

Video Article

Preparation of Acute Spinal Cord Slices for Whole-cell Patch-clamp Recording in Substantia Gelatinosa Neurons

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Abstract

Recent whole-cell patch-clamp studies from substantia gelatinosa (SG) neurons have provided a large body of information about the spinal mechanisms underlying sensory transmission, nociceptive regulation, and chronic pain or itch development. Implementations of electrophysiological recordings together with morphological studies based on the utility of acute spinal cord slices have further improved our understanding of neuronal properties and the composition of local circuitry in SG. Here, we present a detailed and practical guide for the preparation of spinal cord slices and show representative whole-cell recording and morphological results. This protocol permits ideal neuronal preservation and can mimic *in vivo* conditions to a certain extent. In summary, the ability to obtain an *in vitro* preparation of spinal cord slices enables stable current- and voltage-clamp recordings and could thus facilitate detailed investigations into the intrinsic membrane properties, local circuitry and neuronal structure using diverse experimental approaches.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58479/>

Introduction

The substantia gelatinosa (SG, lamina II of the spinal dorsal horn) is an indisputably important relay center for transmitting and regulating sensory information. It is composed of excitatory and inhibitory interneurons, which receive inputs from the primary afferent fibers, local interneurons, and the endogenous descending inhibitory system¹. In recent decades, the development of acute spinal cord slice preparation and the advent of whole-cell patch-clamp recording have enabled various studies on the intrinsic electrophysiological and morphological properties of SG neurons^{2,3,4} as well as studies of the local circuitry in SG^{5,6}. In addition, by using the *in vitro* spinal cord slice preparation, researchers can interpret the changes in neuronal excitabilities^{7,8}, the function of ion channels^{9,10}, and synaptic activities^{11,12} under various pathological conditions. These studies have deepened our understanding of the role that SG neurons play in the development and maintenance of chronic pain and neuropathic itch.

Essentially, the key prerequisite to achieve a clear visualization of neuronal soma and ideal whole-cell patching using acute spinal cord slices is to ensure the excellent quality of slices so healthy and patchable neurons can be obtained. However, preparing spinal cord slices involves several steps, such as performing a ventral laminectomy and removing the pia-arachnoid membrane, which may be obstacles in obtaining healthy slices. Although it is not easy to prepare spinal cord slices, performing recordings *in vitro* on spinal cord slices has several advantages. Compared to cell culture preparations, spinal cord slices can partially preserve inherent synaptic connections that are in a physiologically relevant condition. In addition, whole-cell patch-clamp recording using spinal cord slices could be combined with other techniques, such as double patch clamp^{13,14}, morphological studies^{15,16} and single-cell RT-PCR¹⁷. Therefore, this technique provides more information on characterizing the anatomical and genetic diversities within a specific region and allows for investigation of the composition of local circuitry.

Here, we provide a basic and detailed description of our method for preparing acute spinal cord slices and acquiring whole-cell patch-clamp recordings from SG neurons.

Protocol

All experimental protocols described were approved by the Animal Ethics Committee of Nanchang University (Nanchang, PR China, Ethical No.2017-010). All efforts were made to minimize the stress and pain of the experimental animals. The electrophysiological recordings performed here were carried out at room temperature (RT, 22–25 °C).

1. Animals

1. Use Sprague-Dawley rats (3–5 weeks old) of either sex. House the animals under a 12 h light-dark cycle and give them *ad libitum* access to adequate food and water.

2. Preparation of Solutions and Materials

1. Solutions

1. Prepare artificial cerebrospinal fluid (ACSF) (in mM): 117 NaCl, 3.6 KCl, 1.2 $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 2.5 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.2 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 25 NaHCO_3 , 11 D-glucose, 0.4 ascorbic acid, and 2 sodium pyruvate. See **Table 1**.
2. Prepare sucrose-ACSF (in mM): 240 Sucrose, 2.5 KCl, 1.25 $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.5 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3.5 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 25 NaHCO_3 , 0.4 Ascorbic acid, and 2 sodium pyruvate. See **Table 2**.
3. Prepare K^+ -based intracellular solution (in mM): 130 K-gluconate, 5 KCl, 10 Na_2 -phosphocreatine, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, and 0.3 Li-GTP. See **Table 3**.
4. Prepare Cs^+ -based intracellular solution (in mM): 92 CsMeSO_4 , 43 CsCl, 10 Na_2 -phosphocreatine, 5 tetraethylammonium (TEA)-Cl, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, and 0.3 Li-GTP. See **Table 4**.

Note: All solutions must be prepared using distilled water. ACSF and sucrose-ACSF should be carbogenated (95% O_2 and 5% CO_2 mixture) prior to use to maintain an optimal pH of approximately 7.4, and the osmolality of these two solutions should be adjusted to 300–310 mOsm. Because ascorbic acid could affect calcium channels, this agent must be omitted if one would like to record calcium currents. The osmolality and pH of intracellular solutions should be measured and adjusted to 290–300 mOsm and 7.2–7.3, respectively. It is recommended to filter the intracellular solutions with 0.2 μm filters and store the solutions as 1 mL aliquots at -20°C . Cs^+ and TEA are applied in Cs^+ -based intracellular solution to block potassium channel, which is conducive to using the amplifier to hold the membrane potential steady at 0 mV when recording inhibitory postsynaptic currents (IPSCs).

5. Prepare the 0.05% neurobiotin 488 solution. Dissolve 2 mg of neurobiotin 488 in 4 mL of K^+ -based intracellular solution and adjust the osmolality to 290–300 mOsm by using distilled water or sucrose if needed.

