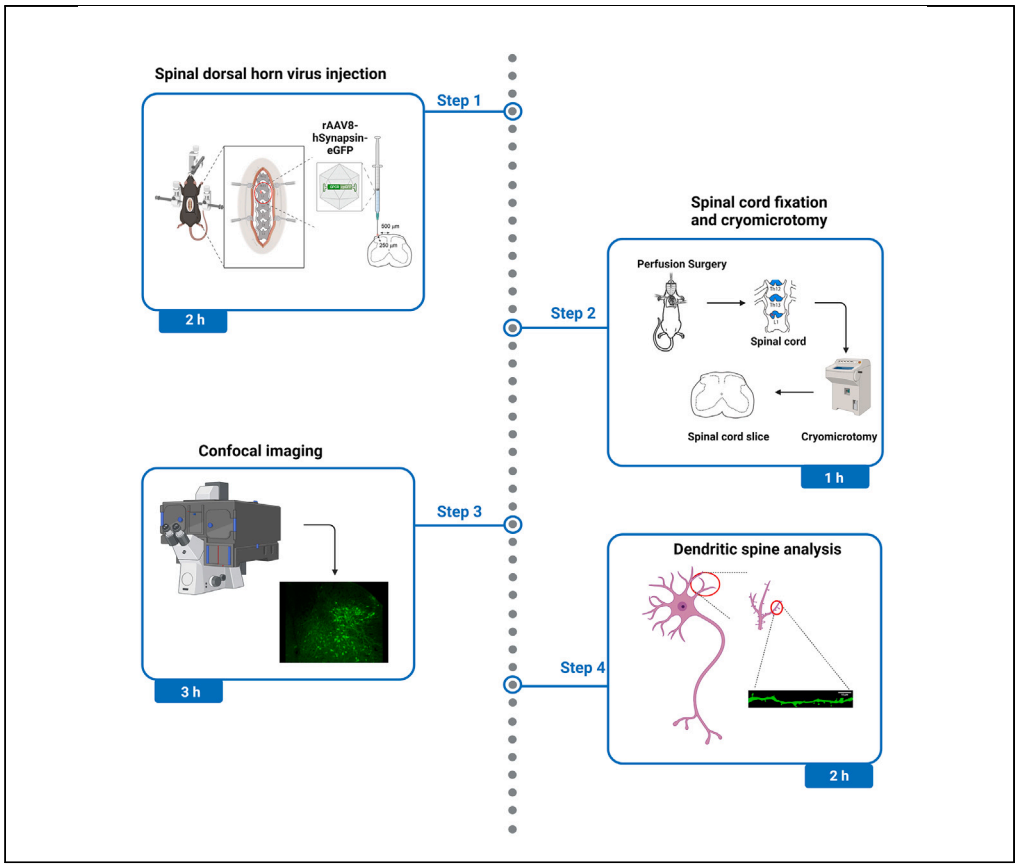
Characterization of synaptic structural plasticity in mouse spinal dorsal horn neurons



Here, we present a pipeline for the characterization of synaptic structural plasticity in mouse spinal dorsal horn (SDH) neurons. We describe steps for the intra-SDH microinjection of the EGFP virus to sparsely label L4 SDH neurons without laminectomy, wide dynamic range neuron imaging, dendritic spine morphometric analysis, and F-actin to G-actin ratio measurement. This protocol can be applied to investigate the synaptic structural plasticity mechanisms in the SDH as well as in the brain.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol characterizes synaptic structural plasticity in spinal dorsal horn neurons

Dendritic spine morphological analysis

F-actin to G-actin ratio analysis

Protocol can be used to characterize synaptic structural plasticity in brain neurons

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Characterization of synaptic structural plasticity in mouse spinal dorsal horn neurons

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SUMMARY

Here, we present a pipeline for the characterization of synaptic structural plasticity in mouse spinal dorsal horn (SDH) neurons. We describe steps for the intra-SDH microinjection of the EGFP virus to sparsely label L4 SDH neurons without laminectomy, wide dynamic range neuron imaging, dendritic spine morphometric analysis, and F-actin to G-actin ratio measurement. This protocol can be applied to investigate the synaptic structural plasticity mechanisms in the SDH as well as in the brain.

