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We want to submit this manuscript for your editorial considerations.

Title: **Muscle Velocity Recovery Cycles (MVRCs) - A novel method to examine muscle membrane properties**

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TITLE:

Muscle Velocity Recovery Cycles to Examine Muscle Membrane Properties

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KEYWORDS:

MVRCs, muscle velocity recovery cycles, muscle membrane depolarization, muscle excitability, myopathy, ion channel function, neurogenic muscles, anterior tibial muscle

SUMMARY:

Presented here is a protocol for the recording of muscle velocity recovery cycles (MVRCs), a new method of examining muscle membrane properties. MVRCs enable in vivo assessment of muscle membrane potential and alterations in muscle ion channel function in relation to pathology, and it enables the demonstration of muscle depolarization in neurogenic muscles.

ABSTRACT:

Although conventional nerve conduction studies (NCS) and electromyography (EMG) are suitable for the diagnosis of neuromuscular disorders, they provide limited information about muscle fiber membrane properties and underlying disease mechanisms. Muscle velocity recovery cycles (MVRCs) illustrate how the velocity of a muscle action potential depends on the time after a preceding action potential. MVRCs are closely related to changes in membrane potential that follow an action potential, thereby providing information about muscle fiber

membrane properties. MVRCs may be recorded quickly and easily by direct stimulation and recording from multi-fiber bundles in vivo. MVRCs have been helpful in understanding disease mechanisms in several neuromuscular disorders. Studies in patients with channelopathies have demonstrated the different effects of specific ion channel mutations on muscle excitability. MVRCs have been previously tested in patients with neurogenic muscles. In this prior study, muscle relative refraction period (MRRP) was prolonged, and early supernormality (ESN) and late supernormality (LSN) were reduced in patients compared to healthy controls. Thereby, MVRCs can provide in vivo evidence of membrane depolarization in intact human muscle fibers that underlie their reduced excitability. The protocol presented here describes how to record MVRCs and analyze the recordings. MVRCs can serve as a fast, simple, and useful method for revealing disease mechanisms across a broad range of neuromuscular disorders.

INTRODUCTION:

Nerve conduction studies (NCS) and electromyography (EMG) are the conventional electrophysiological methods used for the diagnosis of neuromuscular disorders. NCS enables detection of axonal loss and demyelination in the nerves[1], while EMG can differentiate whether myopathy or neurogenic changes are present in the muscle due to nerve damage. However, NCS or EMG provide limited information about muscle fiber membrane properties and underlying disease mechanisms. This information can be achieved using intracellular electrodes in isolated muscles from muscle biopsies[2-4]. However, it is of clinical importance to use methodologies using recordings from intact muscles in patients.

The velocity of a second muscle fiber action potential changes as a function of the delay after the first[5], and this velocity recovery function (or recovery cycle) has been shown to change in dystrophic or denervated muscles. The yield of such recordings from single muscle fibers was, however, too low to be of use as a clinical tool[6]. However, Z'Graggen and Bostock later found that multi-fiber recordings, obtained by direct stimulation and recording from the same bundle of muscle fibers, provide a fast and simple method of obtaining such recordings in vivo[7]. A sequence of paired pulse electrical stimuli with varying interstimulus intervals (ISIs) is used in this method[7-11].

The evaluated MVRC parameters include the following: 1) muscle relative refractory period (MRRP), which is the duration after a muscle action potential until the next action potential can be elicited; 2) early supernormality (ESN); and 3) late supernormality (LSN). ESN and LSN are the periods after the refractory period in which the action potentials are conducted along the muscle membrane faster than normal. Post-depolarization and potassium accumulation in the muscle are hypothesized as the main causes for the two periods of supernormality.

The wide applicability of MVRCs to muscle disorders has been shown in detecting membrane depolarization in ischemia[7,10,12] and renal failure[13], as well as providing information about muscle membrane abnormalities in critical illness myopathy[14] and inclusion body myositis[15]. Frequency ramp and intermittent 15 Hz and 20 Hz simulation protocols have since been introduced. MVRCs, together with these additional protocols, have demonstrated the different effects on muscle membrane excitability related to loss-of-function or gain-of-function mutations in various muscle ion channels in the inherited muscle ion channelopathies (i.e., sodium channel myotonia, paramyotonia congenita[16], myotonic dystrophy[17], Andersen-Tawil syndrome[18], and myotonia congenita[19,20]).

