Video Article

The Preparation of Oblique Spinal Cord Slices for Ventral Root Stimulation

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Abstract

Electrophysiological recordings from spinal cord slices have proven to be a valuable technique to investigate a wide range of questions, from cellular to network properties. We show how to prepare viable oblique slices of the spinal cord of young mice (P2 - P11). In this preparation, the motoneurons retain their axons coming out from the ventral roots of the spinal cord. Stimulation of these axons elicits back-propagating action potentials invading the motoneuron somas and exciting the motoneuron collaterals within the spinal cord. Recording of antidromic action potentials is an immediate, definitive and elegant way to characterize motoneuron identity, which surpasses other identification methods. Furthermore, stimulating the motoneuron collaterals is a simple and reliable way to excite the collateral targets of the motoneurons within the spinal cord, such as other motoneurons or Renshaw cells. In this protocol, we present antidromic recordings from the motoneuron somas as well as Renshaw cell excitation, resulting from ventral root stimulation.

Video Link

The video component of this article can be found at https://www.jove.com/video/54525/

Introduction

Historically, motoneuron recordings using sharp-electrode were conducted *in vivo* on large animals such as cats or rats[1] or on an isolated whole spinal cord in mice[2]. The emergence of the patch-clamp recording technique during the 1980s, called for direct access to the motoneuron somas as sealing needed to be achieved under visual guidance. Thus, spinal cord slice preparation has been readily achieved since the early 1990s[3]. However, early slice preparation often did not allow for the stimulation of the ventral roots. To the best of our knowledge, only two studies have reported successful stimulation of the ventral roots in transverse slices, and none was obtained from mice[4,5].

In this article we present a technique to achieve viable spinal cord slices of neonatal mice (P2 - P11) in which the motoneuron pool retains its ventral root departing axons. Ventral root stimulation triggers antidromic action potential back into the somas of the motoneuron pool exiting from the same ventral root. It also excites the motoneuron collateral targets, other motoneurons[6-10] and the Renshaw cells[11-13]. Since only motoneurons send their axons down the ventral roots, we use the recording of antidromic action potentials as a simple and definitive way to physiologicaly identify motoneurons[10].

In addition to using potentially non-inclusive or misleading electrophysiological and morphological criterions to confirm the motoneuron identity, recent studies on spinal cord motoneurons also relied on tedious and time-consuming *post hoc* stainings[16]. Such identification is usually performed only on a sample of the recorded cells. Other identification strategies rely on mouse lines in which the motoneurons express endogenous fluorescence[17-19]. However, using genetically encoded markers may be difficult at a young age when marker expression is still variable or if the study already requires using a transgenic mouse line. Alternatively, antidromic action potential recordings can be performed routinely on all mice from the onset of cell recording. Experimenters working on intact spinal cord preparations in the cat, rat and mouse, have reliably used such identification techniques since the 1950's[1,2,20,21]. In optimal conditions, we were able to elicit antidromic action potentials from virtually all of the recorded motoneurons.

Furthermore, ventral root stimulation can be used to reliably excite other motoneurons[22,23] or their targets. the Renshaw cells[10,24,25]. We present here applications of the ventral root stimulation in the form of antidromic action potential recordings from motoneuron somas, as well as excitation of Renshaw cells.

Protocol

The experiments were performed in accordance with European directives (86/609/CEE and 2010-63-UE) and French legislation, and were approved by the Paris Descartes University ethics committee.

1. Spinal Cord Slice Preparation

1. Prepare the following solutions daily or one day in advance. If kept overnight, bubble with 95% O_2 and 5% CO_2 and keep refrigerated in tightly closed bottles.
    1. **Prepare Low Na $^+$ artificial cerebrospinal fluid (ACSF):** 3 mM KCl, 1 mM NaH_2PO_4, 230 mM sucrose, 26 mM $NaHCO_3$, 0.8 mM $CaCl_2$, 8 mM $MgCl_2$, 25 mM glucose, 0.4 mM ascorbic acid, 1 mM Na-kynurenate, 2 mM Na-pyruvate. Bubble with 95% O_2 and 5% CO_2 (pH 7.4). Since Na-kynurenate is often hard to dissolve, make sure to purchase the one listed in the materials table.
    2. **Prepare K-gluconate solution:** 130 mM K-gluconate, 15 mM KCl, 0.05 mM EGTA, 20 mM HEPES, 25 mM glucose, 1 mM Na-kynurenate, 2 mM Na-pyruvate, adjusted to pH 7.4 with KOH.
    3. **Prepare ACSF:** 130 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 25 mM glucose, 0.4 mM ascorbic acid, 2 mM Na-pyruvate. Bubble with 95% O_2 and 5% CO_2 (pH 7.4).
    4. Prior to the beginning of the dissection, dissolve 2% agar in 80 ml of the K-gluconate solution, and keep warm at 60 °C.

