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Functional isolation of single motor units of rat medial gastrocnemius muscle

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We would like to resubmit for publication in *JoVE* the manuscript entitled "Functional isolation of single motor units of rat medial gastrocnemius muscle" by Hanna Drzymała-Celichowska and Jan Celichowski. The references were numbered in order of appearance in the manuscript and all additional details were added in the protocol.

All authors were fully involved in the study and preparation of the manuscript. The manuscript have not been published and have not been submitted for publication elsewhere.

Yours sincerely,

Hanna Drzymała-Celichowska

TITLE:

Functional Isolation of Single Motor Units of Rat Medial Gastrocnemius Muscle

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KEYWORDS:

Motor unit, skeletal muscle, gastrocnemius, ventral roots, electrophysiology, rat.

SUMMARY:

This method allows the recording of the force of twitch and tetanic contractions and action potentials in three types of motor units in the rat medial gastrocnemius muscle. The functional isolation of a single motor unit is induced by electrical stimulation of the axon.

ABSTRACT:

This work outlines functional isolation of motor units (MUs), a standard electrophysiological method for determining characteristics of motor units in hindlimb muscles (such as the medial gastrocnemius, soleus, or plantaris muscle) in experimental rats. A crucial element of the method is the application of electrical stimuli delivered to a motor axon isolated from the ventral root. The stimuli may be delivered at constant or variable inter-pulse intervals. This method is suitable for experiments on animals at varying stages of maturity (young, adult or old). Moreover, this protocol can be used in experiments studying variability and plasticity of motor units evoked by a large spectrum of interventions. The results of these experiments may both augment basic knowledge in muscle physiology and be translated into practical applications. This procedure focuses on the surgical preparation for the recording and stimulation of MUs, with an emphasis on the necessary steps to achieve preparation stability and reproducibility of results.

INTRODUCTION:

Motor units (MUs) are the smallest functional units of skeletal muscles. Therefore, understanding their function, plasticity and contractile properties, as well as the mechanisms of their force regulation, is crucial for progress in muscle physiology. The basic contractile properties of MUs and the proportions of their physiological types have been documented for

numerous muscles, predominantly the hindlimb muscles in experimental animals. However, both the plasticity of MU properties and the mechanisms of MU force regulation are still not fully understood.

The principle of the described method is extensive denervation of the hindlimb muscles except the investigated one and laminectomy at the lumbar vertebrae in order to prepare thin ventral rootlets, each one containing a single "functional" motor axon, stimulated electrically to record the force and action potential of the MU. Using the technique described in this paper, it is possible to isolate more than half of the MUs of the medial gastrocnemius muscle in a successful experiment. The rat medial gastrocnemius is composed of on average 52 MUs (females) or 57 MUs (males) of three physiological types: S (slow), FR (fast resistant) and FF (fast fatigable)[1,2], and have variable contractile properties[3]. For experiments comparing mean values for MUs in the control and experimental groups, isolation and recording of 10-30 MUs for each of these groups are necessary. Critically, individual MUs may be accessible for stimulation for time periods exceeding one hour. Moreover, since this technique allows for recording both MU force and action potentials, this method is suitable for studying phenomena associated with force production, assessing the effect of fatigue, and observing the relationship between the force and action potentials.

Previous studies have confirmed that MU contractile properties are plastic and may be modulated by numerous interventions. Experiments using the technique described here have been performed on rat medial gastrocnemius[4] or other hindlimb muscles of the rat[5,6] as well as on cat muscles[7], using a similar method of single MU isolation. Another series of experiments using trains of stimuli delivered at variable inter-pulse intervals provided observations concerning motor control processes, and the results in general turn attention to the history of stimulation, including considerable effects of a shift in time scale of even one stimulus, crucial for force production[8,9].

MUs may also be studied using alternative methods. First, one method is direct stimulation of motoneurons. Burke used intracellular stimulation of motoneurons in cat medial gastrocnemius and soleus with glass microelectrodes used in parallel to determine the electrophysiological properties of these neurons[1,10]. Other methods have been proposed to study MUs in human muscles, which require considerably lower intervention. For all these methods, the stimulating and recordings electrodes are inserted into the muscle or nerve, and force is recorded from the finger or from the foot. The first of these methods was used to study MUs in the first dorsal interosseous muscle. For this muscle, contracting with a low force, in the electromyogram recorded with the needle electrode inserted into the muscle the action potentials of only one active motor unit were identified. Then the fragments of a muscle force recorded in parallel and following each action potential were averaged (spike-triggered averaging). This method enables extraction of the force of one motor unit from the muscle force recording[11]. However, the methodological weakness of this procedure is that no single twitch force but rather fragments of tetanic contractions were averaged. Human MUs may also be studied using the second method of intramuscular electrical microstimulation using an electrode inserted into the muscle[12], which stimulates a fragment of an axonal tree, leading to activation of a single motor

unit. The third method is microstimulation with an electrode inserted into the nerve. When the electrode activates only one motor axon in the nerve, only one motor unit contracts13. These last methods have some limitations, including stability and quality of the recording, ethical restrictions and access to the experimental material. This protocol has been extensively used in cats in the 70's and 80's[14].