Note: 1 mM of sucrose increases osmolality by 1 mOsm.

6. Prepare 3% agar as a block for spinal cord. Dissolve 7.5 g of agar in 250 mL of purified water in a glass beaker, and then use a microwave to heat it until boiling and clear. Swirl the solution and pour the mixture into a 17.5 cm x 10.5 cm x 1.8 cm plastic box for solidification afterwards. Keep the agar at 4°C prior to use.

7. Prepare 4% paraformaldehyde (PFA) for immunohistochemical processing. Mix 40 g of PFA powder to ~800 mL of heated (approximately 60°C) 1x PBS solution (130 mM NaCl, 7 mM Na_2HPO_4 , 3 mM NaH_2PO_4). Slowly adjust the pH by adding 1 N NaOH drops until PFA powder is completely dissolved. Once the solution has become clear, adjust the total volume to 1 L with 1x PBS. Readjust the pH to 7.2–7.4 using 1 N HCl when needed, then filter the 4% PFA solution and store it at -20°C until use.

Note: PFA is toxic, so it is needed to wear masks, gloves as well as safety glasses. Conduct the process of preparing 4% PFA inside a ventilated hood. PFA powder can be completely dissolved at a pH of approximately 9–10.

2. Instruments

1. For a typical electrophysiological system, use an upright microscope equipped with infrared differential interference contrast (IR-DIC) and a high-resolution water-immersion objective, a CCD/CMOS camera, a patch-clamp amplifier, a micropipette holder and a micromanipulator allowing fine adjustment of the pipette position. An XY stage is also needed to move the microscope.
2. Mount all equipment on a vibration isolation table surrounded by a Faraday cage. Connect a video monitor to the video camera to observe the neurons and visualize the micropipettes.

3. Micropipettes

1. Make recording electrodes from borosilicate glass capillaries using a micropipette puller. The typical pipette resistance ranges from 3–6 M Ω when filled with intracellular solution.

4. Agar block

1. Prepare a 1.2 cm x 1.5 cm x 2.0 cm agar block. Trim the block into the shapes shown in **Figure 1** as required.

3. Acute Spinal Cord Slice Preparation

Note: Transverse or parasagittal spinal cord slices are prepared as previously described^{18,19,20}.

1. Prior to transcardial perfusion and spinal cord extraction, prepare ~500 mL sucrose-ACSF equilibrated with 95% O_2 and 5% CO_2 and cool the solution to ice-cold ($0\text{--}4^\circ\text{C}$).
2. Cool down all the dissection tools (e.g., dissecting scissors, iris scissors, toothed forceps, fine forceps, curved forceps) on the ice. The diagram of the preparation of acute spinal cord slice is shown in **Figure 1**.
3. Transcardial perfusion
 1. After a single injection (intraperitoneal, i.p.) of urethane (1.5 g/kg), wait for 2–3 min, and assess the anesthesia depth of rats by testing toe or tail pinch responses. Once a surgical plane of anesthesia is maintained, place the rat on crushed ice in the supine position.
 2. Make an incision (3–4 cm) through the skin from the xiphoid process to the clavicle, and then make a transverse incision beneath the level of the xiphoid process.
 3. Grasp and raise the xiphoid process with a pair of curved forceps to expose the diaphragm completely. Make a transverse incision through the diaphragm, and then cut through the ribcage between the breastbone and ribs bilaterally to open the chest cavity. Use the curved forceps to grasp the xiphoid process so the ribcage is fixed, and the heart is sufficiently exposed.
 4. Hold the heart with another curved forceps gently, and then insert a 22 G needle through the left ventricle to the base of the aortic arch.

5. Cut the right atrium with fine scissors immediately, and then start the perfusion of ice-cold, oxygenated sucrose-ACSF through a gravity system.

Note: Rapid and sufficient transcardial perfusion with ice-cold sucrose-ACSF can facilitate the rapid cooling of the spinal cord, while low sodium and calcium concentration may help alleviate excitatory toxicity and protect neuronal function. In addition, clearing red blood cells would be beneficial for reducing the background staining of biocytin when performing a morphological study. The perfusion is considered sufficient and satisfied as long as the fluid exiting the right atrium is clear and the color of the rat's liver and paws is pale. The tip of the 22 G needle should be blunt to avoid rupturing the heart and aortic arch.

4. Spinal cord dissection and slicing

1. Make a longitudinal incision (5 cm) on the back skin from caudal to rostral, then cut through the ribcage between the spine and ribs on either side in the state of perfusion.
2. Make a cut at the caudal end of the spine, use scissors to cut away surrounding tissues, and isolate the lumbosacral segment of the spine rapidly.
3. Transfer the lumbosacral segment to a glass dish containing ice-cold sucrose-based ACSF. With the ventral side upward, use fine scissors to cut through the vertebral pedicle bilaterally and expose the spinal cord carefully. Isolate a 2-cm long spinal cord with lumbosacral enlargement (L1–S3) and transfer the spinal section to another glass dish filled with cold sucrose-ACSF.
4. Remove the meninges and the pia-arachnoid membrane under a dissecting microscope. Cut all the ventral and dorsal roots away as quickly as possible.
5. Place the spinal cord on a previously trimmed agar block. To prepare transverse slices, attach the ventral side of the spinal cord to the agar and let the dorsal side toward the blade. To prepare parasagittal slices, attach the ventral side with superglue to the agar in a vertical direction as shown in **Figure 1**. Then, mount the agar block to a platform of a vibratome with superglue. Prepare 300–500 μm transverse or parasagittal slices with an advance speed of 0.025 mm/s and a vibration frequency of 80 Hz.
6. Use a plastic-trimmed pipette to transfer the slices onto nylon mesh in a storage chamber containing continuously oxygenated ACSF at 32 °C for at least 30 min prior to recording.

