MOTOR UNITS AS TOOLS TO EVALUATE PROFILE OF HUMAN RENSHAW INHIBITION

Mustafa Görkem Özyurt[1], Maria Piotrkiewicz[2], Betilay Topkara[1], Hans-Werner Weisskircher[3], Kemal Sitki Türker[1*]

1. Koç University, School of Medicine, Istanbul, Turkey.

2. Nalecz Institute of Biocybernetics and Biomedical Engineering, Polish Academy of Sciences, Warsaw, Poland.

3. Dental Practice, Bahnhofstr 7, Igel, Germany.

Running Title: Renshaw inhibition in human

This is an Accepted Article that has been peer-reviewed and approved for publication in the The Journal of Physiology, but has yet to undergo copy-editing and proof correction. Please cite this article as an 'Accepted Article'; doi: 10.1113/JP277129.

***Corresponding Author**
Prof. Dr. Kemal S. Türker
Koç University School of Medicine
Rumelifeneri Yolu, 34450
Sarıyer, Istanbul, Turkey
Tel: +90 212 338 1174
Fax: +90 212 338 1165
Email: kTürker@ku.edu.tr

Key Points:

- To uncover synaptic profile of Renshaw inhibition on motoneurons we stimulated thick motor axons and recorded from voluntarily-activated motor units.
- Stimuli generated direct motor response on the whole muscle and an inhibitory response in active motor units.
- We have estimated the profile of Renshaw inhibition indirectly using the response of motor unit discharge rates to the stimulus.
- We have put forward an idea of extrapolation which may be used to determine genuine synaptic potentials as they develop on motoneurons.
- These optimized techniques can be used in research and in clinics to fully appreciate Renshaw cell function in various neurological disorders.

Abstract

Although Renshaw inhibition (RI) has been extensively studied for decades, its precise role in motor control is yet to be discovered. One of the main handicaps for this is the lack of reliable methods to study RI in conscious human subjects. We stimulated the lowest electrical threshold motor axons (thickest axons) in the tibial nerve and analyzed the stimulus-correlated changes in discharge of voluntarily recruited low-threshold single motor units (SMUs) from the soleus muscle. A total of 54 distinct SMUs from 12 subjects were analyzed. Stimuli that generated only the direct motor response (M-only) on surface electromyography

(SEMG) induced an inhibitory response in the low-threshold SMUs. Since the properties of the RI had to be estimated indirectly using the background discharge rate of SMUs, its profile varied with the discharge rate of the SMU. The duration of the RI was found to be inversely proportional to the discharge rate of SMUs. Using this important finding, we have developed a method of extrapolation to estimate RI as it develops on motoneurons (MNs) in the spinal cord. The frequency methods indicated that the duration of the RI was between 30 to 40 ms depending on the background firing rate of units, and the extrapolation indicated that the RI on silent MNs was around 55 ms. This study establishes a novel methodology for studying RI in human subjects and hence may serve as a tool towards improving our understanding of RI involvement in human motor control.

Keywords: Renshaw circuitry, synaptic potentials, direct motor response, human neuronal networks, single motor units.

Mustafa Görkem Özyurt received his Bachelor's Degree in Bioengineering Department from Ege University. He worked in cellular neuroscience using three-dimensional tissue culture models to mimic blood-brain barrier *in vitro*. During his Bachelor's Degree, he visited Magdeburg to investigate the intracellular signaling pathways involved in neuronal differentiation. Subsequently, he moved to

Koç University to receive his doctoral training in Renshaw interneurons with electrical stimulation of the motor axons in human and optogenetic stimulation in the mouse. Currently, he is studying neuronal circuits and reflex pathways using electrical stimulation of the peripheral nerves, transcranial magnetic stimulation of the motor cortex and the response of the single motor units as a PhD Candidate.