For complete details on the use and execution of this protocol, please refer to Li et al. (2023).[1]

BEFORE YOU BEGIN

Synaptic functional and structural plasticity in the spinal dorsal horn serves as the neural substrate and common feature of chronic pain triggered by diverse pathophysiologies. Functional alterations are accompanied by structural remodeling and reorganization of synapses, cells, and circuits, and this structural plasticity likely accounts for the long-term nature of chronic pain. Morphological remodeling of the dendritic spine, the primary post-synaptic site of the excitatory synapse, in spinal dorsal horn neurons provides a structural-based mechanism for modifying and maintaining long-term changes in synaptic function. Dendritic spine remodeling in spinal dorsal neurons also provides a structural substrate for sensory processing and morphine tolerance. Actin polymerization is a driving force for dendritic spine remodeling. Actin exists in two forms: monomeric globular actin (G-actin) and polymerized filamentous actin (F-actin). The F-actin to G-actin ratio reflects the balance between actin polymerization and depolymerization. Here, we introduce a protocol to analyze the dendritic spine morphology and F-actin to G-actin ratio in L4 spinal dorsal horn neurons in mice. However, this experimental approach can be adapted for use in other rodent species for synaptic structural plasticity characterization in a cell type-, segment-, and region-specific manner.

The existing methods for virus microinjection into the spinal dorsal horn need complicated surgical procedures such as laminectomy or drilling of a hole in the vertebra for direct access to the spinal cord parenchyma, which may result in an inflammatory response and extensive tissue damage that could complex data interpretation. We injected the virus unilaterally into the spinal dorsal horn through the window on the interspace between the Th13 and L1 vertebrae without laminectomy and any complicated surgical procedures. This technique is much easier and less invasive than previously reported methods. Furthermore, intraspinal viral injection is a useful tool to

investigate the function of a gene in a cell type- and segment-specific manner and to visualize neural circuits in the spinal dorsal horn.

Institutional permissions

All animal protocols were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and the University of Alabama at Birmingham and were conducted in accordance with the National Institutes of Health Guidelines. We would like to remind the readers that they will need to acquire permission from the relevant institutions before carrying out the procedures outlined in this protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-actin (1:10,000)	Millipore	MAB1501
Peroxidase goat anti-mouse IgG (1:10,000)	Jackson ImmunoResearch	115-035-003
Isoflurane	VetOne	502017
Povidone-iodine	Medline	MDS093943
Ethanol pads	Fisher Healthcare	22-363-750
Lubricant eye ointment	Dechra	N/A
Pentobarbital sodium	VetOne	503016
NaCl	Sigma	S7653
Aqueous mounting medium	Sigma	F4680
Glass micropipette	WPI	TW150F-4
OCT compound	Sakura	4583
Sucrose	Sigma	S0389
Mineral oil	Sigma	M5310
Bacterial and virus strains		
rAAV8-hSynapsin-eGFP	UNC Vector Core	N/A
Software and algorithms		
Imaris	Imaris	https://imaris.oxinst.com
Prism 9.0	GraphPad Software	https://www.graphpad.com/
Other		
Confocal microscope	Zeiss	880 Airyscan
Stereotaxic apparatus	Kopf	Model 940
Anesthesia induction chamber	Kent Scientific	VetFlo-0530XS
Cryostat	Leica	CM1860
Surgery microscope	Leica	M80
Micropipette puller	Sutter Instrument	P-1000
Microforge	WPI	MF200-1
Microinjection syringe pump	WPI	UMP3T-2
Sub-microliter injection system	NanoFil	10 µL
Joint Teflon tubing	Amuza	JT-10-80
Microliter syringe	Hamilton	700-75