PROTOCOL:

All procedures need to be approved by the local ethics committee and adhered to the European Union guidelines on animal care as well as the national law on the protection of animals.

NOTE: Each experimenter involved in this procedure must be trained in basic surgical procedures and has to obtain a valid license for performing animal experiments.

1. Anesthesia

1.1. Anesthetize the rat with an intraperitoneal injection of sodium pentobarbital (an initial dose of 60 $mg \cdot kg^{-1}$).

1.2. After approximately 5 min, check the depth of anesthesia by pinching the rat's ear or forelimb with blunt forceps. Go to the next steps of the protocol only when no reflex action is observed.

1.3. During the surgery, check the animal for reflex actions every 10-15 minutes and supplement anesthesia if the animal responds to a pinch with movement (usually, 10 $mg \cdot kg^{-1} \cdot h^{-1}$ sodium pentobarbital, IP).

2. Surgery

2.1. Prepare the animal for the surgical procedure by shaving the fur over the left hindlimb from the heel to the hip (the first segment, muscle and nerve isolation), the right hindlimb from the heel to the hip (the second segment, ground electrode), and the backside from the tail to the thoracic segments (the third segment, laminectomy). Antiseptic is not necessary because of the acute nature of the experiment.

2.1.1. Place the rat on its abdomen on a heating pad (37 °C ± 1 °C.)

2.2. Laminectomy

2.2.1. Using sharp-blunt scissors, cut the skin along the spinal column from the sacrum up to the thoracic vertebrae.

2.2.2. Separate the skin from the underlying muscles.

2.2.3. Using blunt tip scissors, cut out the longissimus muscle on both sides of the sacrum and lumbar spinous processes.

2.2.4. Identify the S1 vertebra as the lowest segment. Using sharp-blunt scissors, cut and remove the spinous processes from L6 to L2 vertebrae. Next, using fine rongeurs, remove the transverse processes L6-L2 and perform a laminectomy over L6 – L2 segments (first transverse processes, then lamina, begin with L6 vertebral segment) to expose the lumbar segments of the spinal cord covered by the dura mater. Be careful not to cut the sacral bone and L1 spinous process, which will be used as a fixation point for animal immobilization.

2.2.5. Using sharp scissors, cut the spinal cord (its caudal fragment) and the dorsal and ventral roots at L2 vertebrae segment level, at the upper border of laminectomy. Place small pieces of dried gel foam to stop bleeding. Next, place a thin, saline-soaked cotton wool over the exposed spinal cord segments.

2.3. Isolation of medial gastrocnemius muscle and its nerve

2.3.1. Using sharp-blunt scissors, make a longitudinal cut on the posterior side of the left hind limb, from the Achilles tendon to the hip.

2.3.2. Grasp the skin with the forceps and separate the skin from the underlying muscles on both sides of the incision.

2.3.3. Locate the popliteal fossa at the back of the knee joint, which is covered by the biceps femoris muscle. Using scissors, make a cut between the anterior and posterior part of this muscle.

2.3.4. Moving upwards, cut the two heads of the biceps femoris all the way to the hip to expose the sciatic nerve.

2.3.5. Using blunt forceps and scissors, separate the lateral from the medial head of the gastrocnemius muscle and cut the distal insertion (Achilles tendon) of the medial gastrocnemius muscle. Preserve the fragment of Achilles tendon as long as possible in order to use it to connect to the force transducer.

2.3.6. Identify the medial gastrocnemius (MG) nerve. Using the forceps and scissors, cut all remaining collaterals of the sciatic nerve, including collaterals to posterior biceps and semitendinosus. Leave the supply blood vessels to the medial gastrocnemius intact.

2.3.7. Thread a non-elastic ligature through the Achilles tendon and make three knots.

2.3.8. Place a saline-soaked piece of cotton wool under the exposed nerve and muscle.

2.3.9. Using serrated forceps, close the skin over the operated area.

2.3.10. Using sharp-blunt scissors, make a 2 cm incision in the skin and underlying connective tissue along the anterior side of the left hind limb for immobilization with a metal clamp (3.1.6.).