Note: Take care to avoid injury to the spinal cord, especially the dorsal horn, when removing the meninges and spinal roots. The spinal cord should be sliced dorsal-ventrally. For the best results, the slices should be prepared rapidly (within 15–20 min). The thickness of a spinal slice should be no more than 600 μm to satisfy cell visibility. In addition, the technique described above could be used to obtain horizontal spinal slices.

4. Whole-cell Patch-clamp Recordings

1. To conduct the whole-cell patch-clamp recordings from SG neurons, use K^+ -based intracellular solution for most recording cases, while applying Cs^+ -based solution only for the recording of inhibitory postsynaptic currents.
2. Gently move a spinal cord slice to the recording chamber, and then maintain it with a U-shaped platinum wire attached with nylon threads firmly for optimal slice stability. Steadily perfuse the slice with bubbled ACSF at RT through a gravity system and set the perfusion rate at 2–4 mL/min to achieve sufficient oxygenation.
3. Identify the region of SG (a translucent band) using a low-resolution microscope lens, choose a healthy neuron by using the high-resolution objective as the target cell, and adjust it to the center of the video monitor screen.
4. Fill a micropipette with an appropriate volume of K^+ -based or Cs^+ -based intracellular solution as needed, insert the micropipette into the electrode holder, and ensure that the intracellular solution is contacting the silver wire inside the holder.
5. Bring the micropipette into focus and immerse it into the ACSF using a micromanipulator, and then apply a mild positive pressure (~1 psi when measured with a manometer) to force the micropipette away from any dirt and debris.
6. Move the micropipette towards the targeted neuron gradually. Release the positive pressure once the pipette approaches the neuron and a very small dimple forms on neuronal membrane to form a gigaseal.
7. Alter the holding potential to -70 mV, which is close to the physiological resting membrane potential (RMP) of a cell. Then, apply a transient and gentle suction to the micropipette to rupture the membrane and create a good whole-cell configuration.

Note: After transferring the slice into the recording chamber, ensure steady perfusion for at least 5 min to clear the debris on the slice surface. It is worth noting that the ability to distinguish between healthy and unhealthy/dead neurons is of paramount importance for good sealing and stable recording. An unhealthy/dead neuron has a swollen or shrunken appearance, together with a visible large nucleus, while a healthy neuron is characterized by a 3-dimensional (3D) shape with a bright and smooth membrane, and its nucleus is invisible. To achieve a whole-cell configuration, it is essential to compensate for fast or slow capacitance step-by-step when necessary. At RT, the liquid junction potential is calculated to be 15.1–15.2 mV and 4.3–4.4 mV in K^+ -based and Cs^+ -based intracellular solution, respectively. In our studies, the recorded data were not corrected for liquid junction potential.

8. Recordings of intrinsic membrane properties

1. Record passive intrinsic membrane properties: Record RMP immediately (within 20 s) after break in. Determine the neuronal input resistance by measuring the voltage response to a depolarizing current (10 pA, 500 ms) at RMP in current-clamp mode.
2. Record firing properties: Test the firing pattern of each neuron in current-clamp with a series of 1 s depolarizing current pulses (25–150 pA with 25 pA increment) at RMP. Measure the threshold, amplitude and half-width of a single action potential offline.
3. Record subthreshold current: To assess the somatic subthreshold currents, hold the membrane potential at -50 mV in voltage-clamp mode. Then, apply a series of hyperpolarizing voltage pulses of 1 s duration from -60 mV to -120 mV, with a 10-mV decrement.
4. Record excitatory postsynaptic currents (EPSCs): Record EPSCs with the K^+ -based intracellular solution in voltage-clamp mode at a holding potential of -70 mV.
5. Record IPSCs: Apply Cs^+ -based intracellular solution for recording IPSCs. Once the whole-cell configuration is established, hold the membrane potential at -70 mV for ~5 min, and then change the holding potential to 0 mV gradually. Wait a few minutes for stabilization, and then start to record the IPSC events.

Note: Only neurons with an RMP less than -50 mV and showing overshoot should be selected for further study. The series resistances in our study are typically 10–30 M Ω , and a recording should be excluded once the series resistance changes by more than 20%.

EPSCs could be confirmed with a bath application of 50 μ M APV and 20 μ M CNQX, while IPSCs could be confirmed with 10 μ M bicuculline and 1 μ M strychnine.

5. Morphological study

1. For morphological experiments, use a K^+ -based intracellular solution containing 0.05% neurobiotin 488.
2. After maintaining a stable electrophysiological recording for at least 20 min, slowly remove the micropipette in the upward direction to allow the cell membrane to reseal and transfer the spinal cord slice to a container filled with 4% PFA. Fix the slices in 4% PFA at RT for 1 h and then at 4°C overnight.
3. Rinse the slices in PBS, and then immerse them in 50% ethanol for 30 min. After another three washes in PBS, mount the slices in their original thickness onto slides with a mounting medium.
4. Use a confocal microscope (see **Table of Materials**) for image acquisition and neuronal 3D reconstruction. Scan neurons through a 20X lens with a z-stack of 1.5 μ m.

