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TITLE:

In Vivo Intracellular Recording of Type-Identified Rat Spinal Motoneurons During Trans-Spinal Direct Current Stimulation

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SUMMARY:

This protocol describes in vivo intracellular recording of rat lumbar motoneurons with simultaneous trans-spinal direct current stimulation. The method enables us to measure membrane properties and to record rhythmic firing of motoneurons before, during and after anodal or cathodal polarization of the spinal cord.

ABSTRACT:

Intracellular recording of spinal motoneurons in vivo provides a “gold standard” for determining the cells’ electrophysiological characteristics in the intact spinal network and holds significant advantages relative to classical in vitro or extracellular recording techniques. An advantage of in vivo intracellular recordings is that this method can be performed on adult animals with a fully mature nervous system, and therefore many observed physiological mechanisms can be translated to practical applications. In this methodological paper, we describe this procedure combined with externally applied constant current stimulation, which mimics polarization processes occurring within spinal neuronal networks. Trans-spinal direct current stimulation (tsDCS) is an innovative method increasingly used as a neuromodulatory intervention in rehabilitation after various neurological injuries as well as in sports. The influence of tsDCS on the nervous system remains poorly understood and the physiological mechanisms behind its actions are largely unknown. The application of the tsDCS simultaneously with intracellular recordings enables us to directly observe changes of motoneuron membrane properties and characteristics of rhythmic firing in response to the polarization of the spinal neuronal network, which is crucial for the understanding of tsDCS actions. Moreover, when the presented protocol includes the identification of the motoneuron with respect to an innervated muscle and its

function (flexor versus extensor) as well as the physiological type (fast versus slow) it provides an opportunity to selectively investigate the influence of tsDCS on identified components of spinal circuitry, which seem to be differently affected by polarization. The presented procedure focuses on surgical preparation for intracellular recordings and stimulation with an emphasis on the steps which are necessary to achieve preparation stability and reproducibility of results. The details of the methodology of the anodal or cathodal tsDCS application are discussed while paying attention to practical and safety issues.

INTRODUCTION:

Trans-spinal direct current stimulation (tsDCS) is gaining recognition as a potent method to modify spinal circuit excitability in health and disease[1–3]. In this technique, a constant current is passed between an active electrode located above selected spinal segments, with a reference electrode located either ventrally or more rostrally[4]. Several studies have already confirmed that tsDCS can be used in managing certain pathological conditions, such as neuropathic pain[5], spasticity[6], spinal cord injury[7] or to facilitate rehabilitation[8]. Several studies suggest that tsDCS evokes alterations in the ion distribution between the intracellular and the extracellular space across the cell membrane, and this can either facilitate or inhibit neuronal activity depending on the current orientation[9–11]. However, until recently, a direct confirmation of this influence on motoneurons was lacking.

Here, we describe a detailed protocol to conduct in vivo intracellular recording of electrical potentials from lumbar spinal motoneurons in the anesthetized rat with simultaneous application of tsDCS, in order to observe changes in motoneuron membrane and firing properties in response to anodal or cathodal polarization of the spinal neuronal network. Intracellular recordings open several areas of investigation of neuron properties, unavailable for previously used extracellular techniques[9, 12]. For example, it is possible to precisely measure motoneuron membrane voltage response to direct current flow induced by tsDCS, to indicate voltage threshold for spike generation, or to analyze action potential parameters. Moreover, this technique allows us to determine motoneuron passive membrane properties, such as input resistance, and to observe the relationship between intracellular stimulation current and frequency of rhythmic firing of motoneurons. Antidromic identification of recorded motoneuron, based on the stimulation of functionally identified nerves (i.e., nerves providing efferents to flexors or extensors) allows us to additionally identify types of innervated motor units (fast versus slow), which gives an opportunity to test whether polarization differently influences individual elements of the mature spinal neuronal system. Due to extensive surgery preceding the recording and high requirements on stability and reliability of recordings, this technique is highly challenging but allows direct and long-term assessment of electrophysiological characteristics of one motoneuron: before, during and after application of tsDCS, which is crucial to determine both its acute actions and persistent effects[13]. As a motoneuron directly activates extrafusal muscle fibers[14]and takes part in feedback control of a muscle contraction and developed force[15, 16] any observed influence of tsDCS on the motor unit or muscle contractile properties may be linked to modulations of motoneuron excitability or firing characteristics.

PROTOCOL:

All procedures connected to this protocol have been accepted by the appropriate authorities (e.g., Local Ethics Committee) and follow the national and international rules on animal welfare and management.

NOTE: Each participant involved in the procedure has to be properly trained in basic surgical procedures and has to have a valid license for performing animal experiments.

1. Anesthesia and premedication

1.1. Anesthetize a rat with intraperitoneal injections of sodium pentobarbital (an initial dose of 60 $mg \cdot kg^{-1}$ for 6-month old male Wistar rats weighing 400–550g).

NOTE: This protocol is not limited to the indicated strain, sex or age of rats. Also, alternative anesthesia such as ketamine-xylazine mix, alpha-chloralose or fentanyl+midazolam+medetomidine can be used if more suitable for different research goals or when required by the ethics committee.

1.2. After approximately 5 min, check the depth of anesthesia by pinching the rat's hind limb toe with blunt forceps. Proceed with the next steps of the protocol only when no reflex action is observed.

1.3. Inject 0.05 mL of atropine subcutaneously in order to reduce mucus production after intubation.

1.4. Inject subcutaneously 5 mL of phosphate buffer containing 4% glucose solution, $NaHCO_3$ (1 %) and gelatin (14%). This buffer will be absorbed by the cutaneous vessels throughout an experiment and will help maintain fluid balance.

1.5. Throughout the surgery, periodically check the animal for reflex actions and supplement anesthesia if required (10 $mg \cdot kg^{-1} \cdot h^{-1}$of sodium pentobarbital).

2. Surgery

2.1. Prepare the animal for surgical treatment by shaving fur over the dorsal part of the left hindlimb, from the ankle to the hip, the backside, from tail to the high thoracic segments, the left side of the chest, and the ventral side of the neck area above the sternum

2.2. Placement of the intravenous line

2.2.1. Place the rat on its back on a closed-loop heating pad (and secure it with limb fixations).

2.2.2. Using a 21 blade, make a longitudinal cut through the skin from a sternum to a chin.

2.2.3. Hold the skin with forceps and separate it from the underlying tissue.

2.2.4. Using blunt dissection techniques expose the right jugular vein. Carefully dissect the vein from surrounding tissues.

2.2.5. Locate the part of the vein without branching points, slip two 4-0 ligatures beneath it.

2.2.6. Make one loose knot on the proximal end of the previously identified non-branching segment of the vein and one loose knot on the distal end of this segment of the vein. Clamp the vein proximal to the heart, and then ligate the distal part of the vein.

2.2.7. Using iris scissors, make an incision between the clamp and distant ligature. Hold a flap of the vein, and introduce a pre-filled catheter to the point where it is blocked by the clamp.

2.2.8. While holding the vein and the catheter together with forceps, remove the clamp and push the catheter several millimeters into the vein. Secure both ends of the catheter to the vein, and add an additional fixation point to the skin.

2.3. Introduction of the tracheal tube

2.3.1. Using blunt forceps separate the two mandibular glands covering the sternohyoid muscles.Separate sternohyoid muscles at the midline to expose the trachea.

2.3.2. Slip three 4-0 ligatures beneath the trachea, then make two knots below the tracheal tube insertion point and one knot above.

2.3.3. Locate the cricoid cartilage of the larynx and make an incision below the third tracheal cartilage.

2.3.4. Insert a tracheal tube down the trachea and secure the tube in place with pre-prepared ligatures, then add an additional ligature to the skin.

2.3.5. Place a small piece of cotton wool above the separated muscles, and suture the skin over the operated area.

2.4. Dissection of hind limb nerves

2.4.1. Using 21 blade, make a longitudinal cut on the posterior side of the left hind limb, from the Achilles tendon to the hip.

2.4.2. Grab the skin with forceps, and using blunt dissection techniques separate the skin from underlying muscles on both sides of the incision.

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

2.4.4. Moving upwards cut two heads of the biceps femoris all the way to the hip to expose the sciatic nerve. Cauterize as needed to prevent bleeding.

2.4.5. Identify the sural, tibial and common peroneal branches of the sciatic nerve.

2.4.6. Using scissors, separate the lateral from the medial head of the gastrocnemius muscle to expose the tibial nerve and its branches.

2.4.7. Using 55 forceps grab the distal end of the sural nerve, cut it distally and dissect as far as possible.

2.4.8. repeat the procedure with the common peroneal nerve.

2.4.9. Using a blunt glass rod separate the tibial nerve from surrounding tissues, taking care not to damage the blood vessels, and cut it distally.

2.4.10. Identify the medial gastrocnemius (MG) and the lateral gastrocnemius and soleus (LGS) nerves.

2.4.11. Using 55 forceps, carefully dissect the MG and LGS nerves, disconnecting them from surrounding tissues, but maintaining their connection to the respective muscles.

2.4.12. Place a saline-soaked piece of cotton wool under the exposed nerves.

2.4.13. Close the skin over the operated area.

2.5. Laminectomy

2.5.1. Using 21 blade make a longitudinal incision from the sacrum up to the thoracic vertebrae.

2.5.2. Separate the skin from underlying muscles.

2.5.3. Cut the longissimus muscle on both sides of the thoracic and lumbar spinous processes.

2.5.4. Using blunt scalpel retract the muscles from the spinal column to expose the transverse processes of each vertebra.