3. Preparation for the recording and stimulation

3.1. Vertebral column and leg fixation and muscle arrangement

3.1.1. Using a steel clamp, fix the left hind limb by putting the clamp on the tibia.

3.1.2. Place the rat in the custom-made adjustable frame (isolated copper wire, 1 mm), pull up the skin flaps around the laminectomy using four ligatures and suture the skin to the frame in order to form a pool for paraffin oil (size approximately 50 mm x 50 mm) over the exposed spinal cord.

3.1.3. Using a Dumont #55 forceps, lift the dura mater at the intersection of the spinal cord, cut it caudally up to the sacral bone and retract it.

3.1.4. Using a blunt glass rod, separate left and right dorsal and ventral roots at successive levels, taking care not to damage them.

3.1.5. Fill the pool over the spinal cord with warm (37 °C) paraffin oil, covering the exposed ventral and dorsal roots.

3.1.6. Place the rat on the custom-made aluminum plate (length 260 mm, width 120 mm, height 80 mm) with a pool (length 135 mm, width 100 mm, depth 45 mm) for his hindlimbs connected to the closed-loop heating system. The plate is a place where the animal will be immobilized and the experiment will be performed.

3.1.7. Fix the clamp put on the left hindlimb with the metal bar to immobilize the hindimb.

3.1.8. Fix the vertebral column by putting steel clamps at the sacral bone and the L1 vertebra to immobilize the animal body and eliminate the artefacts in force recordings related to respiratory movements.

3.1.9. Connect the left medial gastrocnemius muscle with the non-elastic ligature to the force transducer (with compliance of 50 µm/250 mN, measurement range 0-1000 mN) via the Achilles tendon.

3.1.10. Fill the chamber for hindlimbs with warm (37 °C) paraffin oil to cover the medial gastrocnemius muscle and maintain the oil temperature at 37 °C ± 1 °C using the temperature probe and the automatic system.

3.2 Placement of electrodes for action potentials recording and stimulation

3.2.1. Insert a bipolar silver-wire electrode through the middle part of the medial gastrocnemius muscle, perpendicular to its long axis. Maintain about 5 mm distance between the two electrodes located along a long axis of the muscle. These electrodes will be used to record motor unit action potentials (MUAPs). Connect the electrodes to the low-noise amplifier.

3.2.2. Stretch the operated muscle to a passive tension of 100 mN, controlled by the force transducer. For rat medial gastrocnemius at this stretch the MUs of three types develop the highest twitch force[15].

3.2.3. Using sharp-blunt scissors, make a 2 cm incision in the skin of the right hind limb and insert a silver-wire electrode to be used as a reference electrode.

3.2.4. Place and fix a custom-made insulated metal plate (size 30 mm x 13 mm) above the exposed spinal roots. Put left pairs of ventral and dorsal roots (L4, L5 and L6) on the plate.

3.2.5. Add saline to the pool formed by the skin around the laminectomy. The saline level should be below the insulated plate.

3.2.6. Place a silver wire stimulating electrode (two silver wires, 0.5 mm diameter, length 50 mm) over the exposed spinal roots, place a positive pole 3 mm above the plate in oil, whereas the negative pole in the saline (added to the pool, below the plate) and connect to the stimulator.

4. Motor unit recordings

4.1. Stimulating with electrical rectangular pulses (0.1 ms duration, amplitude up to 0.5 V), select the ventral roots (L4, L5 and L6); ventral root stimulation evokes contraction of muscles whereas there is no such effect for dorsal roots. Eliminate dorsal roots from the plate. For the medial gastrocnemius, most axons are in the L5 ventral root.

4.2. Using a pair of Dumont #55 forceps and magnifying glasses, split L5 or L4 ventral roots into very fine bundles of axons (grasp the cut end of the ventral rootlet with both forceps and peel the rootlet apart); place one of these bundles on a silver wire electrode and stimulate (0.1 ms rectangular pulses of amplitude up to 0.5 V) to achieve activity of a single MU. A solid support (metal bar) is very useful for manipulating thin bundles of axons, which can be used as a hand support for using forceps. Note also that an additional source of light is necessary.

4.3. By progressively increasing the intensity of the stimulus, identify a single MU on the basis of the evoked “all-or-none” character of the twitch contraction and action potential stimulus. Carefully test the evoked activity at a stimulation around the threshold.

4.3.1. When more than one MU is contracting in the studied muscle and increasing level of the force as well as increasing amplitude or changing shape of the action potential are visible, go back to step 4.2 and split the bundle of axons again. Note that the strongest MUs in rat medial gastrocnemius have about 70 times larger twitch force than the weakest ones and when very strong MU is twitching the second, weak MU may be not evident. Note also that some MUs have their muscle fibers located out of the recording area of the electrode and are not visible in electromyogram; in such a case changes in the stimulus amplitude may have effect in the force but not in action potential.