In a recent study, the applicability of MVRCs to neurogenic muscles was shown for the first time. The term "neurogenic muscle" refers to the secondary changes in skeletal muscles that develop as denervation and reinnervation after any injury to the anterior horn cells or motor axons. Denervation is characterized in EMG as spontaneous activity (i.e., fibrillations [fibs] and positive sharp waves [psws]), while large motor unit potentials with prolonged duration and increased amplitude present reinnervation[21]. EMG changes are evident in denervated muscles, but the underlying cellular changes in muscle fiber membrane potentials have only been demonstrated in experimental studies on isolated muscle tissue[2-4]. MVRCs provide further insight into in vivo human muscle membrane properties regarding the denervation process.

This paper describes the methodology of MVRCs in detail. It also summarizes the changes in neurogenic muscles in a subgroup of patients from a previously reported study[22] and healthy control subjects that enables determination of whether the method is appropriate for a planned study.

The recordings are performing using a recording protocol that is part of a software program. Other equipment used is an isolated linear bipolar constant current stimulator, 50 Hz noise eliminator, isolated electromyography amplifier, and analogue-to-digital converter.

PROTOCOL:

All subjects must provide written consent prior to examination, and the protocol must be approved by the appropriate local ethical review board. All methods described here were approved by the Regional Scientific Ethical Committee and Danish Data Protection Agency.

1. Preparation of the subject

1.1. Assess subjects' medical histories to ensure that they do not have any previous nervous system disorders other than the disease group that will be investigated.

1.2. Inform the subject in detail about the examinations and request to obtain written consent.

1.2.1. Inform the subject about the insertion of two needles in a leg muscle and that the muscle fibers will be stimulated with weak current.

1.2.2. Explain that the sensation may feel slightly unpleasant.

1.2.3. Inform the subject that the stimulation can be turned off immediately at any moment during the recording in case of any discomfort.

1.3. Clean the subject’s lower leg with alcohol.

1.4. Insert the stimulating monopolar needle electrode (25 mm x 26 G) over the anterior tibial muscle and adhesive surface electrode as the anode 1 cm distal to the monopolar needle (**Figure 1**).

1.5. Place a ground electrode distal to the anode.

1.6. Insert the recording concentric needle electrode (25 mm x 30 G) about 2cm proximal to the stimulating monopolar needle electrode along the muscle fibers (**Figure 1**).

1.7. Connect the recording concentric needle and ground electrodes to the preamplifier.

1.8. Ask the subject to remain silent and avoid movement during the examination.

1.9. Zero the output of the stimulator and connect the stimulating electrodes to the stimulator (**Figure 1**).

1.10. Maintain the skin temperature between 32–36 °C using a warming lamp.

2. Recording of the MVRCs

2.1. Start the semi-automated recording software using the muscle excitability recording protocol and turn on the stimulator. Stimulations will start at 2.5 mA with 1 Hz.

2.2. Increase the stimulus intensity manually by hitting the **Insert** key until a response is recorded (max = 10 mA).

2.3.1. Adjust the stimulating and recording needles if necessary, until recording an acceptable response with a stimulus intensity of less than 10 mA. The shape of the muscle action potential should be triphasic, if possible, and stable. Avoid large twitches of the whole muscle.

2.3.2. Invert the muscle action potential by hitting the minus key (-) if the potential appears upside down.

NOTE: A magenta horizontal line appears on the screen indicating the width of the action potential.

2.4. Adjust the position and length of the magenta line by dragging the line with the mouse. The green horizontal line represents the baseline.

2.5. Click **OK** to start recording the MVRCs.

2.6. Select a stimulus response relationship from the main options.

2.7. Increase stimulus intensity by hitting the **Insert** key to a max of 10 mA or tolerable.

2.8. Click **OK t**o start descending the stimulus response curve.

2.9. Click **OK** when the test stimulus reaches zero.

2.10. Set the stimulus intensity to level for stable latency.

2.11. Click **OK** to return to the main menu.

2.12. Select the option **1/2/5 conditioning stims for RC**.

2.13. Select a protocol from recovery cycle options (e.g., start quick recovery cycle [skip alternate delays]), which is the default.

NOTE: The recording continues automatically for 34 steps with decreasing inter-stimulus intervals (ISIs).

2.14. Make sure that muscle action potential is stable during the recording and that the needle has not moved. The screen changes automatically to main options when the 34 steps have completed.

2.16. Click on **Finish recording** | **Close file** | **OK**, unless a ramp-up frequency or 20 Hz s recordings is being performed.

2.17. Finish the recording and save the data by clicking on the **Close file and save data** button.

3. MVRC analyses

3.1. Start the analyzing software program to perform the analysis offline.

3.2. Select the recording that will be analysed and click on the **OK** button.

3.3. Click on **Load parameters** from the **Files** menu.

3.4. Select MANAL9 option for the analysis. If this is not present on the list, click on **Browse** to find this file. Click **OK** to continue.

