bio-protocol

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Published: Jul 20, 2024

Approach for Electrophysiological Studies of Spinal Lamina X Neurons

Volodymyr Krotov[1, *, $], Pavel Belan[1, 2] and Nana Voitenko[2, 3]

[1]Department of Molecular Biophysics, Bogomoletz Institute of Physiology, Kyiv, Ukraine
[2]Department of Biomedicine and Neuroscience, Kyiv Academic University, Kyiv, Ukraine
[3]Dobrobut Academy Medical School, Kyiv, Ukraine
[$]Present address: Dept. Physiology, Pharmacology and Neuroscience, University College London, UK
*For correspondence: vkrotov@biph.kiev.ua

Abstract

Despite playing diverse physiological roles, the area surrounding the central canal, lamina X, remains one of the least studied spinal cord regions. Technical challenges and limitations of the commonly used experimental approaches are the main difficulties that hamper lamina X research. In the current protocol, we describe a reliable method for functional investigation of lamina X neurons that requires neither time-consuming slicing nor sophisticated in vivo experiments. Our approach relies on ex vivo hemisected spinal cord preparation that preserves the rostrocaudal and mediolateral spinal architecture as well as the dorsal roots, and infrared LED oblique illumination for visually guided patch clamp in thick blocks of tissue. When coupled with electric stimulation of the spared dorsal roots, electrophysiological recordings provide information on primary afferent inputs to lamina X neurons from myelinated and non-myelinated fibers and allow estimating primary afferent–driven presynaptic inhibition. Overall, we describe a simple, time-efficient, inexpensive, and versatile approach for lamina X research.

Key features

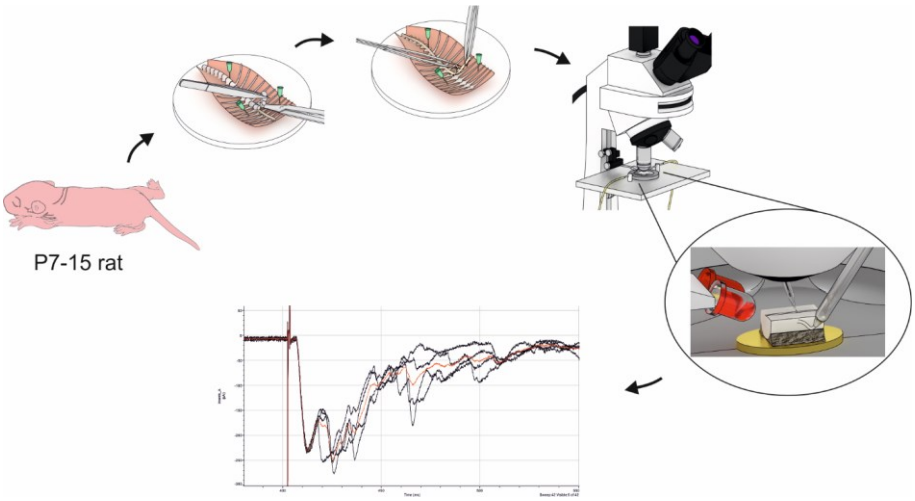
- Quick and easy preparation procedure that grants access to lamina X neurons without spinal cord slicing.
- Preserved rostrocaudal and mediolateral connectivity and preserved primary afferent supply.
- Ability to perform electrophysiological recordings in combination with dorsal root stimulations allowing to study afferent inputs and presynaptic inhibition of lamina X neurons.

Keywords: Spinal cord, Lamina X, Electrophysiology, Dorsal root stimulation, Postsynaptic currents, Presynaptic inhibition

This protocol is used in: Pain (2022), DOI: 10.1097/j.pain.0000000000002597; Frontiers in Cellular Neuroscience (2023), DOI: 10.3389/fncel.2022.1029799 and Pain (2019), DOI: 10.1097/j.pain.0000000000001586

Published: Jul 20, 2024

Graphical overview



Preparation of ex vivo hemisected spinal cord and electrophysiological recordings from lamina X neurons

Background

Based on its cytoarchitectonics, the spinal cord is subdivided into ten regions called laminae [1]. Spinal laminae are studied to a different extent: while superficial ones have been explored in much detail, our knowledge about the deeper laminae is unsatisfactory. In particular, lamina X surrounding the central canal remains one of the least studied spinal regions despite playing diverse physiological roles. Evidence indicates that lamina X is crucial for visceral nociception [2–6] and participates in somatosensory processing [4,7,8], locomotion [9], and autonomic regulation [10]. Yet, reports on lamina X are scarce and derive mostly from morphological and immunohistochemical data. Functional investigations of lamina X are even rarer given that they mainly rely on two approaches. The first one involves in vivo electrophysiological recordings using either an extracellular electrode [4,7] or a blind patch clamp technique [10,11]. Both methods require post hoc tissue analysis to confirm recordings from lamina X cells. The other approach utilizes visually guided patch clamp in spinal cord slices [13–23] in which intrinsic spinal cord connectivity and primary afferent inputs are disrupted. Both mentioned methods are technically challenging and have inherent flaws that limit the functional investigation of lamina X. Thus, an experimental approach circumventing these limitations would greatly boost the lamina X research.

Here, we provide a detailed protocol for electrophysiological studies of lamina X neurons. The described procedure is easy and reliable. It allows recording over prolonged periods (up to 6–8 h) and requires only very basic equipment for ex vivo electrophysiology. The main advantage of our approach is the use of a spinal cord hemisected at the sagittal midline, which fully preserves rostrocaudal and mediolateral spinal architecture and spares the dorsal roots. Unlike spinal cord slices, the hemisected preparation is produced without any specialized device and needs little to no incubation; the preparation is ready for use within half an hour after decapitation. For cell visualization, we use infrared LED oblique illumination, which is specifically designed to work with thick blocks of tissue [24,25]. A combination of hemisected spinal cord preparation together with oblique LED illumination gives a unique opportunity to perform visually guided patch clamp in practically intact tissue. This is particularly beneficial for studying primary afferent inputs to lamina X and their modulation. Patch clamp experiments coupled with electrical dorsal root stimulation via suction electrodes enable recording elicited postsynaptic currents in lamina X neurons. Different parameters of electrical stimuli allow the examination of direct synaptic inputs from thinly myelinated A fibers and non-myelinated C fibers as well as their primary afferent–driven presynaptic inhibition. The current protocol is suitable for using both young rats and mice of various genetic backgrounds. Additionally, the protocol may be applied for studying various cell populations (such as spinothalamic, spinohypothalamic, and sympathetic

preganglionic neurons) preliminary labeled with retrograde dyes [26]. Finally, the preserved rostrocaudal architecture of the hemisected preparation allows complementing the protocol with the stimulation of descending (top-down) fibers of the spinal cord [27].