4.4. Stimulate a motor unit with a stimulation protocol necessary for the aim of experiment. For a basic stimulation protocol necessary to calculate all basic motor unit contractile and action potentials properties, include the following.

4.4.1. Include 5 stimuli at 1 Hz (5 single twitches recorded and averaged; averaging is eliminating noise, which is especially important for the weakest MUs).

4.4.2. Include a series of stimuli at 10, 20, 30, 40, 50, 60, 75, 100 and 150 Hz frequencies with a duration of 500 ms (these recordings enable calculation of the force-frequency relationship, the maximum tetanic force at 150 Hz as well as the sag at 20-40 Hz stimulation).

4.4.3. Include the fatigue test (tetani evoked by trains of 14 stimuli at 40 Hz frequency, repeated every second for 4 minutes).

4.4.4. Include at least 10 s time intervals between all the elements of the protocol above.

4.4.5. Repeat the process with successive isolated motor units.

4.5. Terminate the experiment and euthanize the animal using intravenous administration of a lethal dose of pentobarbital sodium (180 $mg \cdot kg^{-1}$).

5. Electronic apparatus

NOTE: The custom-made computer program controls the stimulator, providing the possibility to create variable patterns of stimulation, including those indicated in step 4.4. The program cooperates with the analog-to-digital converter (at least 10 kHz for the MUAP and force recordings).

5.1. Connect the AC amplifier to the computer by the analog-to-digital converter and in parallel to the oscilloscope.

5.2. Connect the force transducer to the computer by the analog-to-digital converter and in parallel to the oscilloscope. Use the force transducer to control the passive muscle force during experiment. Note that during experiment, the passive force may decrease; therefore, it is necessary to increase the muscle length to keep the passive muscle force constant.

REPRESENTATIVE RESULTS:

Parameters of motor unit contractions and action potentials can be calculated on the basis of recordings when stable conditions of recordings are ensured. **Figure 1** presents a representative recording of the single twitch of a fast MU. The upper trace shows the motor unit action potential. The delay between stimulus delivery and onset of the motor unit action potential is due to conduction time from ventral root to muscle. **Figure 2** shows a representative recording of the unfused tetanus force of a fast MU and a train of motor unit action potentials.

FIGURE AND TABLE LEGENDS:

Figure 1: A representative recording of the single twitch of a fast MU. Over the force track, there is motor unit action potential.

Figure 2: A representative recording of the unfused tetanus force of a fast MU (middle recording), a train of motor unit action potentials (upper recording) and a time position of a train of applied stimuli (below).

DISCUSSION:

If performed correctly by experienced scientists, the surgical component of the described protocol should be completed within approximately two hours. One should take particular care to maintain stable physiological conditions of the animal during the surgery, particularly body temperature and depth of anesthesia, which should be systematically controlled by assessing pinna and withdrawal reflexes. Following the surgery, it should be possible to maintain stable recording conditions for at least six hours.

The crucial experimental procedure begins with the splitting of the ventral root into very thin filaments leading to isolation of a single motor axon to the studied muscle. In fact, the thin filaments of ventral roots contain groups of axons innervating different muscles of the hindlimb; however, because all muscles except the studied one are denervated, when the stimulated bundle of axons contains only one axon to the studied medial gastrocnemius it is possible to evoke the single MU contraction only in this studied muscle. Following successful identification of the evoked activity as single MU contraction, it is possible to record a set of force recordings (single twitch, the unfused tetanus, the fatigue test) crucial for a classification of MU as one of three physiological types. The advantage of this technique is the ability to record up to 30 units in one experiment; additionally, MUs can be immediately classified as fast or slow types on the basis of "sag" presence[1,3]. Furthermore, MUs can be classified as fast-fatigable or fast-resistant with very high accuracy based on a profile of the unfused tetanic contraction force recording[16]. This last method may be used when the classical fatigue test cannot be performed. It is also worth noting that fast/slow MU classification can be also done with a 20 Hz index[17].

The proposed stimulation protocol (step 4.4) may be adapted to needs of the study. This particular set of stimulations enable to record twitch (to calculate basic twitch parameters including the twitch force, contraction as well as relaxation time), the maximum tetanus

(therefore it is possible to calculate the twitch-to-tetanus ratio), unfused tetanic contractions at a set of stimulation frequencies (to classify an MU as slow or fast basing on sag presence or 20 Hz index, as well as to calculate the force-frequency curve) and the fatigue test (necessary to calculate the fatigue index). The fatigue index is a basic method to classify MUs as fatigable or resistant. This method is open to being modified to produce various stimulation patterns; however, a possible limitation is the computer program that generates the time distribution of stimuli delivered to the axon. Moreover, some additional modifications may be introduced to answer specific research questions, such as several stimulating electrodes to activate several MUs in parallel[18], an additional laser sensor to record a mechanomyogram (MMG) from the muscle surface[19] or a recording electrode on a nerve branch to the muscle to calculate nerve conduction velocity[20].