2.5.5. Using blunt tip scissors cut the tendons of muscles connected to the transverse processes along the exposed spinal column. Apply hemostatic agents if necessary.

2.5.6. Identify the Th13 vertebra as the lowest thoracic segment with rib insertion and using fine rongeurs remove spinous processes and laminae from Th13 to L2 vertebrae to expose lumbar segments of the spinal cord. Remember not to damage the L3 spinous process which will be used as a fixation point for spine stabilization.

2.5.7. Remove the Th12 spinous process and smooth the vertebra dorsal surface as much as possible.

2.5.8. Using blunt dissection techniques separate the muscles from Th11 vertebra to create holder insertion points.

2.5.9. Place thin saline-soaked cotton wool over the exposed spinal cord segments.

2.5.10. Move the rat to the custom made metal frame with two parallel bars and two adjustable arms with clamps to support and stabilize the spine.

3. Preparation for the recording and stimulation

3.1. Vertebral column fixation and nerve arrangement

3.1.1. Place the rat in the custom-made frame on a heating pad, connected to the closed-loop heating system to maintain the animal body temperature at 37 ± 1°C.

3.1.2. Insert ECG electrodes under the skin and connect to an amplifier for heart rate monitoring.

3.1.3. Using the skin flaps, form a deep pool over the exposed spinal cord.

3.1.4. Using metal clamps, fix the vertebral column by putting clamps below Th12 transverse processes and at L3 spinous process.

3.1.5. Make sure that the vertebral column is secured and arranged horizontally, and then apply dorso-ventral pressure on both sides of the column to retract the muscles.

3.1.6. Fill the pool with warm (37 °C) mineral oil and maintain it at this temperature.

3.1.7. Thread a 4-0 ligature through the Achilles tendon, lift and stretch the operated left hind limb so that the ankle is leveled with the hip.

3.1.8. Using the skin flaps make a deep pool over the exposed tibial, MG and LGS nerves.

3.1.9. Fill the pool with warm (37 °C) mineral oil.

3.1.10. Place MG and LGS nerves on bipolar silver-wire stimulating electrodes and connect them to a square pulse stimulator. Use separate stimulation channels for each nerve.

3.2. Surface electrode placement

3.2.1. Place a silver ball electrode on the left caudal side of the exposed spinal cord, with a reference electrode inserted in the back muscles, and connect both electrodes to the differential DC amplifier. The surface ball electrode will be used to record afferent volleys from nerves.

3.2.2. Using a constant-current stimulator, stimulate the MG and LGS nerves with square pulses of 0.1 ms duration, repeated at a frequency of 3 Hz, and observe afferent volleys.

3.2.3. Determine the threshold (T) for nerve activation, stimulate each nerve at approximately 3·T intensity, and record amplitude of afferent volley for each nerve.

3.2.4. Move the surface electrode rostral and repeat the procedure to identify spinal segments at which amplitudes of the volleys are the highest for each nerve. After determining the maximum volley location, move the surface electrode to a safe distance from the spinal cord.

3.3. Muscle paralysis and forming a pneumothorax in order to reduce respiratory movements

3.3.1. Paralyze the rat intravenously with a neuromuscular blocker and connect the tracheal tube to an external ventilator in line with a rodent-compatible capnometer (Pancuronium bromide, at an initial dose of 0.4 $mg \cdot kg^{-1}$, supplemented every 30 min in doses of 0.2 $mg \cdot kg^{-1}$)

3.3.2. Monitor the end-tidal CO_2 concentration and maintain it at about 3–4% by adjusting ventilation parameters (frequency, air pressure, and flow volumes).

3.3.3. Make a longitudinal incision in the skin between the 5th and 6th rib on a side of the recording.

3.3.4. Using blunt tip scissors cut the overlying muscles to visualize intercostal space between the ribs.

3.3.5. Using small sharp scissors, make a small incision in the intercostal muscles and in the pleura, then insert a tip of a blunt edge forceps into the opening, taking care not to press on the lungs.

3.3.6. Allow forceps to expand or insert a small tube to keep the pneumothorax open throughout the experiment.

3.3.7. After the neuromuscular block, monitor anesthesia depth by checking ECG frequency, and supplement the anesthetic agent if the heart rate exceeds 400 bpm.

3.4. Opening the dura and pia mater

3.4.1. Using #55 forceps, gently lift the dura mater, and cut it caudally from the L5 segment, rostrally up to the L4 segment.

3.4.2. Using a pair of ultra-thin 5SF forceps make a small patch in the pia covering the dorsal column, between the blood vessels, exactly at the level of the maximum afferent volley from the MG or the LGS nerve.

3.4.3. Use small pieces of saline-soaked and dried gel foam to block bleeding if necessary.

3.5. tsDCS electrode placement

3.5.1. Make a small incision in the skin on the ventral side of a rat abdomen at the rostro-caudal level corresponding to the location of L4-L5 spinal segments.

3.5.2. Grab the exposed skin flap with a metal clip which will serve as a reference electrode.

3.5.3. Place a saline-soaked sponge on the dorsal side of the Th12 vertebra. Make sure that the sponge size is equal to that of an active tsDCS electrode (circle-shaped stainless steel plate of 5 mm in diameter).

3.5.4. Using a fine manipulator, press the sponge with an active tsDCS electrode to the bone and make sure that the entire surface of the electrode is pressed equally.

3.5.5. Connect both reference and active tsDCS electrodes to a constant-current stimulator unit, capable of delivering a continuous flow of direct current.

3.6. Preparation of micropipettes

3.6.1. Using a microelectrode puller, prepare a microelectrode.

NOTE: Both filament and non-filament electrodes can be used, however, remember that the shank of the electrode must be long enough to reach the ventral horn while being thin enough not to compress the spinal cord while descending.

3.6.1.1. Adjust the puller setting so that the shank entering the spinal cord is approximately 3 mm long, while the tip of the electrode is no more than 1–2 µm in diameter and microelectrode resistance is between 10 and 20 MΩ.

3.6.2. Fill the microelectrodes with 2M potassium-citrate electrolyte.

3.6.3. Mount the prepared microelectrode on the micromanipulator allowing 1–2 µm stepping movement and stereotaxic calibration.

3.6.4. Connect the microelectrode to the intracellular amplifier with the reference electrode placed in the back muscles.

4. Motoneuron tracking and penetration

4.1. Place the afferent volley recording electrode back on the dorsal surface of the spinal cord, caudally to the location of the recording site, at the level of the L6 segment.

4.2. Stimulate the MG and LGS nerves with electrical 0.1 ms pulses at a frequency of 3 Hz, and 3T intensity, to activate all the axons of alpha-motoneurons within a selected nerve.

4.3. Drive the micropipette into a selected patch in the pia with a medio-lateral angle of 15–20° (with a tip directed laterally).

4.4. After descending below the surface, calibrate the microelectrode and compensate its capacitance and voltage offset, and continue penetration of the spinal cord when all parameters are stable.An antidromic field potential of the motoneuron pool will be visible at the microelectrode voltage trace while approaching a dedicated motor nucleus during stimulation of the respective nerve.

4.5. Proceed penetration with the microelectrode at 1–2 µm steps, and periodically use the buzz function of the intracellular amplifier to clear the electrode tip from any residue.

4.6. Observe motoneuron penetration which will be characterized by a sudden hyperpolarization of the recorded voltage trace and appearance of an antidromic spike potential.

5. **Recording motoneuron membrane and firing properties**

5.1. In a bridge mode of the intracellular amplifier, identify the motoneuron on the basis of the "all-or-nothing" appearance of the antidromic action potential by stimulating respective nerve branches. Record 20 subsequent traces for later averaging.

5.2. Implement a strict inclusion criterion to ensure high-quality data:resting membrane potential of at least -50 mV in amplitude;action potential amplitudes greater than 50 mV, with a positive overshoot;membrane potential stable for at least 5 min prior to recording.

5.3. In a discontinuous current-clamp mode (current switch rate mode 4–8 kHz) of the intracellular amplifier, evoke an orthodromic action potential in a motoneuron using 0.5 ms intracellular depolarizing current pulses. Repeat at least 20 times for offline averaging.

5.4. Stimulate a motoneuron with 40 short pulses (100 ms) of hyperpolarizing current (1 nA) in order to calculate cell input resistance.

5.5. Stimulate a motoneuron with 50 ms square-wave pulses at increasing amplitudes to determine the rheobase value as the minimum amplitude of depolarizing current required to elicit a single spike.

5.6. Inject 500 ms square-wave pulses of depolarizing current, at increasing amplitudes in steps of 0.1–2 nA to evoke rhythmic discharges of motoneurons.

6. Trans-spinal direct current stimulation (tsDCS)

6.1. While maintaining a stable penetration of the motoneuron, start the polarization procedure by trans-spinal application of direct current. Adjust the current intensity and application time to the experiment design (e.g., 0.1 mA for 15 min).

6.2. Immediately after switching on the DC, observe the motoneuron membrane potential. Anodal polarization (the active electrode as an anode) should result in depolarization of the membrane potential, while cathodal polarization (the active electrode as a cathode) should evoke an opposite effect. Observe whether a change in the resting membrane potential in response to DC stimulation is constant, which ensures that electrical field intensity is not affected.

6.3. During continuous current application, repeat steps5.3–5.6 in 5 min intervals.

6.4. Turn off the DC and continue to repeat steps 5.3–5.6in 5 min intervals until recordings become unstable or inclusion criteria are compromised.

