Video Article

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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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.

Video Link

The video component of this article can be found at https://www.jove.com/video/59234/

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. 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

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. Anesthetize rats. On days 3 to 7 after CFA injection, 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.
3. **Exposure of sciatic nerve trunk for recording**
   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. 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.
   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.
4. **Recording session**
   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. 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.
   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.
   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.
   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

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 Iintact DRG Attached with Sciatic Nerve

1. Prepare surgical tools and Ringer's extracellular solution as described in step 2.1.
2. **Separate the DRG with the attached sciatic nerve.**
   1. Anesthetize the rats as described in step 2.2 (On days 3 to 7 after CFA injection). Cut the hair on back and leg with shearing scissors, and sterilize the skin with tincture of iodine.

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.
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 which is at the proximal end of sciatic nerve. 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. 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 via lifting the dorsal root until it reaches the adjacent part of the sciatic nerve. Thus, isolate the whole preparation of DRG with an attached sciatic nerve.

3. **Clear the surface of the DRG.**
   1. Carefully remove the spinal dura on the surfaces of L4−L6 DRG using tweezers under a stereoscope at 4x magnification.
   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).
   3. Lift the end of the ligation 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. **Recording session**
   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.
   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.
   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. 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.
   5. Deliver repetitive stimuli 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 $p < 0.05$.

5. **Ending the Experiment**
   1. When the experimental task is finished while the rats are still under anaesthetic situation, rats are humanely euthanized with a intracardiac injections of overdose pentobarbital sodium.

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 1** illustrates how the in vivo single-fiber recording was operated (**Figure 1A**) and presents one classical recording from the sciatic nerve of CFA-treated animals (**Figure 1B**).

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 2A** 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 2B**). 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 3A**,**B**). **Figure 3C** 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 3C**,**D**). The presence of AHP in small DRG neurons potentially activates hyperpolarization-activated, cyclic nucleotide-modulated (HCN) channels[13,14,15]. 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 3E**).