However, it is important to be aware of the limitations and challenges of this procedure. First, a considerable part of the experimental setup is custom-made (i.e., clamps for the limb and the vertebrae segments, a plate for ventral roots and electrodes). The experimental setup includes a solid metal plate (thickness 30 mm) for all supporting metal bars (necessary for animal immobilization and the force transducer) to enable stable conditions for the isometric force recording. The application of this method also requires both extensive training in surgery as well as preparation of a complex experimental setup including an electronic apparatus and a computer program.

ACKNOWLEDGMENTS:
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DISCLOSURES:
Authors have no conflict of interest to disclose.

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Figure 1

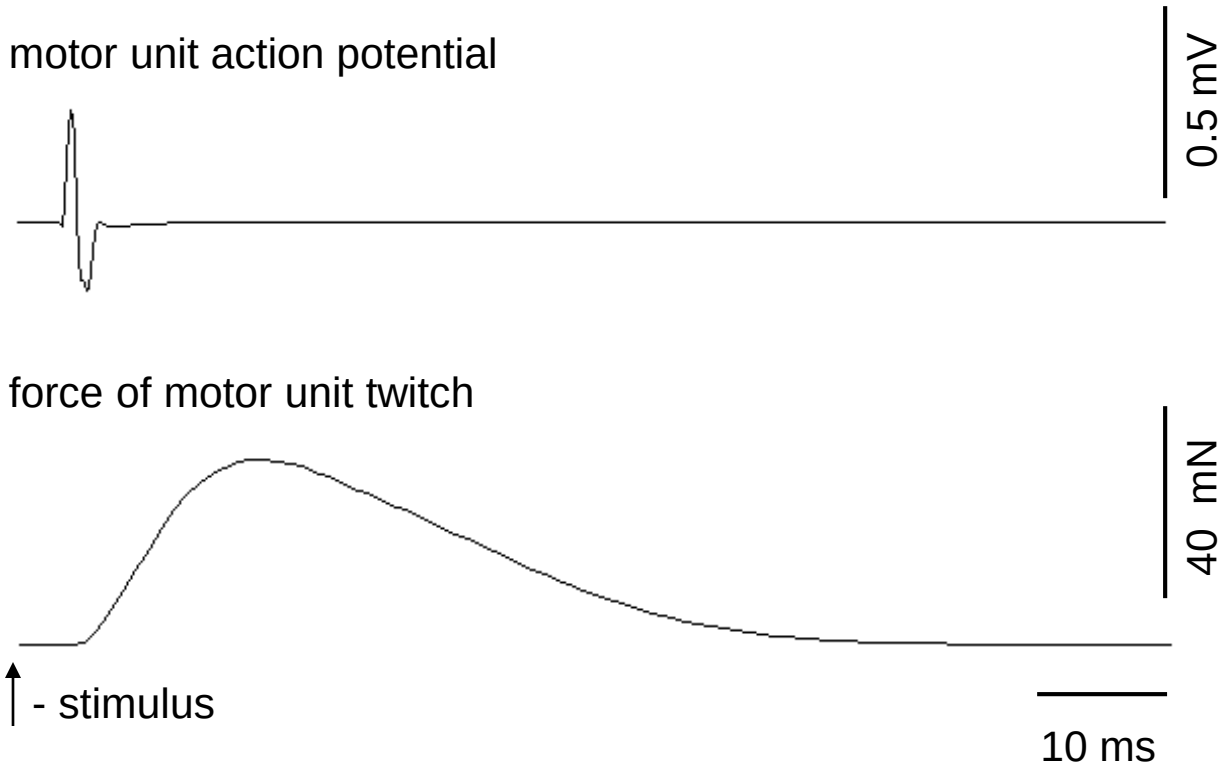

Figure 2

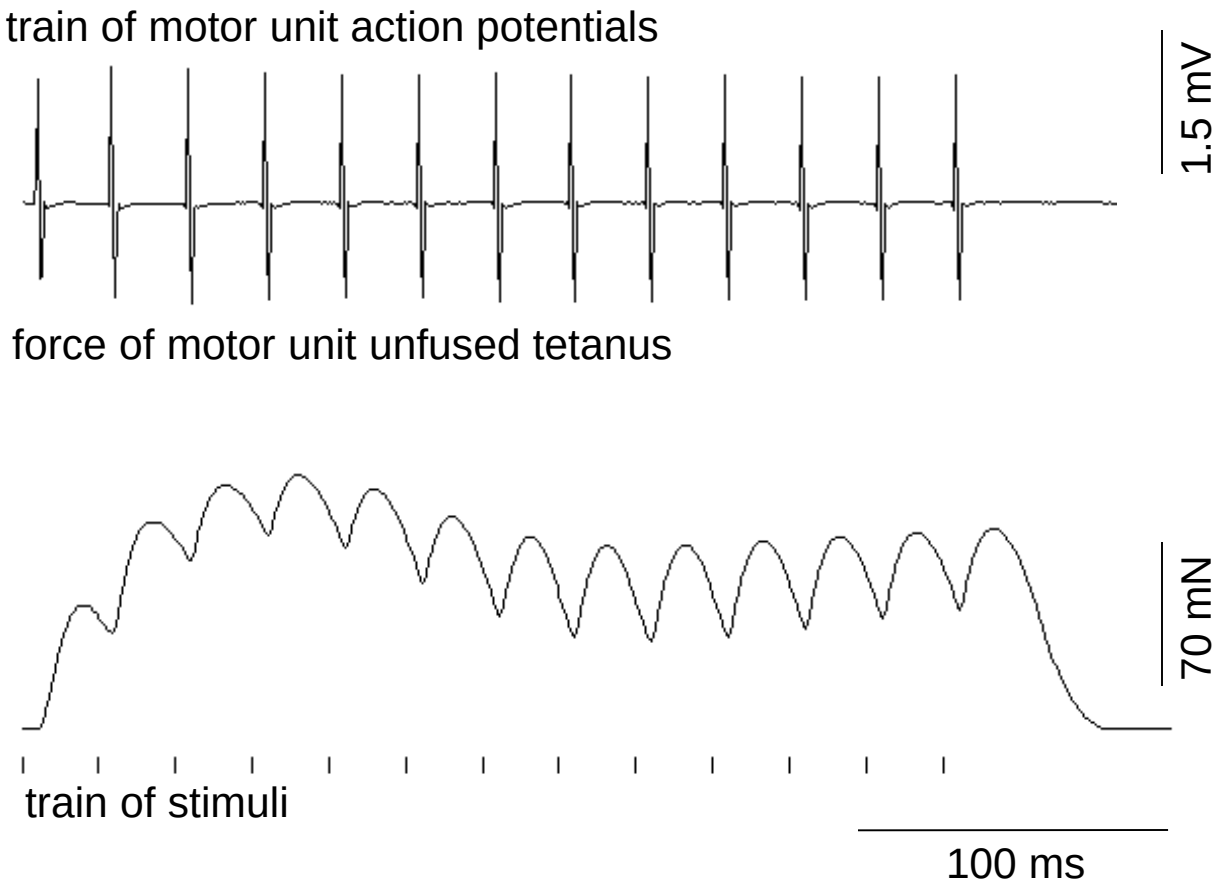

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Force transducer	custom-made	
Forceps	Fine Science Tools	No. 11255-20
Forceps	Fine Science Tools	No. 11150-10
Forceps	Fine Science Tools	No. 11026-15
Forceps	Fine Science Tools	No. 11023-10
Forceps	Fine Science Tools	No. 11251-20
Hemostats	Fine Science Tools	No. 13003-10
Isolation Unit	Grass Instruments	S1U5A
Low Noise Bioamplifer	World Precision Instruments	Order code 74030
Needle holders	Fine Science Tools	No. 12503-15
Rongeurs	Fine Science Tools	No. 16021-14
Scissors	Fine Science Tools	No. 14101-14
Scissors	Fine Science Tools	No. 14075-11
Scissors	Fine Science Tools	No. 14084-08
