

Journal of Visualized Experiments

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

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58479R2
Full Title:	Preparation of Acute Spinal Cord Slices for Whole-cell Patch-clamp Recording in Substantia Gelatinosa Neurons
Keywords:	neuroscience; spinal cord slice; substantia gelatinosa neuron; whole-cell patch-clamp; Electrophysiology; morphology; in vitro
Corresponding Author:	L Dr. Liu
Corresponding Author's Institution:	
Corresponding Author E-Mail:	liutaomm@hotmail.com
Order of Authors:	Mengye Zhu Daying Zhang Sicong Peng Nana Liu Jing Wu Haixia Kuang Tao Liu
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences

TITLE:

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

AUTHORS AND AFFILIATIONS:

Mengye Zhu^{1*}, Daying Zhang^{1*}, Sicong Peng², Nana Liu², Jing Wu², Haixia Kuang², Tao Liu^{2,3}

¹Department of Pain, the First Affiliated Hospital of Nanchang University

²Department of Pediatrics, the First Affiliated Hospital of Nanchang University

³Center for Laboratory Medicine, the First Affiliated Hospital of Nanchang University

*These authors contributed equally.

Corresponding Author:

Tao Liu (liutaomm@hotmail.com, liutao1241@ncu.edu.cn)

Tel: +86-791-88692139

Email Addresses of Co-authors:

Mengye Zhu (zhumengye0403@163.com, menye.zhu@ncu.edu.cn)

Daying Zhang (zdysino@163.com, zdysino@ncu.edu.cn)

Sicong Peng (sicongpeng@163.com)

Nana Liu (liulala1224@163.com)

Jing Wu (448698921@qq.com)

Haixia Kuang (594778316@qq.com)

KEYWORDS:

Neuroscience, spinal cord slice, substantia gelatinosa neuron, whole-cell patch-clamp, electrophysiology, morphology, *in vitro*

SUMMARY:

Here, we describe the essential steps for whole-cell patch-clamp recordings made from substantia gelatinosa (SG) neurons in the *in vitro* spinal cord slice. This method allows the intrinsic membrane properties, synaptic transmission and morphological characterization of SG neurons to be studied.

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.

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 neurons²⁻⁴ 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

1.1. 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

2.1. Solutions

2.1.1. Prepare artificial cerebrospinal fluid (ACSF) (in mM): 117 NaCl, 3.6 KCl, 1.2 NaH₂PO₄·2H₂O, 2.5 CaCl₂·2H₂O, 1.2 MgCl₂·6H₂O, 25 NaHCO₃, 11 D-glucose, 0.4 ascorbic acid, and 2 sodium pyruvate. See **Table 1**.

2.1.2. Prepare sucrose-ACSF (in mM): 240 Sucrose, 2.5 KCl, 1.25 NaH₂PO₄·2H₂O, 0.5 CaCl₂·2H₂O, 3.5 MgCl₂·6H₂O, 25 NaHCO₃, 0.4 Ascorbic acid, and 2 sodium pyruvate. See **Table 2**.

2.1.3. Prepare K⁺-based intracellular solution (in mM): 130 K-gluconate, 5 KCl, 10 Na₂-phosphocreatine, 0.5 EGTA, 10 HEPES, 4 Mg-ATP, and 0.3 Li-GTP. See **Table 3**.

2.1.4. Prepare Cs⁺-based intracellular solution (in mM): 92 CsMeSO₄, 43 CsCl, 10 Na₂-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% O₂ and 5% CO₂ 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).

2.1.5. 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 osmolarity by 1 mOsm.

2.1.6. 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.

2.1.7. 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 Na₂HPO₄, 3 mM NaH₂PO₄). 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.

2.2. Instruments

2.2.1. 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.

2.2.2. 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.

2.3. Micropipettes

2.3.1. Make recording electrodes from borosilicate glass capillaries using a micropipette puller. The typical pipette resistance ranges from 3-6 MΩ when filled with intracellular solution.

2.4. Agar block

2.4.1. 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 described¹⁸⁻²⁰.