Materials and reagents

Biological materials

1. Wistar rats P7–14 (Charles River, strain code: 003)

Reagents

1. Sucrose (Sigma-Aldrich, catalog number: S0389)
2. Glucose (Sigma-Aldrich, catalog number: G8270)
3. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
4. Sodium bicarbonate ($NaHCO_3$) (Sigma-Aldrich, catalog number: S0751)
5. Sodium monophosphate (NaH_2PO_4) (Sigma-Aldrich, catalog number: S6040)
6. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9333)
7. Magnesium chloride ($MgCl_2·6H_2O$) (Sigma-Aldrich, catalog number: M2670)
8. Calcium chloride ($CaCl_2·2H_2O$) (Sigma-Aldrich, catalog number: C7902)
9. Potassium gluconate (Sigma-Aldrich, catalog number: G4500)
10. Sodium ATP (Na_2ATP) (Sigma-Aldrich, catalog number: A3377)
11. Sodium GTP (NaGTP) (Sigma-Aldrich, catalog number: G8877)
12. HEPES (Sigma-Aldrich, catalog number: H3375)
13. EGTA (Sigma-Aldrich, catalog number: E0396)
14. Sodium ascorbate (Sigma-Aldrich, catalog number: 11140)
15. Sodium pyruvate (Sigma-Aldrich, catalog number: P2256)
16. Isoflurane (Abbvie, catalog number: B506)
17. 95% O_2 and 5% CO_2 gas mixture

Solutions

1. Sucrose dissection solution (see Recipes)
2. Krebs bicarbonate solution (see Recipes)
3. Potassium gluconate intracellular solution (see Recipes)

Recipes

1. **Sucrose dissection solution (final volume 0.5 L, in ddH_2O)**

Reagent	Final concentration	Quantity
Sucrose	200 mM	34,230 mg
Glucose	11 mM	991 mg
$NaHCO_3$	26 mM	1092 mg
NaH_2PO_4	1.2 mM	72 mg
KCl	2 mM	74.5 mg
$MgCl_2·6H_2O$	7 mM	712 mg
$CaCl_2·2H_2O$	0.5 mM	37 mg
Sodium ascorbate (optional)	5 mM	495 mg
Sodium pyruvate (optional)	3 mM	168 mg

pH 7.3–7.4 when bubbled with 95% O_2 and 5% CO_2; osmolarity 310–320 mOsm/kg. This solution may be used

for up to two weeks after preparation if kept at 4–8 °C.

2. **Krebs bicarbonate recording solution (final volume 0.5 L, in ddH_2O)**
Critical: To avoid precipitation, the solution must be bubbled with 95% O_2 and 5% CO_2 gas mixture before adding calcium chloride.

Reagent	Final concentration	Quantity or Volume
NaCl	125 mM	3,653 mg
Glucose	10 mM	901 mg
$NaHCO_3$	26 mM	1092 mg
NaH_2PO_4	1.25 mM	75 mg
KCl	2.5 mM	93 mg
$MgCl_2·6H_2O$	1 mM	102 mg
$CaCl_2·2H_2O$	2 mM	147 mg

pH 7.3–7.4 when bubbled with 95% O_2 and 5% CO_2; osmolarity 300–310 mOsm/kg. This solution may be used for up to two weeks after preparation if kept at 4–8 °C.

3. **Potassium gluconate intracellular solution (final volume 50 mL, in ddH_2O)**

Reagent	Final concentration	Quantity or Volume
Potassium gluconate	145 mM	1,698 mg
$MgCl_2·6H_2O$	2.5 mM	25.5 mg
Sodium ATP	2 mM	55 mg
Sodium GTP	0.5 mM	13 mg
HEPES	10 mM	119 mg
EGTA	0.5 mM	9.5 mg

pH 7.3 adjusted with KOH; osmolarity 280–290 mOsm/kg. Make 1 mL aliquots and freeze them at -20 °C.

Laboratory supplies

1. Dissection dish with Sylgard-lined bottom (Living Systems Instrumentation, catalog number: DD-90-S-BLK)
2. 25 G hypodermic needles (BD, catalog number: 300400)
3. 30 G hypodermic needles (BD, catalog number: 304000)
4. Super glue (cyanoacrylate, water-resistant gel)
5. Glass capillaries with filament O.D. 1.5 mm, I.D. 0.86 mm (e.g., Sutter Instruments, catalog number: BF-150-86-10; Harvard Apparatus, catalog number: GC150F-10)
6. Thin-walled glass capillaries without filament O.D. 1.5 mm, I.D. 1.17 mm (Warner Instruments, catalog number: G150T-3)
7. 1 mL Luer-Lok syringes (BD, catalog number: 309628)
8. 2.5 mL Luer-Lok syringes (BD, catalog number: 300185)
9. Silicon tubing (VWR, catalog number: 228-0701)
10. Three-way tap (BD Connecta, catalog number: 394601)
11. Patch pipette fillers (WPI, catalog number: MF28G67-5)
12. Sterile syringe filters 0.22 µm (Thermo Scientific, catalog number: 171-0020)

Equipment

Dissection instruments

1. Dissection pad (could be made from Styrofoam tightly wrapped in foil)

2. Anesthesia induction chamber (VetEquip, US) or any other available
3. Big scissors (F.S.T., catalog number 14000-20)
4. Small scissors (F.S.T., catalog number 14058-11)
5. Coarse forceps (F.S.T., catalog number 11651-10)
6. Big spring scissors (F.S.T., catalog number 15025-10)
7. Small spring scissors (F.S.T., catalog number 15000-12)
8. Two curved forceps (F.S.T., catalog number 11063-07)
9. Small metal plate (preferably gold, should fit in experimental chamber)

Visualization and illumination

1. Stereomicroscope (Olympus, model: SZX7)
2. Light source (AmScope, model: 6 W LED Dual Gooseneck Illuminator)
3. Upright microscope (Olympus, model: BX50WI)
4. 60× water immersion objective (Olympus, model: LUMPlanFl 60×/0.90 W)
5. Low magnification (4–5×) objective (Carl Zeiss, model: Epiplan; Olympus, model Plan N)
6. Eyepiece graticule (any fitting the microscope)
7. Infrared-sensitive camera (Olympus, model: OLY-150IR or Dage-MTI, model: IR-2000)
8. Narrow beam (± 3°) infrared light emitting diode (Osram, models: SFH4550, SFH4545)
9. White light emitting diode (Dialight, model: 5219901802F)
10. Power supply unit (AimTTI, model: QL355T)

Perfusion

1. Gravity-fed perfusion system (any self-made or commercially available)
2. Peristaltic pump (Gilson, model: Minipuls 3)
3. Recording chamber (any fitting the microscope)

Electrophysiology

1. Patch clamp amplifier with respective headstage (Molecular Devices, models: Multiclamp 700B, Axopatch 200B)
2. Digitizer (Molecular Devices, models: Digidata 1440, Digidata 1550)
3. PC computer with Windows operating system
4. Patch clamp micromanipulator (e.g., Scientifica, model: PatchStar; Sutter Instruments, model: MP-225)
5. Constant current stimulator (e.g., A.M.P.I., model: ISO-FLEX; A-M Systems, model: 2100; Digitimer, model: DS3)
6. Vibration isolation table (CleanBench, model: TMC)
7. Faraday cage (Sutter Instruments, model: AT-36FC)
8. Small three-axis micromanipulators (Narishige, model: UN-3C)
9. Pipette holders (Molecular devices, model: 1-HL-U)
10. Pipette puller (Sutter Instruments, models: P-87, P-97)
11. Silver wire for electrodes (WPI, product number: AGW1510)
12. Ag/AgCl pellets (WPI, product number: EP1)
13. BNC cables
14. Bunsen burner

Software and datasets

1. pClamp (version 10.7, Molecular Devices, July 2016)

2. Clampfit (version 10.7, Molecular Devices, July 2016)
3. Origin (version 2022, OriginLab, June 2022)
4. MiniAnalysis (version 6.0.7, Synaptosoft Inc.)

Procedure

Critical: Before the start of the experiments, make sure both sucrose dissection solution and Krebs bicarbonate solution are at room temperature (20–23 °C) and have been bubbled with 95% O_2 and 5% CO_2 gas mixture for at least 15 min. Changes by 2 °C or more significantly impact the conduction velocity of primary afferent fibers, therefore compromising the classification of the monosynaptic components of primary afferent input to lamina X neurons. Cooled (4–8 °C) solutions compromise the quality of ex vivo spinal cord preparation.

