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Use of In Vivo Single-Fiber Recording and Intact Dorsal Root Ganglion with Attached Sciatic Nerve to Examine the Mechanism of Conduction Failure

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We are enclosing here the revision for manuscript (JOVE59234) which is titled "Use of In Vivo Single-fiber Recording and Intact DRG with attached Sciatic Nerve to Examine the Mechanism of Conduction Failure" by Honghui Mao, Xiuchao Wang, Wen Chen, FengYu Liu, You Wan, Sanjue Hu, Junling Xing, in which three authors (Wen Chen, FengYu Liu, You Wan) were added because of the manuscript revision and preparation for the filming of the experiment procedure. The addition of the author has been approved by all authors and Dr. Ronald Myers (Science Editor, JoVE)

We have prepared a response Letter describing how we responded to each point of concern raised by the reviewers. In the new manuscript, about 2 pages of the contents that identifies the essential steps of the protocol for the video were highlighted.

We would be grateful if the manuscript could be considered for publication in JoVE. Thanks a lot!

Best wishes.

Yours sincerely,

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TITLE:

Use of In Vivo Single-Fiber Recording and Intact Dorsal Root Ganglion with Attached Sciatic Nerve to Examine the Mechanism of Conduction Failure

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KEYWORDS:

single-fiber recording, polymodal nociceptive C-fibers, dorsal root ganglion (DRG), conduction failure, after-hyperpolarization potential (AHP), analgesic

SUMMARY:

Single-fiber recording is an effective electrophysiological technique that is applicable to the central and peripheral nervous systems. Along with the preparation of intact DRG with the attached sciatic nerve, the mechanism of conduction failure is examined. Both protocols improve the understanding of the peripheral nervous system's relationship with pain.

ABSTRACT:

Single-fiber recording has been a classical and effective electrophysiological technique over the last few decades because of its specific application for nerve fibers in the central and peripheral nervous systems. This method is particularly applicable to dorsal root ganglia (DRG), which are primary sensory neurons that exhibit a pseudo-unipolar structure of nervous processes. The patterns and features of the action potentials passed along axons are recordable in these neurons. The present study uses in vivo single-fiber recordings to observe the conduction failure of sciatic nerves in complete Freund's adjuvant (CFA)-treated rats. As the underlying mechanism cannot be studied using in vivo single-fiber recordings, patch-clamp-recordings of DRG neurons are performed on preparations of intact DRG with the attached sciatic nerve. These recordings reveal a positive correlation between conduction failure and the rising slope of the after-hyperpolarization potential (AHP) of DRG neurons in CFA-treated animals. The protocol for in vivo single fiber-recordings allows the classification of nerve fibers via the measurement of conduction velocity and monitoring of abnormal conditions in nerve fibers in certain diseases. Intact DRG with attached peripheral nerve allows observation of the activity of DRG neurons in most physiological conditions. Conclusively, single-fiber recording combined with electrophysiological recording of intact DRGs is an effective method to examine the role of conduction failure during the analgesic process.

INTRODUCTION:

The normal transmission of information along nerve fibers guarantees the normal function of the nervous system. Abnormal functioning of the nervous system is also reflected in the electrical signal transmission of nerve fibers. For example, the degree of demyelination in central demyelination lesions can be classified via comparison of changes in nerve conduction velocity before and after intervention application[1]. It is difficult to intracellularly record nerve fibers, except in special preparations such as the squid giant axon[2]. Therefore, electrophysiological activity is only recordable via the extracellular recording of single fibers. As one of the classical electrophysiological methods, single-fiber recording has a longer history than other techniques. However, fewer electrophysiologists grasping this method despite its extensive application. Therefore, a detailed introduction of the standard protocol for single-fiber recording is needed for its appropriate application.

Although various patch-clamp techniques have dominated modern electrophysiological study, single-fiber recording still plays an irreplaceable role in recording the activities of nerve fibers, especially fibers transmitting peripheral sensation with their sensory cell body located in dorsal root ganglion (DRG). The advantage of using single-fiber recording here is that in vivo fiber recording provides a long observation time with the capacity to record responses to natural stimuli in preclinical models without disturbance of the intracellular environment[3,4].

An increasing number of studies over the last two decades has examined complex functions along nerve fibers[5], and conduction failure, which is defined as a state of unsuccessful nerve impulse transmission along the axon, was present in many different peripheral nerves[6,7]. The presence of conduction failure in our investigation served as an intrinsic self-inhibitory mechanism for the modulation of persistent nociceptive input along C-fibers[8]. This conduction failure was significantly attenuated under conditions of hyperalgesia[4,9]. Therefore, targeting the

factors involved in conduction failure may represent a new treatment for neuropathic pain. To observe conduction failure, the firing pattern should be recorded and analyzed on the basis of sequentially discharged spikes based on single-fiber recording.

To thoroughly understand the mechanism of conduction failure, it is necessary to identify the transmission properties of the axon, or more precisely, the membrane properties of DRG neurons, based on their pseudo-unipolar anatomical properties. Many previous studies in this field have been performed on dissociated DRG neurons[10,11], which may not be feasible for the investigation of conduction failure due to two obstacles. First, various mechanical and chemical methods are used in the dissociation process to free DRG neurons, which may result in unhealthy cells or alter the phenotype/properties of the neurons and confound the findings. Second, the attached peripheral nerves are basically removed, and conduction failure phenomena are not observable in these preparations. Therefore, a preparation of intact DRG neurons with an attached nerve has been improved to avoid the abovementioned obstacles.

PROTOCOL:

The current protocol followed the Guide for United States Public Health Service's Policy on the Humane Care and Use of Laboratory Animals, and the Committee on the Ethics of Animal Experiments of the Fourth Military Medical University approved the protocol.

1. Animals

1.1. Divide 24 Sprague-Dawley rats (4−8 weeks old) into two groups. Produce complete Freund's adjuvant (CFA) model by intraplantar injection of 100 µL of CFA in one group of 14 rats and another group of 10 rats by treatment with saline.

NOTE: All of the animals were acquired from the Animal Center of the Fourth Military Medical University. Adult male and female Sprague-Dawley rats (150−200 g) were used for all procedures, and rats were randomly assigned to cages. Two rats were housed per cage under a 12-/12-hour light/dark cycle at a constant temperature (25 ± 1 °C) with free access to food and water.

