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Terminal H-reflex Measurements in Mice

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Abstract

The Hoffmann reflex (H-reflex), as an electrical analog to the stretch reflex, allows electrophysiological validation of the integrity of neural circuits after injuries such as spinal cord damage or stroke. An increase of the H-reflex response, together with symptoms like non-voluntary muscle contractions, pathologically augmented stretch reflex, and hypertonia in the corresponding muscle, is an indicator of post-stroke spasticity (PSS).

In contrast to rather nerve-unspecific transcutaneous measurements, here, we present a protocol to quantify the H-reflex directly at the ulnar and median nerves of the forepaw, which is applicable, with minor modifications, to the tibial and sciatic nerve of the hindpaw. Based on the direct stimulation and the adaptation to different nerves, the method represents a reliable and versatile tool to validate electrophysiological changes in spasticity-related disease models.

Introduction

The Hoffmann reflex (H-reflex), named after the physiologist Paul Hoffmann, can be evoked by electrical stimulation of peripheral nerves, which carry axons of sensory and motor neurons arising from and leading to the same muscles. It is the electrically induced analog of the monosynaptic stretch reflex, and shares the same pathway[1]. Unlike the muscle stretch, the H-reflex results from electrical stimulation. When peripheral nerves are electrically stimulated at low current intensity, the Ia afferent fibers are typically depolarized first due to their large axon diameter[2]. Their action potentials excite alpha motorneurons (αMNs) in the spinal cord, which in turn elicit action potentials that travel down the αMN axons toward the muscle (**Figure 1**). This cascade generates a muscular response with small amplitude, reflected in the so-called H-wave. By gradually increasing the stimulus intensity, the amplitude of the H-wave increases due to the recruitment of additional motor units. From a certain stimulus intensity, action potentials in the thinner axons of the αMNs are elicited directly, which is recorded as the M-wave. This M-wave appears with a shorter latency than the H-wave (**Figure 2**). If the stimulation intensity is further increased, the amplitude of the M-wave becomes larger due to the recruitment of more

αMN axons, whereas the H-wave gradually becomes smaller. The H-wave can be suppressed at high stimulus intensities due to antidromic backpropagation of action potentials in the αMN axons. These triggered action potentials collide with those from the Ia stimulation and can thus cancel each other out. At supramaximal stimulus intensities, orthodromic (toward the muscle) and antidromic (toward the spinal cord) action potentials occur in all MN axons; the former gives rise to the maximal M-wave amplitude (Mmax), whereas the latter results in complete abolition of the H-reflex[3].

For the evaluation of post-stroke spasticity (PSS) or spinal cord injury (SCI), the H-reflex has been used to assess the neural basis of movement and spasticity in humans[1]. An improved quantification of the change in the H-reflex between measurements and between subjects is achieved by using the ratio of the H- and M-wave (H/M ratio). Alternatively, the rate-dependent depression (RDD) is measured, using a set of ascending frequencies (e.g., 0.1, 0.5, 1.0, 2.0, and 5.0 Hz). The RDD reflects the integrity of inhibitory circuits that may be disturbed by stroke or SCI. When all neural circuits are intact, there is a uniform, frequency-independent suppression of the H-reflex. However, if there is reduced neural inhibition as a result of stroke or SCI, the suppression of the H-reflex decreases with increasing stimulation frequency[4].

The correct electrophysiological recording using surface electrodes can be challenging and may be affected by motor tasks, inhibitory mechanisms, and αMN excitability[5]. In the transcutaneous recording in rodents, a stimulus electrode is placed near the tibial nerve, and a recording electrode is placed near the related muscles in the forepaw. According to our experience, however, the correct placement of the transcutaneous electrodes (**Figure 1A**) is even more complex and variable in rodents than surface electrode placement in humans. This can lead to differences in length, frequency, and stimulation intensity necessary to elicit the H-reflex. These methodological challenges could explain why there are only a very limited number of H-reflex measurement studies (e.g., in experimental stroke models[3,4], and other spasticity models[6]. A precise (long-term) stimulation and recording of the H-reflex on individual nerves could, in principle, be achieved using implantable electrodes surrounding the target nerve[7,8]. Due to the challenging surgery with potential side effects for the animal and potential instability of the probe, this approach has not become a standard in the field. The method presented here also requires some surgical expertise. However, it allows a novel, precise stimulation and recording of isolated nerves *in vivo* using low stimulation intensities, which avoids simultaneous stimulation of neighboring nerves.