A. Ex vivo spinal cord preparation

1. Prepare dissection instruments and place them next to the stereomicroscope.
2. Deeply anesthetize the animal with isoflurane. Ensure loss of pedal reflex by pinching the hind paw. Quickly decapitate the animal with big scissors and drain the blood for 10 s.
3. Excise the spinal column with attached ribs.
   a. Place the decapitated rat on the dissection pad and pin its paws with 25G needles as shown in Figure 1.

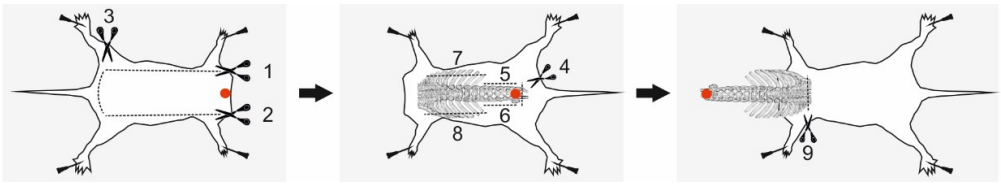


Figure 1. Excision of the spinal cord with attached ribs. Schematic depiction of the excision process. Red dots: sites to grasp using forceps. Dash lines: lines of cut. Numerals denote the order of cuts for a right-handed person.

   b. Remove the skin from the back to expose the spinal column and the ribs. Using coarse forceps, pull the skin up and insert one branch of small scissors underneath it. Push the scissors to either side and cut the skin along the flank holding the scissors parallel to the dissection pad. Then, cut the skin along the other flank. Finally, cut the skin near the tail (Figure 1).
   c. Hold the scissors vertically with the branches open and facing down. Thrust the scissors into the rat's back by 3–5 mm at the level of the hips so that the branches are around the spinal column. Cut the spinal column.
   d. Using coarse forceps, grab the cut part spinal column, pull it up, and cut the tissues rostrally along each side of the spinal column until the ribs are reached.
   e. Cut the ribs on each side and cut any thoracic and abdominal viscera that might be attached to the spinal column or the rib cage.
   Note: The ribs are necessary for pinning the preparation to the dissection dish. Therefore, do not cut them too short.
   f. Hold the spinal column vertically and cut it at the lower cervical/upper thoracic level.
4. Put the excised spinal column into a dissection dish with a Sylgard-lined bottom filled with sucrose solution continuously bubbled with 95% O_2 and 5% CO_2 gas mixture.
5. Using 30G needles, pin the excised tissue dorsal side down (ventral side up) rostrally to the experimenter as depicted in Figure 2.
6. Focus the stereomicroscope on the preparation (8–10× magnification).

7. Using coarse forceps, grab the ribs near the rostral part of the spinal column and slightly pull it up to see the opening. Insert one branch of big spring scissors inside the opening, press the blade against the vertebrae, and cut them. Keeping the branches closed, move the scissors to the side to evacuate them. Then, cut the other side of the vertebrae in a similar manner.
8. Grasp the cut ventral part of the spinal column with coarse forceps and slightly pull it upward and caudally. This allows us to clearly see the space between the spinal cord and the vertebrae where the branch of the scissors needs to be inserted. Continue cutting the vertebrae as described above, keeping the scissors parallel to the dissecting dish (Figure 2). Move the forceps as you cut and take extra care once lumbar enlargement is reached. Cut any *dura mater* (an opaque sheath covering the spinal cord) attached to the ventral part of the spinal column. Once *cauda equina* (a group of nerves and nerve roots stemming from the distal end of the spinal cord) is exposed, cut off the ventral part of the spinal column and remove it from the dish.
9. Using small spring scissors, cut the *dura mater* along the midline all the way to the caudal segment of the spinal cord. Gently pull the *dura mater* with curved forceps if necessary.
10. Remove the spinal cord from the spinal column (Figure 2).
    a. Cut the *cauda equina*.
    b. Using the side of the vertebrae as a rail, cut thoracic dorsal and ventral roots on each side of the spinal cord.
    c. Using curved forceps, pull the spinal cord upward and to the side and cut *dura mater* attached to the dorsal part of the vertebrae. Hold the spinal cord by its very rostral part or, even better, by the *dura mater* or thoracic roots.
    Critical: The spinal cord should always remain in aqueous solution. Do not stretch the preparation.
    d. Cut the lumbar dorsal roots sparing their whole length.
    e. Cut any other tissue that attaches the spinal cord to the inside of the spinal column.
11. Turn the spinal cord ventral side down and cut the *dura mater* on the dorsal side along the midline.

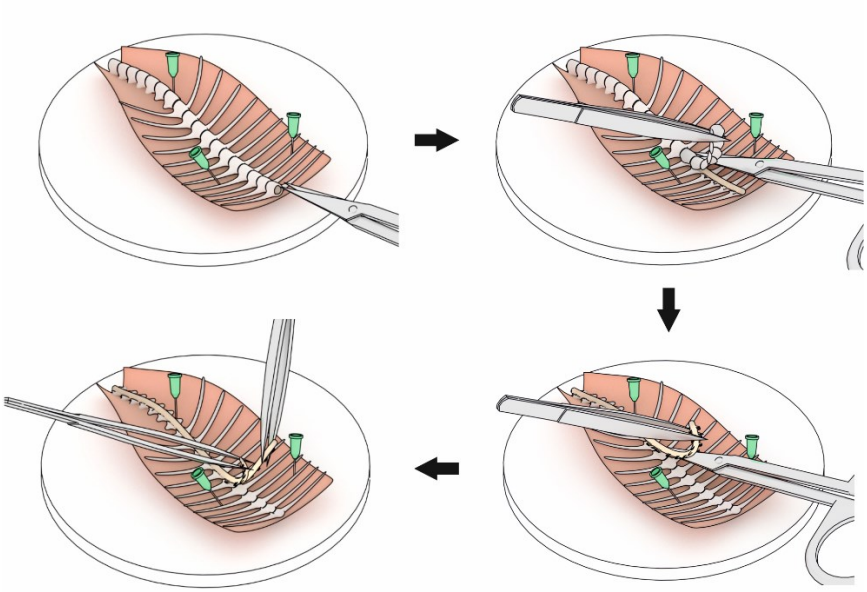


Figure 2. Schemes illustrating stages of making hemisected spinal cord preparation. Modified from Krotov et al. [26].

12. **Optional:** Cut the very rostral part of the spinal cord if it is severely damaged.
13. Turn the spinal cord ventral side up. Take big spring scissors, position the branches along the midline, and make a single cut (2–4 mm long).
    Note: Hemisecting the spinal cord with scissors/scalpel/blade damages lamina X; use the scissors solely

to perform the initial cut.

14. Take each side of the spinal cord with curved forceps and spread them apart as shown in Figure 2 to expose lamina X. This results in two hemisected spinal cord preparations with spared roots. In approximately 25% of cases, the dorsal roots on one of the hemisected cords are severed. That happens more often if the *dura mater* is not cut properly.
    Note: All the above steps should be completed within 7–10 min after decapitation. Longer preparation time decreases the number of lamina X neurons that are fit for recordings.
15. Remove the spinal column from the dissection dish.
16. Cut off damaged parts of the spinal cord, thin sacral segment, remainders of the *dura mater*, and dorsal/ventral roots not destined for stimulations/recordings. Typically, dorsal L3–L5 roots are spared. Avoid crushing the spinal cord with the forceps. Use *dura mater* or unwanted roots to handle the spinal cord.
    Note: The hemisected cord is slightly curved, and the outer curve corresponds to the dorsal side.
17. Position the preparation so that the spinal cord and the dorsal roots form an inverted T letter (Figure 3).