2. In vivo single-fiber recording

2.1. Prepare and disinfect all surgical instruments (scalpel, tweezers, ophthalmic scissors, shearing scissors, glass separating needle, suture needle, bone rongeur) prior to surgery. Prepare 1 L or 2 L of normal Ringer's extracellular solution (in mM: NaCl 124, KCl 3, $MgSO_4$ 1.3, $CaCl_2$ 2, $NaHCO_3$ 26, NaH_2PO_4 1.25, glucose 15; pH 7.4 and 305 mOsm). Store at 4 °C until use.

2.2. Anesthetize rats. Use intraperitoneal injection of a mixed solution (1% chloralose and 17% urethane, 5 mL/kg body weight) to keep the animals in a stable aesthetic condition during the experiment. Apply supplementary injection of anesthetics, if necessary, after checking pupils and the response to pain stimulation. Monitor and maintain a body temperature near 37 °C.

2.3. Exposure of sciatic nerve trunk for recording

2.3.1. Cut open the skin and muscle on the dorsal part of the thigh. Perform a blunt dissection along femoral biceps. Carefully isolate the sciatic nerve trunk using ophthalmic scissors and a glass separation needle. Keep the tissue wet using Ringer's solution.

2.3.2. Fix the animal on a homemade metal hoop (3 cm long, 2 mm wide metal hoop with an iron wire 1 mm in diameter) via sewing the skin into the slot around it. Pull the skin up slightly so as to establish a fluid bath.

2.3.3. Expose 1 cm of sciatic nerve trunk at the proximal side. Place a small brown platform under the nerve trunk to enhance the contrast and observe the fine nerve trunk clearly. Heat liquid paraffin in a water bath to 37 °C and drop it on the top of the nerve trunk to prevent drying of the surface of the fiber. Remove the pia mater spinalis and dura mater around the sciatic nerve.

2.4. Recording session

2.4.1. Select a platinum filament (29 µm in diameter) as the recording electrode. Heat over for easier molding, and create a small hook at the very end. Attach the electrode to a micromanipulator to move the electrode as required.

2.4.2. In the bath, place a reference electrode in adjacent subcutaneous tissue. Split the spinal dura and the pia mater. Separate the sciatic nerve into a single fiber (15−20 µm in diameter) in the recording pool. Then, pick up a fine fascicle of axon and suspend the proximal end of the axon on the hook of the recording electrode under a stereoscope at 25x magnification.

NOTE: The just-dissected filaments tend to be thicker and require further separation until a single unit may be recorded.

2.4.3. Identify the receptive field of a single nociceptive C-fiber using a mechanical stimulus (Von Frey hairs) and thermal stimulus (small cotton ball with 50−55 °C water). Briefly, if the firing of nerve fiber respond to the mechanical stimuli and hot water, then consider it as a polymodal nociceptive C-fiber[4]. Next, insert two needle stimulus electrodes (2 mm interval) into the skin of the identified field for the delivery of electrical stimuli.

2.4.4. Display the waveform of an action potential on oscilloscope and employ a computer A/D board with a signal sampling rate of 20 kHz to amplify and record the spikes.

2.4.5. Collect data using data acquisition software (**Table of Materials**). Save data on a computer and analyze later with professional software (**Table of Materials**).

3. Measurement of conduction failure

3.1. Deliver the repetitive electrical stimuli (0.8 ms duration, 1.5x threshold intensity) in different frequencies (2 Hz, 5 Hz, 10 Hz) to a C-fiber for 60 s[4,8,9]. Allow a 10 min interval for fiber to relax between stimuli. Calculate the ratio of the number of failures to the number of delivered repetitive stimulus pulses and multiply by 100% to obtain the degree of conduction failure.

4. Preparation of intact DRG attached with sciatic nerve

4.1. Prepare surgical tools and Ringer's extracellular solution as described in step 2.1.

4.2. Separate the DRG with the attached sciatic nerve.

4.2.1. Anesthetize the rats as described in step 2.2. Cut the hair on back and leg with shearing scissors, and sterilize the skin with tincture of iodine.

4.2.2. For DRG exposure, first cut open the skin from the midline of the back at the L4 to L5 segment level. Remove muscles, the process of spine, vertebra board, and transverse process using a bone rongeur to expose the spinal cord and DRG body. Cover the exposed spinal cord and DRG with cottons infiltrated by normal Ringer's extracellular solution to maintain neural activity. Stop the bleeding and clear the blood in time.

4.2.3. Expose the sciatic nerve from two directions: remove the L4 to S1 bone structure above the vertebral foramen using ophthalmic scissors to expose the spinal nerve connected to DRG. Cut open the skin to expose the sciatic nerve at the middle thigh. Separate and disconnect the sciatic nerve from the distal end of the nerve where it goes inside the muscle, and ligate the nerve trunk with surgical line at the end of the nerve prior to cutting.

4.2.4. Separate the sciatic nerve from the underlying connective tissue using ophthalmic scissors via lifting of the nerve ligation point. Remove the dura from the spinal cord and separate the DRG from the underlying connective tissue until it reaches the adjacent part of the sciatic nerve. Thus, isolate the whole preparation of DRG with an attached sciatic nerve.

4.3. Clear the surface of the DRG.

4.3.1. Carefully remove the spinal dura on the surfaces of L4–L6 DRG using tweezers under a stereoscope at 4x magnification.

4.3.2. Place the DRG with attached sciatic nerve in a glass tube containing 1 mL of mixed enzymes (0.2% proteinase and 0.32% collagenase) and digest in a 37 °C water bath for 15 min (blow slightly with a plastic dropper at an interval of 5 min).

4.3.3. Lift the end of the surgical line and move the preparation to a dish filled with a normal Ringer's extracellular solution to wash out the enzyme. Then transfer the digested DRG to a container (**Figure 1A**) filled with oxygenated Ringer's extracellular solution for recording.

4.4 Recording session

4.4.1. Prepare intracellular solution (in mM: potassium gluconate 120, KCl 18, $MgCl_2$ 2, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid [EGTA] 5, HEPES 10, Na_2-ATP 5, Na-GTP 0.4, $CaCl_2$ 1; pH 7.2 and 300 mOsm). Keep at 0 °C until use.

4.4.2. Stabilize ganglia using a slice anchor and connect nerve end to a suction-stimulating electrode (**Figure 1A**). Visualize and select a DRG neuron with a water-immersion objective at 40x magnification.