Protocol

All experiments were conducted in compliance with European and National animal care laws and institutional guidelines, and were approved by the Landesamt für Natur-, Umwelt-, und Verbraucherschutz North Rhine-Westphalia (Az: 81-02.04.2019.A309). The protocol is optimized for adult mice (approx. 8-16 weeks old C57Bl/6J mice) and the forelimb recording. It can be easily adapted by stimulating the respective nerves of the hindlimb and recording hindpaw muscles (**Figure 1B**). A description of the recording and stimulation electrodes is added in the **Table of Materials**. Note that the protocol is used for a terminal measurement only.

1. Preparation

1. Weigh the animal and start anesthesia by injecting *via* i.p. a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg).

2. Keep the mouse in the warming box until surgical tolerance is reached. Wait a few minutes until the mouse is calm, the breathing is stable, and reflexes are absent. Check the depth of anesthesia by measuring the lack of response to the toe pinch.

3. Turn the mouse on its back and place it on a heating pad (optimal would be feedback-controlled heating using a rectal temperature probe). Fix the forepaws with tape. Here, ensure that the tape is positioned in such a way that the measuring electrodes are easily inserted into the forepaws.

4. Insert a rectal probe to measure the temperature of the animal and fix it with tape. Apply eye ointment to prevent the eyes from drying out.

2. Surgery

NOTE: The direct nerve H-wave measurement procedure is shown for the radial/ulnar/median nerve of the forepaw (**Figure 3A**). The measurement can also be adapted to the hindpaw (sciatic/tibial nerve) with modifications.

1. For a better overview of the surgical area, remove the hair with an electric razor or a pair of scissors in a seperate place beforehand. For more experienced surgeons this is only optional.
   NOTE: Disinfection is not necessary here, as this is an end experiment. The animal will be euthanized later.

2. Lift the skin with tweezers and make an incision of approximately 1 cm in the skin along the ventro-posterior axis of the forepaw (area above the armpit and thorax) with a pair of fine rounded scissors.

3. Carefully remove the connective tissue and expose the muscle and nerve underneath. Remove the exposed pectoralis profundus muscle using forceps, to access the median nerve (**Figure 3E,F**). Remove small amounts of blood and tissue fluid with soft tissue.

4. In the next step, carefully cut the breast or axillary muscle from the top to the bottom to expose the nerve bundle underneath. Free the nerve bundle from the connective and muscle tissue over a length of approximately 1.5 cm.
   NOTE: Here, particular care should be taken not to damage the blood vessels that run in parallel to the median nerve. When cutting the tissue, always cut along the nerve to avoid injuring it. If this happens, remove leaking fluid and blood with a swab. Astereo microscope is not necessary for the entire experiment, however it can be useful for the preparation of the nerves.

5. Separate the ulnar and median nerve of the forepaw carefully using a bent glass pipette (**Figure 3E**). The upper of the two nerves is the ulnar nerve, and the lower is the median nerve.
   NOTE: When separating the nerves from each other, make sure that the blood vessel underneath is not injured.

3. Electrode placement

1. Arrange the stimulation hook electrodes in parallel at a distance of 0.5-1.0 mm, and use a micromanipulator to position the double hook in close proximity to the nerve.

2. Use the glass hook as a tool to lift the ulnar nerve onto the stimulation hook electrodes. Pull back the electrode with the nerve and separate it from other nerves by approximately 1-2 mm using the micromanipulator (**Figure 3D,F**).

3. Place the electrodes along the long axis of the paw to reduce the crosstalk between the muscles.

NOTE: The placement of the electrodes is very important but difficult to standardize due to the individual anatomy. It requires an experienced surgeon to place the electrodes correctly. The wires can also be repositioned should the signal amplitude be unsatisfactory.

4. Superficially dry the electrode hooks attached to the nerve and apply petroleum jelly using a syringe to provide electrical insulation from the adjacent tissue.

   NOTE: Care should be taken to apply enough petroleum jelly to the electrode and also between the two hooks to ensure electrical insulation and prevent the nerves from drying out.