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

REPRESENTATIVE RESULTS:

Parameters of action potentials and several membrane properties can be calculated on the basis of intracellular recordings when stable conditions of cell penetration are ensured. **Figure 1A** presents a typical orthodromic action potential evoked by intracellular stimulation, which meets all criteria for data inclusion (the resting membrane potential of at least -50 mV, and spike amplitude higher than 50 mV, with a positive overshoot). Action potential parameters, such as the spike amplitude, the afterhyperpolarization amplitude or the afterhyperpolarization half-decay time (AHP-HDT) can be measured. A value of the latter parameter in rat motoneurons serves as a reliable criterion for distinguishing between fast and slow motoneurons (AHP-HDT > 20 ms for slow, while AHP-HDT <20 ms for fast motoneurons)[17]. **Figure 1B** shows a cell response to a 100 ms hyperpolarizing current pulse of 1nA, from which both peak and plateau input resistance (IR) of a motoneuron can be determined from the voltage deflection. **Figure 1C** shows an expanded voltage trace of a rheobasic spike with a clearly marked voltage threshold of the spike, indicating the level of membrane depolarization at which voltage-gated sodium channels are activated to initiate the action potential. All these recordings can be repeated several times during and after tsDCS application, which allows us compare respective parameters as long as

the resting membrane potential is stable and other criteria of stimulation and recording protocol are fulfilled.

Several studies have indirectly shown that tsDCS alters motoneuron excitability and firing pattern[9, 18]. **Figure 2** shows examples of intracellular voltage traces from two motoneurons stimulated intracellularly with 500 ms square pulses of depolarizing current before, during and after tsDCS application. Under stable conditions, recordings repeated several minutes one after another can be performed, and motoneuron firing patterns can be reliably compared. Anodal (+) tsDCS was found to act towards increased motoneuron excitability and higher frequencies of rhythmic firing (Figure 2A), while cathodal (-) tsDCS acted towards firing inhibition (Figure 2B). Moreover, the effects of both types of tsDCS outlasted the period of polarization. It is also worth noting that the observed changes in excitability and firing pattern are not merely a result of cell membrane depolarization or hyperpolarization by anodal or cathodal tsDCS, respectively, but display profound alterations not related to the change of a membrane potential, as they persisted despite the fact that this parameter returned to a baseline after the end of polarization.

Finally, it has to be stressed that any deviations from the presented protocol will likely result in a failed experiment, due to deterioration of preparation and/or a profound decline of data reliability. **Figure 3** shows examples of recordings when data inclusion criteria were compromised either due to imperfect cell penetration (**Figure 3A**), neglection to compensate microelectrode resistance and capacitance (**Figure 3B**) or a spinal cord instability (**Figure 3C**). It is important that researchers identify such non-optimal recordings, and implement proper corrective actions or disregard such results from the data set.

FIGURE AND TABLE LEGENDS:

Figure 1: Parameters of action potentials and membrane properties.(**A**) An orthodromic action potential elicited by intracellular stimulation, with indicated basic parameters which can be calculated from this record. AP ampl = action potential amplitude; AHP ampl = afterhyperpolarization amplitude; AHP-HDT = afterhyperpolarization half-decay time. (**B**) The voltage trace of a membrane response to a short (100 ms) depolarizing current pulse of 1nA intensity, which enables us to calculate input resistance (IR). Notice the peak of a potential deflection (IR Peak) followed by a small decrease and the following plateau phase of the membrane potential (IR plateau). (**C**) The expanded voltage trace of a rheobasic spike (, with a dotted horizontal line indicating the spike voltage threshold.

Figure 2: Effects of polarization on motoneuron firing.(**A**) Intracellular records from one motoneuron stimulated intracellularly with 7.5 nA for 500 ms, made before (left), during anodal tsDCS (0.1 mA, middle), and 10 min after the end of polarization (right). Note the gradual increase in the motoneuron excitability at the same stimulus intensity. (**B**) Intracellular records from another motoneuron stimulated intracellularly with 6 nA for 500 ms, made before (left), during cathodal tsDCS (0.1 mA, middle), and 10 min after the end of polarization (right). Note a gradual inhibition of motoneuron firing frequency at the same stimulus intensity. Below recordings, traces of intracellular stimulation current are provided. The calibration bars in the bottom right

apply to all presented intracellular recordings. The values of the resting membrane potential are provided to the left of each recording. Frequencies of steady-state firing, calculated from the means of the final three interspike intervals, are given above records.

Figure 3: Examples of suboptimal records as a result of deviations from the experimental protocol.(**A**) The antidromic spike recorded from a motoneuron inadequately penetrated. The resting membrane potential is insufficient (-45 mV), and despite an appropriate shape of the spike with all consecutive phases of depolarization, repolarization, and hyperpolarization, its amplitude is too low (41 mV) and without an overshoot. (**B**) A rheobasic spike generated at an unrealistic voltage threshold (membrane depolarized to +68 mV). This kind of error is usually due to a blocked microelectrode, with uncompensated resistance and capacitance. One can also see that this record is strongly contaminated by 50 Hz electrical noise. (**C**) A motoneuron rhythmic firing in response to 500 ms depolarizing current, with large fluctuations of a membrane, predominantly caused by unstable microelectrode penetration, possibly due to excessive respiratory movements. For all the presented cases the calculated membrane or firing properties would be unreliable.

DISCUSSION:

If performed correctly, the surgical part of the described protocol should be completed within approximately three hours. One should take particular care in maintaining stable physiological conditions of an animal during the surgery, in particular body temperature and depth of anesthesia. Apart from obvious ethical considerations, a lack of proper anesthesia can result in excessive limb movements during nerve dissection or laminectomy and lead to damage to the preparation or a premature experiment termination. Upon paralyzing an animal prior to penetrating the spinal cord with a microelectrode, it is crucial to monitor the depth of anesthesia and heart rate and to apply proper ventilation parameters based on animal weight and lung capacity. Any deviations from the desired physiological parameters have to be amended immediately to ensure procedure success. Following the surgery, stable recording conditions should be possible to maintain for at least four hours.

After penetration of a motoneuron, the recording stability is of high importance. It is imperative that a membrane potential remains constant during control recordings, as any fluctuations will significantly influence the rheobase current and a threshold of rhythmic firing. Proper fixation of the vertebral column should provide basic stability, while the goal of a pneumothorax is to decrease the spinal cord movements evoked by respiration. Moreover, one has to be sure that muscle contractions are fully abolished before attempting the penetration and the neuromuscular blocker is administered at regular intervals.

Following a successful penetration antidromic identification of the recorded motoneuron can be performed by stimulation of a respective nerve branch. This is a real advantage of an in vivo preparation, in which motoneuron axons are kept in continuity with the innervated muscles in reference to in vitro intracellular recordings performed on spinal slices, which only recently were possible in adult animals[16], but do not allow identification of recorded motoneuron. However, it is important that the researchers have a clear understanding of the difference between

antidromic and orthodromic activation of motoneuron[19] to avoid misinterpretation of the data. It is important to keep the peripheral nerve stimulation as low as possible (less than 0.5 V) to prevent the activation of additional nerves due to the current spread and to pay attention to a constant and short latency of the antidromic spike[19].

Another advantage of the presented technique is that motoneurons can be additionally classified as fast or slow types on the basis of their action potential parameters, namely the AHP-HDT duration[17]. Differentiation between motoneurons innervating fast-type and slow-type muscle fibers is crucial in regard to their different contribution to muscle performance during movements. Moreover, fast and slow motoneurons can react differently to the polarization[9].

To ensure reliable results of polarization one should pay attention to setting proper parameters of tsDCS. Current intensity should, on one hand, provide a desired field density at the selected area to evoke effects on neuronal networks, while on the other hand should be within safety limits for tissue damage[20]. Size of active and reference electrodes and their placement with regard to a site of recording are also important elements to consider[4], and the tsDCS duration application time should be sufficient to evoke the desired effects[16, 17, 22]. In this methods paper, the representative results were obtained by application of 100 µA cathodal or anodal polarization for 15 min. Taking into account the electrode shape and diameter, the respective electrical field intensity directly under the electrode was 39.25 $\mu A \cdot mm^2$. However, one should understand that the precise value of the electrical field at the recorded motoneuron site is impossible to pre-determine as motoneuron location with respect to polarization electrode varies, and the e-field density drops significantly with increased depth and decreased electrode size[4, 24]. Moreover, the orientation of the motoneuron compartments relative to the applied electric field is important for generation of action potentials[22, 25, 26], and this cannot be predicted for individual cells. In addition, it is highly important to understand that tsDCS effects are not limited to a period of polarization, and that persistent, long-lasting effects are well documented[22, 27]. Therefore, following even a single, brief polarization session all successive recordings in the same preparation would be performed in post-polarization conditions, which limits the number of possible acute polarization recordings to one per animal.

Additional modifications of the presented procedure can be made to answer specific research questions. This protocol with minimal modifications can be used as a standard for several experimental designs, e.g., when testing various duration and/or amplitudes of applied tsDCS or when comparing short or long-term effects of tsDCS in various pools motoneurons. Use of several genetic disease models (for example SOD1 G93A rat model of amyotrophic lateral sclerosis) or different nerves for antidromic nerve activation (peroneal, tibial, saphenous, etc.) is acceptable. However, one should also be aware of procedure limitations. For example, the use of barbiturates for anesthesia inhibits the activity of persistent inward currents[28], while the systemic introduction of specific blockers commonly used in in vitro preparations (e.g., strychnine to block nicotinic receptors) can prove fatal to the animal. It is advisable for researchers to consider these limitations before selecting the proper experimental protocol.