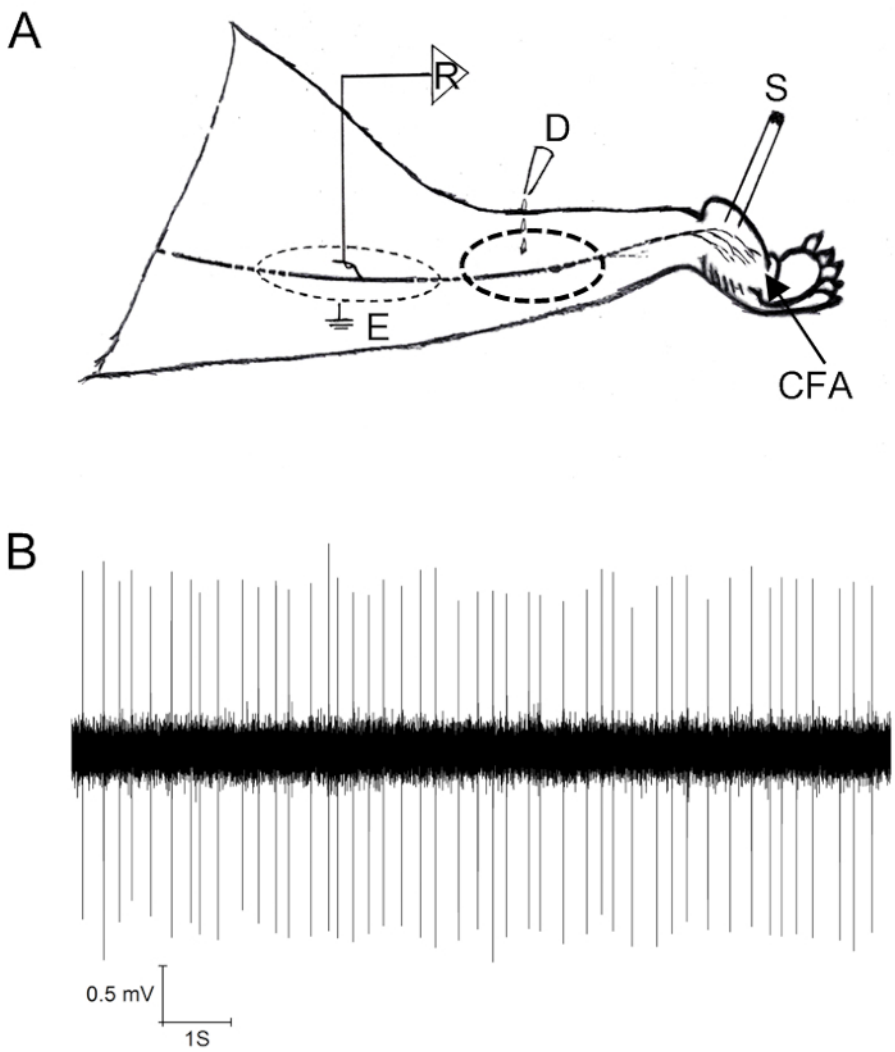

Figure 1: In vivo single-fiber recordings of rat sciatic nerves. (**A**) Schematic diagram of single-fiber recording indicating the regions for recording (R) (before splitting a filament for recording, the pia mater spinalis and dura mater were removed here), drug application (D), stimulation (S), and 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] Please click here to view a larger version of this figure.

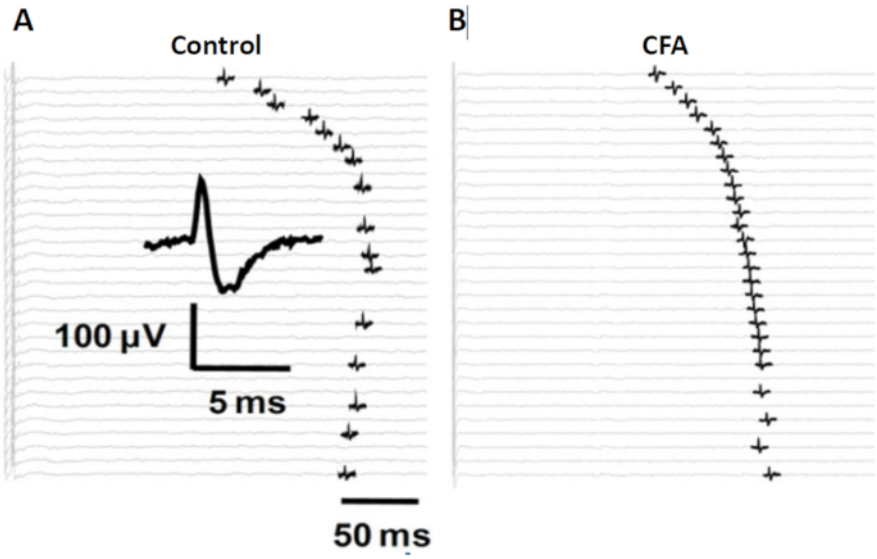

Figure 2: Conduction failure in CFA-treated rats was attenuated compared to control rats. (**A**) Original consecutive recordings of single C-fiber firings from control rats in response to 10-Hz electrical stimulation. Every 20th sweep is shown (consecutive sweeps were at 2-s intervals) and displayed 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]. Please click here to view a larger version of this figure.