3.1. Prior to transcardial perfusion and spinal cord extraction, prepare ~500 mL sucrose-ACSF equilibrated with 95% O₂ and 5% CO₂ and cool the solution to ice-cold (0-4 °C).

3.2. 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**.

3.3. Transcardial perfusion

3.3.1. 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.

3.3.2. 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.

3.3.3. 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.

3.3.4. Hold the heart with another curved forceps gently, and then insert a 22G needle through the left ventricle to the base of the aortic arch.

3.3.5. 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.

3.4. Spinal cord dissection and slicing

3.4.1. 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.

3.4.2. 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.

3.4.3. 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.

3.4.4. Remove the meninges and the pia-arachnoid membrane under a dissecting microscope. Cut all the ventral and dorsal roots away as quickly as possible.

3.4.5. 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.

3.4.6. 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

4.1. 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.

4.2. 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.

4.3. 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.

4.4. 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.

4.5. 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.

4.6. 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.

4.7. 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.

4.8. Recordings of intrinsic membrane properties

4.8.1. 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.

4.8.2. 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.

4.8.3. 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.

4.8.4. Record excitatory postsynaptic currents (EPSCs): Record EPSCs with the K^+ -based intracellular solution in voltage-clamp mode at a holding potential of -70 mV.

4.8.5. 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

5.1. For morphological experiments, use a K^+ -based intracellular solution containing 0.05% neurobiotin 488.

5.2. 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.

5.3. 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.

5.4. 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. **Figures 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 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 AND TABLE LEGENDS:

Figure 1. Diagram for acute spinal cord slice preparation. After being deeply anesthetized with urethane (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.

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.

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.

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.

Table 1. Recipe for ACSF.

Table 2. Recipe for sucrose-ACSF.

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

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 neurons¹⁸⁻²¹. 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'²⁷⁻²⁹. 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 slices³⁰⁻³³. 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.

ACKNOWLEDGMENTS:

This work was supported by grants from the National Natural Science Foundation of China (No. 81560198, 31660289).

DISCLOSURES:

The authors declare no conflicts of interest.

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).

485 16. Yasaka, T., Tiong, S.Y., Hughes, D.I., Riddell, J.S. and Todd, A.J. Populations of inhibitory and
486 excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined
487 electrophysiological and anatomical approach. *Pain*. **151** (2), 475-488 (2010).

488 17. Yin, H., Park, S.A., Han, S.K. and Park, S.J. Effects of 5-hydroxytryptamine on substantia
489 gelatinosa neurons of the trigeminal subnucleus caudalis in immature mice. *Brain Research*.
490 **1368**, 91-101 (2011).

491 18. Hu, T., *et al.* Lidocaine Inhibits HCN Currents in Rat Spinal Substantia Gelatinosa Neurons.
492 *Anesthesia and Analgesia*. **122** (4), 1048-1059 (2016).

493 19. Peng, H.Z., Ma, L.X., Lv, M.H., Hu, T. and Liu, T. Minocycline enhances inhibitory transmission
494 to substantia gelatinosa neurons of the rat spinal dorsal horn. *Neuroscience*. **319**, 183-193 (2016).

495 20. Peng, S.C., *et al.* Contribution of presynaptic HCN channels to excitatory inputs of spinal
496 substantia gelatinosa neurons. *Neuroscience*. **358**, 146-157 (2017).

497 21. Liu, N., Zhang, D., Zhu, M., Luo, S. and Liu, T. Minocycline inhibits hyperpolarization-activated
498 currents in rat substantia gelatinosa neurons. *Neuropharmacology*. **95**, 110-120 (2015).

499 22. Moyer, J.R., Jr. and Brown, T.H. Methods for whole-cell recording from visually preselected
500 neurons of perirhinal cortex in brain slices from young and aging rats. *Journal of Neuroscience*
501 *Methods*. **86** (1), 35-54 (1998).

502 23. Rothman, S.M. The neurotoxicity of excitatory amino acids is produced by passive chloride
503 influx. *The Journal of Neuroscience*. **5** (6), 1483-1489 (1985).

504 24. Rice, M.E. Use of ascorbate in the preparation and maintenance of brain slices. *Methods*. **18**
505 (2), 144-149 (1999).