Introduction

Recurrent inhibition was one of the first identified neuronal circuits with feedback in the spinal cord. It is mediated by a special type of inhibitory interneurons, which are nowadays called Renshaw cells (RCs) (Eccles *et al.*, 1954). Properties of RC have been extensively investigated over the last 70 years, making them a uniquely well-defined class of spinal interneurons. RCs are excited by motoneuron (MN) axon collaterals and synapse on MNs. Certainly, one of the most intriguing parts of Renshaw inhibition (RI) is the exertion of its effect back on the same MN pool. This self-inhibiting recurrent circuit is analogous to negative feedback control on complex systems known in technology, and perhaps due to this resemblance so many researchers became attracted to studying RI. However, despite years of extensive research, the role that RI plays in motor control still remains obscure.

The vast majority of knowledge about RCs and their interaction with MNs were collected in acute animal experiments, and their limitations (to mention only the influence of anesthetics on synaptic potentials) may be among the factors hindering the explanation of the physiological role of RI. In animals RI was studied essentially by 3 methods: (i) morphological study of the recurrent axon collaterals after intracellular staining with horseradish peroxidase (Cullheim & Kellerth, 1978], (ii) *in vivo* intracellular recordings under anesthesia, where antidromic RC activation was induced via stimulation of the ventral roots (Hultborn et al., 1971; McCurdy & Hamm, 1994), and (iii) *in vitro* in spinal cord slices (Lamotte d'Incamps *et al.*, 2012; Moore *et al.*, 2015). These studies are remarkable to understand the role of RCs in isolation; however, usage of anesthetics and lack of descending inputs limit the use of these findings towards understanding of the physiological role that the RI circuits play under conscious conditions (Fung *et al.*, 1987; Mazzocchio *et al.*, 1994).

On the other hand, human experiments have recently gained attention among scientists who previously worked with animals (Jankowska & Hammar, 2002; Heckman *et al.*, 2008; Gorassini *et al.*, 2009). Moreover, with respect to the possibility of defining a role for RI,

human experiments are more promising as here intact MNs are studied in their physiological environment. Majority of human studies used paired Hoffmann reflex (H-reflex) technique of Pierrot-Deseilligny and Bussel (1975). This technique uses graded activation of Ia afferents to induce H-reflex (Hoffmann, 1910), followed by supramaximal nerve stimulation and seeks to detect the RI response using surface electromyography (SEMG). Using this method, studies on RI in human have been performed, which revealed some RC properties (Rossi *et al.*, 1987; Raynor & Shefner, 1994; Rossi *et al.*, 2003).

Although double stimulation method was used commonly to investigate the RI in different conditions (Rossi *et al.*, 1987; Mazzocchio & Rossi, 2010), a number of variables such as the post-activation depression (Crone & Nielsen, 1989) limit its reliability (Kudina & Pantseva, 1988). This technique has been criticized to be nonspecific for examining the RC systems (Mazzocchio & Rossi, 1989; Rossi & Mazzocchio, 1991; Mazzocchio & Rossi, 1997). Moreover, double stimulation procedure reveals only the net effect of the RC system on large MN pools of resting muscles, which is far from studying RC impact on normally functioning individual MNs. The problems arise from the fact that the double stimulation technique involves stimulating a mixed nerve using two high-intensity stimuli with an interstimulus interval of 10 ms. Beside activating the spindle primary afferents, such stimulation can also generate tendon organ (Ib afferents) activation, presynaptic inhibition of homonymous spindle primary afferent synapses and post-activation depression of MNs (Mazzocchio & Rossi, 1989). Success of this technique also depends upon afterhyperpolarization (AHP) duration of active MNs (Mazzocchio & Rossi, 1989; Rossi & Mazzocchio, 1991). Therefore, a more reliable RI technique must be developed to support, re-evaluate and understand the background of the diseases.