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DISCLOSURES:

Authors have no conflict of interest to disclose.

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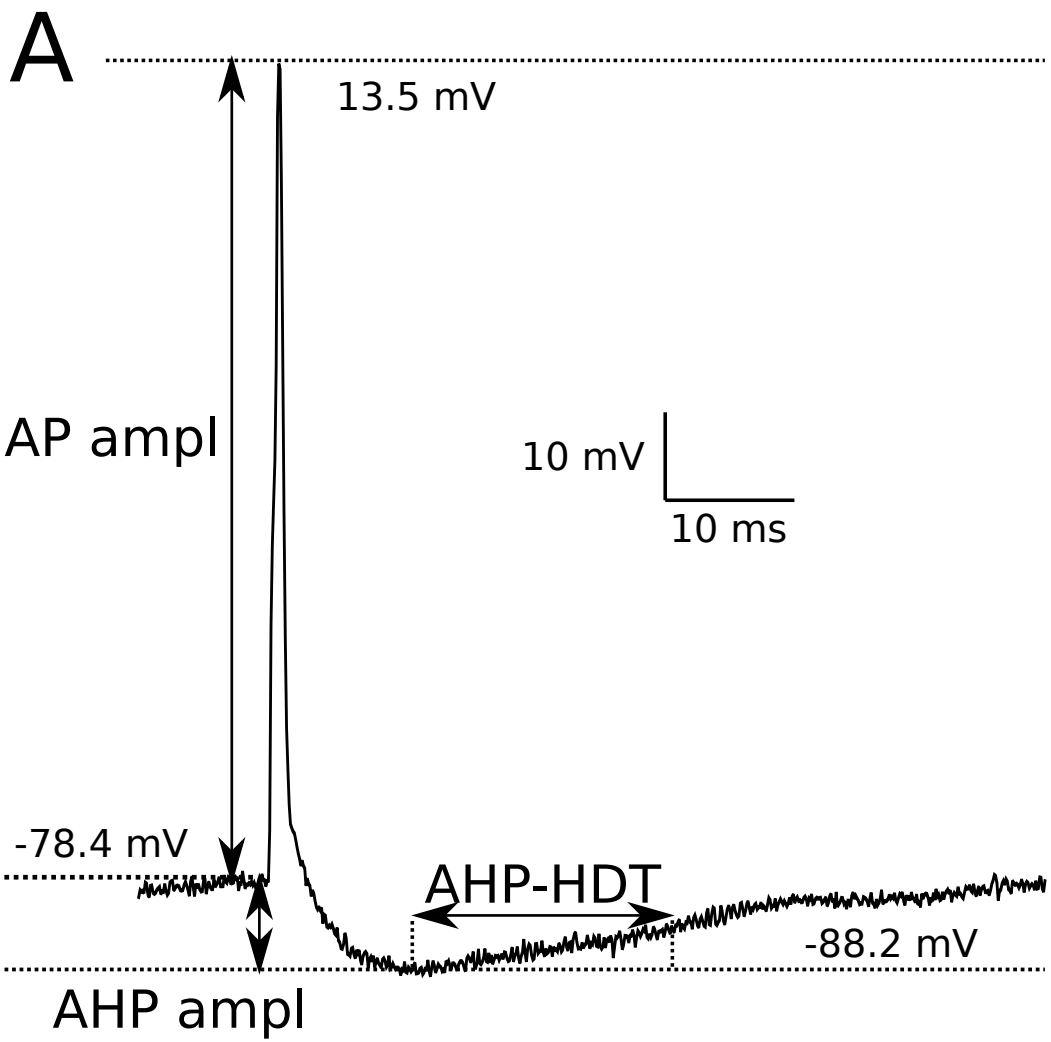
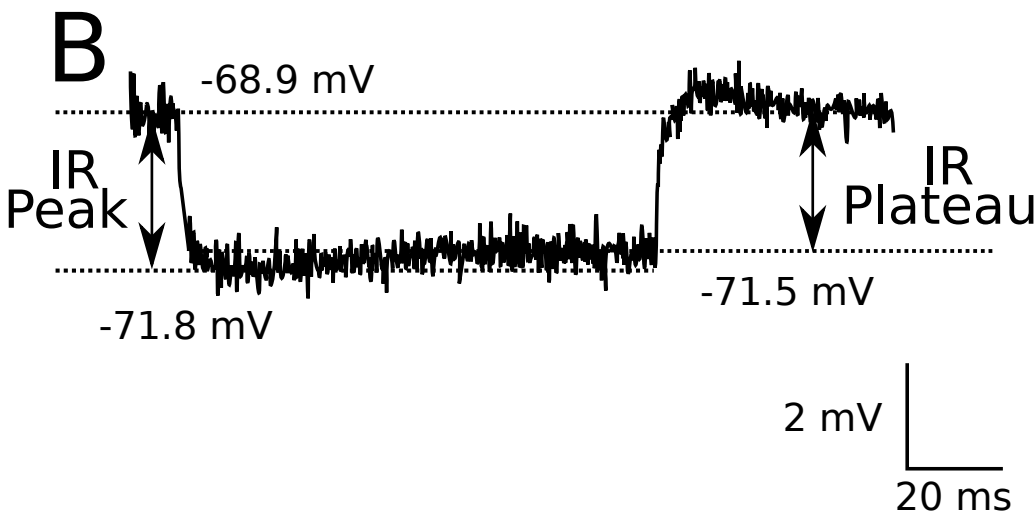
Figure 1