506 25. Takasu, K., Ogawa, K., Minami, K., Shinohara, S. and Kato, A. Injury-specific functional
507 alteration of N-type voltage-gated calcium channels in synaptic transmission of primary afferent
508 C-fibers in the rat spinal superficial dorsal horn. *European Journal of Pharmacology*. **772**, 11-21
509 (2016).

510 26. Tian, L., *et al.* Excitatory synaptic transmission in the spinal substantia gelatinosa is under an
511 inhibitory tone of endogenous adenosine. *Neuroscience Letters*. **477** (1), 28-32 (2010).

512 27. Funai, Y., *et al.* Systemic dexmedetomidine augments inhibitory synaptic transmission in the
513 superficial dorsal horn through activation of descending noradrenergic control: an *in vivo* patch-
514 clamp analysis of analgesic mechanisms. *Pain*. **155** (3), 617-628 (2014).

515 28. Yamasaki, H., Funai, Y., Funao, T., Mori, T. and Nishikawa, K. Effects of tramadol on substantia
516 gelatinosa neurons in the rat spinal cord: an *in vivo* patch-clamp analysis. *PLoS One*. **10** (5),
517 e0125147 (2015).

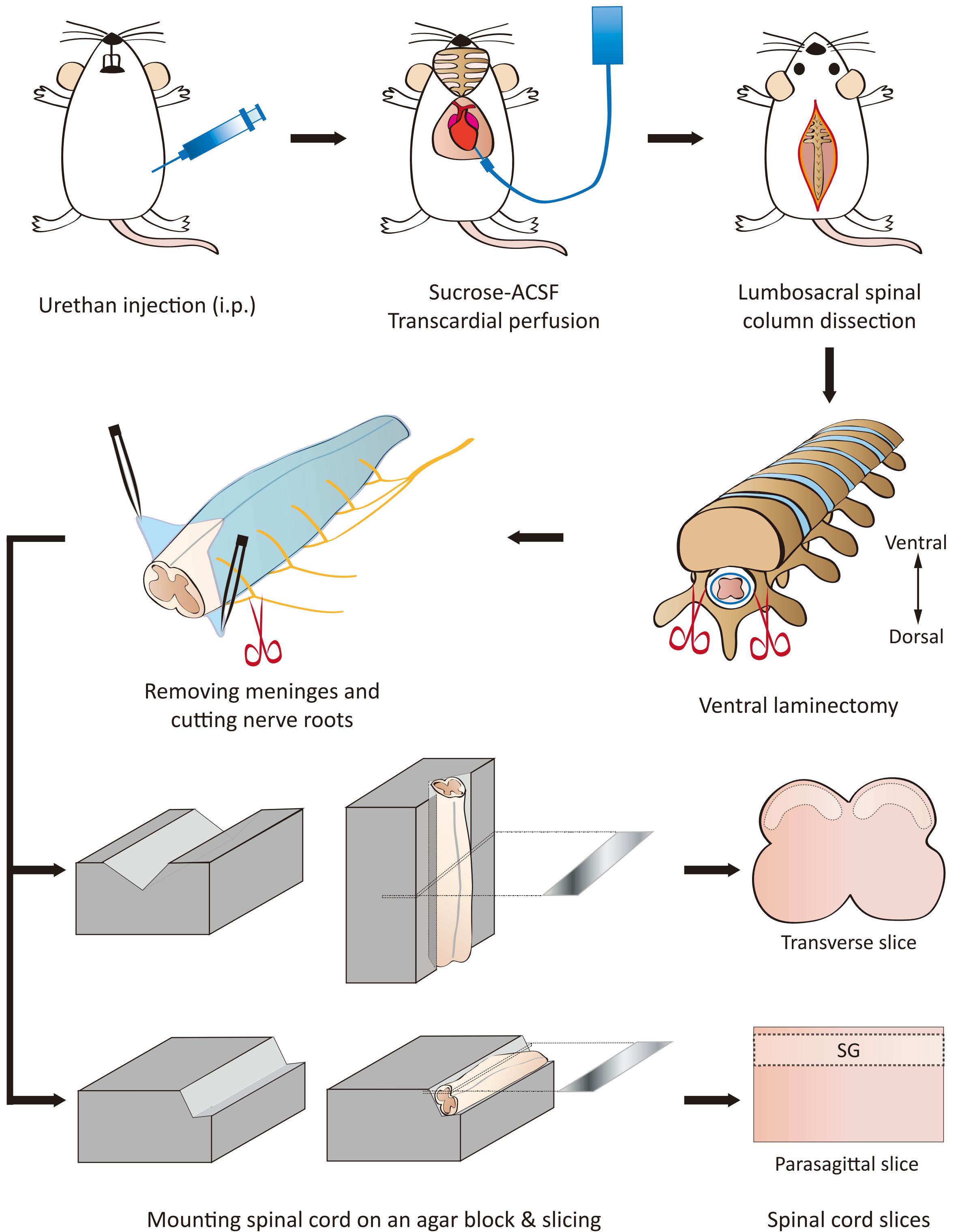
518 29. Furue, H., Narikawa, K., Kumamoto, E. and Yoshimura, M. Responsiveness of rat substantia
519 gelatinosa neurones to mechanical but not thermal stimuli revealed by *in vivo* patch-clamp
520 recording. *The Journal of Physiology*. **521** (Pt 2), 529-535 (1999).