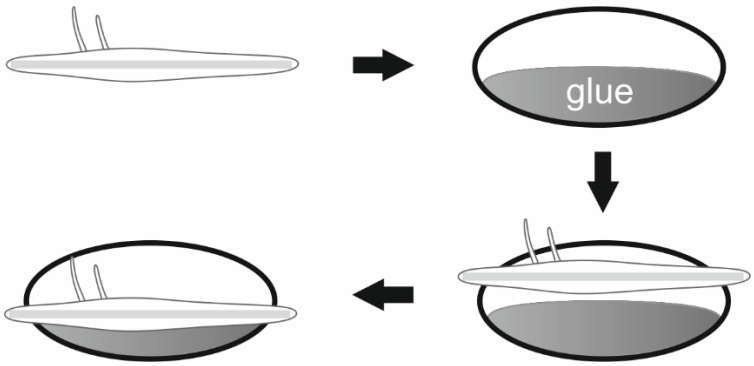


Figure 3. Scheme illustrating the stages of gluing hemisected spinal cord preparation to a metal plate

18. Glue the preparation to the metal plate.
    a. Place the metal plate on the base of the stereomicroscope close to the dissection dish.
    b. Use 25 G needle to spread cyanoacrylate glue. Create a thin layer covering half of the plate (Figure 3).
    c. Using two curved forceps, grab the hemisected spinal cord by its most rostral and caudal parts. Remove the preparation from the aqueous solution and delicately transfer it to the part of the metal plate that is NOT covered with glue. Ensure the gray matter (i.e., the lamina X) faces upward. Do not let the forceps go.
    d. Slightly stretch the preparation, slightly lift above the metal plate, and put the hemisected cord on the glue-covered part of the plate. Do not lift the preparation too high; dorsal roots should stay on the glue-free part of the plate and the drag should help to avoid getting the roots glued (Figure 3).
    e. Use the curved forceps to grab the metal plate and transfer it to the dissection dish.
    Note: Minimize exposure to air. Ideally, steps A18c–e should be completed within 10–20 s.
19. Use a 30 G needle to detach the roots from the plate. The roots should float freely (Figure 4).

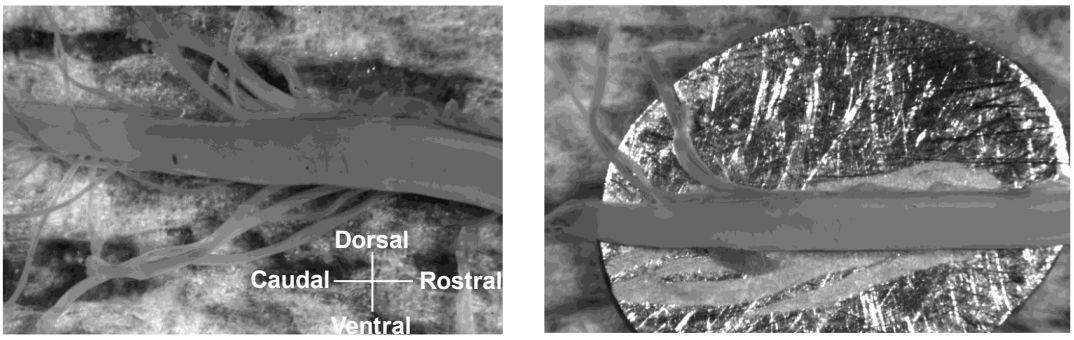


Figure 4. Ex vivo hemisected spinal cord preparation. Left: Lumbar segment of hemisected spinal cord with spared dorsal and ventral roots. Right: Ex vivo preparation glued to the metal plate. Notice spared L4 and L5 dorsal roots. Typically, lumbar and lower thoracic (T6–13) segments are available for recordings. The length of the dorsal roots is expected to be 4–6 mm. Modified from Krotov et al. [26].

20. To avoid unnecessary vibration during recordings, cut caudal and rostral (unless descending stimulation is to be performed) parts of the hemisected cord that stick out of the metal plate or are not glued properly.
21. **Optional:** Cut off any damaged ends of the dorsal roots. Cut the root to 2–3 mm length for dorsal root potential recordings. Use sharp scissors to avoid crushing the roots when cutting.

B. Setting up the perfusion and transferring the preparation to the recording rig

1. Install the experimental chamber and position the patch clamp reference electrode.
2. Fill the perfusion system with Krebs bicarbonate solution; at least 30–40 mL is necessary if the solution is recycled.
   Note: Incorporate drippers into the outflow to avoid peristaltic pump–associated electrical noise.
3. Set the flow rate to 1.5–3 mL/min. To achieve that, use glass capillaries for inflow and outflow and fire polish their tips to an appropriate diameter. Alternatively, a flow regulator may be used.
4. Ensure that the flow through the chamber is stable and continuous. No ripples should be observed in the experimental chamber.
5. Before transferring the preparation, make sure the solution is thoroughly bubbled with 95% O_2 and 5% CO_2 gas mixture for at least 5 min.
6. Bring the dissection dish containing the preparation close to the recording chamber. Use the forceps to grab the metal plate to which the preparation is glued and transfer it to the chamber.
   Important: Once in the recording chamber, the entire preparation including the dorsal roots should always be covered with the solution.
7. Position the metal plate so that the rostrocaudal axis of the preparation is along either the x- or y-axis of the microscope table.
8. Allow the preparation to accommodate the Krebs bicarbonate recording solution for 10 min before any recordings.

C. Attaching dorsal roots to suction electrodes

1. Using white LED as a source of light and a low magnification objective (4–5×), focus on the tip of the dorsal root and assess its width using eyepiece graticule or camera software.
   Note: It is possible to use a portable white LED flashlight for attaching dorsal roots and positioning the preparation. However, having a designated stationary white LED is more convenient.
2. Fabricate a suction electrode by fire-polishing a glass capillary. The opening of the suction electrode should be slightly narrower than the width of the root.
   Note 1: It is recommended to manufacture a calibrated set of suction electrodes with various opening

diameters beforehand and choose an appropriate one during the experiment, similarly to the recordings from the sciatic nerve [28].

Note 2: Use thin-walled glass capillaries to manufacture suction electrodes with narrow openings.

3. Insert the suction electrode into the pipette holder mounted on a manipulator (compact one with a magnet base is preferable) and connected to the anode of the constant current stimulator.
4. Position the manipulator and dip the suction electrode into the experimental chamber. Connect a 2.5- or 5-mL syringe to the pipette holder using silicone tubing; apply negative pressure to draw the solution into the suction electrode until it reaches the AgCl wire.
5. Position the opening of the suction electrode right next to the tip of the dorsal root. The suction electrode and the root should be aligned; if necessary, push the root with the forceps to achieve this configuration. Apply negative pressure sufficient for the root to go inside the electrode.
6. Visually check the tightness of the contact between the root and the electrode; it should be as shown in Figure 5. If a substantial proportion of the root enters the suction electrode creating excessive tension, the opening is too wide, and the glass electrode should be fire-polished further. If the root bulges inside the suction electrode, the opening is too narrow.