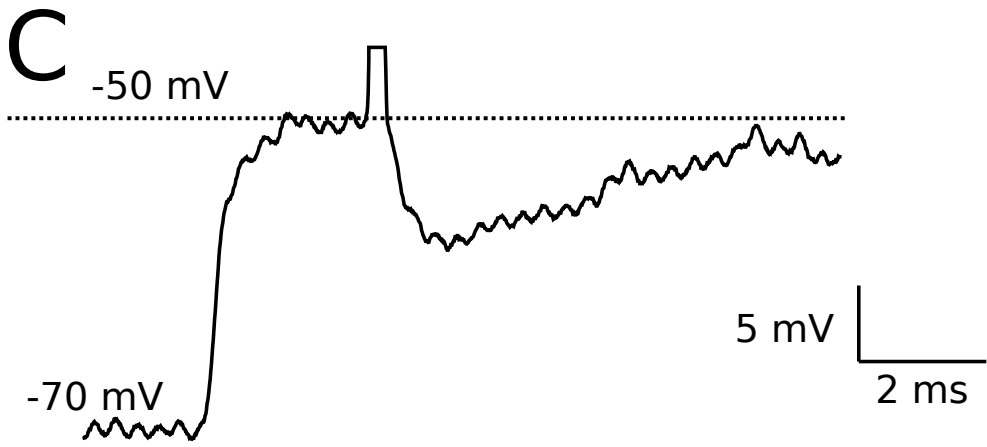


Figure 2

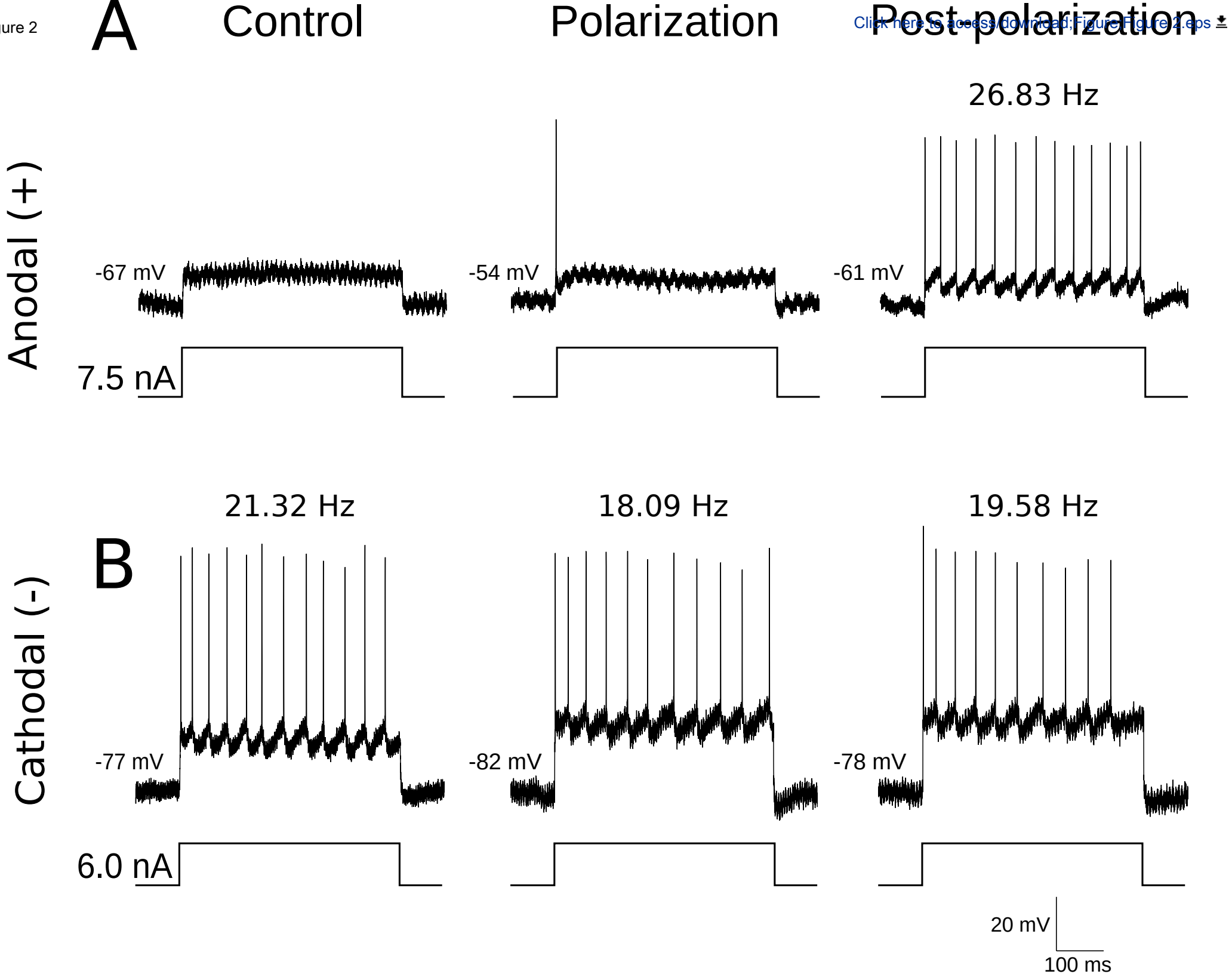


Figure 3

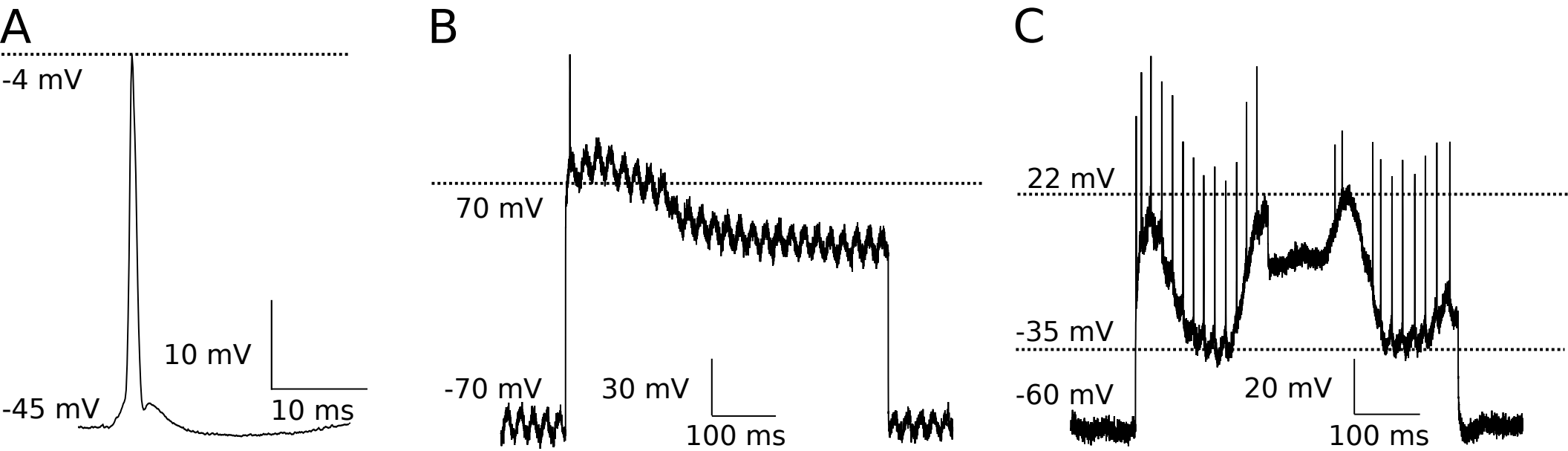


Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Durgs and solutions			
Atropinum sulfuricum	Polfa Warszawa	-	-
Glucose	Merck	346351	-
NaHCO3	Merck	106329	-
Pancuronium Jelfa	PharmaSwiss/Valeant	-	Neuromuscular blocker
Pentobarbital sodium	Biowet Puławy Sp. z o.o	-	Main anesthetic agent
Pottasium citrate	Chempur	6100-05-06	-
Tetraspan	Braun	-	HES solution
Surgical equipment			
21 Blade	FST	10021-00	Scalpel blade
Cauterizer	FST	18010-00	-
Chest Tubes	Mila	CT1215	-
Dumont #4 Forceps	FST	11241-30	Muscle forceps
Dumont #5 Forceps	FST	11254-20	Dura forceps
Dumont #5F Forceps	FST	11255-20	Nerve forceps
Dumont #5SF Forceps	FST	11252-00	Pia forceps
Forceps	FST	11008-13	Blunt forceps
Forceps	FST	11053-10	Skin forceps
Hemostat	FST	13013-14	-
Rongeur	FST	16021-14	For laminectomy
Scissors	FST	15000-08	Vein scissors
Scissors	FST	15002-08	Dura scissors
Scissors	FST	14184-09	For trachea cut
Scissors	FST	104075-11	Muscle scissors
Scissors	FST	14002-13	Skin scissors
Tracheal tube	-	-	Custom made
Vein catheter	Vygon	1261.201	-
Vessel cannulation forceps	FST	18403-11	-
Vessel clamp	FST	18320-11	For vein clamping
Vessel Dilating Probe	FST	10160-13	For vein dissection

Sugrgical materials			
Gel foam	Pfizer	GTIN 00300090315085	Hemostatic agent
Silk suture 4.0	FST	18020-40	
Silk suture 6.0	FST	18020-60	
Equipment			
Axoclamp 2B	Molecular devices	discontinued	Intracellular amplifier/ new model Axoclamp 900A
CapStar-100 End-tidal CO2 Monitor	CWE	11-10000	Gas analyzer
Grass S-88	A-M Systems	discontinued	Constant current stimulator
Homeothermic Blanket Systems with Flexible Probe	Harvard Apparatus	507222F	Heating system
ISO-DAM8A	WPI	74020	Extracellular amplifier
Microdrive	-	-	Custom
P-1000 Microelectrode puller	Sutter Instruments	P-1000	Microelectrode puller
SAR-830/AP Small Animal Ventilator	CWE	12-02100	Respirator
Support frame	-	-	Custom made/replacement lab standard base
Spinal clamps	-	-	51601/Stoelting Custom made/replacement Rat spinal adaptor
TP-1 DC stimulator	WiNUE	-	51695/Stoelting tsDCS stimulator

Miscellaneous			
1B150-4 glass capillaries	WPI	1B150-4	For microelectrodes production
Cotton wool	-	-	-
flexible tubing	-	-	For respirator and CO2 analyzer connection
MicroFil	WPI	MF28G67-5	For filling micropipettes
Silver wire	-	-	For nerve electrodes

Dear Dr. Bączyk,

Your manuscript, JoVE61439 "In vivo intracellular recording of type-identified rat spinal motoneurons during trans-spinal direct current stimulation," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **Apr 11, 2020**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Alisha DSouza, Ph.D.

Senior Review Editor

JoVE

617.674.1888

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You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.

NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Done

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Done

• **Protocol Numbering:** Please add a one-line space after each protocol step.

Added

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. 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 included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4) Notes cannot be filmed and should be excluded from highlighting.

5) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Confirmed

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Capstar, SAR-830, CWE, Dumond, Dumond 5SF, (P-1000 Micropipette Puller, Sutter,

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

The commercial language, including trademark or registered trademark symbols, has been removed from the text

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Figures and their legends are original and have been created exclusively for this article.

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:

This manuscript describes a protocol allowing the intracellular recording of spinal motoneurons in vivo in anesthetized rats, while applying trans-spinal direct current stimulation (tsDCS).

The protocol is well described. I only have mostly minor comments to help clarify the text.

We are glad that our protocol was found satisfactory by the Reviewer. We have corrected the obvious mistakes and introduced all the required corrections to clarify the procedure when necessary.

Major Concerns:

My only concern, although not quite "major" is that the authors describe the frame and spinal clamps as "custom made", but provide no more information, which could hinder any other groups trying to reproduce this method. It would be useful to provide a diagram or a photograph of the frame as a figure of the paper.

Indeed the custom-frame does not give much information. However, it is relatively simple and electrophysiological labs usually mount their own metal frames to fit space with the experimental design and setup arrangement. We have added information about its shape and purpose (point 3.5.10, lines 263-264). Regardless, this frame will be presented on film, which is part of this article.

Minor Concerns:

There are some minor mistakes in the english

- abstract (l. 35) "gold standard"

Corrected

- (l. 39) a fully mature (...) nervous system

Corrected

- 3.2.1 (l.119) Place the rat on its back

Corrected

- 3.4.2 (l.156) "Capture" -> Grab ? or Hold ?

Grab, replaced

- 5.6 (l.324° residuals -> residue ?

Residue, replaced

Some steps need clarification:

- 3.4.3 (l.158) why the popliteal fossa? Isn't it covered by the Biceps at this point? Wouldn't it make more sense to locate the knee?

The preferable approach is to locate the popliteal fossa at the back of the knee joint and then cut the Biceps muscle. However, in line with your comment, we have rewritten this point to make it more understandable to the reader.

- 3.4.5 (l.162) "cut the Planters muscle to expose Triceps Surae" (?) Isn't the planters muscle below the Triceps, how does cutting that muscle expose the Triceps?

We cannot really explain this obvious mistake. The sentence has been removed.

- 3.5.1 (l.182) "from the pelvis" (?) Do you mean the sacrum?

Corrected, we are sorry for this error.