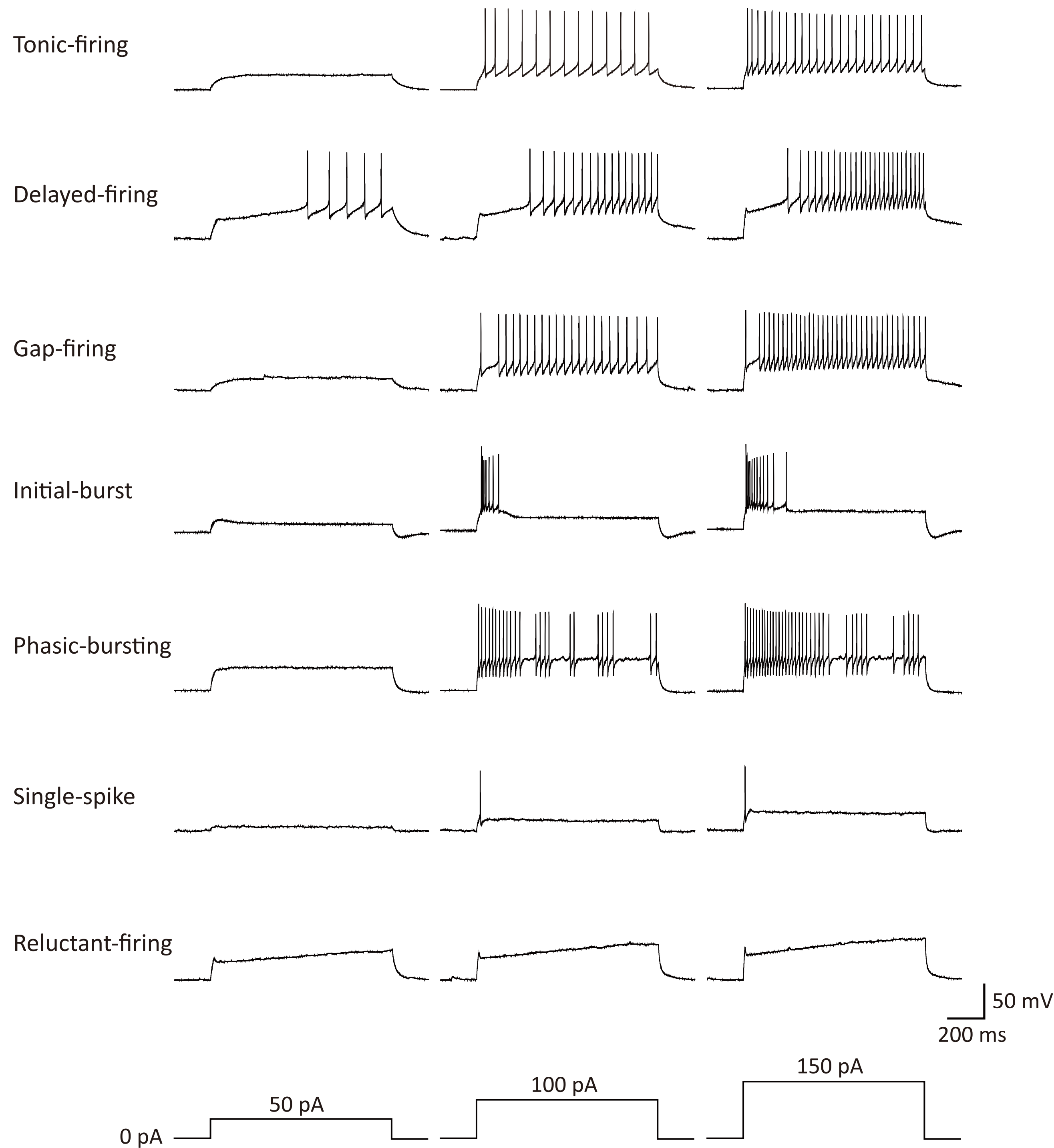
521 30. Ting, J.T., Daigle, T.L., Chen, Q. and Feng, G. Acute brain slice methods for adult and aging
522 animals: application of targeted patch clamp analysis and optogenetics. *Methods in Molecular*
523 *Biology*. **1183**, 221-242 (2014).