4.4.3. Pull an electrode (**Table of Materials**) and fill it with intracellular solution. Insert electrode on holder and apply positive pressure in the pipette with a final resistance of 4−7 MΩ.

4.4.4. Bring electrode close to the cell and touch it. Give a negative pressure in the pipette, once GΩ seal is reached, set the membrane potential at about -60 mV and then establish whole-cell recording mode.

4.4.5. Deliver repetitive discharges of 5−50 Hz to the sciatic nerve through the suction electrode to screen for conduction failure. Measure the amplitude of afterhyperpolarization potential (AHP) from baseline to peak, and the 80% AHP duration.

NOTE: One-way analysis of variance (ANOVA; for more than two groups) or Student's t-test (for only two groups) was used to analyze the data. Data are presented as means ± standard error of the mean (SEM). The statistical significant level was set at $p < 0.05$.

REPRESENTATIVE RESULTS:

The outcome of the single-fiber recording protocol depends on the quality of the fiber dissection. The animal for in vivo experiments must be in a good situation to keep the nerve trunk healthy for easy dissection (see advice in the discussion section). A drug application bath is needed in many cases for drug delivery on fibers. **Figure 2** illustrates how the in vivo single-fiber recording was operated (**Figure 2A**) and presents one classical recording from the sciatic nerve of CFA-treated animals (**Figure 2B**).

The following experiments investigated the existence of conduction failure in CFA-treated animals. This investigation was based on the assumption that conduction failure along the nociceptive C-fibers was a common phenomenon, and the degree of conduction failure was significantly attenuated under conditions of hyperalgesia, which are supported by our previous studies[4,8,9,12]. **Figure 3A** shows that C-fiber conduction failure was observed in normal animals. However, the degree of conduction failure was reduced significantly after the establishment of CFA-induced hyperalgesia following CFA injection into the foot compared to control (**Figure 3B**). These data demonstrate that the conduction failure of pain-relevant polymodal nociceptive C-fibers is attenuated in the CFA model of inflammatory pain.

To examine intracellular mechanism during conduction failure, the preparation of intact DRG with attached sciatic nerve was used (**Figure 1A,B**). **Figure 1C** shows that within the stimulus series, spikes in response to repetitive stimuli piled up on the previous after-hyperpolarization potential (AHP) and resulted in a decrease in the rising slope of the following AHP (**Figure 1C,D**). The presence of AHP in small DRG neurons potentially activates hyperpolarization-activated, cyclic nucleotide-modulated(HCN) channels[13-15]. The cumulative effect of AHP plays a role in the occurrence of conduction failure. Therefore, we hypothesized that blocking HCN channels would significantly enhance the conduction failure effect. The following experiment used a blocker of HCN channels, ZD7288. Continuous recordings revealed an increase in conduction failure in the presence of ZD7288 in a concentration-dependent manner. Insets show expanded traces for the specified intervals. A positive correlation between conduction failure and the rising slope of the AHP in small DRG neurons of CFA-treated animals was observed (**Figure 1E**).

FIGURE LEGENDS:

Figure 1: Measurement of C-fiber conduction failure using preparations of intact DRG with attached peripheral nerve. (**A**) Schematic diagram illustrating the set-up and placement of DRG preparations. SE: stimulation electrode; SN: sciatic nerve; FM: fluorescence microscope; RE: recording electrode. (**B**) Whole DRG specimen observed under 40x magnification, and a microelectrode (right shadow) was used for patching a small DRG neuron. (**C**) Continuous recordings of series firing responses to 5 Hz stimulation under control conditions or administration of different concentrations of ZD7288 in a small-diameter DRG neuron from CFA-treated rats. The insets show expanded traces for the specified recording periods. Dark spots represent spike failures. (**D**) Representative traces used to measure the rising slope of the AHP. The rising slope was equal to the amplitude difference between the maximum and minimum AHP voltages (mV) divided by duration (interval of stimuli, in seconds). The left panel illustrates a bigger rising slope (from the first trace in panel C marked with an asterisk), and the right panel shows a smaller rising slope (from the fourth trace in panel C marked with "#") after ZD7288 application (125 μM). (**E**) Relationship between the degree of conduction failure and the rising slope of the AHP in response to different concentrations of ZD7288. $^{*}p < 0.05$ and $^{\#}p < 0.05$ vs. control. This figure has been modified from Wang et al.[9].

Figure 2: In vivo single-fiber recordings of rat sciatic nerves. (**A**) Schematic diagram of single-fiber recording indicating the regions for recording (R) (before splitting a filament for recording, the pia mater spinalis and dura mater were removed here), drug application (D), stimulation (S), and the site of CFA injection. (**B**) Representative recording of a sciatic single fiber exhibiting a tonic firing pattern. This figure has been modified from Wang et al.[9].

Figure 3: Conduction failure in CFA-treated rats was attenuated compared to control rats. (**A**) Original consecutive recordings of single C-fiber firings from control rats in response to 10 Hz electrical stimulation. Every twentieth sweep is shown (consecutive sweeps were at 2 s intervals) and displayed top-to-bottom. The inset shows a representative action potential. (**B**)

Recordings of single C-fibers from CFA-injected rats in response to the same stimulation as in panel A. This figure has been modified from Wang et al.[9].

DISCUSSION:

Although recent studies have achieved calcium imaging of DRG neurons in vivo[16], performing in vivo patch-clamp recording from individual DRG nociceptors remains extremely challenging. Therefore, an in vivo single-fiber approach for the pain field is of continuing importance. Single-fiber recording in the present protocol allow objective observation of conduction failure phenomena, and the combination of this technique with the ex vivo preparation developed in the current study allows examination of underlying mechanisms in nociceptor excitability changes in preclinical models. Three steps of the single-fiber recording protocol are critical for successful recordings. First, it is critical to pay attention to the anesthesia of the animal. In elaborate in vivo recording experiments, the length of the thin fiber that is wrapped around the 29 µm platinum electrode is only 2−3 mm, which is easily interfered during the recording process. If the anesthetic condition is not particularly stable, tiny movements of the animals may lead to recording failures of electrophysiological activities.