   Note: Given that action potential initiation in response to electrical stimulation of the dorsal root occurs at the opening of the suction electrode [29,30], just the distal part of the root needs to go inside. Longer dorsal roots allow us to more easily distinguish between postsynaptic currents driven by fast-conducting (myelinated) A-fiber and slow-conducting (non-myelinated) C-fiber primary afferents.
7. Ensure the dorsal root does not pull the hemisected spinal cord. Otherwise, the preparation may move during recordings.
8. Position the AgCl reference electrode (connected to the cathode of the stimulator) next to the suction electrode.
9. Repeat steps C1–8 for other root(s).
10. **Optional:** For recording dorsal root potentials that spread passively, the proximal part of the root needs to be inside the recording suction electrode (Figure 5) to maximize signal-to-noise ratio.

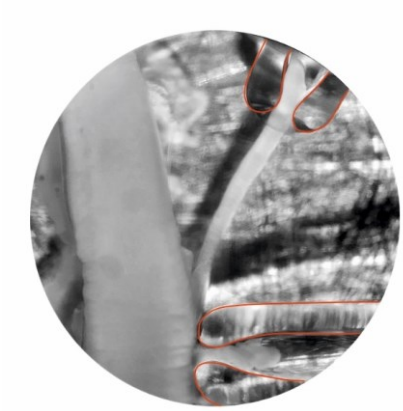


Figure 5. Ex vivo spinal cord preparation with dorsal roots attached to suction electrodes. The walls of the suction electrodes are highlighted in red. Notice the tight contact between the roots and the electrodes. Top electrode: arrangement for dorsal root stimulation; the distal part of the root is inside the electrode. Bottom electrode: arrangement for dorsal root potential recordings; the proximal part of the root is inside the electrode. The configuration shown in the figure allows recording afferent-driven dorsal root potentials, a generalized readout of primary afferent–driven presynaptic inhibition.

D. Visualization of lamina X neurons

1. Visualization of lamina X neurons is performed using oblique infrared illumination (Figure 6) for which the following is necessary:

a. Narrow beam (± 3–5°) infrared light-emitting diode (IR-LED) connected to an adjusted power supply unit and mounted on a micromanipulator (or a gooseneck) allowing IR-LED to change its position in relation to the ex vivo spinal cord preparation.
Note: For convenience, it is better to mount IR and white LEDs on the same micromanipulator.
b. 60× objective.
Note: It is possible to use a 40× objective; yet, it is less convenient than the 60× one and digital zooming might be required for patching small lamina X neurons.
c. CCD-camera coupled to a computer (or a separate display in case of an analog camera).
Note: Make sure that the spectral characteristics of IR-LED and CCD-camera match.

Turn these devices on for visualization.

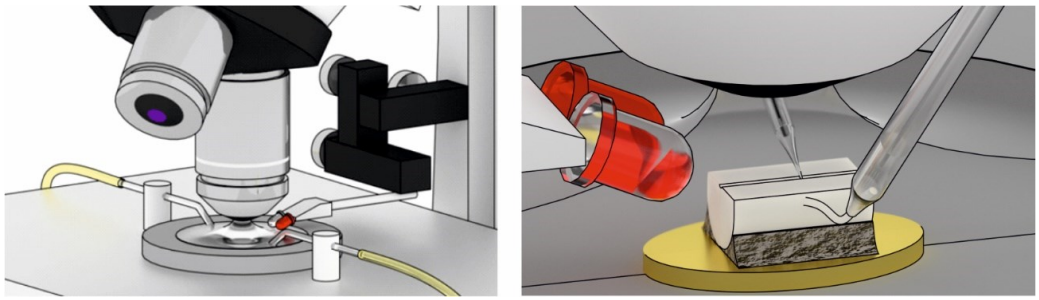


Figure 6. Schematic illustration of the experimental setup for oblique LED illumination. Left: notice the experimental chamber with perfusion inflow and outflow tubes, low-magnification objective, high-magnification water-immersion objective, and white and IR-LED mounted together on a micromanipulator. The LEDs should not be immersed in the solution. Right: notice two LEDs: white LED used together with a low-magnification objective for rough positioning and attaching dorsal roots to suction electrodes, and IR-LED used with a high-magnification objective for cell visualization. The IR-LED should be positioned at an acute (10–20°) angle in relation to the spinal cord preparation. Also, notice ex vivo spinal cord preparation glued to a metal plate; spared dorsal root is attached to the suction electrode. The patch pipette for electrophysiological recordings from lamina X neurons is located in the middle of the dorsoventral axis of the preparation. Modified from Krotov et al. [26].

2. Install IR-LED so that its beam forms a 10–20° angle with the plane of the experimental chamber (Figure 6).
Note: The exact position of the LED greatly depends on its properties and needs to be found experimentally.
3. Using a white LED and low-magnification objective, focus on the desired spinal segment approximately at the middle of the dorsoventral axis of the preparation. Once done, turn off the white LED.
4. Switch to the 60× objective and focus on the surface of the preparation to see the cells. Typically, the cells of the ependyma (Figure 7A), a thin (5–10 μm) neuroepithelial lining of the central canal, are observed first. The ependymal layer is not uniform, and some lamina X neurons may be observed on the very surface of the preparation (Figure 7B). Lamina X neurons are mostly located underneath the ependyma (Figure 7C) and are approximately 20–40 μm long, although some may amount to 70–80 μm.
Critical: Make sure the image is still, i.e., the preparation does not move or wobble. Slow lateral movement indicates that the dorsal root attached to a suction electrode is pulling the preparation. A wobbly image indicates that a) the flow of the solution is unstable or b) the preparation is not glued to the metal plate properly. Apply additional glue and/or stabilize the flow before proceeding further.
Note: The auto-contrast option of the camera should be turned on.

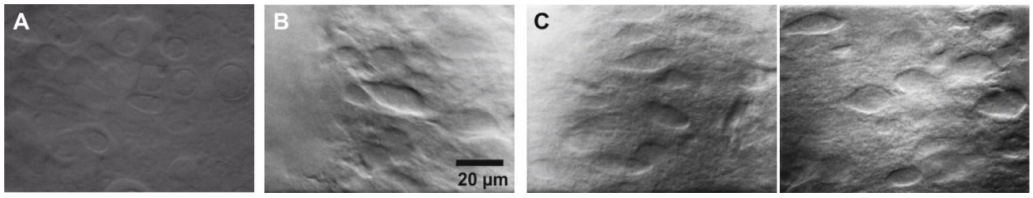


Figure 7. Lamina X cells observed using IR-LED oblique illumination. A. Ependymal cells lining the central canal. B. Lamina X neurons on the surface of hemisected spinal cord preparation. C. Lamina X neurons located underneath the ependymal layer. Modified from Krotov et al. [26].

5. Adjust the position of the IR-LED for the best quality of the picture. Change the intensity of LED emission if necessary. When adjusted correctly, it is possible to visualize the cells up to 60 µm deep. In younger (P7–9) animals in which the ependyma is less pronounced, it is possible to visualize cells up to 80–100 µm deep.
   Note: In oblique illumination, healthy-looking cells are usually perceived as convex, which is convenient for further patching. In case the cells appear concave, mirror the image using camera software or physically turn the camera 180 degrees.
6. Choose a lamina X neuron for patch clamping. The dorsal and ventral boundaries of lamina X are defined by bundles of parallel fibers spanning in the rostrocaudal direction (Figure 8). If the dorsal root is to be stimulated, choose neurons from the same (in respect to the dorsal root) or rostrally adjacent segments (i.e., if L5 root is stimulated, choose neurons from L4–5 spinal segments), since they receive most of the primary afferent input.