Scissors	Fine Science Tools	No. 15000-00
Stimulator	Grass Instruments	S88

Comments/Description

Dumont #55 with extra light and fine shanks
Extra Fine Greafe Forceps
Special cupped pattern for superior grip
Slim 1x2 teeth
Dumont #5
Hartman

With tungsten carbide jaws
Friedman-Pearson
Straight sharp/blunt with large finger loops
Curved blunt/blunt
Extra fine bonn
Straight, ideal for cutting nerves
Dual Output Square Pulse Stimulator

Answers to Reviewers' comments

Reviewer #1

This manuscript describes the protocol allowing investigators to functionally isolate a single motor axon in a ventral rootlet, and then to stimulate the motor axon in order to activate the innervated muscle fibers and to record the contractile force developped by the motor unit at tendon in rats. This protocol allows to investigate, in each experiment, a substantial fraction of the motor units from a single hindlimb muscle. This protocol is used in order to study the diversity of the motor units (there are several types of motor units that differ according to their contractile properties) and to investigate the motor units plasticity under various physical constraints or under pathological conditions. The authors are the best international experts of this technique. The paper precisely describes the protocol step by step.
I have a few suggestions for the authors:
Thank you for the valuable corrections and suggestions which may help to improve this paper and help us in our research work in the future.
Below we have explained the respective corrections and additions introduced in the paper on the basis of your comments.