More reliable information on the RC system and its functions may be obtained in single motor unit (SMU) studies in human subjects. The latency, duration and distribution of RI on MNs are the essential parameters to be investigated. To find these parameters, the isolated stimulation of the motor fibers (M-only stimulation) was used to study antidromic activation of RCs through MN collaterals either in healthy subjects (Kudina & Pantseva, 1988) or in a deafferented patient (Mattei *et al.*, 2003). This method was based on stimulating the thickest axons originating from the largest MNs while recording stimulus-evoked responses from the low threshold voluntarily-activated MNs.

In this study, we investigated the properties of the RI in human experiments using a similar experimental protocol. Our analysis differed from the earlier work as we utilized both the probability and frequency-based methods (Türker & Powers, 1999, 2005). Furthermore, we evaluated stimulus-correlated changes in SMU activity at different levels of background discharge rates. Since we have obtained consistent and reliable data our approach to investigate RI may become a valuable tool for investigating normal functions of RCs and their impairment in patients suffering from various MN disorders. Our hypothesis therefore is that a new and reliable method can be developed to be used for studying RC function in human MNs in basic and clinical research.

Methods

Experiments were performed in the neurophysiology laboratory of Koç University. The Human Ethics Committee of Koç University approved the experimental procedure, which conformed to the Declaration of Helsinki. Study participated by 14 subjects (8 males and 6 females; age 18-35 years) with no known neuromuscular disorders. Twelve subjects attended SMU study while 2 subjects were recruited for paired H-reflex trials. In addition, exclusion criteria included having medication for any type of neuromuscular or psychological problems and ongoing chronic back or leg pain. Before the experiments, subjects signed an informed written consent form.

Setup: For recording and analysis, software Spike2 7.20 was used (Cambridge Electronic Design, England). Recording was performed by the hardware of CED 1902 Quad System MKIII amplifier and CED 3601 Power 1401 MKII DAC. A constant current stimulator (model DS7A, Digitimer Ltd, Hertfordshire, UK) was used for electrical stimulation of the motor axons.

SEMG recording: SEMG were recorded bipolarly from the soleus and tibialis anterior muscles of the right leg with 20-10,000 Hz band pass filter and 20,000 Hz sampling rate. For soleus muscle, subjects were asked to perform plantar flexion. After determining the lateral portion of the soleus muscle which was the posterolateral part of the leg, the routine preparation procedures were used that involved gently rubbing with sandpaper, cleaning with alcohol and applying ultrasound gel. Two standard SEMG electrodes (Ag/AgCl) were placed

on the muscle, one on the muscle belly and the other 4 cm distal to the muscle belly (Tucker & Türker, 2005) and both electrodes were stabilized with tape. For tibialis anterior muscle, subjects were asked to perform dorsiflexion and the same preparation procedure and electrode placement were followed.

Intramuscular EMG recording: For SMU recording, we used tip active silver fine-wire electrodes coated with Teflon (75 μm in core diameter; Medwire, USA). Two 25 cm long wires were placed into the 25G needle and sterilized. The sterile needle was inserted close to the soleus muscle belly in between two SEMG electrodes and immediately withdrawn, leaving a pair of fish-hooked wires inside the soleus muscle. Intramuscular recordings were amplified, band-pass filtered (100-10,000 Hz), sampled at 20,000 Hz, and stored for off-line analysis. For both surface and intramuscular EMG recordings, sterile lip-clip was used as ground electrode (Türker *et al.*, 1988). Averaging the response of intramuscular EMG represents the muscle activity in an accurate manner as many unit fires continuously most of the time. Therefore, average response of SMUs involves 2 or more units which are referred as multi-motor unit (MMU). For better observation of the inhibition, the SMU channel was full wave rectified before averaging.

Force recording: The right foot of each subject was stabilized to a force transducer which located around their sole. This linear strain gauge (Model LC1205-K020, A & D Co. Ltd., Tokyo, Japan: linear to 196 N) was used to quantify the amount of twitch produced after electrical stimulation. Force signals were amplified by 1,000 times, filtered with DC-100 Hz and sampled at 2,000 Hz.