Second, the preparation must be continuously covered with paraffin. The purpose of this manipulation is to maintain the activity of the fibers. An appropriate recording slot was generally constructed using the skin of animals. To prevent paraffin oil leakage, the wall of the slot may be strengthened using super glue, and paraffin oil should be added whenever necessary. The fiber cannot dry during the entire test. Finally, the environment around the nerve trunk must be healthily maintained. There is always some effusion liquid present around the recording area, and this effusion is an obstacle for good quality recordings. The amplitude of the fiber activity will continue to decline and ultimately become indistinguishable from extreme baseline noise, which causes a recording failure. A homemade syringe tube is required to reach deep into the bottom of the slot to suck out the effusion liquid. Sometimes, a semidry cotton ball soaked in saline is also helpful.

The present study introduces an in vivo single-fiber recording technique to observe alterations in the transmission process that occur in nociceptive C-fibers provided with repetitive electrical stimuli. It was demonstrated that the degree of conduction failure was significantly attenuated in hyperalgesic conditions, but the underlying mechanism using single-fiber recording was not investigated because of technical difficulties in patch-clamping C-fibers. Therefore, the investigation of the relationship between conduction failure and changes in membrane potential of small-diameter DRG neurons were detected using preparation of intact DRG with the attached sciatic nerve. Instead of single-fiber recording, patch-clamp using such preparations explores AHP-dependent mechanisms for the production of conduction failure. Using this protocol, though only a few surface neurons could be selected, the degree of conduction failure at the level of DRG neurons was still able to be recorded, even with the drug administration.

DRG have two outer membranes: the pia mater spinalis and dura mater. The dura mater must be removed using hairspring tweezers, and the pia mater spinalis must be digested (moderate

digestion, not as series as used in the isolation of single DRG cells) to ensure that the patch-clamp electrodes can reach the surface of the DRG cells to form a seal; otherwise, it is impossible to obtain patch-clamp recordings. The current approach more completely preserves the peripheral nerve input compared to slices of DRG plus nerve and ensures that the patch-clamp recording of DRG neurons is easily achieved. This protocol has broad application prospects to improve the understanding of the peripheral nervous system's associations with pain, such as the investigation of electrophysiological changes in different DRG neurons in different chronic pain models[17,18] and molecular mechanisms underlying abnormal spontaneous activity in DRG with myelinated or unmyelinated axons[19,20].

The preparation of intact DRG with attached sciatic nerve presented here has many advantages compared to the traditional dissociated ganglion method, because the structure of the DRG remains basically unbroken in this preparation. Therefore, it simulates real conditions in vivo and provides a preferable microenvironment for physiological activity. The preparation of intact DRG with attached sciatic nerve produces less neuronal damage than the dissociated DRG preparation because the latter process uses more digestive enzymes and external physical actions (e.g., shearing and blowing of the cells), which causes more damage to the cells. Most electrophysiological studies are still performed on dissociated DRG neurons[21,22], and the dissociation process itself damages the cells, which results in abnormal hyperexcitations of neurons[23]. Another advantage of this protocol is that extracellular afferent electrophysiological activities are also obtained because the nerve projections remain, which allows investigations of interactions between afferent spikes and somatic DRG spontaneous discharges. Finally, this preparation preserves DRG neurons and satellite glial cells, and only DRG neurons remain in dissociation protocols. Satellite glial cells, which are essential for maintaining the microenvironment of the DRG, are a barrier that protect individual DRG neurons[24], and these cells warrant further study.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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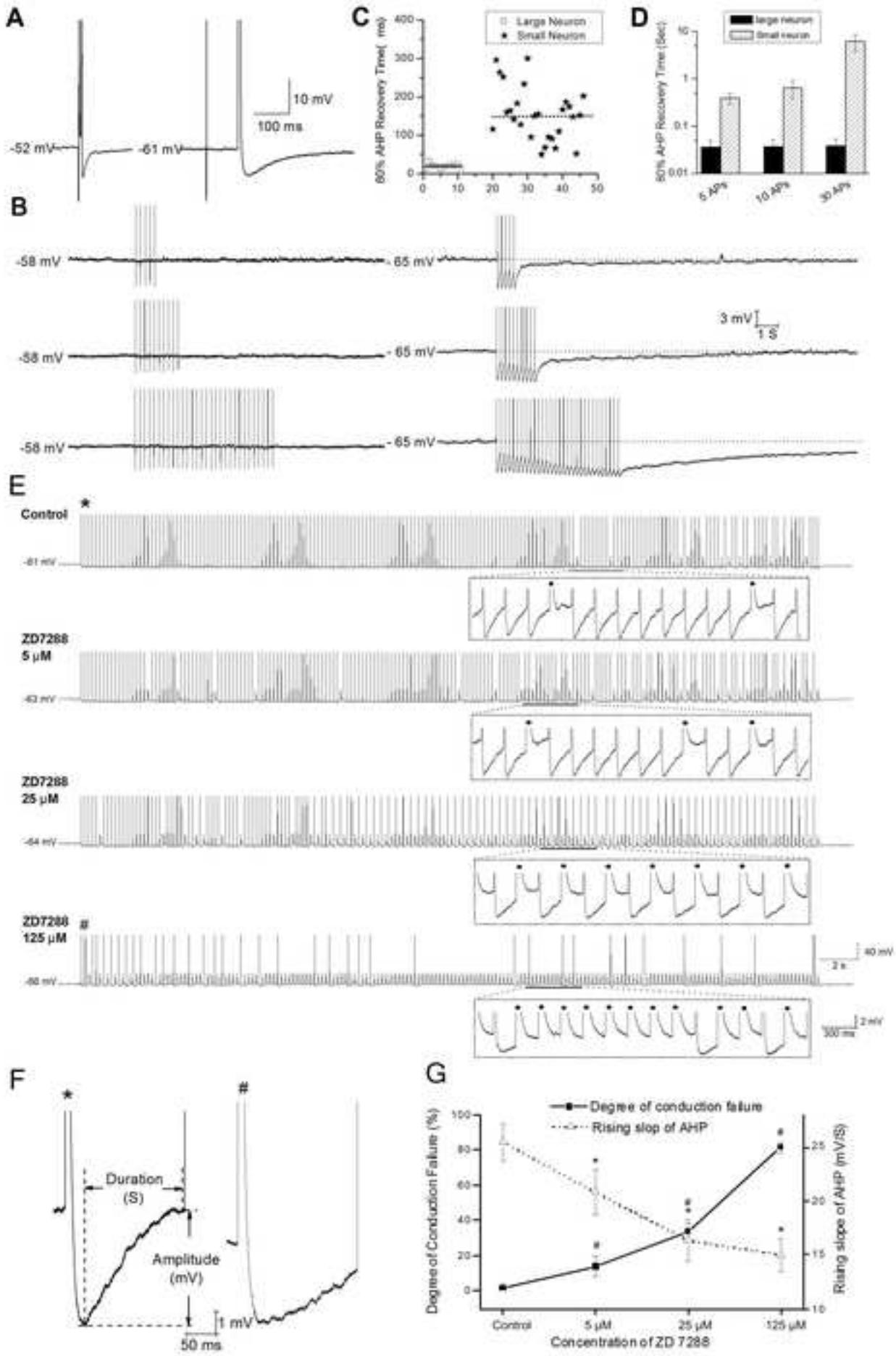