MATERIALS AND EQUIPMENT

Lysis buffer 1

Reagent	Final concentration (mM)	Amount (g/L ddH_2O)
K_2HPO_4	10	1.742
NaF	100	4.199

(*Continued on next page*)

Continued		
Reagent	Final concentration (mM)	Amount (g/L ddH_2O)
KCl	50	3.728
$MgCl_2$	2	0.1904
EGTA	1	0.380
Triton X-100	0.5%	5 mL/L ddH_2O
Sucrose	1	0.342
PH = 7.0		
Freshly prepare the solution right before experiments.		

Lysis buffer 2		
Reagent	Final concentration (mM)	Amount (g/L ddH_2O)
$C_2H_3NaO_2$	1.5	0.123
$CaCl_2$	1	0.111
ATP	1	0.507
Tris-HCl	20	2.423
EGTA	1	0.380
CH_5N_3-HCl	1.5	0.143
PH = 7.5		
Freshly prepare the solution right before experiments.		

STEP-BY-STEP METHOD DETAILS

Microinjection system preparation

Timing: about 30 min

This section describes how to assemble the microinjection system for virus injection.

1. Prepare the glass micropipette. Pull the glass capillaries with a P-1000 micropipette puller with heat: 850, vel: 30, and time: 250.

   Note: Store the pulled glass capillaries in a closed container to avoid dust, which may clog the micropipette. Autoclaving of micropipettes is not necessary.

2. Trim the tip of the glass micropipette with microscissors.
   a. Cut off about 1–2 mm of excess glass.
   b. Bevel the micropipette tip with a microforge.
   c. Refine the length of the micropipette tip to about 4.5–5 mm.

   Note: The approximate aperture size of the glass micropipette was measured under a microscope with a microscope graduated slide, using a micropipette chipper to create a tip with a 30–40 μm aperture.

   Note: Smaller tips may easily cause clogging; larger tips may cause tissue damage and make it difficult to penetrate the spinal cord.

3. Assemble the microinjection system (Figure 1B).
   a. Set up the stereotaxic frame.
   b. Mount the microsyringe injector into the manipulator of the micropump.
   c. Connect the injector to the controller.
   d. Fix the microsyringe so that the vernier scale is visible (Figure 1A).

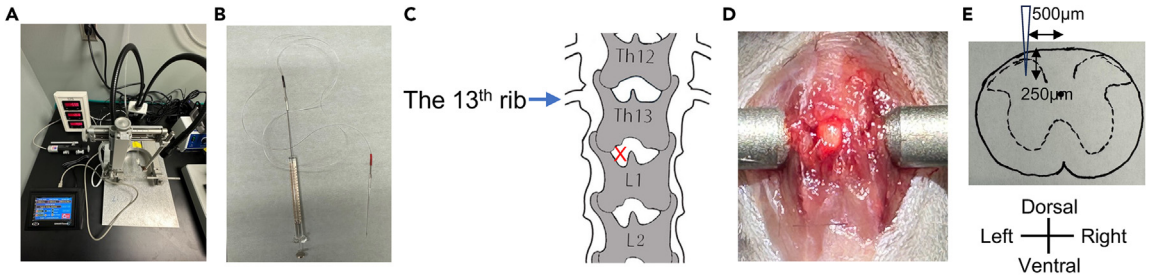

Figure 1. Intra-spinal dorsal horn microinjection

(A) The components of the microinjection system. This microinjection system comprises a stereotaxic instrument, microsyringe, micropump, spine clamps, and illumination lights.

(B) Joint Teflon tubing connects the glass micropipette with the microsyringe.

(C) The schematic showing the intra-spinal dorsal horn injection site (red X).

(D) Photograph showing intra-spinal dorsal horn injection site.