3.5. When a description of MAnal9 muscle excitability analysis appears, click **OK** to continue.

3.6.1. Invert the muscle action potential by typing MM-1 if the potential appears upside down.

3.6.2. Right-click the mouse to make the magenta line visible. Set the window to the base of the peak response and with a width corresponding roughly to the width of the action potential at that height. Drag with the mouse to adjust the window. The window determines the latencies within which the height and latency are measured, as indicated by the pale blue lines, and green line indicates the baseline. Click **OK** to continue.

3.7. Click **OK** to remeasure the latencies and peaks. This will be done automatically.

NOTE: In the display of the remeasured latencies, the latencies are measured to shorter delays than original ones. This is because the responses to conditioning stimuli alone were subtracted from responses to the conditioning plus the test. This ensures that conditioning stimuli do not interfere with latency measurements. As is indicated in the prompt box, single bad points can be eliminated by positioning the cursor (vertical red line) over the point and hitting the **~** key. The bad point is replaced with mean of values on either side in same channel. If there are no bad points, set DE (display end) to just after the last latency required.

3.8. Click **OK** to create an RMC file.

3.9. Ignore most of the options appearing in the "Create RCC or RMC" form, since these are concerned with measurements of C-fiber rather than MVRCs. Click **Save and Exit** to continue. After saving the RMC file, the prompt box provides different options

3.11. If frequency ramp and/or repetitive stimulation data have been recorded, follow the instructions to analyse these. Otherwise, select **Go straight to create MEM file option** to create a MEM file. Click **OK** to continue.

3.12. Click **Save and Exit** to continue.

3.13. Click **OK** to add the RMC data to MEM file.

3.14. Click **Add from Input RMC file** to add this data to the MEM file, then change the directory to save the composite MEM file. Then, click **Save and Exit** to save it.

3.15. Click **OK** to save the remeasured QZD file to allow differentiation from the original QZD file using a **#** sign.

REPRESENTATIVE RESULTS:

The following results were obtained in a subgroup of patients from a recent study[22], in which there were fibs/psws in all sites showing profuse denervation activity. The results showed that changes in muscle fibers after denervation were assessed in vivo using the MVRC technique described in this protocol. MVRCs showed changes consistent with depolarization of the resting membrane potential in the neurogenic muscle fibers.

Fourteen patients were compared with 29 healthy subjects. Subject demographics are shown in **Table 1**. **Figure 2** illustrates recordings from a healthy subject and patient. **Figure 3** and **Table 2** illustrate comparison of patients' MVRCs with healthy subjects. MRRP was prolonged, and ESN and LSN were reduced in patients compared to healthy controls (**Table 2**, **Figure 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: Picture of MVRCs set-up. (**A**) Isolated linear bipolar constant-current stimulator, (B) 50 Hz noise eliminator, (**C**) isolated EMG amplifier, and (**D**) analogue-to-digital converter.

Figure 2: Examples of MVRC recordings. Recordings after one conditioning stimulus (red), two conditioning stimuli (green), and five conditioning stimuli (blue) from a (**A**) healthy subject and (**B**) patient with L5 radiculopathy.

Figure 3: MVRCs with one, two, and five conditioning stimuli. (**A**) MVRCs in 14 patients (grey lines) compared to mean value of 29 healthy controls (filled black squares). Graphical representation of percentage change in latency is plotted against ISIs from 2–1,000 ms (logarithmic scale). (**B,C**): Same as (A), but with two and five conditioning stimuli.

Table 1: Demographics and clinical characteristics. Values are listed as means ± standard deviation. This table has been modified from Witt et al.[22].

Table 2: **Comparison of MVRC parameters between healthy controls and patients.** MRRP = muscle relative refractive period; ESN (%) = latency reduction of muscle action potential after one conditioning stimulus as percentage of unconditioned stimulus at ISI of <15 ms. ESN (ms), ISI corresponding to ESN (%). 5ESN = peak early supernormality after five conditioning stimuli. LSN (%) = latency reduction of muscle action potential after one conditioning stimulus as percentage of unconditioned stimulus at ISI between 100–150 ms. XLSN (%) = latency reduction of muscle action potential after two conditioning stimuli as percentage of one conditioning stimulus at ISI between 100–150 ms. 5XLSN (%) = latency reduction of muscle action potential after five conditioning stimuli as percentage of one conditioning stimulus at ISI between 100–150 ms. Values are listed as means ± standard deviation.