2. **Intracardiac perfusion**
    1. Perform this preparation on female and male mice, ranging in age from P2 to P11.
    2. Anesthetize the mouse with an intraperitoneal injection of 0.1 ml of 25 mM pentobarbital sodium (50 mg/kg).
    3. Using needles or tape, immobilize the mouse on its back on a large petri dish filled with silicon. Use a dissecting microscope for the rest of the dissection.
    4. Holding the tip of the sternum, lift the chest and cut the diaphragm using fine scissors. Then open the chest on both sides by cutting through the ribs to expose the heart.
    5. Cut the right atrium before puncturing the left ventricle with a 27G needle.
    6. Perfuse with ice-cold low Na$^+$ ACSF. After 30 sec, low Na$^+$ ACSF should be seen flowing out of the atrium. Low amounts of sodium prevent the cells from spiking and reduce cellular death during dissection.
    7. Keep holding the needle in the heart under the dissecting microscope until the liver turns yellow when the blood is drained.

3. **Spinal cord dissection**
    1. Decapitate the animal and put it on its stomach.
    2. Quickly remove the skin of the back (**Figure 1A1**). Make two cuts through the shoulders and going down the chest cage (**Figure 1A2**). Then cut the cord as low as possible in the caudal section in order to isolate the vertebral column with the beginning of the ribs from the lower part of the animal. Flip the animal again and remove the viscera still attached to the ribs.
    3. Transfer the vertebral column to another, smaller silicon-filled petri dish and use 4 insect pins to hold it dorsal side up (**Figure 1A**).
    4. Continuously perfuse the animal with carbogen-bubbled ACSF (at about 4 °C) while performing a laminectomy of the dorsal side, and exposing the spinal cord from the rostral end (**Figure 1B**). To do so, insert the tip of fine scissors between the bone and the spinal cord and cut the bone little by little from the rostral end, making sure to stay away from the white matter. Alternate on each side while using tweezers to keep away the band of bone already cut (**Figure 1B1**).
    5. Using the smallest available spring scissors and tweezers, lift the dura and cut on both sides while holding the loose part of the dura in order to avoid damaging the spinal cord with the scissors. Cut along the rostro-caudal axis.
    NOTE: The dura is a semi transparent continuous membrane; at this age the pia mater is too fragile and will come apart during the dissection and slicing (**Figure 1B2**).
    6. Once the dura is removed use a blunt glass or plastic tip to gently push the cord on the left side of the groove formed by the half-cut spinal column, and cut the ventral and dorsal roots on the right side, starting from the rostral side, furthest from where they enter the cord (a few mm at least, **Figure 1B2**).
    7. Repeat the operation on the left side, always going from rostral to caudal. If left-handed, start from the left side and then move to the right side.

4. **Embedding in agar**
    1. Slip the cord out of the vertebral column. Use a smaller insect pin to pin down the cord with the dorsal surface up and delicately remove any piece of membrane still attached to it (**Figure 1C1**).
    2. Once cleaned, trim both ends (**Figure 1C2**). Insert a bended insect pin in the anterior part of the cord to manipulate the cord and note its orientation (arrow in **Figure 1C2**). Then transfer the spinal cord to an ice-cold K-gluconate solution.
    NOTE: This solution mimics the intracellular composition of the CSF and will prevent the cells from dying of osmotic shock once the motoneurons will be cut[26].
    3. Once the spinal cord is within the intra-cellular solution, take the beaker with the agar out of the dry bath and cool it down on a mixture of ice and water.
    4. Keep stirring while measuring the temperature. When the temperature reaches 38 °C immerse the spinal cord, holding it by the insect pin and place it rostral side down. Make sure the spinal cord is as straight as possible, away from the walls, caudal part slightly upward (**Figure 1D1**).
    5. Leave the beaker in the mixture of ice and water to allow the agar to solidify as quickly as possible. Make sure it stays in place and that the cord is as straight as possible (**Figure 1D1**).