(E) Schematic illustration of intra-spinal dorsal horn injection.

e. Attach one of the glass micropipettes to the microsyringe using the Joint Teflon tubing.
f. Carefully fill the microsyringe and micropipette with mineral oil and make sure that no air bubbles remain in the micropipette.
g. Use aseptic techniques when handling the micropipette, avoiding contact with the tip.

4. Prepare the virus solution. On the day of the injection, defrost a stock aliquot of the required purified virus on the ice and keep it on the ice before the injection.

⚠ CRITICAL: Avoid repeated freeze-thaw cycles, which may reduce the effective titer of the virus. If necessary, the defrosted virus aliquot can be kept at 4°C for up to 3 days.

Note: Different serotypes of AAVs have different tropisms. There is no known universal serotype that works for all neurons. There are 15 different types of both excitatory and inhibitory neurons in the spinal dorsal horn. For the specific type of neurons, if low or no expression is observed with one serotype, one should test other serotypes.

Note: Based on our experience and literature, rAAV8 under the control of a *hSyn* promoter could drive gene expression in spinal dorsal horn neurons, and rAAV2/5, a serotype showing a preferential tropism for astrocytes, under the control of a *gfap* promoter was used to drive gene expression in spinal dorsal horn astrocytes.

5. Dilute the virus particles with sterile 1x phosphate-buffered saline (1x PBS) if needed and prepare the appropriate amount of virus solution as required by the experiment.

⚠ CRITICAL: The appropriate titer depends on the purpose of the experiment and should be determined by preliminary experiments. The titer used in the current protocol was 2.6E+11 GC/mL.

Note: Taking excess and some loss while loading the capillary into account, therefore, if each mouse needs 1 mL of virus solution, about 2.5 mL of virus solution will be needed for each mouse. Viruses are infectious reagents and should be handled according to the relevant guidelines. In most cases, rAAVs-related experiments can be handled at biosafety level 1 laboratories. Transport and store the virus on the ice when not in use.

6. Load the virus. Move the tip of the micropipette carefully into the virus solution, and control the micropump connected to the micro syringe to load the virus solution at a rate of 200 nL/s.

Anesthetize animals and surgical site preparation

Timing: about 15 min

This section describes animal anesthesia and pre-surgery preparation for virus injection.

7. Weigh the animal using a digital scale. Record the preoperative weight to determine the required anesthetic dose and to facilitate postoperative weight monitoring.

   Note: Mice aged 10–12 weeks (approximately 22–25 g) were selected and used in this protocol.

   Note: Injections can be given to young or old animals if the experimental design requires. In principle, any strain and both sexes can be used, but the same background strain should be used to compare the behavior between different transgenic mouse strains. If you expect or want to investigate sex differences, analyze male and female animals in different groups.

8. Anesthetize mice with 5% isoflurane. Place the mice in the anesthesia induction chamber at 5% and then keep them under 2%–3% isoflurane anesthesia once asleep.

   ⚠ CRITICAL: Monitor the respiration rate throughout the surgery and adjust anesthesia as required.

9. Pre-surgery preparation.
   a. Place the animal in the stereotaxic frame on a heat mat and maintain anesthesia at 2%–3% isoflurane.
   b. Shave the fur from the lower back to the neck of the mouse and remove the hair carefully.
   c. Disinfect the shaved skin with 75% ethanol pads followed by 1% povidone-iodine (3 times).
   d. Isolate the aseptically prepared site with a surgical drape.
   e. Place lubricant eye ointment on each eye to protect the eyes from drying during the operation. Reapply lubricant as necessary.

Intraspinal dorsal horn microinjection without laminectomy

Timing: about 40 min

This section describes how to perform minimally invasive methods for microinjection of the virus into the mouse spinal dorsal horn.