DISCUSSION:

MVRCs, as programmed in the recording software, is a highly automated procedure, but care is needed to obtain reliable results. In the recording stage, while adjusting the needles, it is important to avoid stimulating the end-plate zone or nerve. This usually leads to large twitches of the whole muscle, which increases the risk of displacement of the stimulation and/or recording needle during recording MVRCs. To date, the method has been applied to several muscles that have better described end-plate zone; however, the endplates may be scattered (i.e., in the anterior tibial muscle). Therefore, particular attention is required.

In order to avoid stimulation of the endplate or nerve instead of muscle fibers, care should be taken when observing the muscle for twitches. The stimulating monopolar needle should be moved, as well as the recording concentric needle, to locate a site that does not cause twitches. Additionally, subjects should be asked whether or not they feel pain. MVRC recordings do not cause any unpleasantness, unless the end-plate zone or the nerve is stimulated instead of muscle fibers.

A limitation of the MVRCs method is performing the recording in only one site and examination of only a few muscle fibers, which does not necessarily represent the whole muscle. This limitation is particularly important in disorders where the pathology is not diffuse. A previous study found surprisingly no difference between patients with amyotrophic lateral sclerosis and healthy controls despite denervated muscles. This was probably because denervation activity was not recorded at the site where MVRCs were recorded[23]. It also cannot be excluded that the needle could have been adjusted to a healthier spot with a more optimal response.

Another limitation of MVRCs is that one may have a tendency to spot the healthy muscle fibers while adjusting the recording needle to obtain a stable response for measurements. One way to overcome this limitation may be to do the recordings from polyphasic potentials. However, this may pose problems for determining an accurate latency if there are undifferentiated peaks. Additionally, although we intend to stimulate and record from the same bundle of muscle fibers, these may not be exactly the same. The stimulated bundle may contain different fibers during ongoing experiment[24].

MVRCs provide information that cannot be obtained by the conventional electrophysiological methods. Thus, there is no other method in current use that can be compared to MVRCs. The earlier report[6], using single fiber needle electrodes to record at two sites from the same muscle fiber, was much more difficult. Good recordings were only obtained from 43 out of 118 muscle fiber studies, and this method has not been adopted in research labs or clinics. Another similar but unautomated approach used eight different ISIs from 20 ms to 2 ms[25]. The authors reported that a recording took 20–60 min, whereas this method records MVRCs with 34 ISIs in about 10 min. The analysis is also fast and highly automated.

In conclusion, MVRCs is a method that may provide invaluable information to understand the underlying mechanisms of neuromuscular disorders. For patients in which a mutation in an ion channel gene has been identified, this method also provides data on the effects of those specific mutations on muscle membrane excitability in vivo. This, together with in vitro expression studies, enables a more accurate understanding of muscle pathophysiology in these patients. This method has the potential to provide insight into the role of those channels in normal muscle physiology, thus improving the understanding of muscle disease in general. Further studies with other patient groups and larger groups are necessary. Studies recording MVRCs in different muscles are also warranted.

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DISCLOSURES:

H.B. receives royalties from UCL for sales of his Qtrac software used in this study. The other authors have no potential conflicts of interest. All authors have approved the final article.

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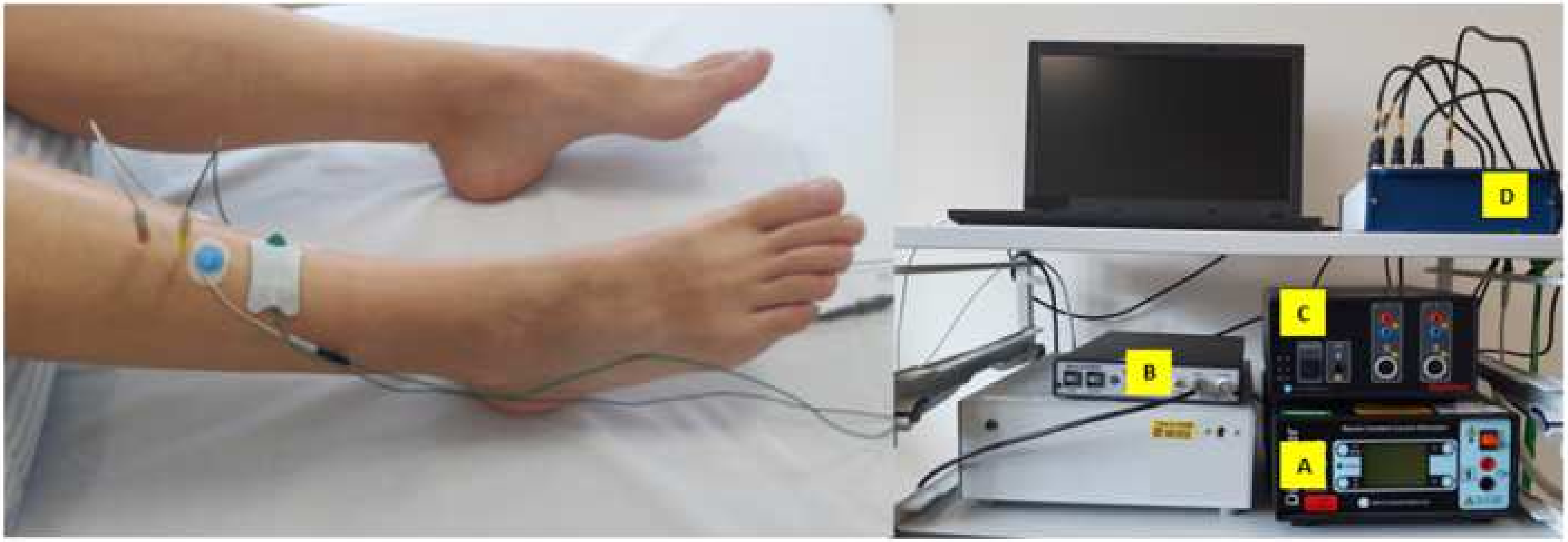
Figure