5. **Slicing**
    1. After solidification, cut the agar block containing the spinal cord in such a way that the base of the block is at a 35° angle with the lumbar part of the cord (arrow in **Figure 1D2**). The dorsal surface should be facing away from the base (**Figure 1D2**).
    NOTE: This is a critical step in the procedure, for maintaining the continuity of the motoneuron pools to the ventral roots from which they exit.
    2. Glue the block into the chamber of the vibratome using cyanoacrylate glue. Immerse it in K-gluconate solution and add slushed frozen K-gluconate solution to maintain the bath chilled (below 2 °C).
    3. Cut 350 - 400 µm thick slices of the lumbar region (identifiable by its curvature and larger diameter). In its proper orientation (see 1.4.1.), use the blade to cut from the dorsal to the ventral surface and make slices continuously more rostral. Typically, there are 4-5

suitable slices with ventral roots extending 2 mm or more. Use the following parameters: 10° angle, 70 Hz vibration frequency and 10 mm/min speed of slicing.

6. **Incubation**
   1. Transfer the slices to ACSF at 34 °C. After approximately 30 min, cool the slices down to RT and begin the recording session.
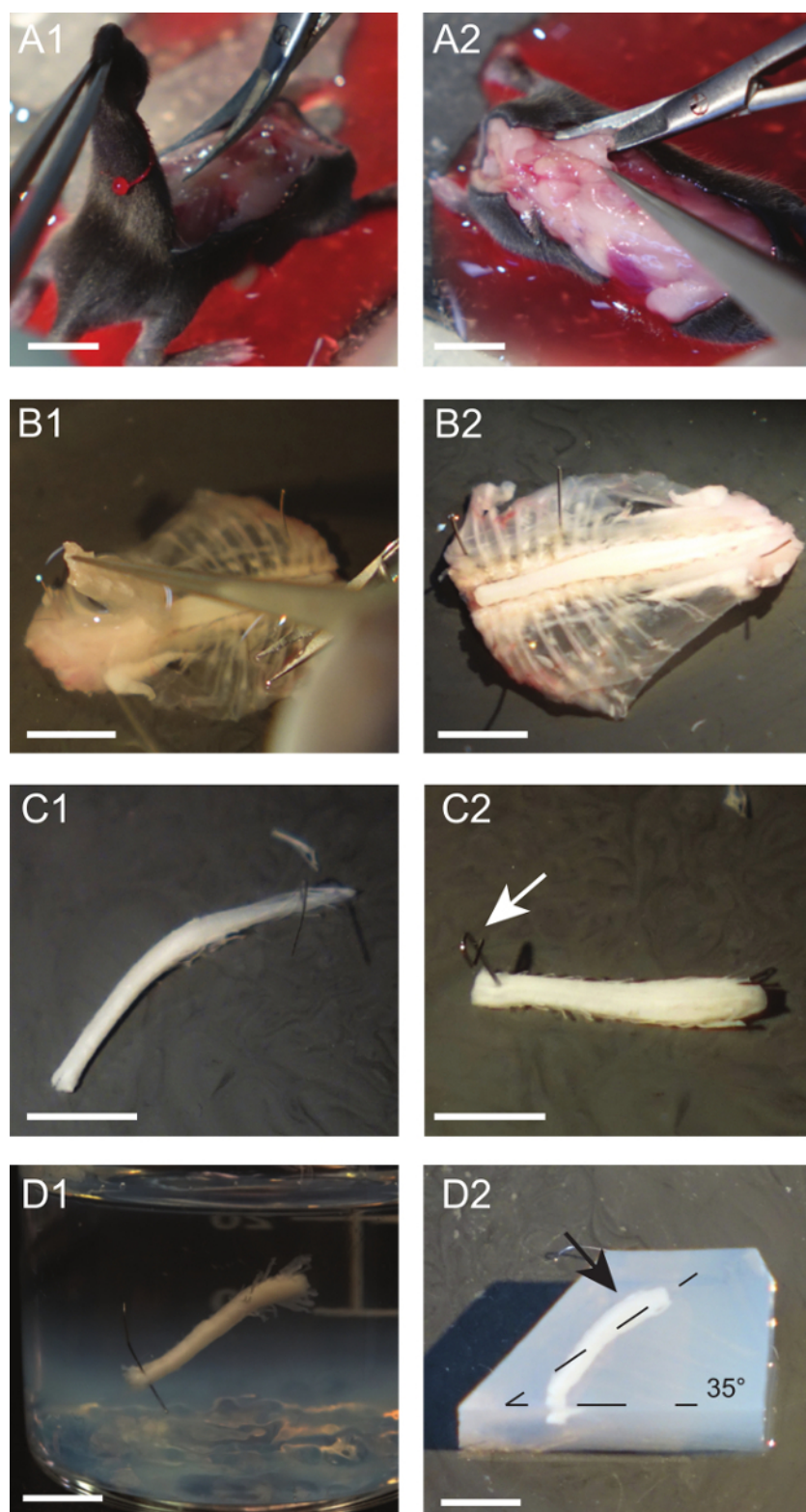
Figure 1. Dissection
A1: Removal of the skin of the back to expose the dorsal column. **A2**: Cutting of the shoulders and ribs to free the dorsal column. **B1**: Vertebral column pinned onto the silicon-filled petri dish (dorsal side up, caudal side left). **B2**: Same with the spinal cord exposed and dissected. **C1**: Spinal cord isolated (rostral side left). **C2**: Spinal cord ready to be embedded (ventral side up, rostral side left). Note the smaller insect pin on the rostral side. **D1**: Spinal cord in the agar beaker (rostral side dow, ventral side facing the bottom). **D2**: Cut agar block with the embedded spinal cord. Note the 35° angle the spinal cord forms with the base of the block and the lumbar enlargement (Arrow). Scale bars 1 cm. Please click here to view a larger version of this figure.