- 3.5.5 (l.191) "Perineal membrane"? Next to the spinal column? Not sure what the authors meant here

Of course, it was a spelling error, should be “peritoneal membrane”. However, we are aware that it is possible to puncture the peritoneal membrane only in exceptional situations if the scissors are inserted deeply at a steep angle. Therefore, we have removed this comment in the revised manuscript to avoid confusion.

- 3.5.6 (l.195) "Remember not to damage the L3 spinous process." Why?

As indicated in point 4.1.4 of the procedure, the L3 spinous process is used as one of the fixation points for the spine stabilization. We’ve added a respective sentence to the protocol in order to clarify this point (point 3.5.6 lines 251-252)

- representative results (l.372) "with an overshoot" should be after the spike amplitude.

Corrected

Reviewer #2:

This manuscript describes the methodological steps for the in vivo intracellular recording from motoneurons during the trans spinal direct current stimulation (tsDCS). Animal studies are necessary to further investigate the underlying mechanism of the neuromodulation modalities. In this study, authors used a rat model and described the experimental steps for rat spinal surgeries to record the intracellular activity from the motoneurons. They used this activity as a measure to investigate how motoneurons are modulated by the tsDCS. The replication of this experimental procedure can also be used to investigate how different stimulation methods modulate the activity in the spinal cord as well.

Thank you for all the comments, which helped to improve the manuscript. We have addressed all of your criticism, and have made respective corrections and additions in the protocol, text of results and discussion.
We also regret if we have not answered fully to all your questions going beyond the frames of methodology of experiments. Some of these questions could not be simply answered in a few sentences and, which seems crucial at this point, the discussion on these issues has already been presented in recently published original papers. Below we provide detailed answers to all the major and minor points, with references to research reports, if necessary.

Major criticisms

1. Several papers from the Ahmed lab have described the effects of tsDC using extracellular recordings. The authors should be specific about what could be learned from intracellular recordings that is not already known.

Indeed the works of Ahmed lab have provided significant advances in our understanding of tsDCS effects. However as the Reviewer indicated, they are primarily based on extracellular records, which, while being highly important to the field, are not sufficient for precise analysis of membrane and firing properties of individual neurons. For example, it is impossible to record motoneuron membrane potential using extracellular techniques, to trigger an orthodromic action potential, to analyze motoneuron input resistance, to determine the voltage threshold for firing, to measure the activity of Ih current, to apply bias current to hyperpolarize or depolarize motoneuron membrane, etc.

As these points were not clear enough in the initial version of our manuscript, the additional paragraph has been added to the Introduction of the revised manuscript to fully address the Reviewer's concerns. Lines 75-82 in Introduction.

2. tsDC is designed as a non-invasive form of stimulation. But the surgery to expose the spinal cord for recording forces the application of tsDC within deep tissues. Given this limitation, what will be learned that is clinically meaningful?

Indeed tsDCS (usually referred to as trans-cutaneous spinal direct current stimulation) is intended as a non-invasive stimulation. However, our protocol describes the procedure which allows us to study physiological mechanisms at the level of individual neurons. Although direct clinical applications are difficult, such intracellular experiments can explain in more detail mechanisms behind the effects of DC stimulation. It is impossible to look for the electrophysiological effects of tsDCS at the level of individual, functionally identified neurons with a non-invasive technique. We believe that the response of the neuronal network to direct current will be similar regardless of whether the electrical field reaching the network was generated transcutaneously or at the deep tissue level. Moreover, comparisons between studies performed with different methods lead to more reliable conclusions, and actually, it has already been proven that DC stimulation acts similarly on the neuronal activity, independently if it was applied trans-cutaneously or locally (for example see Bączyk and Jankowska 2014). Several references to conclusions drawn from both invasive and non-invasive tsDCS studies in animals and humans have been given in the Introduction and Discussion sections.

The introduction of an electrode into the electric field perturbs it. How do the authors account for these effects?

We appreciate the Reviewer's concern for electrical field unity. However, we find it highly unlikely that our recording electrode can perturb the electrical field in a significant manner. First, as described in section 5 to this protocol, the microelectrode is inserted into the spinal cord before DC is switched on. Second, a principal feature of a thin glass microelectrode is that it is isolated from the extracellular environment, with a tip about 1-2 µm in diameter in the intracellular space. If we theoretically assume that this 1-2 µm electrode tip could induce some field perturbation, this perturbation would be limited to the area directly around electrode tip inserted in the cytoplasm and should be neglectable for a motoneuron membrane response to

tsDCS, especially taking into account the overall motoneuron size (with dendrites branching even 1 mm from the soma) or even the motoneuron soma size (which is in rat 30-60µm).

Minor criticisms

The manuscript would benefit from a more detailed description of certain experimental steps.

4. In this study, silver electrodes have been employed for stimulation. However, it is highly preferable to use Ag/AgCl electrodes since they have much better charge injection capacity.

We are not sure whether the Reviewer refers to the nerve stimulation electrodes or the circle electrode used to deliver tsDCS? With regard to tsDCS electrode, we are aware of the advantages of Ag/AgCl in applying trans-cutaneous tsDCS. However, please note that experimental conditions of the described preparation (the animal size, surgical preparation, stimulation and recording equipment, size and location of electrodes) were fundamentally different from tsDCS methods used in humans and in this protocol the active tsDCS electrode was located on the vertebra rostral to the recording site and not on the skin.

As for the nerve stimulating electrodes, they were only used to evoke motoneuron antidromic activation and it is known that the intensity of current to evoke this activation may vary significantly between experiments, and also depends a lot on the nerve dissection quality and overall animal conditions. Therefore using Ag/AgCl would not significantly improve the preparation. Also please note that numerous studies of several labs used silver electrodes for nerve stimulation (Baczyk et al. 2013; Cormery et al. 2005; P. Gardiner, Beaumont, and Cormery 2005; P. F. Gardiner 1993; P. Krutki et al. 2015; Manuel et al. 2009, etc.), one might even say that it was a standard procedure for intracellular recording experiments on rats, mice, and cats for most laboratories.

5. Authors noted that the current direction is important to determine the effect of the tsDCS; however, the primary mechanism of action is the orientation of the neural structures relative to the applied electric field (e-field). Hence, the e-field needs more emphasis when addressing the effect of tsDCS.

We agree that apart from the intensity and direction of the current, the orientation of the neuronal structures in relation to ie-field is also an important factor when analyzing tsDCS effects. In our protocol, we aimed to provide critical steps of the preparation in the same manner to provide as much as possible repetitive conditions. However, one should keep in mind that orientation of motoneurons and their axons within the spinal grey matter may differ between cells, despite the fact that all axons eventually run in the ventral roots. These differences might partly explain uneven responses of individual neurons to tsDCS, and this problem has been addressed and discussed in several previous papers (Rahman et al. 2013; Bikson et al. 2004; M. Bączyk et al. 2019). In line with this Reviewer's comment the respective sentence has been added to the Discussion (lines 603-612).

6. In addition to providing applied current, e-field values should be given.

E-field value has been added. To comply with the Reviewer's request we have added several lines in the Discussion to further address e-field importance (Lines 603-612).

7. What are the impedance values of the stimulation electrodes? Did they change after the stimulation? This is important to show since this may affect the e-field intensity during the experiment.

The impedance of the whole circuit was 5.7 kOhm, and we did not notice it change during stimulation (current intensity and voltage across the path were continuously monitored, so resistance could be calculated from Ohm's law, in line with suggestions of daSilva et al. 2011). Moreover, the deflection in the resting membrane potential in response to DC stimulation was monitored in the motoneuron, and when kept at a constant level during stimulation it indicated no changes in resistance of stimulating electrodes and therefore no changes of e-field intensity. In line with this comment we have supplemented point 7.2 with additional notice.

8. Even though this is a methodological paper, it would be better to repeat the experiment in another animal and show how stable the setup is.

We understand the reviewer's concern for setup stability. However, we feel that a fast glance on some of the papers published by our group (Baczyk et al. 2013; Krutki et al. 2015; Krutki et al. 2017) should ensure the Reviewer that intracellular recording of rat spinal motoneurons is indeed a very stable and reproducible procedure in our lab. We also hope that two recently

published papers on tsDCS influence on rat spinal motoneuron electrophysiological properties (Bączyk et al. 2019a, b) provide information on repeatability and stability of our tsDCS approach. The identical methodological approach as presented in this manuscript in detail was tested successfully and with repeatable results on 21 male Wistar rats (Bączyk et al. 2019a), and the same setup with slightly modified time frames of recording on 26 rats (Bączyk et al. 2019b).

9. The temperature of the recording site should be measured during the experiment; even though the core temperature is measured, temperature change can affect the activity of neurons.

The recording site of motoneuron activity is in the ventral horns of the lumbar spinal cord, at the depth of about 1.5 - 2.5 mm. We are not exactly sure how we are supposed to measure temperature precisely at this location. The closest estimate of this temperature is actually the core body temperature, and this was indeed monitored and maintained by the automatic heating system at 37 degrees Celsius throughout the experiment. Moreover, the exposed regions of the spinal cord are covered with paraffin oil, and its temperature was also kept at 37 degrees Celsius. To emphasize the importance of the stable temperature of the preparation we have mentioned this in points 4.1.1 and 4.1.6 of the protocol that the paraffin oil should be at 37±1 degrees Celsius. A sentence referring to this was added to point 4.1.6 of the protocol.

Line 66-69: "indirect evidence suggests that tsDCS evokes alterations in the ion distribution between the intracellular and the extracellular space across the cell membrane, and this can either facilitate or inhibit neuronal activity depending on the current orientation"

There is much direct evidence that low intensity direct current stimulation shifts the resting membrane potential and changes the excitability of neurons.