Figure 2

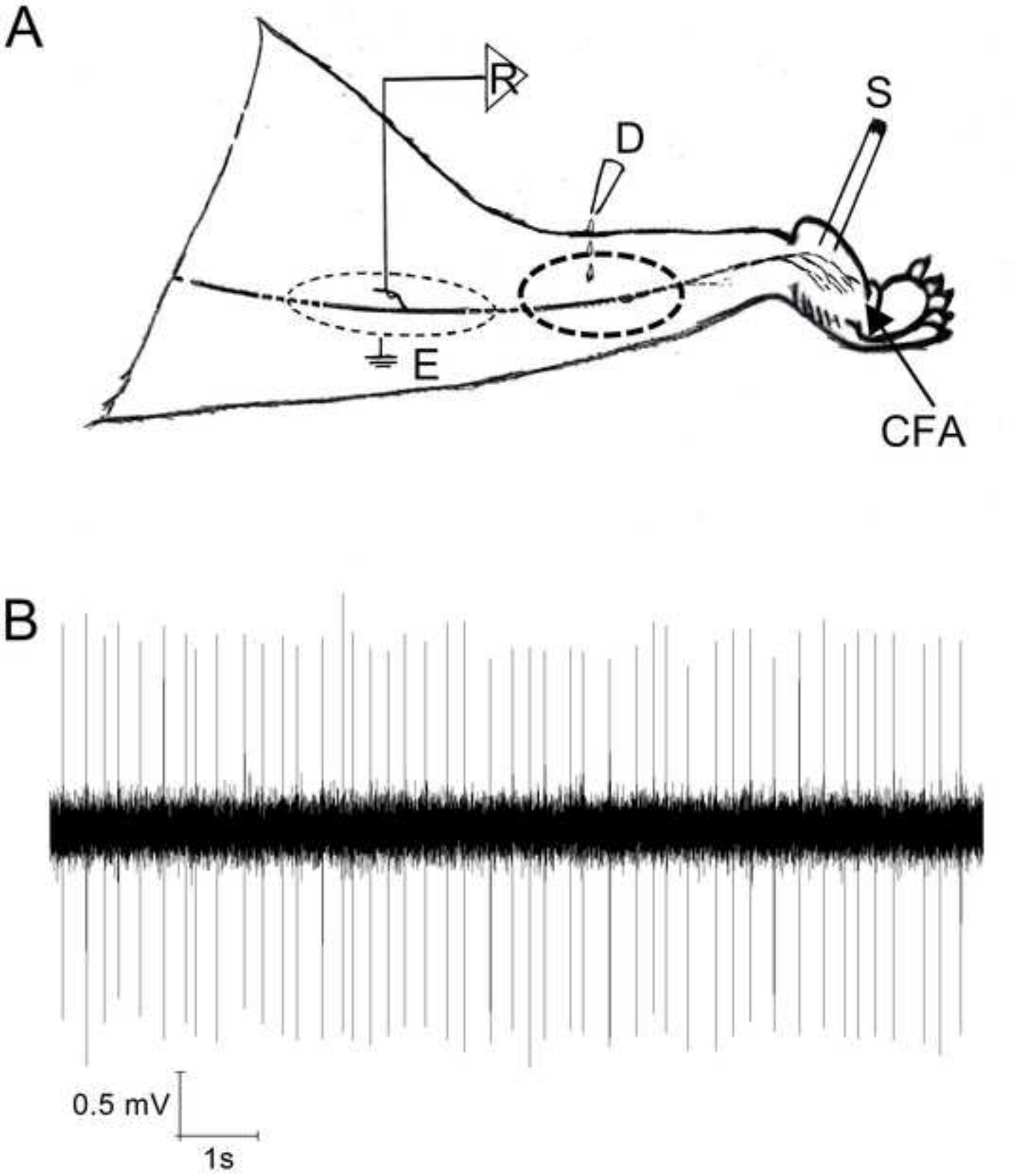

Figure 3

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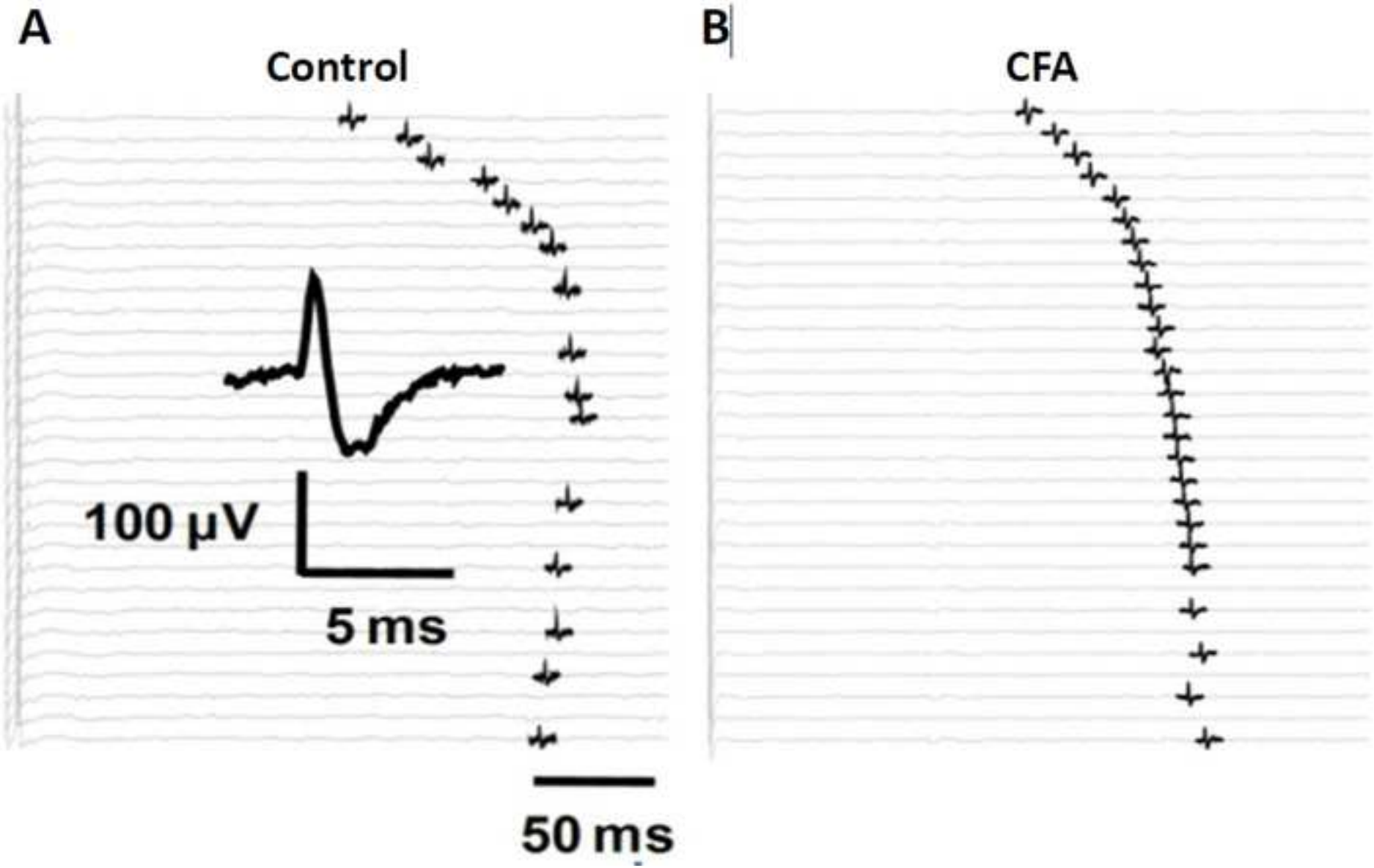

Materials

Name	Company	Catalog Number	Comments
Instruments and software used in single			
Amplifier	Nihon kohden	MEZ-8201	Amplification of the electrophysiological signals
Bioelectric amplifier monitor	ShangHai JiaLong Teaching instrument	SZF-1	Monitor firing process via sound which is transformed from
Data acquisition and analysis system	CED	Spike-2	Software for data acquisition and analysis
Electrode manipulator	Narishige	SM-21	Contro the movement of the electrode as required
Hairspring tweezers	A.Dumont	5#	Separate the single fiber
Isolator	Nihon kohden	SS-220J	
Memory oscilloscope	Nihon kohden	VC-9	Display recorded discharge during experiment
Stereomicroscope	ZEISS	SV-11	Have clear observation when separate the local tissue and single fiber
Stimulator	Nihon kohden	SEZ-7203	Delivery of the electrical stimuli
Von Frey Hair	Stoelting accompany		Delivery of the mechanical stimuli
Water bath	Scientz biotechnology Co., Ltd.	SC-15	Heating paroline to maintain at 37^{o}C
Instruments and software used in patch clamp recording			
Amplifier	Axon Instruments	Multiclamp 700B	Monitors the currents flowing through the recording electrode and also controls the stimuli by sending a signal to the electrode
Anti-vibration table	Optical Technology Co., Ltd.		Isolates the recording system from vibrations induced by the environment
Camera	Olympus	TH4-200	See the neurons in bright field; the controlling software allows to take pictures and do live camera image to monitor the approach of the electrode to the cell

Clampex	Axon	Clampex 9.2	Software for data acquisition and delivery of stimuli
Clampfit	Axon	Clampfit 10.0	Software for data analysis
Electrode puller	Sutter	P-97	Prepare recording pipettes of about 2μm diameter with resistance about 5 to 8 MΩ
Glass pipette	Sutter	BF 150-75-10	
Micromanipulator	Sutter	MP225	Give a precise control of the microelectrode