2. Placing the Slice into the Chamber

NOTE: Ventral roots are of variable size.

1. Prepare a box of various pipettes with tip diameters ranging from 40 to 170 μm in advance. To prepare suction pipettes, prepare many pipettes with a long taper. Using a diamond knife, make a cut at different positions. Then under a dissecting microscope, break it by hitting the tip with tweezers.
2. Remove the chamber from the recording microscope and place it under a dissecting microscope.
3. Select a slice that contains a ventral root of sufficient length (2 mm or more) to be mounted on a suction stimulation electrode. Select the proper orientation of the slice with the ventral roots upward (**Figure 2A**) and delicately cut the agar around the ventral roots while leaving the rest of the agar around the slice (**Figure 2B**).
   NOTE: Because the agar is firmer than the slice, this will allow the threads of the slice's anchor to rest on the agar rather than on the slice and thus the anchor will not damage the tissue. Make sure the threads of the slice's anchor are above the motoneuron pool (shown in red in **Figure 2C**).
4. Mount the chamber back onto the microscope and continuously perfuse the recording chamber with ACSF at a rate of 1 - 2 ml/min, at RT. Using a glass pipette filled with ACSF and connected to a syringe, suck one of the ventral root (arrow in **Figure 2C**). In order to achieve good stimulation of the ventral root, the pipette tip needs to be tight around the ventral root. One pole should be in the stimulating electrode and the other one in the bath (or connected to the patch-clamping electrode reference).
5. Achieve patch-clamp recording of the desired cell-type and record the effect of the ventral root stimulation as described previsouly[10].
   1. Here, use an amplifier for data acquisition. Filter whole-cell recordings at 3 kHz. Digitize at 10 kHz. Compensate bridge resistance in current-clamp mode.
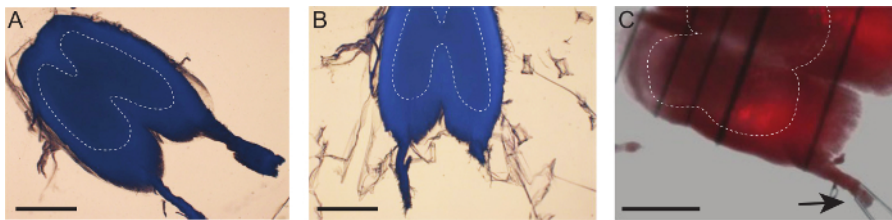