Summary (line 22) : This method allows to record... (instead of "to register"...)
Corrected

Abstract
It is stated that "A crucial element of the method is the application of electrical stimuli delivered to a motor axon isolated from the ventral root, or a single stimulus or trains of pulses at constant or variable inter-pulse intervals." Why opposing single stimulus and trains with electrical stimuli ? Indeed, all of them are electrical stimuli! Please rephrase.
The phrase was rewritten

Introduction
- It would be good to acknowledge in the Introduction that this protocol has been extensively used in cats in the 70's and 80's.
Acknowledged, lines 93-94

- I think that the principle of the method (extensive denervation of the hindlimb muscles but the investigated one, in order to prepare thin ventral rootlets, each one containing a single "functional" motor axon) would be better located in the Introduction than in the Discussion (lines 314-317).
The respective phrase was added to Introduction (lines 48-51)

-line 50 : ".. more than 50 MUs of the three physiological types ...." (instead of "...which represent three their..")
Corrected

Protocol:
1.3 Anesthesia: please add the route through which the supplemental doses of anaesthetic is delivered (IP , IV). If IV route, please precise which vein is used and whether a permanent catheter is placed in the vein?

IP, indicated, line 114

2.1 Why shaving the right hindlimb since only the left one is going to be dissected?
This is necessary to insert the ground electrode (later described 3.2.3), changes in lines 119-121

2.2.5 I guess that the authors mean that they are cutting the dorsal and ventral roots close to their entry into the spinal cord? (not very clear as it stands).
We are cutting low caudal segments of the spinal cord and all ventral/dorsal root at the same level. For clarity we have added "and", line 142

2.2.5 (line 139) "... to stop bleeding." (instead of "... to block bleeding").
Corrected

2.3.6 Are the gluteal muscles also denervated? Is the femoral nerve (on the ventral side) cut?
The gluteal muscles are not denervated and the femoral nerve is intact.

3.2. Placement of electrodes for motor unit action potentials recording and ventral rootlets stimulation
Corrected, lines 224 and 241-243

4.3 (line 256) "...the strongest MUs in rat medial gastrocnemius have about 70 times larger higher twitch force than the weakest ones..." (larger instead of higher).
Corrected

4.3 (line 257) I guess that there is a summation of MUAPS that might also help to determine whether there are several MUs or just a single one. This might be added ?
Yes, it is true that shape of action potentials are indicators of number of MUs activated, and we have mentioned: "When more than one MU is contracting in the studied muscle and increasing level of the force as well as increasing amplitude or changing shape of the action potential are visible…" . Nevertheless, some MUs have their muscle fibers located in some distance to the recording electrode and are not visible in electromyogram, in such a case changing the stimulus amplitude we can see changes in the force but not in MUAP. This possibility was explained in the text, lines 268-271.

5.3 (line 286): "... to keep constant the passive muscle force." (instead of "... to keep constant stretch the muscle."
Corrected

Discussion:
Line 329 "... to calculate basic twitch parameters ..." (instead of "... to calculate basing twitch parameters...").
Corrected

Reviewer #2
Major comments:

In the current work, the authors describe an elegant approach to allow physiological and electrophysiological assessment of individual motor units in the hindlimb of the rat. While the authors provide a nice high-level view of the technique, it is not clear that sufficient detail is

provided so that readers would truly understand how to perform this technique. For a publication such as JoVE, this seems to defeat the purpose of publication. Can the level of detail and instruction be increased? Several steps seem rather vague and nonspecific. The authors point out that a considerable part of the setup is custom-made. Thus instructions (or references) should be provided for all critical steps. It seems pointless to provide instructions that do not provide sufficient detail to perform a technique. If this is not possible, maybe there is no benefit of such a publication?
Thank you for the valuable corrections and suggestions which may help to improve this paper and help us in our research work in the future. As suggested by Reviewer, we have added some detailed information concerning custom-made elements of setup (frame for the skin over laminectomy – line 187 and size of the metal frame - line 189, plate with the pool for the hindlimb – lines 200-201, force transducer – line 212, plate for the ventral roots – line 235, stimulating electrode – 241-lines 242, the amplifier – lines 225-226, and thickness of the metal plate used to install the experimental setup – line 362). Note that all these elements will be visible on video recording being a part of this article.
Below we have explained the remaining respective corrections and additions introduced in the paper on the basis of your comments.