Representative Results

Acute spinal cord slices were prepared according to the diagram shown in **Figure 1**. After slicing and recovery, a spinal cord slice was transferred to the recording chamber. Healthy neurons were identified based on soma appearance using IR-DIC microscopy. Next, the action potentials of SG neurons were elicited by a series of depolarizing current pulses (1 s duration) when neurons were held at RMP. As shown in **Figure 2**, the firing patterns observed in SG neurons included tonic-firing, delayed-firing, gap-firing, initial-burst, phasic-bursting, single-spike and reluctant-firing, which have been described and categorized by previous studies.

Implementing this preparation, we also recorded subthreshold currents and spontaneously appearing currents in voltage clamp. Representative traces of subthreshold currents, including hyperpolarization-activated current (I_h), T-type calcium current (I_T) and A-type potassium current (I_A), are given in **Figure 3A**. These currents were obtained by holding cells at -50 mV and gradually stepping in 10-mV decrements from -60 to -120 mV. I_h was activated by hyperpolarizing voltage steps. However, I_T and I_A were activated by hyperpolarizing prepulses to release from inactivation followed with a depolarized voltage. **Figure 3B, 3C** show representative spontaneous EPSCs (sEPSCs) and IPSCs (sIPSCs) recorded from SG neurons, respectively. The amplitude and frequency of these synaptic events could be analyzed using the Mini-analysis software offline.

To characterize neuronal morphological features, parasagittal slices were applied because most of the SG neurons have significantly rostrocaudal spread of dendritic trees, and neurobiotin 488 was added to intracellular solutions. The size of neuronal soma and the extent and dimensions of their dendritic processes were evaluated after confocal microscopy imaging. As reported previously, SG neurons show morphological distinctions and could be categorized into central cells, radial cells, vertical cells, islet cells and unclassified cells. Representative micrographs of these cells are shown in **Figure 4**.

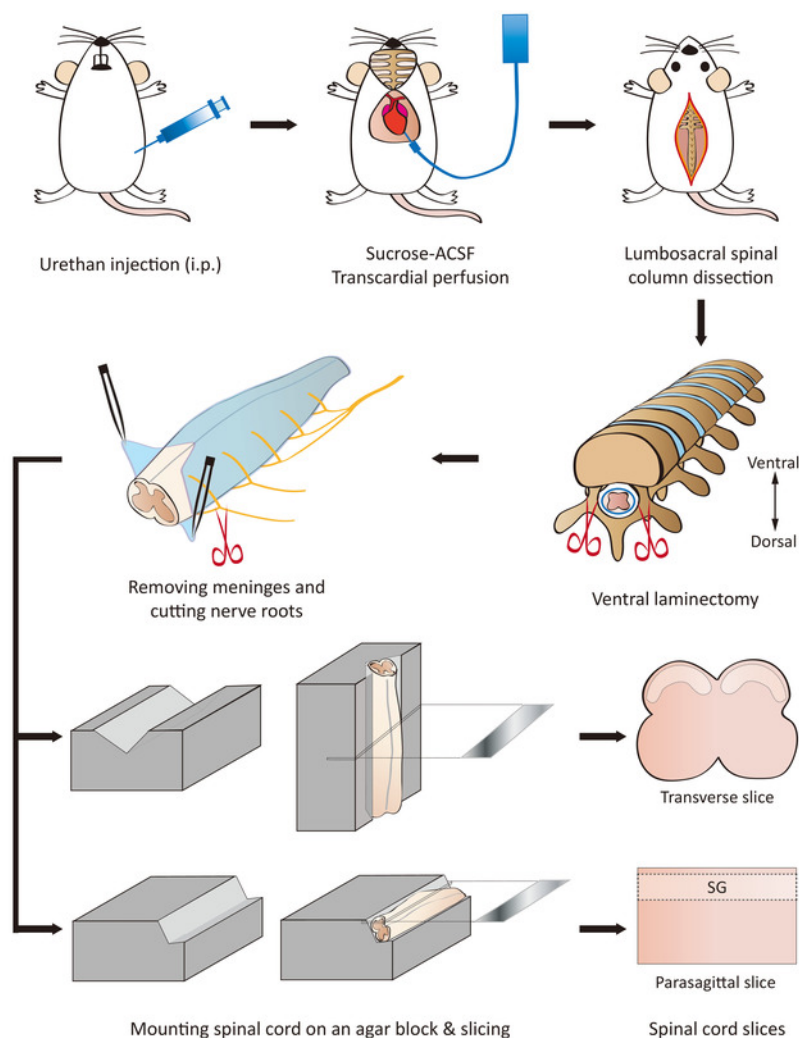


Figure 1: Diagram for acute spinal cord slice preparation. After being deeply anesthetized with urethan (i.p.), rats are transcardially perfused with ice-cold carbogenated sucrose-ACSF. The spinal column is then quickly dissected, and a ventral laminectomy is performed. The meninges, pia-arachnoid membrane and attached spinal nerve roots are removed. Then, the spinal cord specimen is mounted on an agarose block. Transverse or parasagittal slices are cut with a vibratome as needed. [Please click here to view a larger version of this figure.](#)