Figure

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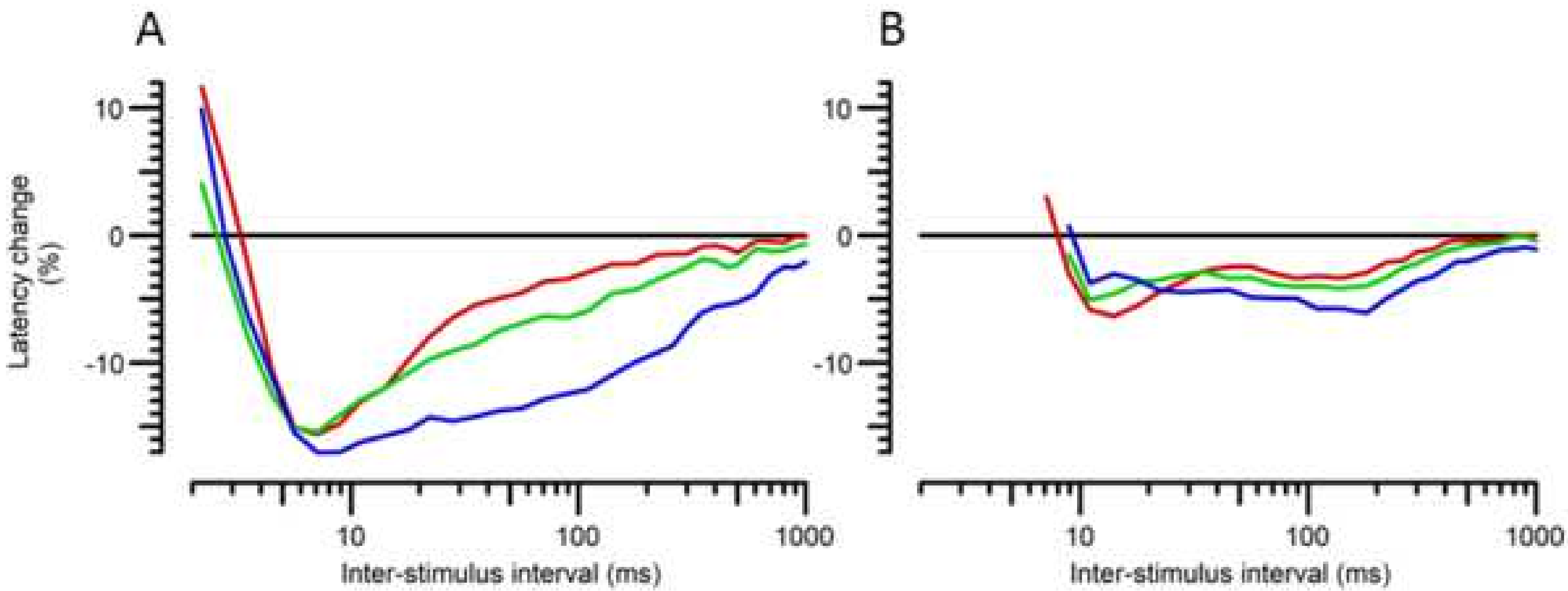