10. Incise the skin at the Th12-L3 (critical step).
    a. Fix the mouse on a stereoscope frame with lug rods and keep it under 2%–3% isoflurane anesthesia.
    b. Gently press fingers on the rib of the mouse to find the Th12-L3 vertebra through the skin.[2] When the mouse is lying on a table, the highest point of the back is around the Th13 vertebrate (the last rib), as marked in Figure 1C, which is used to locate the 13 thoracic vertebrates.
    c. Use a No. 10 scalpel to make a 3–4 cm incision in the skin at the Th12-L3, exposing the muscle. Gently stretch the skin and press firmly with the scalpel blade to make sure the incision is clean.

    Note: The spinal cord does not extend the entire length of the spinal vertebrae because the spinal cord stops growing earlier in postpartum development. This means that the target spinal level may be under different named vertebrae. The L4 spinal segment is located below the first lumbar spine (L1). L1 is located caudally in the vertebra that supports the last pair of ribs (Figure 1C).

11. Stabilize the target vertebra.
    a. Place the animal on a rolled-up tissue pad and lift it onto the spine clamp of the stereoscopic frame.
    b. Secure the animal to a stereotaxic frame with the spinal clamps apparatus at the rostral and caudal sites of the incision respectively.

⚠ CRITICAL: Fixation of the target vertebra prevents movement of the vertebra due to breathing and allows for an accurate virus injection.

12. Locate the injection site (critical step).
    a. Separate the fascia covering the spine and remove the paraspinal muscle around the left side of the interspace between the Th13 and L1 vertebrae.
    b. Using the 13th rib, the last rib, to locate the 13th thoracic vertebrate. L1 is located caudally in the vertebra that supports the 13th ribs) (Figure 1C).
    c. Incise the dura mater and the arachnoid membrane with the tip of a 30G needle to make a small hole that allows the micropipette to insert directly into the spinal dorsal horn through the hole (Figure 1D).
    d. Move the glass micropipette approximately 500 μm lateral from the midline.
    e. Insert the tip into the spinal dorsal horn 250 μm in depth from the surface of the dorsal root entry zone (Figure 1E).

⚠ CRITICAL: Throughout the surgery, keep the exposed tissues moist with sterile 0.9% NaCl.

13. Virus injection.
    a. Wait for 2 min after the glass micropipette is in place to allow the nervous tissue to accommodate the glass micropipette.
    b. Set the injection volume of the pump as 500 nL.
    c. Program the injection speed as 100 nL/min.
    d. Press the start button of the pump to start the injection.
14. After the injection is complete, check the scale to see whether the virus level decreases, leave the micropipette for 5 min to balance the pressure, and then slowly withdraw the syringe.
15. After microinjection, suture the superficial tissue layers with absorbable 6-0 sutures, close the skin with 6-0 non-absorbable nylon sutures, and keep the animal on a heating pad until recovery.

Spinal cord fixation and cryosection

Timing: about 15 min

This section describes the procedures of fixation, dehydration, cryosection, and mounting of the spinal cord tissues for imaging.

16. Three weeks after the virus injection, anesthetize the animals by the injection of pentobarbital sodium (50 mg/kg, i.p.), perfuse transcardially with about 20 mL 1x PBS, and fix with ice-cold 4% paraformaldehyde/1x PBS.
17. Remove the whole spine.
    a. Postfix with ice-cold 4% paraformaldehyde/1x PBS for about 12 h at 4°C.
    b. Place in 30% sucrose solution for 24 h at 4°C.

Note: The spinal cord is ready for processing when saturated with 30% sucrose – as indicated when the spinal cord sinks to the bottom of the solution.

18. Section the spinal cord.

a. Dissect the L3-L5 segments of the spinal cord.
b. Embed the spinal cord section in the OCT compound.
c. Section 40 μm thick transverse slices from anterior to posterior with the cryostat.

19. Mount the spinal cord sections on the adhesive slide with cover glass with the aqueous mounting medium containing DAPI. Once the mounting solution solidifies, seal it with nail polish.

Confocal imaging and dendritic spine analysis

Timing: 1–2 days

This section describes how to identify wide dynamic range neurons and how to analyze dendritic spine morphology in the spinal dorsal horn.