We are aware that the fact that electrical fields modify the resting membrane potential of neurons is not a new discovery. Possibly the phrase “indirect evidence” could be differently understood and thus confusing. Our intention was to point that before the research of Bolzoni and Jankowska (2015), in which polarization was applied locally by tungsten electrode located in the proximity of motoneuron pool, and our recent experiments (Bączyk et al. 2019a, b) there were no in vivo studies measuring changes of motoneuron resting membrane properties during intracellular recordings (i.e. by direct comparison of the voltage difference between intracellular

and extracellular space) from neurons during tsDCS stimulation. However, we acknowledge that there is a lot of evidence of such phenomenon provided on different neuron types, or with the use of different techniques. The earlies would be one of classical works of Eccles (Eccles, Kostyuk, and Schmidt 1962) in which polarization was applied by electrodes located on the dorsal and ventral surface of the cat spinal cord to modify Ia synaptic excitability, multiple in vitro studies also tackled this issue (Bikson et al. 2004) and it was indeed the possibility to control cell membrane potential (a principle of voltage-clamp techniques) that allowed the discovery of several types of voltage-gated ion channels (Armstrong and Hille 1998), and moreover modeling studies were also implemented to predict how spinal neurons would behave in the presence of externally induced electric fields (Elbasiouny and Mushahwar 2007; Tranchina and Nicholson 1986).
To avoid further confusion we have removed the phrase “indirect evidence” and we have rewritten the indicated sentence to make our point clear: “Several studies suggests that tsDCS evokes alterations in the ion distribution between the intracellular and the extracellular space across the cell membrane,”

Line 74-78: "Antidromic identification of recorded motoneuron, based on the stimulation of functionally identified nerves (i.e. nerves providing efferents to flexors or extensors), with a later analysis, allows us to additionally identify types of innervated motor units (fast vs. slow), which gives an opportunity to test whether f polarization differently influences individual elements of the mature spinal neuronal system."

What types of later analysis? There is an extra "f" in the line 77.

We apologize for not making this point clearer. The later analysis referred to the analysis of the action potential parameters with the main focus on the after hyperpolarization half-decay time (AHP-HDT) which cannot be made immediately during the experiment, but rather during analysis on the records after the experiment is completed. We have removed this phrase “later analysis” from the sentence in the introduction, however, this “later” analysis has already been described in further parts of the text, when explaining calculation of the parameters presented in Fig. 1. in lines 487-491.

Line 90: I don't know the journal policy about this, but I think it would be helpful to write a brief explanation about each section before going into details. This would help the reader to better understand why that step is needed.

According to the detailed instructions of the journal, the protocol should not consist of explanations and the following steps should form a logical flow. Also, the Editor did not ask to provide such explanations in the revised version.

Line 100: Please provide the strain, sex, and weight of the rat.

In line with the Reviewer's request, we have provided the details of the animals used for this study (line 110). However, please note that this protocol is not restricted to only one strain, sex, weight or age of rats. We believe that this methodological paper describes the procedure that could be adapted to the requirements of individual researchers, and this notice has also been added at point 2.1. (lines 110-114).

Line 100: Is there a specific reason for using the sodium pentobarbital as an anesthesia regimen since most of the studies are using ketamine/xylazine or urethane for the intra- and extra- cellular recordings.

There is no specific reason for the use of sodium pentobarbital as an anesthesia regiment. The use of barbiturates was chosen due to our vast experience in using this kind of anesthesia for rat intracellular motoneuron recording and that it does not jeopardize the goals of the experiment. Another type of anesthesia is certainly possible (though actually, urethane is not allowed by our local ethical committee due to its high cancerogenic actions), and a respective notice about such possibilities has been added to the protocol 2.1. (lines 110-114).

Line 125-131: "slip two 4.0 ligatures beneath the vein, and make knots on ligatures at both distal and proximal parts of the vein. Clamp the vein proximal to the heart, and then ligate the distal part of the vein."

Suggest clarifying this language; proximal and distal and used to refer to two different structures.

The sentence has been rewritten to clarify this step of the procedure (lines 152-154)

Line 164: Why are some nerves transected and others preserved?

Anatomical organization makes it extremely difficult to place the MG and LGS nerves on the bipolar stimulating electrodes if the Sural, Common Peroneal and lower parts of the Tibial nerves are not transected. Dissection of other nerves such as posterior biceps, semitendinosus or quadriceps nerves is not necessary as these nerves are located far away from MG and LGS nerves stimulation sites. On the other hand, when MG and LGS nerves are dissected but not transected they are still connected to the muscles they innervate and they are less injured, which is in favor of the optimal conditions for antidromic stimulation during experiments.

Line 193: "Identify the Th13 vertebra, and using fine rongeurs remove spinous processes and laminae from Th13 to L2 vertebrae to expose lumbar segments of the spinal cord."

How do you identify Th13 (lowest segment with rib insertion)?

Indeed, that was one of the methods. Another option is to locate the last L6 vertebra before laminectomy and count spinous processes rostrally. The explanation has been added to the point 3.5.6 of the protocol (line 248-252)

Line 207: Must a custom spinal frame be used? What about commercial frames, such as the Kopf spinal frame?

It is not necessary to use a custom frame. Kopf or Stoelting frames can also be used and this possibility was given in the material list, being a part of this article. However, electrophysiological labs usually mount their own metal frames to fit space with the experimental design and setup arrangement. We have added information about the shape and purpose of this metal frame (point 3.5.10, lines 263-264). Regardless, this frame will be presented on film, which is part of this article.

Line 233: "Using constant-current stimulator (GRASS Instruments; model S88), stimulate the nerves with square pulses of 0.1 ms duration and observe afferent volleys."

Are you using pulse train here for stimulation? If yes, what is the burst duration?

The nerves were stimulated with 0.1 ms pulses repeated at 3 Hz. This was already specified at point 5.2 of the protocol. We have added additional information in line 309 to clarify this point.

Line 232- 234: "Using constant-current stimulator (GRASS Instruments; model S88), stimulate the nerves with square pulses of 0.1 ms duration and observe afferent volleys."

Which nerves are you stimulating? Are the afferent volleys recorded from the ball electrode? Does the position of the electrode change with the nerve being stimulated? What is the rationale for the suggestion of stimulation at 3T? Does the stimulus intensity change during the experiment based on new threshold measurements?

The information that MG and LGS nerves were stimulated has been added to the point 4.2.2. (originally it was given later in point 5.2.).
A note about afferent volleys recorded from the ball electrode has been added to the point 4.2.1. (lines 306-307).
As indicated in the points 4.2.3. and 4.2.4 the surface electrode was moved in order to identify spinal segments at which amplitudes of the volleys are the highest for each nerve, and then left at a safe distance from the spine, and as indicated later in the point 5.1 the electrode was placed back on the dorsal surface of the spinal cord, caudally to the location of the identified recording site.
The rationale for the suggestion of stimulation at 3T was explained at the point 5.2: 3T intensity is necessary to activate all the axons of alpha-motoneurons within a selected nerve. Such intensity of the stimulation during descending the micropipette assures us that antidromic activation of all motoneurons can be achieved.

Line 228: "Surface electrode placement"

To make it clearer where the recording is made, make more descriptive (e.g. dorsal spinal cord recording at specific spinal level). Specify model of amplifier.

Points 4.2.1, 4.2.3, 4.2.4 and 5.1 describe the volley electrode placement procedure. The points 4.4.1 and 4.4.2. indicate that the sites for micropipette insertion are in segments L4 and L5. Therefore, the ball electrode should be located caudal to L4-L5 (point 5.1), which means that it is usually at the L6 level. In line with the comment, this information has been added to point 5.1.

It seems important to note that slight variations in location of the surface ball electrode will result in minor changes in the amplitudes afferent volleys from nerves, but will not affect the results of intracellular recordings. Moreover, the exact locations of recording sites may slightly differ in each preparation, as they depend on the course of blood vessels on the surface of the spinal cord (which is highly variable between rats).

The reference to the amplifier type was given only in the material list. The policy of the journal is against indicating this in the protocol: “JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent.”

Line 260: "insert a small tube to keep the pneumothorax open."

Will this small tube stay there for the rest of the surgery?

Indeed the tube will remain in the chest cavity during the entire surgery and later during the recording procedure “to keep the pneumothorax open”. As indicated in the discussion (lines 579-580), the chest tube is used to stabilize the recording conditions by minimizing respiratory movements. This is crucial for stable intracellular recordings, and therefore it is imperative that the tube stays in place throughout the experiment. In line with the Reviewer’s comment this statement has been added to point 4.3.6.

Line 267: "Using Dumond 55 forceps lift gently the dura mater, caudally from the L5 segment, rostrally up to the L4 segment."

After lifting, are you cutting the dura?

We apologize for this obvious omission. The sentence has been supplemented. Point 4.4.1.

Line 269: Maximum afferent volley from stimulation of which nerve?

Point 4.4.2. has been supplemented with the respective information.

Line 279: Metal clip is not a good return electrode. Use Ag/AgCl.

We are aware of the advantage of Ag/AgCl electrodes over a metal clip. However, please see our response above to question 4.

Line 281: Need more detail for the electrode placement. Was the electrode placed over the skin?