4. Placement of the recording and reference electrodes

1. To measure the H-reflex, position the EMG electrodes intramuscularly in the forepaw. In addition, position the reference electrode subcutaneously in the hindlimb (e.g., using a minute pin), held by a miniature alligator clip (**Figure 3B**).
2. When the stimulator is switched on, observe a successful stimulation as tiny twitches of the forepaw. The minimum stimulation current to elicit the M-wave and tiny visible twitches in the forepaw should be in the range of 10-50 µA.

   NOTE: If no twitches are visible at 50 µA, adjust the stimulation electrodes and re-apply petroleum jelly. Also, in mice, it is not uncommon that the M-wave appears at lower stimulation intensities than the H-wave[5].

5. Measurement

1. Repeat the stimulation of the nerve 15 times with 0.2 ms long pulses each. With pauses of 2 min between sets of stimuli, the frequency is increased from 0.1, 0.5, 1.0, 2.0, to 5 Hz.

   NOTE: These frequencies are necessary if calculating the RDD afterwards. All EMG data are recorded, digitized, and analyzed using software e.g., Spike2 software (CED, version 7.19). The largest H-wave amplitude is expected at 0.1 Hz. The higher the frequency, the smaller the amplitude of the H-wave becomes due to the RDD.
2. After the experiment, sacrifice the animal as per the IACUC protocol of the institute. In this experiment mouse transcardial perfusion was performed using PBS and 4% PFA under deep anesthesia.

Representative Results

From the n = 15 stimulation trials per stimulation frequency and paw, select at least n = 10 successful recordings for the analysis. Trials with measurement errors (e.g., missing M-wave) are excluded from the analysis. Analyze each trial separately and generate an average for group/time comparisons later on. The latency between stimulation and appearance of the M-wave and H-wave is recorded for each trial. In our experience, the M-wave occurs approximately 2 ms after stimulation, and the H-wave after 6-8 ms, due to the longer transit time through the spinal cord (**Figure 1A** and **Figure 2B**). Measure the amplitude of the M- and H-waves as peak-to-peak.

To evaluate the physiological changes that occur in spinal cord injury or stroke, the ratio between the H- and M-wave amplitude (H/M ratio, **Figure 2**) is less prone to experimental variability, which would be reflected, for example, in amplitude differences. The ratio thus provides a more reliable evaluation of disease-related electrophysiological changes. For example, in mice with a stroke in the primary and

secondary motor cortex, the H-wave is increased, whereas the M-wave remains unchanged (**Figure 2**), suggesting an increased excitability of αMN. Furthermore, there is a reduced RDD (i.e., a reduced decrease in the suppression of the H-wave with increasing stimulation frequency). The decreased RDD is the result of reduced spinal cord inhibition[4]. Thus, RDD can validate the activation of spinal inhibitory circuits, whose interruption may result in spasticity. To calculate the RDD of the H-reflex, the method described by Lee et al. is recommended[4]. Briefly, the H-reflex stimulation at 0.1 Hz is averaged and set to 100%. The H-reflex obtained for the other stimulation frequencies is expressed as relative values to 0.1 Hz. From each stimulation train, the first three stimulations are discarded.