Figure 2. Ventral Root Preparation
A: Lumbar spinal cord slice embedded in agar with the ventral root facing up. **B**: Lumbar spinal cord slice with the ventral roots freed from the agar. **C**: Lumbar spinal cord slice with a stimulating electrode tightly placed around the ventral roots (arrow). Note the location of the Renshaw cells expressing red fluorescence in the chrna2-Cre mouse[28] crossed with the mouse reporter R26[Tom 17]. Scale bars 1 mm. Please click here to view a larger version of this figure.

Representative Results

Confirmation of Motoneuron Identity Using Antidromic Action Potentials

Cell targeting

Motoneurons are found in the ventral horn (visible in red in **Figure 2C**). Start from the bundle of axons forming the ventral root and go up until the bundle disperses fully and one starts seeing large cells (long soma axis, above 20 μm). Achieve whole-cell recording of a round healthy looking cell using an electrode of initial resistance of 3 to 4 MΩ. The internal solution used contained: 140 mM K-gluconate, 6 mM KCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM $CaCl_2$, 4 mM Mg-ATP, 0.3 mM Na_2GTP. Adjust the pH to 7.3 with KOH, and the osmolarity to 285 - 295 mOsm by adding distilled water.

Confirmation of the motoneuron identity

Once whole-cell recording is achieved, apply a single biphasic stimulation of the ventral root (1 - 50 V, 0.1 - 0.3 msec) to elicit an antidromic action potential in the recorded cells of the ventral cord. **Figures 3A1** and **3A2** show antidromic action potentials in voltage-clamp or current-clamp, respectively. When increasing the stimulation intensity from 1 to 5 V (**Figure 3A1**) and from 10 to 15 V (**Figure 3A2**) the antidromic action potential appears in an all-or-nothing fashion with a latency of 0.9 msec and 1.1 msec, respectively. Given that only motoneurons send their axons through the ventral roots, the antidromic action potential is a proof of the cell identity.

In a minority of motoneurons (about 10%), ventral root stimulation failed to elicit antidromic action potential but rather elicited orthodromic action potentials. **Figures 3B1** and **3B2** show orthodromic action potentials in voltage-clamp or current-clamp, respectively. When increasing the stimulation from 20 to 30 V (**Figure 3B1**) and from 25 to 40 V (**Figure 3B2**), an action potential appears, following a previous excitatory post-synaptic current (EPSC) or excitatory post-synaptic potential (EPSP). The latencies of the action potentials are longer (5.1 msec and 5.3 msec, respectively). In these two cells, stimulating the ventral root elicited a feed-forward excitation (motoneurons send axon collaterals to themselves and to other motoneurons) that was strong enough to elicit an orthodromic action potential. In such cells, the failure to elicit an antidromic action potential (characterized by its all-or-nothing fashion and a latency shorter than 5 msec) does not allow us to conclude that these cells are motoneurons. Note that the required stimulation intensity to elicit feed-forward excitation is higher than the one needed to elicit an antidromic action potential. All stimulations shown here are 0.1 msec but sometimes, longer stimulation (up to 0.3 msec) may be required to elicit an antidromic action potential.
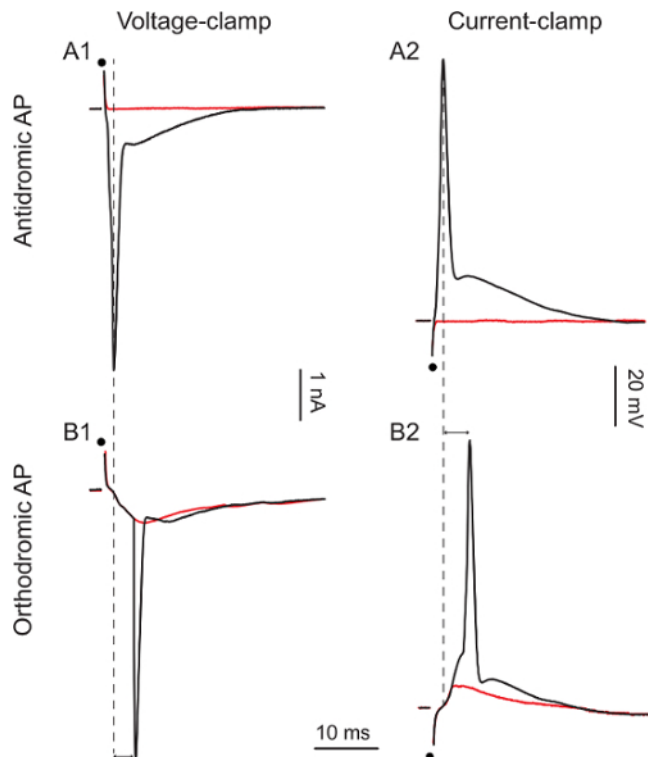
Figure 3. Motoneuron Responses to Ventral Root Stimulation
A1: 1 V (red trace) and 5 V (black trace) stimulations. **A2**: 10 V (red trace) and 15 V (black trace) stimulations. **B1**: 20 V (red trace) and 30 V (black trace) stimulations. **B2**: 25 V (red trace) and 40 V (black trace) stimulations. Black dots indicate stimulus timing. Double arrowheads emphasize the difference in latencies between ortho- and antidromic spikes. Please click here to view a larger version of this figure.