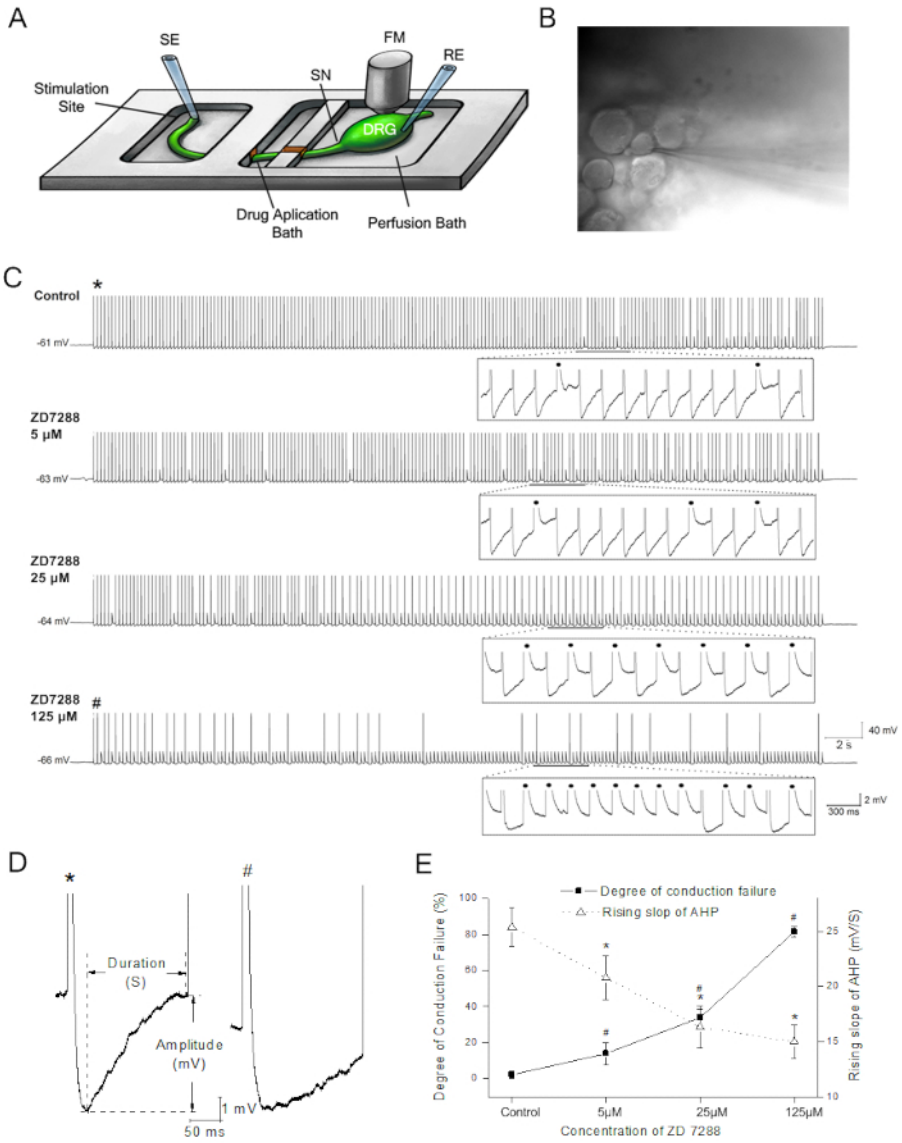

Figure 3: Measurement of C-fiber conduction failure using preparations of intact DRG with attached sciatic nerve. (**A**) Schematic diagram illustrating the setup and placement of DRG preparations. SE: stimulation electrode; SN: sciatic nerve; FM: fluorescence microscope; RE: recording electrode. (**B**) Whole DRG specimen observed under a 40x view, 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 "*"), 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. * and # $P < 0.05$ vs. control. This figure has been modified from Wang et al.[9]. Please click here to view a larger version of this figure.

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.

In the present study, CFA model was applied which produces foot inflammation and hyperalgesia following CFA injection. In order to investigate the properties of peripheral afferent discharge as well as the underlying mechanisms, no analgesics were used in the experiment, which is a routine practice in pain research and is approved by the IACUC/Ethics committee. 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 we could not investigate the underlying mechanism using single-fiber recording 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 pertaining to 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.

Disclosures

The authors have nothing to disclose.

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