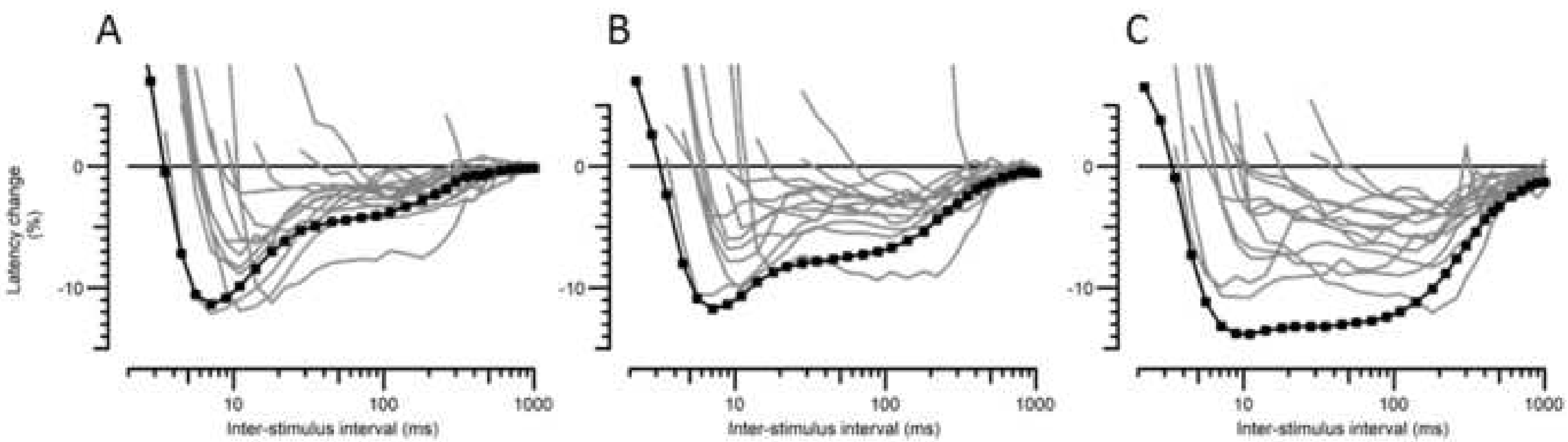
A
B
C
Latency change (%)
0
-10
10
100
1000
Inter-stimulus interval (ms)

Table 1: Demographics and clinical characteristics

	Healthy controls ***(n=29)***	**Patients** ***(n=14)***
Age (years)	55.7 ± 14.9	58.9 ± 16.3
Gender (M/F)	14/15	9/5
Disease duration (months)	-	3.4 ± 2.7
MRC score	-	3.0 ± 1.1
Etiology	-	Peronal neuropathy (9) L5 root afflication

Table 2: Comparison of MVRC parameters between healthy controls and patien

	Healthy Controls *(n=29)*	Patients *(n=14)*	p-value for t-test
MRRP (ms)	3.5 ± 0.4	7.6 ± 3.1	p = 6.8 $^{-8}$
ESN (%)	11.3 ± 2.1	7.6 ± 2.3	p = 5.5 $^{-5}$
ESN (ms)	7.8 ± 1.3	12.7 ± 2.5	p = 1.6 $^{-8}$
5ESN (%)	13.7 ± 2.5	1.0 ± 0.6	p = 9.3 $^{-10}$
LSN (%)	4.1 ± 1.4	2.8 ± 1.7	p = 0.017
XLSN (%)	2.9 ± 0.7	1.0 ± 1.6	p = 1.8 $^{-10}$
5XLSN (%)	8.0 ± 1.4	2.8 ± 1.6	p = 2.2 $^{-11}$

ıts

Name of Material/Equipment
50 Hz Noise Eliminator
Analogue-to-Digital Converter
Analysing software program
Disposable concentric needle electrode, 25 mm x 30G
Disposable monopolar needle electrode, 25 mm x 26G
Isolated EMG amplifier
Isolated linear bipolar constant-current stimulator
Software and recording protocol

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Humbug
NI-6221
QtracP, MANAL9
Dantec DCN
TECA elite
D440
DS5
QtracW software, M3REC3 recording protocol written by Hugh Bostock, Istitute of Neurology, London, UK)

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Comment: Reference list has been updated according to the citation style.

8. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example D440 preamplifier, QtracS, M3REC3 protocol, DS5 bipolar stimulator, HumBug 50 Hz noise eliminator, D440 amplifier, analog-to-digital (A/D) board NI-6221, Dantec, QtracP analysis program, etc.
Comment: This has been corrected throughout the manuscript and the Table of Materials and Reagents has been revised.

9. JoVE's policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please remove "QtrecS" within your text. Please refer to the term using generic language.