Microscope	Olympus	BX51WI	Upright microcope equipped with epifluorescence for clearly observe the cells which would be patched
Origin	Origin lab	Origin 8	Software for drawing picture
Perfusion Pump	BaoDing LanGe Co., Ltd.	BT100-1J	Perfusion of DRG in whole-cell patch clamp
Other instruments			
Electronic balance	Sartorius	BS 124S	Weighing reagent
pH Modulator	Denver Instrument	UB7	Adjust pH to 7.4
Solutions/perfusion/chemicals			
Calcium chloride	Sigma-Aldrich	C5670	Extracellular solution
Chloralose	Shanghai Meryer Chemical Technology Co., Ltd.	M07752	Mixed solution for Anesthesia
Collagenase	Sigma-Aldrich	SLBQ1885V	Enzyme used for clearing the surface of DRG
D (+) Glucose	Sigma-Aldrich	G7528	Extracellular solution
Liquid Paraffin	TianJin HongYan Reagent Co., Ltd.		Maintain fiber wetting
Magnesium sulfate	Sigma-Aldrich	M7506	Extracellular solution
Potassium chloride	Sigma-Aldrich	P3911	Extracellular solution
Protease	Sigma-Aldrich	62H0351	Enzyme used for clearing the surface of DRG
Sodium bicarbonate	Sigma-Aldrich	S5671	Extracellular solution
Sodium chloride	Sigma-Aldrich	S5886	Extracellular solution
Sodium phosphate monobasic	Sigma-Aldrich	S0751	Extracellular solution
Sucrose	Sigma-Aldrich	S0389	Extracellular solution
Urethane	Sigma-Aldrich	U2500	Mixed solution for Anesthesia

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Title of Article: Use of In Vivo Single-fiber Recording and Intact DRG with attached Sciatic Nerve to Examine the Mechanism of Conduction Failure

Author(s): Honghui Mao, Xiuchao Wang, Wen Chen, FengYu Liu, You Wan, Sanjue Hu , Junling Xing

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Article Title: Use of In Vivo Single-fiber Recording and Intact DRG with attached Sciatic Nerve to Examine the Mechanism of Conduction Failure

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Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thanks for the suggestion. The language polish has been performed.