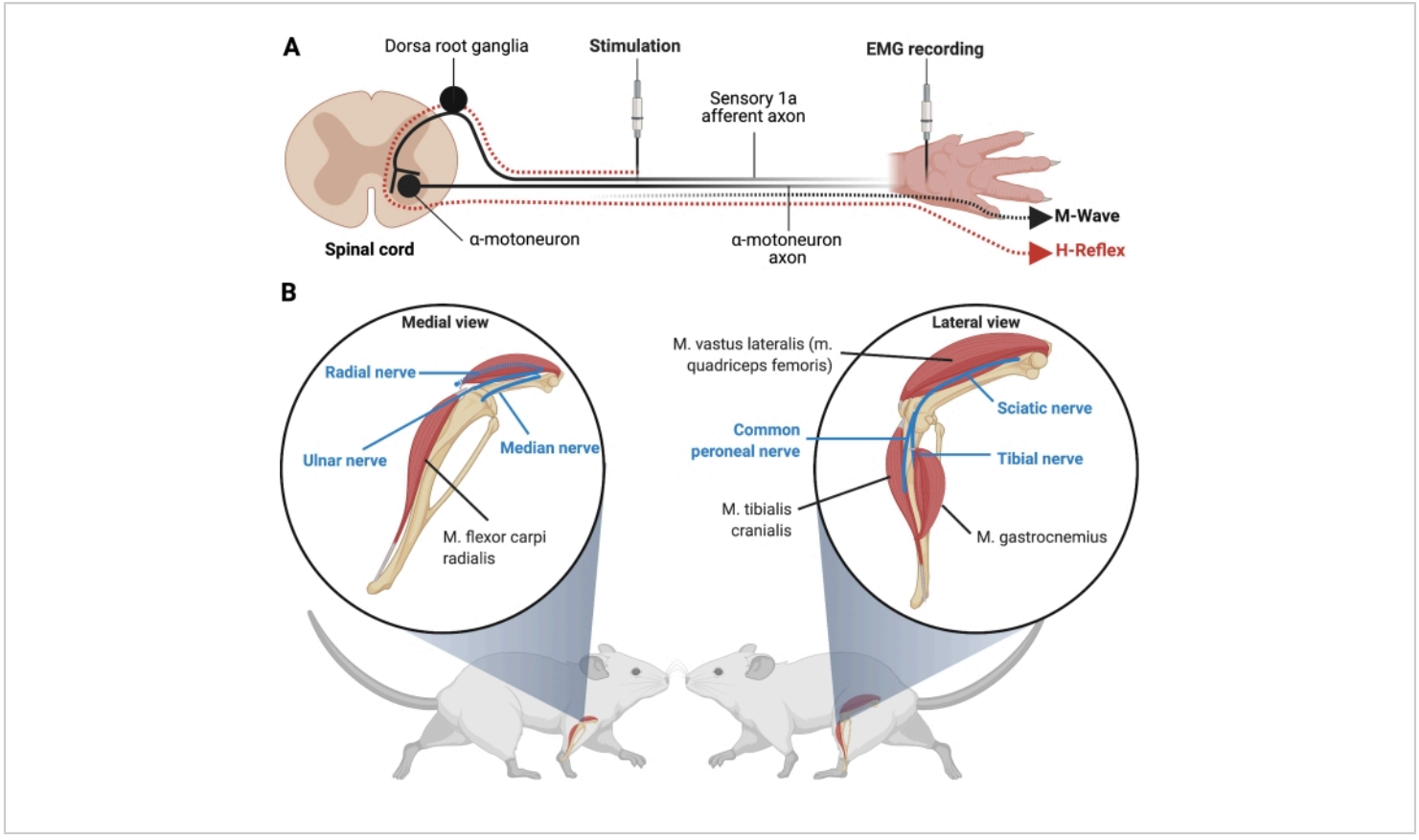

Figure 1: Illustration of the recording setup and pathways to measure the Hoffman reflex (H-reflex) and muscle response (M-wave). (**A**) The H-reflex is induced by stimulation of Ia afferents, which activate corresponding alpha motoneurons in the spinal cord and subsequently evoke muscle contractions in the innervated forepaw muscles. (**B**) Locations of the electrically stimulated radial/ulnar/median nerves in the forepaw and sciatic/tibial nerves in the hindpaw. Created with BioRender.com. Please click here to view a larger version of this figure.

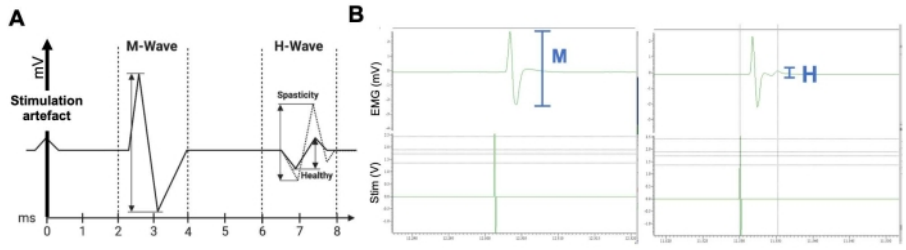

Figure 2: Schematic and representative electrical recording results. (**A**) Schematic of a recording. The stimulus and respective stimulation artifact are set to 0 ms, which is followed by the direct muscular response (M-wave) and the subsequent smaller peak representing the H-wave. In spasticity models, the H-reflex will be larger compared to the healthy control. (**B**) Screenshots from a representative recording with the software showing original data with a stimulus artifact (lower traces) and the appearance of the M-wave alone vs. an example where both the M- and H-waves are visible in the recording (upper trace, middle and right panel, respectively). Please click here to view a larger version of this figure.