Minor comments:
Abstract:
Second sentence of abstract: "or a single stimulus.." should this be "as a single stimulus.."?
Corrected, error indicated also by Reviewer #1.

Introduction:
The phrase "The rate medial gastrocnemius is composed of more than 50 Mus…" is vague. Can the authors provide a range than that saying more than 50 or an estimate? More than could imply 50-1000? What is meant by this statement?
Corrected, error indicated also by Reviewer #1.

Reviewer #3:

Manuscript Summary:

This manuscript describes the procedures needed to stimulate single motor axons and to record the evoked force and electromyographic responses in rat hindlimb muscles. These procedures are important to document and will be useful for many other investigators.
Thank you for the valuable corrections and suggestions which may help to improve this paper and help us in our research work in the future.
Below we have explained the respective corrections and additions introduced in the paper on the basis of your comments.

Major Concerns:
None

Minor Concerns:

1. (ln 31) "ventral root, or a single stimulus" Change to "ventral root, using a single stimulus"
Phrase corrected, error indicated also by Reviewer #1.

2. (ln 115) It is unclear why both hindlimbs are prepped. Please explain.

This is necessary to insert the ground electrode (later described 3.2.3), changes in lines 119-121

3. (ln 132) Explain how the laminectomy is performed.
Explained with more details, lines 137-138

4. (ln 173) Explain why the incision is made on the anterior side of the limb
Explained, lines 178-179

5. (ln 181) Describe and perhaps include a figure of the custom-made frame
Described, line 187, more details will be visible on video recording

6. (ln 194) "Place the rat on the custom-made metal plate with a pool" Describe this custom-made plate. Does the plate have a pool (as is implied) or is the pool made by from the skin of the hindlimb? Please make this clearer.
Corrected, the plate and pool are described with some more details, lines 200-201

7. (ln 204) Describe the characteristics of the force transducer (e.g. sensitivity).
Described, line 212

8. (ln 217) "Connect the electrode" change to "Connect the electrodes"
Corrected

9. (ln 217) Describe the amplifier
The amplifier is described with more details, lines 225-226

10. (ln 223) "Grasp the exposed skin flap of the right hind limb" The exposure of a skin flap on the right limb was not described. Why does the reference need to be placed in the contralateral limb?
Phrase was corrected, line 232; the electrode helps to decrease the noise level

11. (ln 226) "custom-made insulated metal plate" Please describe in detail this plate.
The size of the plate is indicated (line 235)

12. (ln 232) "Place a silver wire stimulating electrode" Describe the approximate dimension of the stimulating electrode.
The electrode is described, lines 241-242

13. (ln 233) "with a reference electrode inserted in the saline (added to the pool) as a negative pole" Why do you use anodal stimulation? This is quite unconventional.
The description was corrected, lines 241-243

14. (ln 239) "Stimulating with electrical pulses" Describe the characteristics of the stimulus pulses.
The stimuli were described, line 249

15. (ln 244) "split L5 or L4 ventral roots" Describe this process. For example, to you grasp the cut end of the ventral rootlet with both forceps and peel the rootlet apart?
Described as suggested, lines 255-256

16. (ln 268) You should indicate here that the process is then repeated to isolate additional motor units
The respective phrase was added, lines 281-282

17. (ln 285) "the passive force is progressively decreasing" Change to "the passive force may decrease".
Corrected

18. (ln 286) "it is necessary to increase the muscle length to keep constant stretch of the muscle" Change to "it is necessary to increase the muscle length to keep the passive tension constant"
Corrected

19. (ln 293 and 299) "Over the force track, motor unit action potential is shown" Change to "The upper trace shows the motor unit action potential"
Corrected

20. Is the delay between stimulus delivery and onset of the motor unit action potential (Figure 1) due to conduction time from ventral root to muscle? Please explain.
Respective explanation was added, lines 308-309

21. (ln 318 and 321) Change "register" to "record"
Corrected

22. (ln 320) "and another necessary force and action potential recordings." It is not clear what this phrase refers to.
Yes, this phrase was not clear, we have deleted it.

23. (ln 344) "a considerable part of the experimental setup is custom-made" Therefore, it is important that these items be describe and/or shown as figures.
We have described with more details some crucial custom-made elements of our setup (frame for the skin over laminectomy – line 187 and size of the metal frame - line 189, plate with the pool for the hindlimb – lines 200-201, force transducer – line 212, plate for the ventral roots – line 235, stimulating electrode – 241-lines 242, the amplifier – lines 225-226 and thickness of the metal plate used to install the experimental setup – line 362). Note that all these elements will be visible on video recording being a part of this article.

24. (ln 346) "for all columns" Not clear what columns refers to.
Corrected, line 361

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