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Response: Thanks for the suggestion. Revised as suggested. The copyright permission will be uploaded accordingly.

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Response: Thanks for the suggestion. Revised as suggested.

4. Figure 1B: Is "1S" meant for the time unit? If so, please change it to "1 s".

Response: Revised as suggested.

5. Please revise the title to avoid the use of colon.

Response: Revised as suggested.

6. Please provide an institutional email address for each author.

Response: Revised as suggested except for one author.

7. Keywords: Please provide at least 6 keywords or phrases.

Response: "analgesic" has been added to the key words.

8. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

Response: The first paragraph of introduction has been rephrased as suggested.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Response: The protocol section has been readjusted.

10. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout

the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Response: The protocol has been revised as suggested.

11. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

12. Line 104: Please specify the age and gender of Sprague-Dawley rats in this step.

13. Line 105: How many animals are included in each group?

14. Lines 125, 179, 180, etc.: Please specify the surgical instrument used.

15. Line 145: Please describe how to deliver a certain electrical stimulus.

16. Line 151: Please describe the collection procedure.

Response: All above questions (11-16) has revised as suggested.

17. Line 179: It is unclear what means by "cut the back and leg hair". Please clarify.

Response: The sentence has rephrased to make the meaning clear.

18. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

19. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

20. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Response: The 2.75 pages of the Protocol has been highlighted as required above (18-20).

21. References: Please do not abbreviate journal titles.

Response: The journal title are given as required in the JoVE EndNote style file .

22. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Response: Revised as suggested.

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Manuscript Summary:

The authors Mao et al. highlight 2 noteworthy electrophysiology techniques/protocols used to study C fibre nociceptors. The strength of this study is the development and description of the ex vivo patch clamp recording of dorsal root ganglion cell bodies in an ex vivo DRG with attached sciatic nerve preparation to enable study of mechanisms underlying altered activity patterns observed in in vivo single fibre recording.

I have the following comments on the manuscript listed by section:

Title/Summary

Neither title nor summary accurately reflect study:

This study describes both in vivo single C fibre recording AND ex vivo patch clamp recording of sensory neuron cell bodies in an ex vivo dorsal root ganglion with attached sciatic nerve preparation. Title says 'in vivo' only whereas summary implies ex vivo only. Given study does both this needs to be reflected in both title and summary.

Response: Thanks for the comments. Revision has been made both in title and summary.

Abstract - should clearly state that patch clamp recording was employed in the ex vivo DRG attached sciatic nerve prep.

The abstract could be improved by clearly stating that cannot study the underlying mechanism using the in vivo single fibre recording hence the development of the ex vivo patch clamp recording of dorsal root ganglion cell bodies in an ex vivo DRG with attached sciatic nerve preparation.

Response: Thanks for the comments. Revision has been made as suggested.

Introduction:

2nd paragraph states 'The advantage of using single fiber recording here is that the variations of fiber responsiveness to the external mechanical, temperature or chemical stimuli is very small and can be observed for a long time3,4.'.

It is unclear what advantage is being highlighted here? The capacity to record responses to natural stimuli in vivo, and ex vivo (skin-nerve prep) in naïve and preclinical models without disturbance of intracellular environment would be reasonable to highlight.

Response: Thanks for the suggestion. Revised as suggested.

Last paragraph introduction - 'it is necessary to identify the transduction property of the axon,' I do not think that 'transduction' is an appropriate word here as studying DRG cell body and axon only in this ex vivo preparation - the peripheral nerve terminals are not present therefore 'transduction' is not studied.

Response: Yes, the use of “transduction” is not correct and we changed it to “transmission” in order to not repeat with conduction.

Last paragraph introduction: 'On one side, various mechanical and chemical methods were used in dissociation process to free the DRG neurons from their intrinsic environment, and this process always made the cells to be recorded unhealthy.' Too strong language - more reasonable to say 'may result in unhealthy cells' and it could also be highlighted that this may in fact alter phenotype/properties of neurons under investigation that could

confound findings.

Response: Thanks for the suggestion. Revised as suggested.

Methods:

Animals:

Please confirm the volume of CFA injected. It is stated that only 10 microlitres of CFA were injected. Typically in adult rats a 50/50 mix of CFA/saline in a total volume of at least 100 microlitres is employed?

Response: Thanks for the comment. The volume of 100 μl has been corrected.

In vivo protocol:

2.3 Expose sciatic nerve truck for recording - is it planned that this section will be recorded as otherwise this section could be difficult for others to replicate? For example point 2 - regarding the homemade metal hoop this is very hard to visualise. Details should be provided of the dissection microscope magnification required to perform this

2.4.2 Recording session - again detail of microscope settings required should be detailed - presumably the Olympus detailed in table is used here? Also there is no indication of what spinal level or which dorsal roots? Please detail

Response: In rat, single fiber recording is easy to be replicated as long as the nerve is fully exposed and the capsule around the nerve trunk is removed totally. In the recording session, separation of single fiber should be finished under stereoscope with 25 times magnification. Single fiber recording here can be performed on the sciatic nerve or on the dorsal root. Since the dorsal root recording requires a relatively high corresponding relationship between the sensory field and the dorsal root, we just do the sciatic nerve recording here. The original text is incorrect and has been revised.

2.4.3 Recording session re electrical stimulation. No detail is provided about where applied (presume exposed sciatic nerve?) or strength and duration of stimulus.

Response: Thanks for the comment. The necessary contents has been added in Protocol 2.4.3 and 3.

2.4.4 Recording session - more detail required re the amplifier component and 'collection procedure' designed by labview

Response: Thanks for the comment. The necessary contents has been added.

3.1 'Recognise the polymodal C fibre' - NB it should be detailed where the natural stimuli are applied to presumably hindlimb? In point 3.3.1 electrical stimuli are applied via pin electrodes in skin - but in 3.2.2. Electrical stimuli are applied 'on nerve' - more detail is required re stimulation location - and the necessity for these 2 different stimulus locations. Also in point 3.3.1 it states that the 2 pin electrodes are placed in receptive field 5mm apart - please confirm this is correct as receptive fields are likely to be smaller than this?