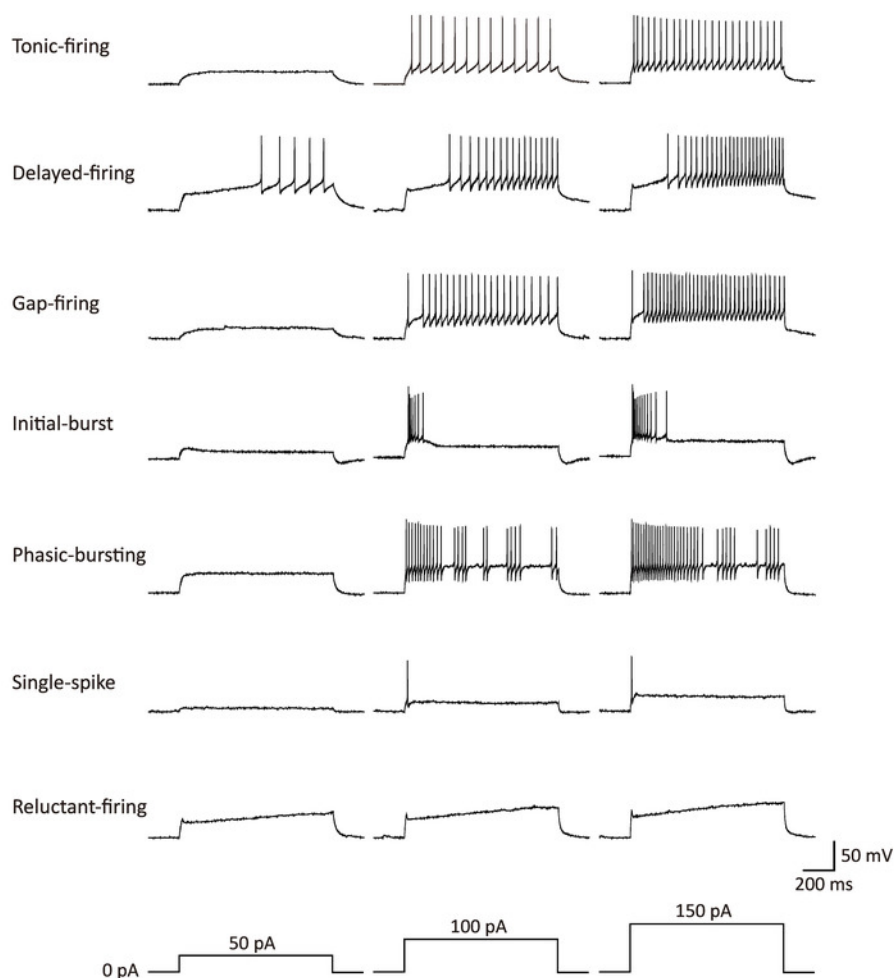


Figure 2: Firing patterns of SG neurons. Firing patterns are determined by injecting a series of 1 s depolarizing current pulses into an SG neuron at RMP. The firing patterns may be classified as tonic-firing, delayed-firing, gap-firing, initial-burst, phasic-bursting, single-spike, and reluctant-firing. [Please click here to view a larger version of this figure.](#)