Comment: This has been done throughout the manuscript.
QtracP is corrected to: "Analysing software program"
QtracS is corrected to "Recording software program"
M3REC is corrected to "Recording protocol"

10. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use the original language throughout the manuscript. Please see lines: 75-78, 98-101, 124-125, 130-131, 145-146, 151-152, 294-297

Comment: These lines have been rephrased.

11. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
Comment: This has been done.
Citation: 3.2 has been rephrased: "Avoid…"

12. The protocol needs click-by-click instructions for each software program used. Describe how the user interacts with the software. Please include all button clicks, the knob turns, etc.
Comment: This has been done.

13. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
Comment: We have rephrased the sections and divided into more steps when necessary.
Citation: Step 2.3 and 3.6 are subdivided into two steps

14. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
Comment: This has been done

15. Please move the equipment details to the table of Materials.
Comment: This has been done.

16. Please do not repeat/duplicate the protocol steps.
Comment: We have tried to avoid repeating the protocol steps.

17. 1: Please include age and sex-specific bias if any for patient recruitment. Please include a table showing clinical details.
Comment: Clinical details of healthy controls and patients are displayed in Table 1. Patients were included prospectively to avoid exclusion bias.

18. 1.2: where will the insertion happen.
Comment: Insertion site has been added to section 1.2.
Citation: " 2 needles in a leg muscle and.."

19. 1.6-1.9: How is this done?
Comment: We believe for people in the field of neurophysiology, these sections are clear.

20. 2.3: How do you ensure this?
Comment: More information has been added to this section.
Citation: "2.3.1. Adjust the stimulating and recording needles if necessary, until recording an acceptable response with a stimulus intensity of less than 10 mA. The shape of the muscle action potential should be triphasic, if possible, and stable. Avoid large twitches of the whole muscle. "

21. 2.6, 2.10: How is this done?
Comment: More information has been added to this section. We believe how these procedures will be done is clear for neurophysiologists.

22. 3.5: What is being checked here?
Comment: This part is information and then clicking on OK to continue rather than checking something. This has been clarified.
Citation: "A description of MAnal9 muscle excitability analysis appears. Click OK to continue."

23. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Comment: The protocol is 5 pages and the highlighted part has been shortened to 2.75 pages.

24. Please ensure that the results are described with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.
Comment: This had already been considered.

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Comment: All figures and tables displayed in this paper are original

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Comment: Table1 and 2 have been moved to separate .xls files and titles have been provided.

27. Please number the citations in the order of it being referenced in the manuscript.
A new reference list has been conducted.

28. Figure 1B: Please remove the commercial term.
Figure 1.B has been revised.

29. Please upload high-resolution figures.
Figure 2 and 3 are uploaded in high-resolution

30. Please sort the Materials table in alphabetical order.
The Material table has been revised.

31. Since some of the authors are from the UK, please sign the UK ALA and upload it to your editorial manager account.
The UK ALA is completed and uploaded.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors described a protocol for recording Muscle Velocity Recovery Cycles (MVRCs) as a new method of examining muscle membrane properties using direct muscle stimulation. The authors tested this method in patients with neurogenic muscles, they found that MRRP was prolonged and ESN and LSN were reduced in patients compared to the healthy controls. The authors concluded that MVRCs provided in vivo evidence of membrane depolarization in intact human muscle fibres that could underlie their reduced excitability. The authors claimed that this simple method can improve the understanding of the mechanisms underlying various neuromuscular disorders including muscle channelopathies. I have no major comment as the data are novel and believable.

Comment: We thank the Reviewer for the positive comments.

Reviewer #2:
Manuscript Summary:
This submission is quite important to detail the methodology of MVRC recording using the Qtrac protocol. The method has a few pitfalls, and it would helpful for future researchers to be able to refer to this, and to access any previous studies in the area.

Major Concerns:
Nil major

Minor Concerns:
* Replication of the ms in parts 1 and 2 of the methodology that appears highlighted on this version
Comment: These parts have been repeated and highlighted as a requirement of the journal.

* 3.8. As is indicated.
Comment: This has been corrected.

* Table 1 affliction (rather than affection)
Comment: L5 root affection has been changed to affliction.

* Discussion: 'One way to overcome this limitation may be to do the recordings from polyphasic potentials, although this may pose problems for determining an accurate latency if there are undifferentiated peaks.
Comment: We agree with the reviewer. This has been added to the discussion.
Citation: "One way to overcome this limitation may be to do the recordings from polyphasic potentials. However, this may pose problems for determining an accurate latency if there are undifferentiated peaks."

* Will there be any abbreviations listed? It would be helpful to know the meaning of all those such as 5XLSN etc..
Comment: We thank the Reviewer for reminding this. An abbreviation list has been added.