Response: Thanks for the comment. Actually, the stimuli are applied on receptive field of hindlimb. While the recording location is on the Sciatic nerve. The corresponding detail has been corrected or supplemented in either 2.4.3 or 3 (measurement of conduction failure).

4. Preparation of intact DRG attached with sciatic nerve - I presume this would be video recorded?

Response: Yes, I think so.

4.1 - would be good to list surgical tools (and for in vivo surgery above also)

Response: Thanks for suggestions. The surgical tools has been listed at the very beginning of section 2.1.

4.3 'Clear the surface of the DRG - again would be good to know what magnification is useful here

Response: The magnification has been added.

'Representative results'

First paragraph states that to get optimal recordings tissue needs to be optimally dissected - it would be more helpful to directly highlight what pitfalls to avoid or advice to maximise chances of healthy tissue (or direct reader to the discussion section on this topic). It also states 'In many cases, drug application bath is needed to make the intervention on fibers possible' it is unclear what is meant by this sentence.

Response: Thanks for the suggestion. A description of "see advice in the discussion section" has been added in the second sentence of this paragraph, and "drug delivery" instead of "intervention" were used to make the meaning to be clear.

'These data suggest that the conduction failure of pain-relevant polymodal nociceptive C-fibers is attenuated in the CFA model of inflammatory pain.' - 'suggests' would imply to the reader that this figure is the only data comparison - whereas you have published this finding previously - please reword so clearer to reader.

Response: Thanks for the suggestion. "suggest" has been changed to "demonstrate" to make the meaning to be clearer to reader.

Discussion

1st paragraph is a bit general / vague. Could state more directly that while recent studies have achieved calcium imaging of DRG neurons in vivo (Kim et al., 2016, Neuron 91, 1085-1096, 2016) in vivo patch clamp recording from individual DRG nociceptors would be extremely challenging. Therefore can highlight the continuing importance of the in vivo single fibre approach for the pain field - and here demonstrate how this in combination with the ex vivo prep developed by the authors can be used to explore mechanisms underlying changes in nociceptor excitability in preclinical models.

Response: Thanks for the suggestion. The first paragraph has been rephrased according to the very constructive comments.

2nd last paragraph

'we introduced the in vivo single-fiber recording to observe the alteration in the transduction process' - I do not think that 'transduction' is an appropriate word here (same argument as for introduction).

2nd last paragraph - when describing the ex vivo patch recording - would be good to give more detail to the reader about 'only a little surface neurons could be selected' - explain why this is the case and why you needed to employ the digestion step. Also explain why you did a 'digest' approach versus trying to prepare slices of DRG plus nerve.

Response: Thanks for the comments. "transduction" has been changed into "transmission" in the 3rd paragraph. The 'digest' related issue has been added also in 3rd paragraph.

Last paragraph - 1st sentence is stating that this ex vivo intact DRG nerve prep has many advantages over the

dissociated DRG prep - I think the authors are stating that in the ex vivo 'intact' prep that there is less neuronal damage than there is in the dissociated DRG prep following digestion. This may be slightly confusing to reader given there is a digestion step for the ex prep also - this should be discussed and clarified further as detailed above

Basically the authors need to clearly describe why they use enzymes - presumably to 'slightly' dissociate DRG so can access cells for patch clamp recordings.

Response: Thanks for the comments. The reason of giving digestion in the ex vivo 'intact' prep has been discussed in the 2nd paragraph of discussion section.

In addition I would be cautious about proposing to use the DRG-attached sciatic nerve prep to study mechanisms underlying spontaneous firing - at least in the case of inflammatory pain models - the removal of the peripheral inflamed site in the tissue preparation may abolish the spontaneous firing as the 'inflammatory soup' driving spontaneous activity is no longer present.

Response: Thanks for the comments. Spontaneous discharge of DRG caused by inflammation is not easy to exist, especially in vitro, but in pathological pain situation, such as in CCD (chronic compression of dorsal root ganglion) treated model, spontaneous discharge from DRG neurons can be recorded in vitro. Thus study mechanisms underlying spontaneous firing can be studied accordingly.

I would also propose that this protocol is edited for English language

Response: Thanks for the suggestion. English editing has also been done.

Reviewer #2:

Manuscript Summary:

This manuscript describes methods for single fiber recordings from somatosensory neurons an in vivo preparation of rat sciatic nerve and in an explant of rat sciatic nerve with DRG attached. The authors used a model of CFA-induced inflammatory pain to verify their hypothesis that increased afferent activity could be due to attenuation of conduction failure in treated animals. Attenuation of conduction failure could thus be considered a target for treatment persistent pain conditions.

The manuscript is of scientific interest because recordings from single fibres are important for determining the properties of somatosensory neurons in normal and pathophysiological conditions and assessing the functional changes in individual somatosensory neurons in pathological states could help to understand the disease process.

Major Concerns:

1. In the section 4 "Recording session" lines 134-152, the description of the in vivo single fiber recording is not clear. I think the Fig. 1 is meant to represent this technique, but it is not referenced in the text. The authors speak of "split the spinal dura and the pia mater" these are not shown in Figure 1, (改图) nor is the DRG shown in the figure. I suppose the recording is done from the teased fibers from the dorsal root, but it is not clear if it is proximal or distal to the DRG. Thus the method is difficult to follow. The figure should be modified to show the structures that are mentioned in the text. Indeed , the authors state that the Figures 1 and 2 are modified from their previous publication (Wang et al, 2016). I find the diagram in the original paper is of better quality than that shown in the present manuscript.

Response: Thanks for the comment. Actually, in present study, the stimuli are applied on receptive field of

hindlimb. While the recording location is on the sciatic nerve. In the last version of manuscript, we made some mistakes. The corresponding detail has been corrected or supplemented in either 2.4.3 or 3 (measurement of conduction failure).

Notes: single fiber recording applied in present study can be performed on the sciatic nerve or on the dorsal root. Since the dorsal root recording requires a relatively highly-recognized relationship between the sensory field and the dorsal root, we just do the sciatic nerve recording here. Thus, the Figure 1 is just the case. Figure 1 is referenced in the section of 'representative results' but not in the section of 'Protocol'. And in the legend of Figure 1, the information for removing dura mater was added.

2. The quality of English in the manuscript needs major improvement. Throughout the manuscript, it is often difficult to understand the meaning that the authors would like to communicate.

Response: Thanks for the suggestion. English editing has also been done.

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