524 31. Ting, J.T., *et al.* Preparation of Acute Brain Slices Using an Optimized N-Methyl-D-glucamine
525 Protective Recovery Method. *Journal of Visualized Experiments*. (132), e53825,
526 doi:10.3791/53825 (2018).

527 32. Li, J. and Baccei, M.L. Neonatal Tissue Damage Promotes Spike Timing-Dependent Synaptic
528 Long-Term Potentiation in Adult Spinal Projection Neurons. *The Journal of Neuroscience*. **36** (19),

529 5405-5416 (2016).
530 33. Ford, N.C., Ren, D. and Baccei, M.L. NALCN channels enhance the intrinsic excitability of spinal
531 projection neurons. *Pain*. doi: 10.1097/j.pain.0000000000001258 (2018).
532 34. Cui, L., *et al.* Modulation of synaptic transmission from primary afferents to spinal substantia
533 gelatinosa neurons by group III mGluRs in GAD65-EGFP transgenic mice. *Journal of*
534 *Neurophysiology*. **105** (3), 1102-1111 (2011).
535 35. Yang, K., Ma, R., Wang, Q., Jiang, P. and Li, Y.Q. Optoactivation of parvalbumin neurons in the
536 spinal dorsal horn evokes GABA release that is regulated by presynaptic GABAB receptors.
537 *Neuroscience Letters*. **594**, 55-59 (2015).
538