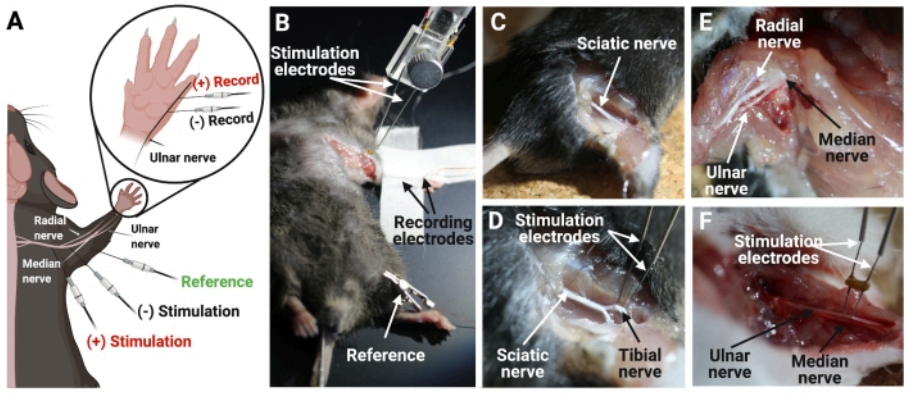

Figure 3: Positioning of the electrodes for terminal electrophysiological measurement. (**A,B**) Overview of the terminal H-reflex measurement with the hook stimulation electrodes, the recording electrodes within the forepaw, and the reference electrode inserted into the hindlimb. (**C,D**) In the hindlimb, after skin and muscle removal, the sciatic nerve becomes visible and can be divided into the sciatic and tibial nerves. (**E**) In the forelimb, the radial, median, and ulnar nerves become visible. (**F**) The ulnar nerve can be stimulated with the hook electrode without stimulation of neighboring nerves. Created with BioRender.com. Please click here to view a larger version of this figure.

Discussion

In contrast to previously described transcutaneous H-reflex measurements in the mouse[6], we provide a more direct and nerve-specific measurement. This new approach can be applied to the nerves of the fore- and hindlimb (e.g., the median, ulnar, and radial nerves, and the tibial, and sciatic nerves, respectively), rendering this method adaptable as a diagnostic tool to many disease models (e.g., stroke, multiple sclerosis, amyotrophic lateral sclerosis, traumatic brain injury, and spinal cord injury). Depending on the selected nerve, validation of the amplitude of the H-wave as a function of the stimulation intensity is recommended. The amplitude can vary due to the nerve diameter and motoneuron excitability, as well as electrical contact. By measuring the H/M ratio and RDD, experimental influences such as the positioning of the needle can be reduced, which significantly increases the reliability of the values obtained.

The main limitation of the protocol presented here is the terminal application without the possibility for longitudinal measurements. Further, several methodological details should be considered. Constant anesthesia with minimal muscle relaxation is critical for reliable measurement and should be validated for each specific model/application. In contrast to isoflurane anesthesia which causes a strong suppression of muscle reflexes (i.e., the H-reflex[9,10,11], the combination of ketamine-xylazine provides a safe anesthesia and is widely used for EMG recordings[12]. In line with measurements of motor evoked potentials in the rat[13], in our experience 100 mg/kg ketamine and 10 mg/kg xylazine provides the best protocol for stable and reliable recordings. For a skilled experimenter it is possible to perform fore- and hindpaw measurements in one final experiment. The procedure as described here for the forepaw, including animal preparation, and measurement of all frequencies for rate-dependent depression, can be performed in about 30-40 min. It is strongly recommended to practice the nerve dissection technique before performing *in vivo* experiments. In unilateral disease models (e.g., cortical stroke), we recommend repeating the stimulation 15 times on both contralateral paws to include the non-affected paw as an internal control. As only one nerve is to be stimulated in the method shown here, special care must be taken to distribute sufficient petroleum jelly around the stimulation electrodes so that no stimulation of neighboring nerves occurs.

Disclosures

The authors declare no competing financial interests.

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