Renshaw Cell Stimulation

Cell targeting

Renshaw cells are found in the ventral horn[27,28]. Visualize the axon bundles with differential interference contrast (DIC) imaging using a 20X and a 63X objectives. Start from the bundle of axons forming the ventral root and go up until the axons start to disperse but are still discernible (**Figure 4A**, arrows). Aim for the cells of intermediate size (about 10 - 15 µm in diameter, **Figure 4A**, arrowheads). Achieve whole-cell recording of a round healthy-looking cell using an electrode of initial resistance of 5 to 7 MΩ. Depending on the experiment, we used either Cs$^+$-based or K$^+$-based internal solutions[10]. The Cs-based solution prevented large potassium currents that were visible around -45 mV. In some experiments, we also added 5 mM QX-314 to block the sodium channels responsible for spiking in the recorded cell. The Cs$^+$-based solution contains: 125 mM Cs-gluconate, 5 mM QX-314 Cl, 10 mM HEPES, 10 mM EGTA, 1 mM CaCl$_2$, 4 mM Mg-ATP, and 0.4 mM Na-GTP, with the pH adjusted to 7.3 with CsOH. The K$^+$-based solution should be used to analyze, both in voltage- and current-clamp, the Renshaw cell responses to ventral root stimulation in conditions approximating as much as possible the physiological ones. This solution contains: 125 mM K-gluconate, 10 mM HEPES, 1 mM EGTA, 0.1 mM CaCl$_2$, 4 mM Mg-ATP, 0.4 mM Na-GTP, with the pH adjusted to 7.3 with KOH. The stimulus intensity varied between 10 and 100 V and its duration varied between 50 and 300 µsec. Bipolar pulses were used in all cases.