* Reference should be made on past studies on demographic data like age (1) and (2) selection of muscles. These would be relevant to any study in future and are important considerations.

References (important):
1. Lee J, Boland-Freitas R, Ng K. Sarcolemmal excitability changes in normal human aging. Muscle Nerve. 2018 ;57:981-988.

2. Lee J, Boland-Freitas R, Ng K. Physiological differences in sarcolemmal excitability between muscles. Muscle Nerve. 2019 Oct;60(4):433-436. doi: 10.1002/mus.26645.

References (optional):
3. Lee J, Boland-Freitas R, Liang C, Ng K. Sarcolemmal depolarization in sporadic inclusion body myositis assessed with muscle velocity recovery cycles. Clin Neurophysiol. 2019 Aug 31. pii: S1388-2457(19)31205-2. doi: 10.1016/j.clinph.2019.08.019.

Comment: We agree with the Reviewer about the importance of these references. These references have been added.

Reviewer #3:
Manuscript Summary:
The events after the passage of an impulse (nerve or muscle) reflects ion channel mechanisms. This can be detected in routine neurophysiological studies and is a daily concern in Single Fiber EMG and also often seen in microneurography. In muscle fiber this was first described 1966, but has, except for the above, not attracted attention. In this manuscript a multi-fiber test is described to measure the velocity changes after a conditioning stimulus. Buchthal in the 1950ties tried the 2 needle technique for measuring velocity in muscle fibers, but could not prove that the same muscle was both stimulated and recorded from. So further studies did not follow.

Major Concerns:
In this technique a number of pitfalls are present, some of which are detailed by Marrero et al 2026 (should be mentioned) in a double electrode study of other parameters. Such problems are:
The bundle of fibers stimulated, may not be exactly the same as those recorded. The stimulated bundle may contain different fibers during ongoing experiment.
Comment: We agree with the Reviewer about this pitfall. This has been added to the discussion.
Citation: Additionally, although we intend to stimulate and record from the same bundle of muscle fibers, these may not be exactly the same. The stimulated bundle may contain different fibers during ongoing experiment.

Initiation of the depolarization may occur after different delays depending on muscle fiber characteristics and probably distance to stimulator. Latency is more than velocity in the muscle fiber.
Comment: We agree with the Reviewer. This limitation has been eliminated by applying test alone and conditioning+test stimuli in MVRCs method.

In tibial anterior muscle, end-plates may be scattered, not easily defined from surface anatomy. Test to ascertain direct stimulation is crucial, not discussed
A number of technical details are missing
Comment: We agree with the Reviewer. This has been discussed.

Citation: "Up to date, the method has been applied to a few muscles that have better described end-plate zone, however the end-plates may be scattered for instance in anterior tibial muscle, therefore particular attention is required. "

Minor Concerns:
In the manuscript, over all clearly written and easy to follow. A few questions may need to be commented:
Title and line 23. Novel and new. The new is the application for routine in EMG. The phenomenon itself is well known and taken into account in daily routine SFEMG, Repetitive nerve stimulation and in interpretation of certain EMG phenomena (double discharges).
Comment: Novel has been removed from the title.

line 47. Abbreviations not yet explained
Comment: An abbreviation list has been added and we have explained the abbreviations in "Long abstract"

Line 71. Same muscle fibers. How can you be sure
Comment: We agree with the reviewer that we can not be exactly sure about this. As also indicated above, this has been added as a challenge of the method.

Line 134. Reference…Do the authors mean anode?
Comment: We mean the anode by reference. This has been changed.

Line 168- Acceptable response. What is that, particularly in abnormal states.
Comment: More information has been added like requirement of triphasic potential, if possible and a stable response.

Line 169 invert funny signals. This is worrisome and underlines the recording problem. Even summated MUPs have main peak in negative direction. May indicate a cannula recording. Should not be accepted, or explained in detail with examples.
Comment: This is simply due to the amplifier settings and not a recording problem or a cannula recording.

Line 171 magenta and green line cannot be understood in text.
Comment: This has been indicated to show in the video. We do not expect this to be understood in the text.

Stimulation frequency not given for each run is not given
Comment: This has been added in Section 2.1

Discussion, section 1. Line 4 "important to avoid stimulating". How is this controlled
Comment: This has been described in the following sentences.

Section 3, last sentence. "polyphasic potentials". Is one or several data obtained from such a recording? The earliest part is probably the fastest conducting fibers (normal?). Late and early components behave differently (MVCR dependent on CV in individual fibers). Latency measured to earliest? Amplitude measured to that?
Comment: For this study, polyphasic potentials have not been analysed.

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