20. Capture the wide dynamic range (WDR) neurons with only the EGFP channel visible to the experimenter using a confocal microscope with a 63x oil immersion objective. Take the Z-series at an interval of 0.37 mm for each dendrite.

Note: Identify WDR neurons with the five criteria based on previous studies[3–5]: Neurons are located within lamina 4 and 5; EGFP-positive neurons must have had dendrites and spines that were completely impregnated, appearing as a continuous length; At least one dendrite extended into an adjacent lamina relative to the origin of the cell body; At least one-half of the primary dendritic branches remained within the thickness of the tissue section, such that their endings were not cut and instead appeared to taper into an ending; The cell body diameter fell between 20 and 50 mm.

21. Measure the dendritic spines.
a. Obtain and visualize the dendritic spine image from EGFP-expressing neurons with 3D confocal stacks using Imaris software (Bitplane Scientific Software).
b. Classify the spines emanating from dendrites with a clear head structure.[3,6]
c. Count the dendritic spines from the secondary and tertiary segments of the dendrite using the Imaris filament tool.
22. Record the length of selected dendrites and the number of dendritic spines. Determine the dendrite spine density by calculating the number of spines of dendrite length and express as the spine density per 10 μm of dendrites.

F-actin to G-actin ratio measurement

Timing: about 2 days

This section describes how to prepare and measure the F-actin and G-actin from spinal dorsal horn tissues.

In parallel with morphology analysis, we used a separate group of animals to measure F-actin to G-actin ratio in the spinal dorsal horn.

23. Isolate the spinal dorsal horn from the 4^{th} lumbar of mice, homogenize in cold lysis buffer 1, centrifuge at 15,000 g for 30 min at 4°C, and measure the soluble actin (G-actin) in the supernatant.
24. Resuspend the insoluble F-actin in the pellet in lysis buffer plus an equal volume of buffer 2 and incubate on ice for 1 h to convert F-actin into soluble G-actin, with gentle mixing every 15 min.
25. Centrifuge the samples at 15,000 g for 30 min at 4°C, and measure the F-actin in this supernatant. Proportionally load the samples from the supernatant (G-actin) and pellet (F-actin) fractions and analyze them by western blotting.

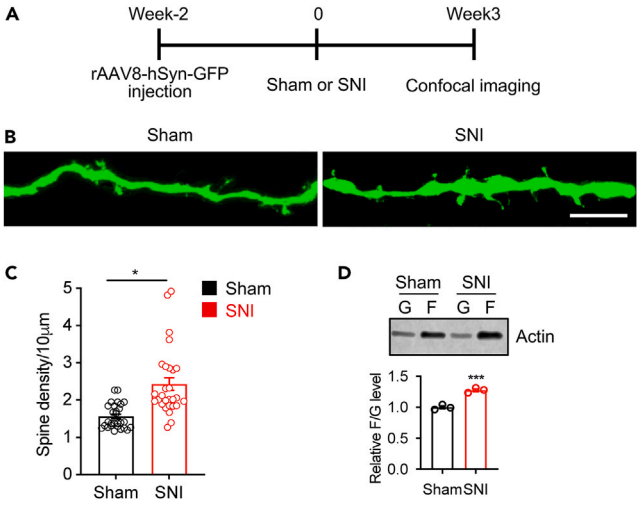

Figure 2. Nerve injury induces synaptic structural plasticity in spinal dorsal horn neurons

(A) Experimental paradigm for dendritic spine analysis. To visualize neuron morphology, we sparsely labeled spinal dorsal horn neurons by performing intra-spinal dorsal horn microinjections of low titer GFP-expressing AAV virus (rAAV8-hSyn-GFP) in wild-type mice. Two weeks after virus injections, we performed sham or spare nerve injury (SNI) surgeries on the mice. Then, mice were perfused three weeks after SNI surgery, and spinal cords were sectioned.