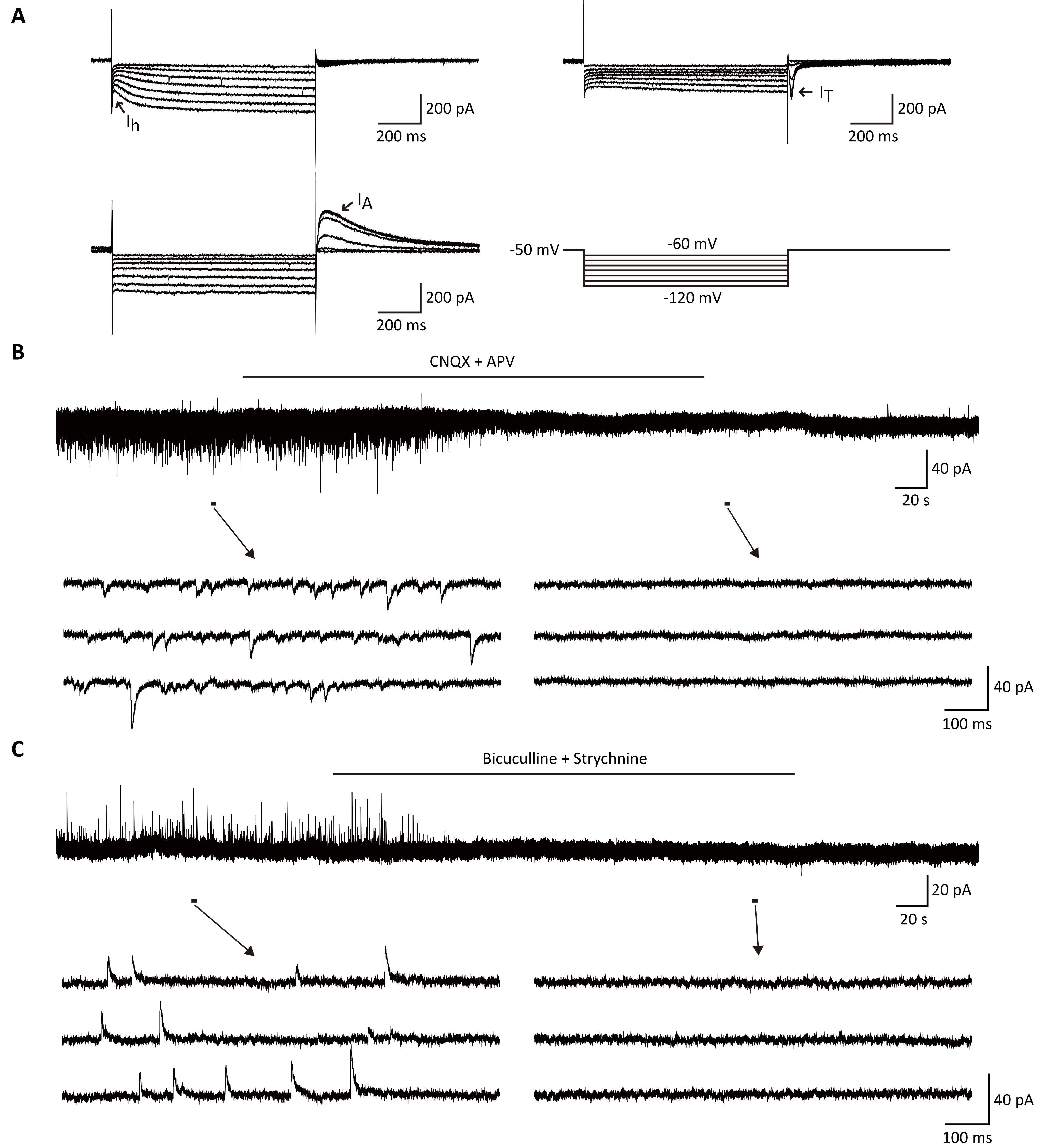
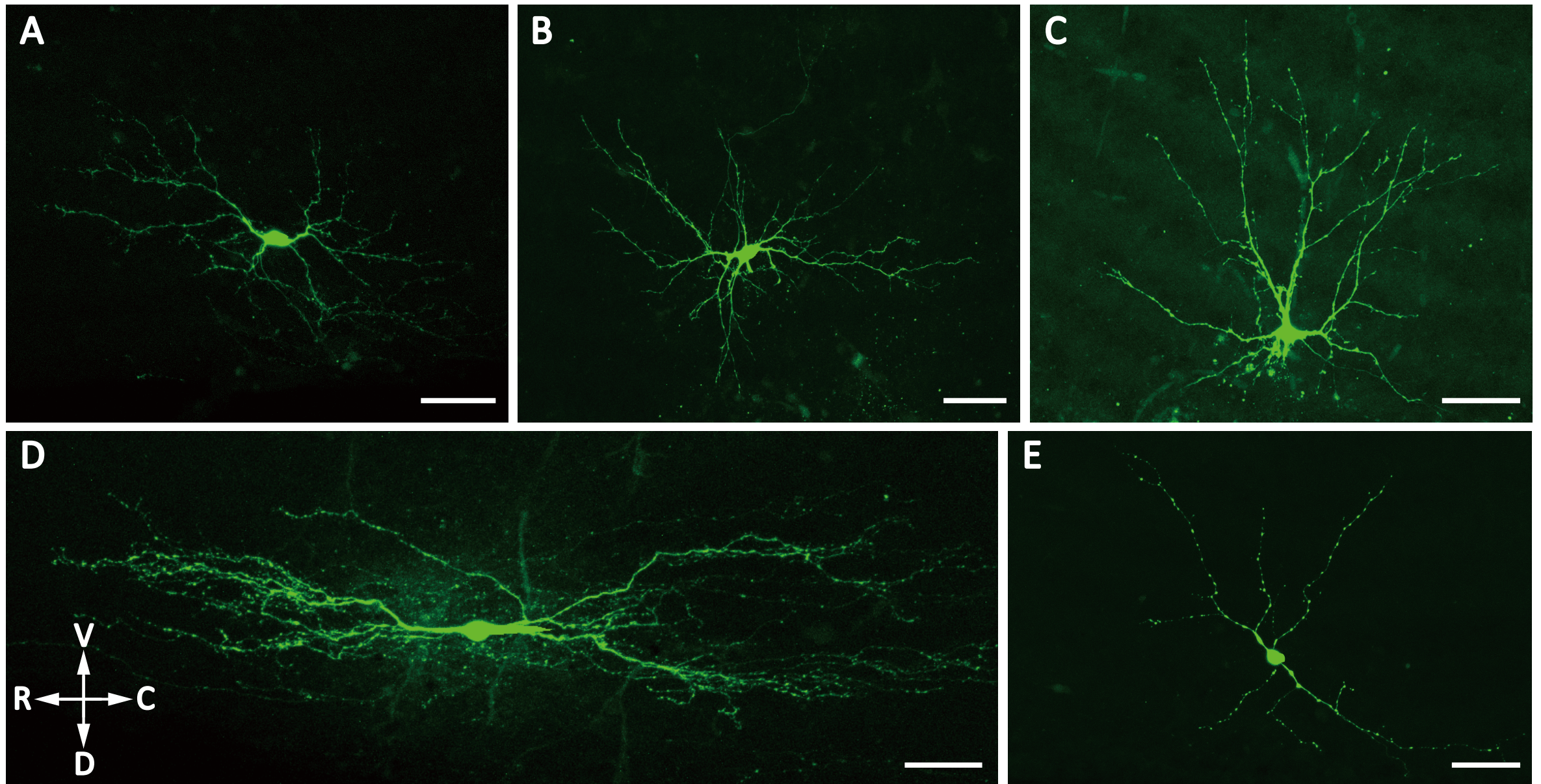