Response of the Renshaw cells to ventral root stimulation

Stimulation of the ventral root triggers the corelease of glutamate and acetylcholine from motoneuron collaterals onto the Renshaw cells[10]. Feed-forward inhibition mediated by GABA and glycine is also recruited[10]. **Figure 4B** displays responses to the single stimulation of the ventral root. The cell was recorded in a voltage-clamp mode with the cesium-based solution, and maintained at a voltage of -45 mV. QX-314 in the intracellular solution prevented the cell from firing and GABA and glycine responses were blocked by 3 µM gabazine and 1 µM strychnine, respectively. The inward synaptic current in **Figure 4B** is the sum of the glutamatergic and nicotinic currents.
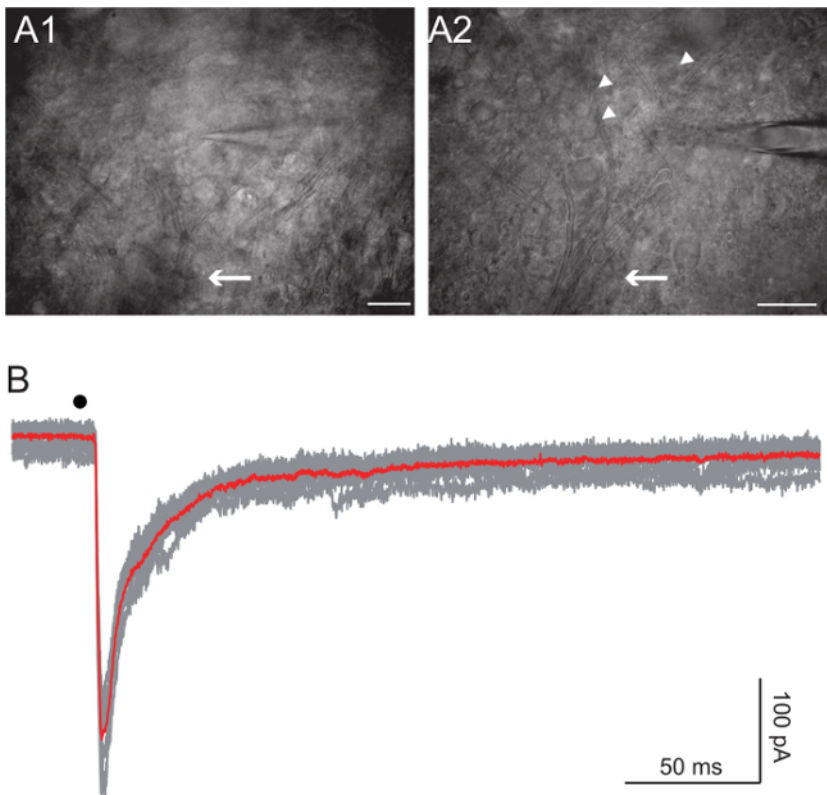
Figure 4. Renshaw Cell Stimulation from the Ventral Root
A: Images of the Renshaw cell pool acquired using an oblique condenser in the same slice taken at two different magnifications (20X and 40X objectives). Note the motoneuron axons merging into the ventral root (arrow). Putative Renshaw cells are indicated with arrowheads. Scale bars: 100 µm in **A1**, 50 µm in **A2**. **B**: Voltage-clamp recordings of a Renshaw cell following stimulation of the ventral root (black dot). The red trace is the average of the grey ones. Holding voltage was set at -45 mV. QX-314 in the intra-cellular solution prevented the cell from firing and GABA and glycine responses were blocked by 3 µM gabazine and 1 µM strychnine, respectively. Black dot indicates stimulus timing. Please click here to view a larger version of this figure.

Discussion

Oblique slicing of the spinal cord is important since it allows for unilateral stimulation of motoneuron pools and Renshaw cells at a single vertebral segment in a reliable, comprehensive and specific way. Furthermore, it allows for a quick, elegant and non-ambiguous identification of recorded motoneurons. Next, we will highlight the advantages of this technique compared to other slice preparation methods, and then we will stress out the most common pitfalls to avoid while performing this procedure.

Most studies using transverse slices base their identification on intrinsic electrical properties [3,14,15,29]. However, these vary greatly between motoneuron pools as well as between motoneuron subtypes within a given pool (alpha-, beta- and gamma-motoneurons[30]). Therefore, studies using size parameters are likely to exclude smaller gamma-motoneurons. On the other hand, Cooper and Sherrington described in 1940 a group of large nerve cells in the ventro-lateral grey matter of the lumbar spinal cord of monkeys and cats which had crossed ascending axons[31,32]. In addition to being close to the motoneuron somas, they noted that they appeared histologically indistinguishable from motoneurons. A recent article confirmed the existence of such cells in the mouse[33]. Due to the large size of these cells and their location, they could have been mistakenly included in former studies. Our identification criterion excludes putative spinal border cells, since their axon does not project in the ventral root, while allowing reliable identification of all motoneurons regardless of their size. Furthermore, analyzing the intrinsic electrical properties, (*e.g.*, input resistance), is more time consuming than the simple observation of an antidromic action potential.

In addition to using intrinsic electrical properties, some studies using transverse slices, confirm their identification by performing *post hoc* analysis of the molecular marker for motoneurons (such as Islet-1/2[16]) or by visualizing the morphology using biocytin labelings[14,17]. Such identifications are however tedious and do not provide a direct identification. They are therefore rarely systematically performed.