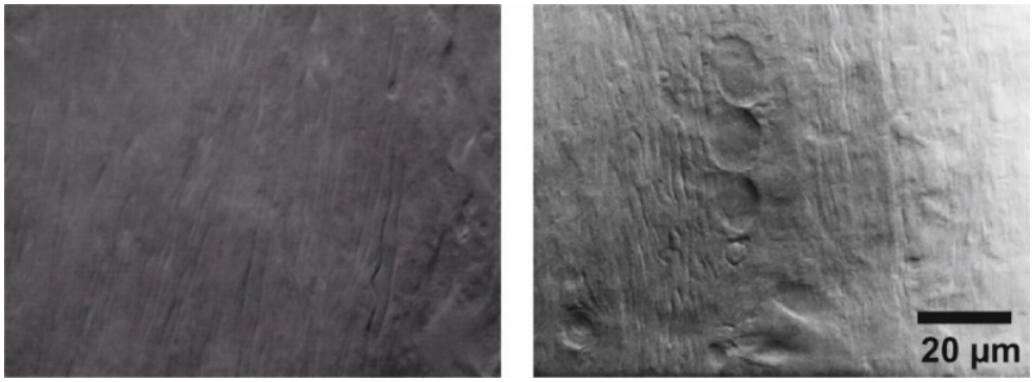


Figure 8. Rostrocaudal fibers of the spinal cord (parallel bundles) defining dorsal and ventral boundaries of the lamina X. Occasionally, cells may be observed in between the parallel fibers (right). Such cells do not belong to lamina X. Modified from Krotov et al. [26].

E. Patch clamping lamina X neurons

1. Turn on the amplifier, digitizer, constant current stimulator, and manipulator. Run their respective software. Set the amplifier to voltage-clamp mode.
2. Using a silicon tube, connect the pipette holder to a three-way tap with an attached 2.5 (or 5 mL) syringe and a mouthpiece (1 mL syringe without the plunger).
3. Thaw 1 mL vial of potassium gluconate intracellular solution. Fill a 1 mL syringe and put on a syringe filter and a patch pipette filler.
   Note: Keep the syringe on ice or in the refrigerator (4–8 °C) during the experiment to avoid aggregation of potassium gluconate and/or NaGTP that may cause pipette blockage.
4. Use glass capillaries and a pipette puller to fabricate patch pipettes.
5. Fill a patch pipette with potassium gluconate solution. Insert the pipette into the holder connected to the

headstage of the amplifier.

6. Lift the water immersion objective to create a meniscus that would accommodate the conical part of the patch pipette. Introduce the pipette and focus on its tip.
7. Visually ensure the opening of the pipette is not blocked. Assess the resistance of the pipette using Membrane test (Bath) in pClamp software. The pipette needs to have a resistance of 3–5 MΩ when filled with potassium gluconate solution.
8. Continuously apply positive pressure using a 2.5 mL syringe (displace the plunger by 1–1.5 mL). Ensure there is an outflow of solution from the pipette. Compensate offset potential.
   Note: When using potassium gluconate solution, applying positive pressure increases the resistance between the patch pipette and the reference electrode.
9. Approach the surface of the preparation with the pipette: iteratively lower the objective first and catch up with the pipette. Once done, switch the manipulator to a lower speed and position the tip of the pipette right above the center of the soma of a chosen neuron.
10. Descend the pipette toward the neuron. The flow from the pipette should visibly spread the tissue. In case the ependymal layer prevents that, gently tap on the headstage and reposition the tip of the pipette above the center of the neuron.
11. Keep descending to the surface of the desired neuron. At some point, the flow from the pipette is going to indent the membrane. Once the indentation is approximately twice the size of the pipette opening, use the mouthpiece to apply negative pressure (as if you were drinking through a straw). Switch the tap to the mouthpiece while applying the negative pressure. At this point, the resistance between the patch pipette and the reference electrode should abruptly increase. When it reaches 200–300 MΩ, release the negative pressure. From this point, the gigaseal (the resistance over 1 GΩ), which defines the cell-attached patch clamp configuration, should establish on its own. If that does not happen, facilitate the gigaseal formation by switching to negative potentials and applying negative pressure with the mouthpiece.
    Important: Insufficient indentation compromises the gigaseal formation. Excessive indentation compromises establishing whole-cell patch clamp configuration. In case the gigaseal cannot be achieved on a regular basis, change the puller settings to increase the resistance of the patch pipettes by additional 1–2 MΩ.
    Critical: The flow of the potassium gluconate solution from the patch pipette depolarizes surrounding neurons. Therefore, keep the time between penetrating the tissue and establishing the gigaseal to a minimum.
    Note: The flow of the solution from the pipette may move the cell. Use the manipulator to always keep the tip of the patch pipette above the center of the cell.
12. Compensate pipette capacitance and let the contact between the cell and the pipette improve for a couple of minutes. Ideally, the absolute value of the observed current should be below 20 pA or, even better, 10 pA.
13. **Optional:** Perform action-potential recordings in the cell-attached configuration. Recordings in the current clamp mode (I = 0) allow further analysis of action potential parameters and are more sensitive to sub-threshold events. Recordings in the voltage clamp mode (V = 0) are less sensitive, hence providing a smoother baseline, and are mostly used for assessing the number of action potentials (see [31] for a detailed description of cell-attached recordings). Typically, recordings in the cell-attached mode are coupled with dorsal root stimulation (see section F).
    Note: Ideally, the recording pipette should be filled with filtered extracellular solution to assess genuine unperturbed activity of a neuron. Yet, that approach makes any further whole-cell experiments impossible. Using a high-potassium internal solution may be a reasonable compromise, allowing both to estimate neuronal activity and to conduct further intracellular recordings. Given that in our experiments dorsal root stimulation evoked the same number of spikes in cell-attached and whole-cell configurations, we assume that depolarization of a small membrane patch does not affect the spike generation significantly.
14. Set the holding potential to -60 (or -70) mV and establish whole-cell patch clamp configuration. For that, apply negative pressure through a mouthpiece and gradually increase it until the flat line on the membrane test (Patch) indicating gigaseal contact changes to a classic whole-cell transient. Once the transient appears, release the negative pressure immediately. Alternatively, apply a small amount of negative pressure and

use the zap function of the amplifier. Typically, 50–250 μs zap duration is enough for a breakthrough.

15. **Critical:** Monitor series resistance often, as it tends to increase over time (generally, the higher the pipette resistance, the greater the increase in series resistance). Make sure that series resistance does not change by more than 20% to avoid compromising collected data.
    Note: Series resistance may vary substantially from cell to cell; larger lamina X neurons exhibit 15–25 MΩ series resistance, and smaller ones exhibit ~25–35 MΩ. Typical values of series resistance are an order of magnitude lower than those of membrane resistance.
    Optional: Compensate whole-cell transient and compensate series resistance. That does not always work and, often, setting compensation and/or prediction over 70% results in oscillations that may ruin the whole cell configuration.