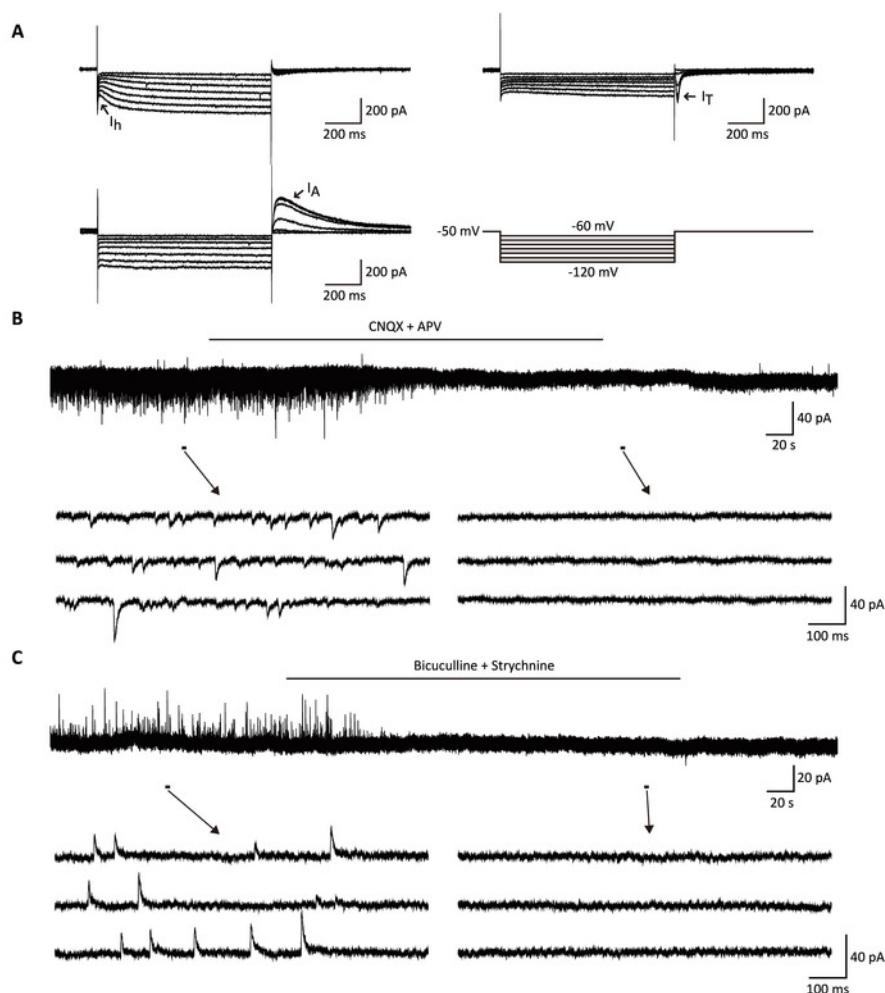


Figure 3: Voltage-clamp recordings in SG neurons. (A) Representative traces showing the response to hyperpolarizing current injection classified as I_h , I_A and I_T . The lower panel shows the evoking protocol for sub-threshold currents in voltage-clamp. (B) Representative traces of sEPSCs recorded from SG neurons at -70 mV in the absence and presence of 50 μ M APV and 20 μ M CNQX. Lower consecutive traces, which are shown in an expanded time scale before (left) and under (right) the action of APV and CNQX, correspond to a period indicated by a bar shown below the chart recording. (C) Representative traces of sIPSCs recorded from SG neurons in the absence and presence of 10 μ M bicuculline and 1 μ M strychnine at 0 mV. [Please click here to view a larger version of this figure.](#)

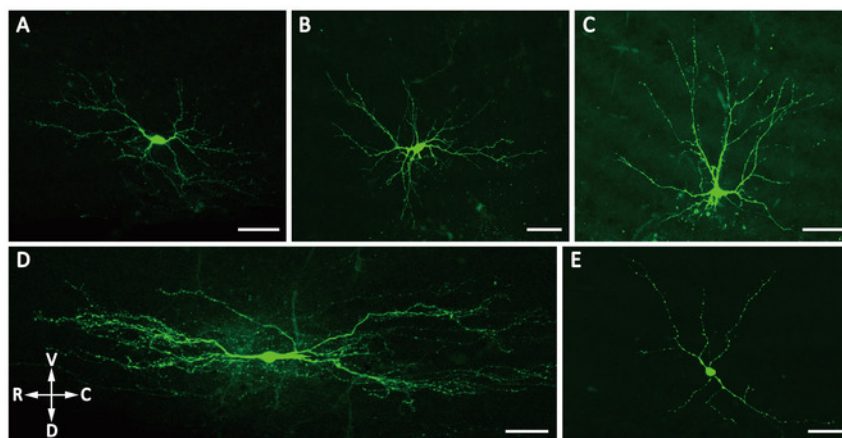


Figure 4: Representative morphology of rat SG neurons. According to soma sizes and dendrite properties shown in confocal microscopy images, SG neurons may be classified as the central cell (A), radial cell (B), vertical cell (C), islet cell (D) and unclassified cell (E). V: ventral; D: dorsal; R: rostral; C: caudal. Scale bar = 50 μ m. [Please click here to view a larger version of this figure.](#)

Component	Molecular Weight	Concentration (mM)	g/L
NaCl	58.5	117	6.84
KCl	74.5	3.6	0.27
NaH ₂ PO ₄ ·2H ₂ O	156	1.2	0.19
CaCl ₂ ·2H ₂ O	147	2.5	0.37
MgCl ₂ ·6H ₂ O	203	1.2	0.24
NaHCO ₃	84	25	2.1
D-Glucose	180	11	1.98
Ascorbic acid	198.11	0.4	0.08
Sodium pyruvate	110	2	0.22

Table 1: Recipe for ACSF.

Component	Molecular Weight	Concentration (mM)	g/L
NaCl	58.5	117	6.84
KCl	74.5	3.6	0.27
NaH ₂ PO ₄ ·2H ₂ O	156	1.2	0.19
CaCl ₂ ·2H ₂ O	147	2.5	0.37
MgCl ₂ ·6H ₂ O	203	1.2	0.24
NaHCO ₃	84	25	2.1
D-Glucose	180	11	1.98
Ascorbic acid	198.11	0.4	0.08
Sodium pyruvate	110	2	0.22

Table 2: Recipe for sucrose-ACSF.

Component	Molecular Weight	Concentration (mM)	mg/100 mL
K-gluconate	234.2	130	3044.6
KCl	74.5	5	37.28
Na ₂ -Phosphocreatine	453.38	10	453.38
EGTA	380.35	0.5	19.02
HEPES	238.31	10	238.3
Mg-ATP	507.18	4	202.9
Li-GTP	523.18	0.3	15.7

Table 3: Recipe for K⁺-based intracellular solution.

Component	Molecular Weight	Concentration (mM)	mg/100 mL
CsMeSO ₄	228	92	2097.6
CsCl	168.36	43	723.95
Na ₂ -Phosphocreatine	453.38	10	453.38
TEA-Cl	165.71	5	82.86
EGTA	380.35	0.5	19.02
HEPES	238.31	10	238.3
Mg-ATP	507.18	4	202.9
Li-GTP	523.18	0.3	15.7

Table 4: Recipe for Cs⁺-based intracellular solution.

Discussion

This protocol details the steps for preparing spinal cord slices, which we have used successfully when performing whole-cell patch-clamp experiments on SG neurons^{18,19,20,21}. By implementing this method, we recently reported that minocycline, a second generation of tetracycline, could markedly enhance inhibitory synaptic transmission through a presynaptic mechanism in SG neurons¹⁹. In addition, this agent could decrease the amplitude of I_h and further inhibit the excitability of SG neurons²¹. In support of these published data and the representative results that we show here, the currently described method is suitable for use in a wide range of electrophysiological studies.