(B and C) Representative confocal images and quantification of dendritic spine density show that nerve injury increases dendritic spines in dorsal horn spinal neurons (scale bar, 10 μm). High-resolution confocal imaging was performed to analyze dendritic spines on GFP-expressing wide dynamic range (WDR) neurons from the spinal dorsal horn. Consistent with previous findings, nerve injury increased the density of dendritic spines on WDR neurons from wild-type mice. Two-tailed Student's t-test (n = 24-28 neurons from 3 mice/group, $*P < 0.05$).

(D) Nerve injury stimulates actin polymerization in the spinal dorsal horn. Western blots and quantification revealed that SNI increased the F- to G-actin ratio in the spinal dorsal horn of wild-type mice (D). Mann-Whitney U-test (n = 3, $***P < 0.001$).

EXPECTED OUTCOMES

This protocol will allow one to characterize synaptic structural plasticity (dendritic spine density of spinal dorsal horn neurons and F-actin to G-actin ratio) in the spinal cord as well as in the brain. Example data from the spinal dorsal horn are shown in Figure 2, as well as in Li et al., (2023).[1]

LIMITATIONS

The success of the labeling of spinal dorsal horn neurons is critical for dendritic spine morphology analysis and is highly dependent on the location and amount of the virus injection. Thus, the intra-spinal dorsal virus injections are highly variable but can be improved with practice.

The F-actin to G-actin ratio is measured from the spinal dorsal horn tissue, including both neurons and microglia. Therefore, the results may not exactly reflect the balance between actin polymerization and depolymerization of spinal dorsal horn neurons.

TROUBLESHOOTING

Problem 1

Intra-spinal dorsal horn Injection of 500–600 nL virus through the small window between Th13 and L1 vertebrae (approximately 500 μm lateral from the midline) only labels the L4 segment of the spinal dorsal horns. It can't label other spinal dorsal horn neurons in the upper or lower segment (step 12).

Potential solution

Multiple injections through Th12 and Th13 or L1 and L2 in addition to Th13 and L1 vertebrae can label longer segments of the spinal dorsal horn.

Problem 2
The micropipette tip is transparent and hard to localize the correct injection site (step 12).

Potential solution
Use a surgery microscope to help localize the micropipette tip location.

Problem 3
The tip of the glass micropipette is blocked between animals if doing more than one injection in a session (step 13).

Potential solution
When finding the glass micropipette is blocked between animals, carefully put a small piece of cotton ball soaked in 1x PBS under the pipette tip and start the pump briefly to press the clogging.

Problem 4
No desired virus volume is injected into the spinal dorsal horn (step 14).

Potential solution
Make sure there is no leakage or air from the connected parts between the tubing and glass micropipette or between the tubing and the micro syringe. After connecting the glass micropipette to the tubing from the Hamilton syringe using a 2–3 cm tube as a connector, test by pushing the syringe to see if mineral oil is coming out from the pipette tip. This procedure is critical to ensure no leakage or air from the connected parts.

Problem 5
Too many or too few GFP-expressing WDR neurons are found within lamina 4 and 5 (step 20).

Potential solution
The suitable virus titer for injection needs to be determined in advance by preliminary experiments.

Problem 6
GFP signal is weak for some virus-mediated GFP-expressing WDR neurons within lamina 4 and 5 (step 20).

Potential solution
The immunofluorescence with anti-GFP antibody will strengthen the GFP signal. However, the GFP signal from rAAV8-mediated GFP-expressing spinal dorsal horn neurons is strong enough to visualize and analyze dendritic spine morphology using confocal microscopy two weeks after virus injection. If the GFP signal is weak for some virus-mediated GFP-expressing neurons, GFP immunofluorescence will help visualize and analyze neuron morphology.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lingyong Li (lingyongli@uabmc.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Methodology, Q.R. and L.L.; writing, Q.R., J.M., and L.L.; funding acquisition, L.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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