F. Electrophysiological recordings and dorsal root stimulation

1. For recordings, set the digitization at 10–20 kHz and the Bessel filter at 2.4–3 kHz.
2. In the first minute after establishing whole-cell configuration, run the protocols for determining membrane resistance and capacitance as well as the firing properties of the cell. This information might help in the classification of studied neurons.
    a. For determining resistance and capacitance of the membrane, apply -10 mV hyperpolarizing step for 200 ms at 1 Hz (10–15 trials).
    b. For determining the firing pattern of a neuron, switch to the current clamp mode and inject a negative current sufficient to hyperpolarize the cell to -80 mV (necessary to recover potassium voltage–gated channels from inactivation). Apply incrementally increasing 500 ms steps of current at 0.3 Hz; start with -20 pA and increase the current by 10 or 20 pA with each trial. Do not exceed 400–500 pA current.
3. Before proceeding to the next recordings, check the series resistance and adjust it if necessary.
4. For assessing network activity, allow cell dialysis for at least 5 min after establishing whole-cell configuration. Then, record spontaneous excitatory postsynaptic currents (sEPSCs) at -60 or -70 mV in a gap-free mode. At these potentials, sEPSCs appear as negative deflections of current. Positive deflections correspond to spontaneous inhibitory postsynaptic currents; however, they are unlikely to appear, given that the reversal potential for chloride ions is around -80 mV for the combination of external Krebs bicarbonate and internal potassium gluconate solutions. Add TTX (final concentration 1 μm) and D-AP5 (final concentration 20–40 μm) to Krebs bicarbonate solution for recordings of AMPA receptor-mediated miniature postsynaptic currents.
5. For assessing primary afferent supply of lamina X neurons, combine recordings at -60 or -70 mV with dorsal root stimulation. Connect the stimulator to the digital output of the digitizer. Set the stimulator to square pulses of current. Set the stimulation frequency to 0.1 Hz to avoid slowing down of conduction [32] and the wind-up phenomenon [33]. Apply the following values of stimulus intensity and duration to activate the primary afferents in the dorsal roots:
    a. Use +30–50 μA × 50 μs stimuli to activate thick fast-conducting myelinated low-threshold Aβ-fiber primary afferents.
    b. Use +60–100 μA × 50 μs stimuli to activate Aβ-, thinly myelinated Aδ-, and slow-conducting low-threshold C-fiber primary afferents.
    c. Use +20–150 μA × 1 ms stimuli to activate all primary afferents including high-threshold Aβ, Aδ, and C fibers.
    Note: In physiological conditions, primary afferent–driven postsynaptic currents are saturated at 70–100 μA; thus, the 100–150 μA range corresponds to supramaximal stimulation.
    d. Use negative 20–150 μA × 1 ms stimuli to exclusively activate C-fiber primary afferents. Stimuli of negative polarity are necessary to induce an anodal block of fast-conducting A fibers [29,30,35], thus ensuring exclusive C-fiber activation.
    Note: The exact stimulus duration necessary to induce anodal block of A fibers depends on the configuration of the suction electrode. In case A fiber–driven postsynaptic currents are observed when stimulating with negative current, adjust the duration in 0.8–1.2 ms range.
    Record at least 12–15 trials for any selected stimulation.

6. For assessing homosegmental A fiber–driven presynaptic inhibition (i.e., decrease of C fiber–driven postsynaptic currents mediated by A fibers from the same dorsal root), perform the recordings as described in the step above. Stimulate the dorsal root at +150 μA × 1 ms first and then at -150 μA × 1 ms.
7. For assessing heterosegmental C fiber–driven presynaptic inhibition (i.e., decrease of A and C fiber–driven postsynaptic currents mediated by C fibers of the adjacent dorsal root), make paired stimulations. First, perform conditioning C-fiber stimulation (-150 μA × 1 ms) and record elicited postsynaptic currents (this is necessary for subtracting postsynaptic current elicited by conditioning stimulation from the one elicited by the test stimulation). Then, stimulate the adjacent dorsal root (choose one of the patterns from step F5) and record test postsynaptic currents in the absence of conditioning stimulation. Finally, make recordings with paired stimulations; apply conditioning stimulus 80–100 ms before the test one (Figure 9).
8. Protocols described in steps F3–7 may be repeated in the presence of a desired pharmacological agent.
9. Record at -10 mV using the protocols described in steps F3–7 to assess spontaneous and primary afferent–driven postsynaptic inhibitory currents.
10. **Critical:** Monitor series resistance often. Do not take data for quantitative analysis if series resistance changes by more than 20% during recording.

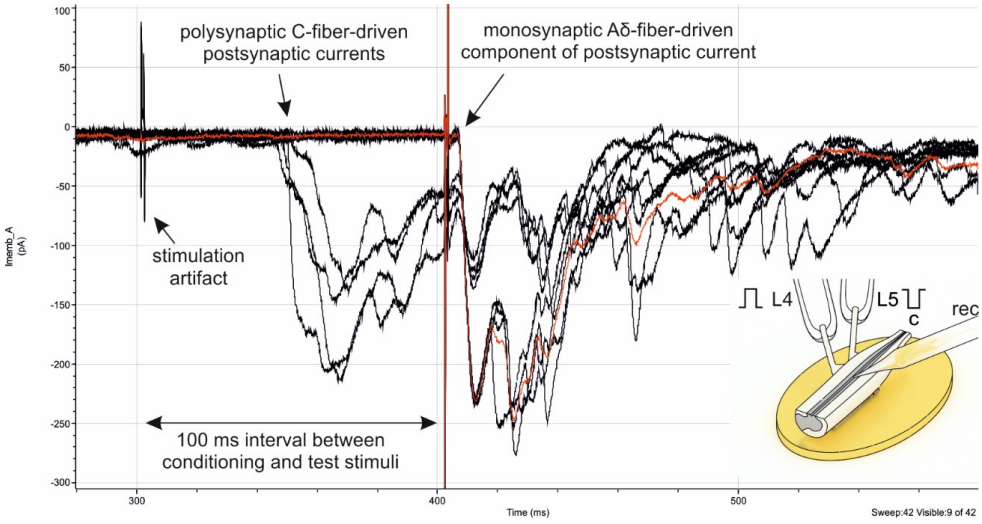


Figure 9. Electrophysiological recordings for assessing heterosegmental C fiber–driven presynaptic inhibition of the afferent input to lamina X neuron. Unprocessed recording (unfiltered, unadjusted baseline) showing individual traces. The red line is an average of traces recorded in the absence of conditioning stimulation. Notice the decreased amplitude of the monosynaptic component of primary afferent input in the presence of conditioning stimulation. Inset: experimental design of paired dorsal root stimulation coupled with patch clamp recordings. Test stimulation of L4 dorsal root (+150 μA × 1 ms) was performed to induce primary afferent–driven postsynaptic currents. Conditioning stimulation (-150 μA × 1 ms) of L5 dorsal root was performed to observe C fiber–mediated presynaptic inhibition of the primary afferent input from L4 dorsal root. Conditioning stimulus was applied 100 ms prior to the test one. Recordings were made from a lamina X neuron of L4 spinal segment. Modified from Krotov et al. [30].

11. **Optional:** To assess generalized presynaptic inhibition in the entire spinal segment, use the dorsal root to record passively spreading dorsal root potentials. For that, ensure the recording suction electrode is attached to the dorsal root as described in step C10. Set the amplifier to current clamp mode. Given that dorsal root potentials rarely exceed 0.2–0.3 mV, use high-gain settings. To elicit primary afferent–driven dorsal root potentials, use stimulation patterns from step F4. Although dorsal root potentials are highly reproducible, record at least 5–10 trials for averaging. Typically, dorsal root potentials peak 50–100 ms after stimulation (Figure 10).