Figure 4

[Click here to download Figure Figure 4. Representative morphology of rat SG neurons.ai](#) 



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

Component	Molecular Weight	Concentration (mM)	g/L
Sucrose	342.3	240	82.15
KCl	74.5	2.5	0.186
NaH ₂ PO ₄ ·2H ₂ O	156	1.25	0.195
CaCl ₂ ·2H ₂ O	147	0.5	0.075
MgCl ₂ ·6H ₂ O	203	3.5	0.71
NaHCO ₃	84	25	2.1
Ascorbic acid	198.11	0.4	0.08
Sodium pyruvate	110	2	0.22

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

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

Name of Material/ Equipment	Company	Catalog Number
NaCl	Sigma	S7653
KCl	Sigma	60130
NaH ₂ PO ₄ ·2H ₂ O	Sigma	71500
CaCl ₂ ·2H ₂ O	Sigma	C5080
MgCl ₂ ·6H ₂ O	Sigma	M2670
NaHCO ₃	Sigma	S5761
D-Glucose	Sigma	G7021
Ascorbic acid	Sigma	P5280
Sodium pyruvate	Sigma	A7631
Sucrose	Sigma	S7903
K-gluconate	Wako	169-11835
Na ₂ -Phosphocreatine	Sigma	P1937
EGTA	Sigma	E3889
HEPES	Sigma	H4034
Mg-ATP	Sigma	A9187
Li-GTP	Sigma	G5884
CsMeSO ₄	Sigma	C1426
CsCl	Sigma	C3011
TEA-Cl	Sigma	T2265
Neurobiotin 488	Vector	SP-1145
Agar	Sigma	A7002
Paraformaldehyde	Sigma	P6148
Na ₂ HPO ₄	Hengxing Chemical Reagents	
Mount Coverslipping Medium	Polyscience	18606
Urethan	National Institute for Food and Drug Control	30191228
Borosilicate glass capillaries	World Precision Instruments	TW150F-4
Micropipette puller	Sutter Instrument	P-97
Vibratome	Leica	VT1000S
Vibration isolation table	Technical Manufacturing Corporation	63544