As we noted previously, transcardial perfusion is a crucial element for obtaining healthy specimens. First, we use ice-cold solution for perfusion so the spinal cord can be rapidly cooled and the neuronal metabolism can be slowed²². Second, sucrose-substituted ACSF, a 'protective cutting' solution with low Na⁺ concentration, can ameliorate passive Na⁺ influx and thus decrease neuronal edema through water entry²³. Third, it is beneficial to obtain and analyze neuronal morphology because perfusion could minimize the background caused by biocytin²². For successful preparations, it is also important to use some antioxidants to reduce oxidative damage, which allows neuronal preservation²⁴. Hence, in our protocol, we supplement ascorbic acid and sodium pyruvate, which are powerful antioxidants and can ameliorate edema in spinal cord slices effectively, in both ACSF and sucrose-ACSF. Also, in our experience, we can obtain healthy spinal cord slices successfully from neonatal as well as 3–10 weeks old SD rats. Thus, for this protocol to be successful, we recommend using SD rats that are less than 10 weeks old.

While performing 'ventral' laminectomy and removing the meninges and spinal nerves, one should be patient and careful to avoid cutting, stretching or splitting the spinal cord. In some studies, spinal cord slices with attached dorsal roots have been used to evaluate the synaptic transmission SG neurons received peripherally^{25,26}. The procedure of removing pia-arachnoid membrane is of technical difficulty in this case, and it requires a lot of patience.

This slice preparing technique also has some limitations. One clear drawback is that although acute slices preserve abundant synaptic connections, it could not reflect the real state and address what exactly happens *in vivo*. Thus, some studies have implemented *in vivo* recordings that are normally performed 'blind'^{27,28,29}. However, this *in vivo* approach is technically challenging, and it is difficult to tell whether a recording is performed from the soma or dendrite without sufficient experience. Another limitation of our current method is that sucrose-ACSF may not be sufficient for neuronal preservation when preparing slices from aging rodents. An updated approach using N-methyl-D-glucamine as a Na⁺ substitute has been proposed, and this optimized methodology could markedly improve morphological and functional preservation of neurons in acute slices^{30,31,32,33}. Finally, SG neurons show different morphological and electrophysiological properties³. It seems difficult to interpret data obtained from whole-cell recordings while overlooking the heterogeneity. This limitation may be sidestepped by further verifying the morphological details of recorded neurons⁵ or using transgenic mice, which could help researchers identify specific neurons^{20,34}. Furthermore, optogenetics, a novel tool allowing control of a sub-population of cells³⁵, could be combined with whole-cell patch-clamp recording to study the role of specific ion channels or proteins and to investigate specific neuronal circuitry.

Overall, this preparation technique is an ideal way to investigate the electrophysiological, morphological, pharmacological, and biological characteristics of SG neurons, complemented by patch-clamp recording, immunofluorescent staining, specific agonists or antagonists, and the single-cell RT-PCR technique. Moreover, this approach can be applied together with paired patch-clamp recordings or optogenetics, and it is thus a valuable tool for illuminating the neuronal microcircuits.

Disclosures

The authors declare no conflicts of interest.