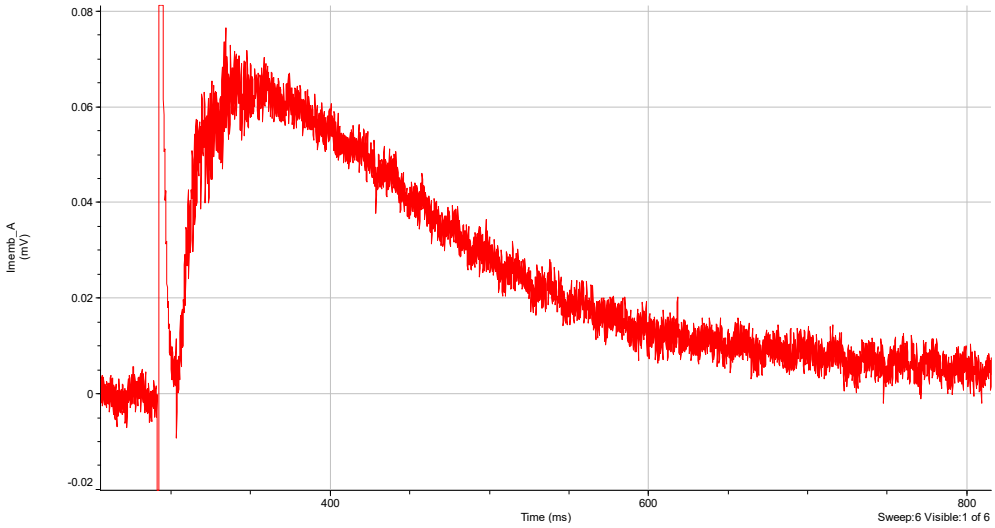


Figure 10. Dorsal root potential recorded from L5 dorsal root. The potential was elicited by supramaximal (+150 μA × 1 ms) L4 dorsal root stimulation. Average of five traces, baseline adjusted to zero.

Data analysis

Data obtained from electrophysiological experiments on lamina X neurons may include recordings of spontaneous/miniature excitatory and inhibitory postsynaptic currents, primary afferent–driven postsynaptic currents elicited by dorsal root stimulation, and action potentials evoked by current injection via patch pipette or dorsal root stimulation. Hemisected spinal cord with spared dorsal roots also allows to perform recordings of compound action potentials driven by primary afferent activation as well as passively spreading dorsal root potentials caused by primary afferent depolarization elicited by the activation of spinal neurons. Spontaneous/miniature currents are better analyzed with MiniAnalysis software, which automatically returns amplitudes and kinetics of individual events. Other types of data such as the integrals and the amplitudes of evoked postsynaptic currents may be analyzed using either Clampfit or Origin software. Identification and classification of monosynaptic components of primary afferent inputs elicited by dorsal root stimulation are performed based on the following criteria [36,37]:

1. Low failure rates (less than 30%).
2. Small latency variations (less than 2 ms).
3. Conduction velocity (CV), calculated as the length of the dorsal root from the opening of the suction electrode to the dorsal root entry zone, divided by the latency of the monosynaptic response with a 1 ms allowance for synaptic transmission:
   a. CV < 0.5 m/s corresponds to C-fiber afferents.
   b. 0.6 m/s < CV < 1.4 m/s corresponds to Aδ-fiber afferents.
   c. CV > 3.5 m/s corresponds to Aβ-fiber afferents.
   Note: Typically, Aβ afferents do not supply lamina X neurons [38].

Analysis of the amplitudes of monosynaptic components of primary afferent input is necessary for studying presynaptic inhibition.
Homosegmental A fiber–driven presynaptic inhibition: compare monosynaptic inputs to a particular lamina X neuron elicited by 150 μA × 1 ms stimuli of positive and negative polarities. After switching to negative polarity, faster A fiber–driven components disappear while C fiber–driven ones are time-shifted to the right by several milliseconds. Emergence (unmasking) of a new (previously unobserved) monosynaptic C fiber–driven input

corresponds to full presynaptic block. An increase in the amplitude of existing monosynaptic C fiber–driven components corresponds to partial presynaptic block.
Heterosegmental presynaptic inhibition: compare monosynaptic components of postsynaptic currents before and after conditioning stimulation of the adjacent dorsal root. Reduced monosynaptic amplitude corresponds to partial presynaptic inhibition, and disappeared monosynaptic component indicates full presynaptic block.
Typically, obtained datasets do not follow normal distribution. Therefore, it is recommended to use non-parametric tests (for instance Mann-Whitney test) for statistical comparisons.
For presentation purposes, the signals may be slightly smoothed (Savitzky-Golay filter) to eliminate fast noise from the peristaltic pump. Dorsal root potential recordings may be filtered for sine wave noise.

Validation of protocol

This protocol has been used and validated in the following research articles:

- Krotov et al. (2019). High-threshold primary afferent supply of spinal lamina X neurons. PAIN (Figures 1–4).
- Krotov et al. (2022). Segmental and descending control of primary afferent input to the spinal lamina X. PAIN (Figures 1–5).
- Krotov et al. (2023). Elucidating afferent-driven presynaptic inhibition of primary afferent input to spinal laminae I and X. Frontiers in Cellular Neuroscience (Figures 1–6).

The part of the current protocol regarding dorsal root stimulations has also been validated in the following articles: Agashkov et al. [39]; Krotov et al. [34]; Tadokoro et al. [40])

General notes and troubleshooting

General notes

1. Electrophysiological recordings from lamina X neurons may be coupled with electrical [27] or optogenetic activation of descending (top-down) spinal cord tracts or calcium transient measurements using fluorescent indicators [26].
2. It is possible to work with defined populations of lamina X neurons using the following techniques: Injections of fluorescent tracer Fluorogold (or its analogues) into the thalamus or hypothalamus retrogradely labels spinothalamic or spinohypothalamic projection neurons in the lamina X [26]. Intraperitoneal injections of Fluorogold retrogradely label sympathetic preganglionic neurons located in thoracic and upper lumbar spinal segments [26]. The use of respective transgenic animals [41] allows to work with cholinergic lamina X neurons that are known to be involved in locomotion [9].
3. The current protocol may be applicable for electrophysiological studies of lamina X neurons in P7–P15 mice. Yet, the use of mice is more challenging given the smaller size of their spinal cord and dorsal roots in comparison with rats.
4. P10–12 Sprague-Dowley rats (Charles River, strain code: 001) may be used for the experiments. However, these animals possess a more pronounced ependymal layer and exhibit weaker primary afferent input to lamina X neurons than their age-matched Wistar counterparts.
5. Visualization of lamina X neurons in animals older than P15 becomes challenging due to the thicker ependymal layer and higher number of commissural fibers.
6. The current protocol is also suited for the electrophysiological investigation of Clarke column neurons (spinal lamina VII). Their cell bodies may be visualized in the thoracic segments of the spinal cord right next to the dorsal border of lamina X [42].
7. The current protocol may also be utilized for electrophysiological recordings of dorsal root potentials from hemisected spinal cords of adult mice. A similar approach was used by Zimmerman and colleagues [43].

Acknowledgments

This protocol is adapted from the previously published papers [26,27,30, 38].
The authors would like to thank Prof. Safronov (University of Porto), Dr. Oleh Halaidych and Mr. Andrew Dromaretsky (Bogomoletz Institute of Physiology). The drawing of the rat pup used in the graphical abstract was modified from the original image by Antonis Asiminas (doi.org/10.5281/zenodo.3926273) uploaded to SciDraw. Funding sources: this work was supported by NIH-1R01NS113189-01 grant (PB and NV), NASU 0124U001557, and 0124U001556 grants (PB).

Competing interests

The authors declare no competing interests.

Ethical considerations

All procedures involving animals were carried out with the approval of the Animal Ethics Committee of the Bogomoletz Institute of Physiology (Kyiv, Ukraine) in accordance with the European Commission Directive (86/609/EEC), ethical guidelines of the International Association for the Study of Pain, and the Society for Neuroscience Policies on the Use of Animals and Humans in Neuroscience Research.

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