Infrared CCD camera
Patch-clamp amplifier
Micromanipulator
X-Y stage
Upright microscope
Osmometer
PH meter
Confocal microscope

Dage-MIT
HEKA
Sutter Instrument
Burleigh
Olympus
Advanced
Mettler Toledo
Zeiss

IR-1000
EPC-10
MP-285
GIBRALTAR X-Y
BX51WI
FISKE 210
FE20
LSM 700

Comments/Description

Used for the preparation of ACSF and PBS

Used for the preparation of ACSF, sucrose-ACSF, and K^+ -based intracellular solution

Used for the preparation of ACSF, sucrose-ACSF and PBS

Used for the preparation of ACSF and sucrose-ACSF

Used for the preparation of ACSF and sucrose-ACSF

Used for the preparation of ACSF and sucrose-ACSF

Used for the preparation of ACSF

Used for the preparation of ACSF and sucrose-ACSF

Used for the preparation of ACSF and sucrose-ACSF

Used for the preparation of sucrose-ACSF

Used for the preparation of K^+ -based intracellular solution

Used for the preparation of intracellular solution

Used for the preparation of intracellular solution

Used for the preparation of intracellular solution

Used for the preparation of intracellular solution

Used for the preparation of intracellular solution

Used for the preparation of Cs^+ -based intracellular solution

Used for the preparation of Cs^+ -based intracellular solution

Used for the preparation of Cs^+ -based intracellular solution

0.05% neurobiotin 488 could be used for morphological studies

3% agar block was used in our protocol

4% paraformaldehyde was used for immunohistochemical processing

Used for the preparation of PBS

1.5 g/kg, i.p.

1.5 mm OD, 1.12 mm ID

Used for the preparation of micropipettes



1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

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

Author(s):

Mengye Zhu, Daying Zhang, Sicong Peng, Nana Liu, Jing Wu, Haixin Kong, Tao Lin

Item 1 (check one box): The Author elects to have the Materials be made available (as described at

<http://www.jove.com/author>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
- ☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
- ☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's

expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Tao Lin		
Department:	Department of Pediatrics		
Institution:	the First Affiliated Hospital of Nanchang University		
Article Title:	Preparation of Acute Spinal Cord Slices for Whole-cell Patch-clamp Recording in Substantia Gelatinosa Neurons		
Signature:	Tao Lin	Date:	2018/5/15

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

July 13, 2018

Dear Dr. Phillip Steindel and Referees,

We thank the editor and the three reviewers again for your constructive and very helpful comments. Accordingly, we made corrections to the text and the figure legends corresponding to the suggestions.

Editorial comments:

Note that some formatting changes have already been made.

1. Per your response to Reviewer 1, could you give more specific details as to how old mice should be for this protocol to be successful?

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 less than 10 weeks old.

2. 3.3.5 (current numbering): Can you indicate how the cord is attached to the agar block (not just how it fits into it)? Superglue?

We apologize for not making this statement clear. We have now added the following description in the text in section 3.3.5: 'use superglue to adhere the spinal cord tissue to the block'.

3. The legend for Figure 3B+C no longer applies to the current figure. Additionally; why did you remove the original 3B+C?

We thank the editor for pointing this out. We have now updated the legend for Figure 3B+C in the revised version as follows '**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.'

As Reviewer 1 suggested us to add traces showing EPSCs and IPSCs could be confirmed with specific blockers, we substituted the original 3B+C with traces showing that EPSCs and IPSCs could be confirmed with APV and CNQX, bicuculline and strychnine, respectively.