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References

1. Todd, A.J. Neuronal circuitry for pain processing in the dorsal horn. *Nature Reviews Neuroscience*. **11** (12), 823-836 (2010).
2. Yoshimura, M. and Nishi, S. Blind patch-clamp recordings from substantia gelatinosa neurons in adult rat spinal cord slices: pharmacological properties of synaptic currents. *Neuroscience*. **53** (2), 519-526 (1993).
3. Maxwell, D.J., Belle, M.D., Cheunsuang, O., Stewart, A. and Morris, R. Morphology of inhibitory and excitatory interneurons in superficial laminae of the rat dorsal horn. *The Journal of Physiology*. **584** (Pt 2), 521-533 (2007).
4. Grudt, T.J. and Perl, E.R. Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. *The Journal of Physiology*. **540** (Pt 1), 189-207 (2002).
5. Lu, Y., et al. A feed-forward spinal cord glycinergic neural circuit gates mechanical allodynia. *Journal of Clinical Investigation*. **123** (9), 4050-4062 (2013).
6. Zheng, J., Lu, Y. and Perl, E.R. Inhibitory neurones of the spinal substantia gelatinosa mediate interaction of signals from primary afferents. *The Journal of Physiology*. **588** (Pt 12), 2065-2075 (2010).
7. Balasubramanyan, S., Stemkowski, P.L., Stebbing, M.J. and Smith, P.A. Sciatic chronic constriction injury produces cell-type-specific changes in the electrophysiological properties of rat substantia gelatinosa neurons. *Journal of Neurophysiology*. **96** (2), 579-590 (2006).
8. Zhang, L., et al. Extracellular signal-regulated kinase (ERK) activation is required for itch sensation in the spinal cord. *Molecular Brain*. **7**, 25 (2014).
9. Kopach, O., et al. Inflammation alters trafficking of extrasynaptic AMPA receptors in tonically firing lamina II neurons of the rat spinal dorsal horn. *Pain*. **152** (4), 912-923 (2011).
10. Takasu, K., Ono, H. and Tanabe, M. Spinal hyperpolarization-activated cyclic nucleotide-gated cation channels at primary afferent terminals contribute to chronic pain. *Pain*. **151** (1), 87-96 (2010).
11. Iura, A., Takahashi, A., Hakata, S., Mashimo, T. and Fujino, Y. Reductions in tonic GABAergic current in substantia gelatinosa neurons and GABAA receptor delta subunit expression after chronic constriction injury of the sciatic nerve in mice. *European Journal of Pain*. **20** (10), 1678-1688 (2016).
12. Alles, S.R., et al. Peripheral nerve injury increases contribution of L-type calcium channels to synaptic transmission in spinal lamina II: Role of alpha2delta-1 subunits. *Molecular Pain*. **14**, 1-12 (2018).
13. Santos, S.F., Rebelo, S., Derkach, V.A. and Safronov, B.V. Excitatory interneurons dominate sensory processing in the spinal substantia gelatinosa of rat. *The Journal of Physiology*. **581** (Pt 1), 241-254 (2007).
14. Lu, Y. and Perl, E.R. Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (laminae I and II). *The Journal of Neuroscience*. **25** (15), 3900-3907 (2005).
15. Hantman, A.W., van den Pol, A.N. and Perl, E.R. Morphological and physiological features of a set of spinal substantia gelatinosa neurons defined by green fluorescent protein expression. *The Journal of Neuroscience*. **24** (4), 836-842 (2004).
16. Yasaka, T., Tiong, S.Y., Hughes, D.I., Riddell, J.S. and Todd, A.J. Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. *Pain*. **151** (2), 475-488 (2010).
17. Yin, H., Park, S.A., Han, S.K. and Park, S.J. Effects of 5-hydroxytryptamine on substantia gelatinosa neurons of the trigeminal subnucleus caudalis in immature mice. *Brain Research*. **1368**, 91-101 (2011).
18. Hu, T., et al. Lidocaine Inhibits HCN Currents in Rat Spinal Substantia Gelatinosa Neurons. *Anesthesia and Analgesia*. **122** (4), 1048-1059 (2016).
19. Peng, H.Z., Ma, L.X., Lv, M.H., Hu, T. and Liu, T. Minocycline enhances inhibitory transmission to substantia gelatinosa neurons of the rat spinal dorsal horn. *Neuroscience*. **319**, 183-193 (2016).
20. Peng, S.C., et al. Contribution of presynaptic HCN channels to excitatory inputs of spinal substantia gelatinosa neurons. *Neuroscience*. **358**, 146-157 (2017).
21. Liu, N., Zhang, D., Zhu, M., Luo, S. and Liu, T. Minocycline inhibits hyperpolarization-activated currents in rat substantia gelatinosa neurons. *Neuropharmacology*. **95**, 110-120 (2015).
22. Moyer, J.R., Jr. and Brown, T.H. Methods for whole-cell recording from visually preselected neurons of perirhinal cortex in brain slices from young and aging rats. *Journal of Neuroscience Methods*. **86** (1), 35-54 (1998).
23. Rothman, S.M. The neurotoxicity of excitatory amino acids is produced by passive chloride influx. *The Journal of Neuroscience*. **5** (6), 1483-1489 (1985).
24. Rice, M.E. Use of ascorbate in the preparation and maintenance of brain slices. *Methods*. **18** (2), 144-149 (1999).
25. Takasu, K., Ogawa, K., Minami, K., Shinohara, S. and Kato, A. Injury-specific functional alteration of N-type voltage-gated calcium channels in synaptic transmission of primary afferent C-fibers in the rat spinal superficial dorsal horn. *European Journal of Pharmacology*. **772**, 11-21 (2016).
26. Tian, L., et al. Excitatory synaptic transmission in the spinal substantia gelatinosa is under an inhibitory tone of endogenous adenosine. *Neuroscience Letters*. **477** (1), 28-32 (2010).
27. Funai, Y., et al. Systemic dexmedetomidine augments inhibitory synaptic transmission in the superficial dorsal horn through activation of descending noradrenergic control: an *in vivo* patch-clamp analysis of analgesic mechanisms. *Pain*. **155** (3), 617-628 (2014).
28. Yamasaki, H., Funai, Y., Funao, T., Mori, T. and Nishikawa, K. Effects of tramadol on substantia gelatinosa neurons in the rat spinal cord: an *in vivo* patch-clamp analysis. *PLoS One*. **10** (5), e0125147 (2015).

29. Furue, H., Narikawa, K., Kumamoto, E. and Yoshimura, M. Responsiveness of rat substantia gelatinosa neurones to mechanical but not thermal stimuli revealed by *in vivo* patch-clamp recording. *The Journal of Physiology*. **521** (Pt 2), 529-535 (1999).
30. Ting, J.T., Daigle, T.L., Chen, Q. and Feng, G. Acute brain slice methods for adult and aging animals: application of targeted patch clamp analysis and optogenetics. *Methods in Molecular Biology*. **1183**, 221-242 (2014).
31. Ting, J.T., *et al.* Preparation of Acute Brain Slices Using an Optimized N-Methyl-D-glucamine Protective Recovery Method. *Journal of Visualized Experiments*. (132), e53825 (2018).
32. Li, J. and Baccei, M.L. Neonatal Tissue Damage Promotes Spike Timing-Dependent Synaptic Long-Term Potentiation in Adult Spinal Projection Neurons. *The Journal of Neuroscience*. **36** (19), 5405-5416 (2016).
33. Ford, N.C., Ren, D. and Baccei, M.L. NALCN channels enhance the intrinsic excitability of spinal projection neurons. *Pain*. (2018).
34. Cui, L., *et al.* Modulation of synaptic transmission from primary afferents to spinal substantia gelatinosa neurons by group III mGluRs in GAD65-EGFP transgenic mice. *Journal of Neurophysiology*. **105** (3), 1102-1111 (2011).
35. Yang, K., Ma, R., Wang, Q., Jiang, P. and Li, Y.Q. Optoactivation of parvalbumin neurons in the spinal dorsal horn evokes GABA release that is regulated by presynaptic GABAB receptors. *Neuroscience Letters*. **594**, 55-59 (2015).