Some studies tried to make use of mouse lines expressing a genetically-encoded motoneuron fluorescent marker (Hb9-GFP mouse line[18], ChAT-EGFP transgenic mice, which express eGFP in cholinergic cells including motoneurons[19], or G85R SOD1-YFP transgenic mice, which strongly express the YFP fusion protein in motoneurons[17]). However, because the genetic marker expression is usually conditioned in time and since the slices are usually taken from embryos or juveniles, the expression of the marker may not be adequate at the age the study is performed. Other studies have used retrograde agent injection to visualize the motoneuron pool[34]. We also performed Cholera toxin beta injections into the soleus or the EDL (personal observation). Such labeling techniques are of great value to label a particular motoneuron pool but require tedious surgery a few days prior to the experiment. Furthermore, the young age at which the slice experiments are being done, makes it significantly harder to specifically target the muscles. Finally, there is a real concern of perturbing the motoneuron by injecting an exogenous agent.

Under optimal conditions we were able to obtain antidromic action potentials in virtually all of the recorded motoneurons. The 35° angle used is critical to obtain ventral roots of sufficient length. In conclusion, the oblique slice preparation offers a quick and reliable way to identify every motoneuron, which is superior to other identification techniques.

As stated in the introduction, only two studies reported successful stimulation of the ventral roots in transverse slices. The first study recorded cervical neurons in the rat and stimulated the white matter where the ventral root stub emerges[4]. They successfully induced antidromic action potential in 85% of the recorded neurons. We believe their high success rate relied on the fact that at the cervical level, the axons and somas of motoneurons are on the same plane. This is not the case in more caudal slices. Even at the cervical level, their transverse slices only retained stubs of the ventral roots, which are less reliable to stimulate. The second study stimulated the chick embryo dorso-lumbar segment[5]. However, they used voltage sensitive dye recording, which lacked the spatial resolution to indicate the success of inducing an antidromic action potentials at the single cell level.

Our slice preparation also offers a superior way to stimulate Renshaw cells as stated in a recent paper[28]. Using the oblique slices, they were able to record monosynaptic Renshaw cells responses in 46% of the cases, which was a major improvement from the 10% success rate encountered using transverse slices[35]. Although we could not find a similar comparison for the stimulation of the motoneuron pools, this observation in the Renshaw cell leads us to believe that motoneurons also retain more connectivity.

Oblique slicing of the spinal cord is a highly demanding technique that requires much practice before reliably obtaining a preparation from which one can record. Using very fine and sharp tweezers and micro-scissors is essential. The time spent dissecting can be quite long (up to 1 hr) as long as the entire procedure is conducted in chilled carbogen-bubbled dissection medium. While some authors did not acknowledge any benefit of intracardiac perfusion in the preparation of slices[36], we decided to maintain this step of the procedure. It is hard to objectively measure the benefit of intracardiac perfusion for the quality of the recordings, especially because it varies with age.

The most critical factor is the mouse age. We successfully recorded from mice between P2 and P11 using the above protocol. Past this age, the spinal cord myelin becomes too dense and the biggest cells (the motoneurons) die before slicing, most likely due to oxygen deprivation. After that window, we found it increasingly difficult to obtain healthy slices. Recently, new techniques have been reported to obtain healthy slices in older animals [17,36]. They used extra additives to their dissecting and recording medium such as supplementary sources of energy (ethyl-pyruvate), lower Na^+ and Cl^- concentrations to prevent oncosis of the cells[37,38] and polyethylene glycol to limit the effects of the extensive membrane transections that occur during slicing. With such enhancements, they were able to record from up to 6 month old animals.

In the future it would be interesting to attempt oblique slice preparations in adult animals to combine the advantages of the two techniques. Combining the two protocols should prove relatively easy and will provide a reliable tool to confirm motoneuron identity and study the population targeted by axon collaterals of motoneurons in adult mice. Additionally, one could try obtaining oblique slices in the embryo mouse. Noteworthy, our preparation also retains the dorsal roots, which can be stimulated to study the activation monosynaptic I_a innervation to the motoneurons, as well as the activation of dorsal spinal cord neuronal populations. In conclusion, ventral root stimulations offer a reliable tool to confirm motoneuron identity and to study the population targeted by motoneuron axon collaterals.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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