No, the active electrode was placed on the dorsal side of Th12 vertebrae, and not on the skin. Please, see point 4.5.3 of the protocol: "Place a saline-soaked sponge on the dorsal side of the Th12 vertebra. Make sure that the sponge size is equal to that of an active tsDCS electrode (circle-shaped stainless steel plate of 5 mm in diameter)", and point 4.5.4: "Using a fine manipulator press the sponge with an active tsDCS electrode to the bone and make sure that the entire surface of the electrode is pressed equally."

Line 291: "Preparation of micropipettes"

What are the specifications of the used glass pipette (inner and outer diameter)?

The glass used for micropipette was the WPI 1B150-4 Single-Barrel Standard Borosilicate Glass Tubing with the inner diameter of 0.84 mm and outer diameter of 1.5 mm. However this information is meaningless with respect to the experimental protocol, as the different microelectrode pullers produce different microelectrode shapes, and they can equally be used and give comparable results. Also giving the puller settings would be misleading as pull parameters always have to be adjusted when a new batch of tubing is introduced, a new heating filament is installed or even when the humidity removal part of the puller is replaced. Moreover, the policy of the journal is against indicating company or product names in the protocol: "JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent."
The meaningful parameters of the microelectrodes which influence recording stability and precision are its resistance, tip diameter and shank length (all are already specified in point 4.6.1).

Line 337: For the experiments during which motoneurons are stimulate, please specify if this is with the recording electrode. For each, please specify the recording site.

We are very sorry, but we do not understand this question. The protocol describes motoneuron intracellular recordings which as the principle are made with sharp microelectrodes, and both recording and intracellular stimulation are accomplished simultaneously with the same electrode. Also, we are not sure about specifying the recording site, as it was specified in point 4.6.1 (that the recording electrode must reach the ventral horns of the spinal cord), and point 4.4.2 (that the patch for microelectrodes insertion should be made at the level of a maximum afferent volley from the stimulated nerves), and point 5.3 (describing driving the microelectrode down into the spinal cord). The precise depth or the recording within the spinal segment is different for each motoneuron (usually between 1.5-2.5 mm below the surface), it depends on an angle of micropipette insertion and in fact, the essential is antidromic identification of a motoneuron.
Moreover, as indicated in the lines 613-615 of the discussion: " following even a single, brief polarization session all successive recordings in the same preparation would be performed in post-polarization conditions, which limits the number of possible acute polarization recordings to one per animal". With regard to the above, we do not understand how we should specify the recording site for "each" motoneuron.

Line 341: "Stimulate a motoneuron with 40 short pulses (100 ms) of hyperpolarizing current (1 nA) in order to calculate cell input resistance."

How do you calculate the cell input resistance?

The input resistance is calculated from the voltage deflection in response to 1nA hyperpolarizing current. The respective sentence in the results section has been supplemented (lines 493-494). We hope that this supplement in the text together with the graphical example shown in Figure 1B are sufficient to explain this calculation.

Provide more detail about the recording amplifier specifications (Cut-off, gain, etc.).

The amplifiers parameters were set to meet the specific requirements of our recording setup and experimental design. It has to be noted that amplifier settings may vary significantly in different setups, so we believe that it is not necessary to indicate the specifications in detail in the protocol, especially when the policy of the journal is against indicating company or product names in the protocol: "JoVE is unable to publish manuscripts containing commercial sounding

language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent.”

For the Reviewer’s information, below we provide the detailed amplifier specifications:

For volley recording:
Low filter: 10 Hz
High Filter: 3 kHz
Notch Filter 50 Hz
Signal gain: 10^3

For the microelectrode amplifier:
Preamplifier Type: HS-2A x 1LU
Precision resistor: 10 MΩ
Max. Bridge Balance 100 MΩ
Max. Step Command: ±199.9 nA
Max. DC Current Command: ±100 nA
Ext. Command: 10 nA/V
Max Total Current(2): 600 nA
I Output 1: 10 mV/nA
Max. Gain in dSEVC: 100 nA/mV
Max. Gain in cSEVC: 1000 nA/mV
Max. Gain in TEVC: 10000 V/V
CAP NEUT RANGE: -1 to 7 pF
Input leakage current: 10 pA

Internal filter:
Single-pole low-pass filter at 3 dB 30 kHz frequency
The time constant of the exponential filtered signal: 0.0053

What is the length of the neural recording and tsDCS (pre-stim, post-stim, and stimulation)?

It is impossible to perform a full recording protocol of motoneuron electrophysiological properties in a single step. The reason for that is that different settings have to used to record different parameters (for example BRIDGE mode for antidromic action potential recording, DCC mode for

input resistance recording, 0.5 ms pulses for orthodromic cell activation, 50 ms stimulation for rheobase, 500 ms stimulation for SSF recording, etc.). Therefore the methodologically correct way of performing intracellular recordings is to divide the recording into several steps covering different aspects of cell physiology (points 6.3-6.6.). It is also very difficult to determine the precise duration of each step because it is impossible to predict how a motoneuron reacts to intracellular stimulation protocol (for example how many steps of increasing depolarization current will have to be used to evoke minimal and maximal SSF). In very general terms, we can say that the full intracellular recording/stimulation protocol lasts on average 3-4 minutes. Then we are able to repeat the procedures every 5 min. Therefore, during tsDCS lasting for 15 minutes (we have accordingly supplemented point 7.1 of the protocol) we can safely repeat recording procedures 3 times, in 5-min. intervals (as indicated in point 7.3 of the protocol).
As for the post-stim period, there is no rule on how long it can last. From our experience, it is extremely difficult to maintain stable intracellular recordings of the same motoneuron longer than 30 minutes. Therefore we suggest in point 7.4 of this protocol to maintain the recordings until a cell becomes unstable or inclusion criteria are compromised.
However, the exact time frames seem not critically important, as if for some reason the consecutive steps 6.3-6.6 would last by a few minutes longer than suggested, it still would be possible to make at least one recording before, one during and one after polarization.

REPRESENTATIVE RESULTS:

Line 392-396 : "It is also worth noting that the observed changes in excitability and firing pattern are not merely a result of cell membrane depolarization or hyperpolarization by anodal or cathodal tsDCS, respectively, but display profound alterations not related to the change of a membrane potential, as they persisted despite the fact that this parameter returned to a baseline after the end of polarization."

What are the other reasons?

This is an excellent question, and we would be very happy to be able to provide a definite answer to that. It is well known that multiple factors can affect motoneuron firing. Cell membrane polarization, input resistance, and synaptic inputs are only the most obvious ones. A substantial number of additional factors could be mentioned, such as the activity of persistent inward currents (both calcium and sodium-dependent), voltage-gated ion channel activity, long term potentiation or long term depression phenomena, or sodium inactivation properties. However,

this is a methodological paper in purpose, and the clear instructions from the Editors define that the representative results and discussion should focus on methodological aspects of the protocol only, and the presented results should not be discussed. However, we encourage the Reviewer to look for hypotheses and possible explanations of the observed phenomena in our previous papers (M. Bączyk et al. 2019a,b) or papers of the E. Jankowska group, in which an extensive discussion on neuronal mechanisms of tsDCS influence had taken place.

Fig 2 needs more explanation, or I have misunderstood their point.

* What are the number of spikes during the pre, post, and stimulation periods? Please provide a bar plot or just add a text on the figure for each case.

For analysis of rhythmic firing of motoneurons in response to long (500 ms) square-wave pulses, steady-state firing frequency measured from the last three spike intervals in a series seems more accurate than a number of spikes (Button et al. 2006). This information has been added to Figure 2 (lines 545-546).

* Control recording means there is no tsDCS but pulse stimulation; hence, both cells should have a similar firing pattern. However, one of the cells is completely quite while other the one has a strong response to the stimulation. Why?

While analyzing motoneuron intracellular recordings, one should be aware of the significant variation of motoneuron threshold properties. Rheobase current may vary in a population of motoneurons in a very wide range, e.g. between 1.5 and 21.90 nA (Bączyk et al. 2019a), the same concerns the minimum threshold and frequency of rhythmic firing. Such widespread values are common in all motoneuron studies (Baczyk et al. 2013; Cormery et al. 2005; Gardiner, Beaumont, and Cormery 2005; Gardiner 1993; Krutki et al. 2015; Manuel et al. 2009, etc.). The reason is that motoneurons are highly variable with respect to cell size, number and density of ion channels which influence input resistance, as well as passive and active membrane properties. There are literally no two identical motoneurons that would have identical firing patterns at the same stimulation intensities. Therefore the only acceptable way of presenting acute tsDCS effects is to show how it modulates the activity of a single motoneuron by comparison between pre-, during, and post- recordings. For this reason, the examples selected in figure 2A were supposed to show the facilitation of firing by anodal polarization, and the cell selected for figure 2B was supposed to show a modest inhibitory effect of cathodal

polarization. As this paper is not intended to discuss these differences between the two kinds of polarization, for details see Bączyk et al. 2019a,b.

* For anodal stimulation, when the stimulation is started, the membrane potential went up to -54, which is higher than the pre and post polarization periods and expected. However, the number of spikes occurred during the post stimulation period is significantly higher than the stimulation period even though it has a lower membrane potential. Why?

Once again, this is a great question for which we cannot provide an extensive answer in this paper which has a purely methodological purpose. Moreover, such an answer needs a couple of paragraphs, and discussion on facilitation or inhibition, and persistent effects of tsDCS would be here a repetition regarding our previous publications. Therefore please look for possible explanations and respective discussion in our recent paper (Bączyk et al. 2019a,b).

* For cathodal stimulation, activity decreases a little during the tsDCS; however, it does not come back after 10 min; oppositely, activity further decreases. Why?

Please, see our answer above.

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