Experimental procedure for M-only technique: The subjects lay comfortably on a bed in prone position. After placement of the recording electrodes and fixing the right foot to the force transducer, an anode (10 cm × 12 cm) of the constant current stimulator was placed immediately proximal to the patella and a small cathode (3 × 3 mm) was placed on slightly lateral part of the popliteal fossa (Ozyurt *et al.*, 2018). Anode and cathode were secured in place using a tight knee pad. The tibial nerve was stimulated with a 1 ms pulse width and 1-2 seconds in interstimulus interval delivered-randomly. Simultaneously, tibialis anterior SEMG was recorded to examine any cross-talk during tibial nerve stimulation. After the exact stimulation point was determined, subjects were asked to perform three maximum voluntary contractions for 3 seconds with a minute rest in between each attempt. While subjects were at

rest, M-response threshold and maximum M-response (M_{max}) was detected. M-response threshold was determined by lowering the stimulus intensity until obtaining minimum detectable M-response. M_{max}, on the other hand, was found by increasing the stimulus intensity above supramaximal level which produced with no further increase in M-response amplitude. Subjects were then asked to perform plantar flexion to recruit a steadily firing SMU. While SMU was active and discharging at a predetermined rate using sound and visual feedback, M-only stimulation was delivered to the tibial nerve. At least two different SMUs discharging at varying rates were obtained from each subject. The stimulus intensities were standardized as percentages of the stimulus strength that induced M_{max}. The predefined stimulus intensities were randomly applied, and the number of stimuli delivered ranged between 250 and 1,000 (454 ± 144; mean ± standard deviation).

Analysis: SMU potentials were extracted based on their shapes using a template matching Spike2 software. SMU firing times were recorded and subjected to further analysis by application of the following output measures: peristimulus time histogram (PSTH), peristimulus frequencygram (PSF) and their cumulative sums (CUSUMs) (Ellaway, 1978). The bin size of 0.1 ms was used in both PSTH and PSF to clearly see the latency and the duration of inhibition. Averaged SEMG for both muscles and rectified-averaged intramuscular MMU were obtained in all experiments.

Muscle twitch generated by electrical stimulation of tibial nerve was recorded by force transducer. Similar to SEMG; force recording was also spike-triggered-averaged. Peak-to-peak amplitude of the level between baseline and highest point of the twitch trace was measured.

Waveform template-based Spike2 software was used to recognize and convert SMUs into acceptance pulses. Interval histogram was constructed (**Figure 1A**) to validate the distribution of the interspike intervals of SMUs. After confirming the acceptance pulses using interval histograms, PSTHs and PSFs were constructed. PSF, discharge rate-based method, superimposes instantaneous discharge rates around the time of the stimuli (±250 ms in this study). Since excitatory currents increase and inhibitory currents reduce the discharge rate of action potentials, PSF demonstrates type of postsynaptic potentials (PSPs) occurring on MNs in terms of discharge rate change (**Figure 1B**). PSTH, probability-based stimulus triggered method, superimposes the timing of the accepted pulses pre and post stimulus and detect

probability of occurrence of units as counts at certain time point referenced to the stimulus (**Figure 1C**). Also, to investigate the gross response of several SMUs detected by the intramuscular electrodes, we have full-wave rectified and averaged the SMU channel which had a number of motor unit potentials (hence this analysis is called multi motor unit: MMU) **(Figure 1D).** To make sure that the stimulus delivered did not induce an H-reflex, averaged SEMG channel was examined online at M-response (around 5-20 ms) and H-reflex latency (around 30-40 ms) in all experiments **(Figure 1E)**.

However, due to synaptic noise, fluctuations occur in PSTH, PSF and MMU records. Therefore, significance of inhibitory and excitatory events (IPSP and EPSP, respectively) at poststimulus time was determined using CUSUM error box method (Türker *et al.*, 1997). To generate error box for these records, average prestimulus bin value was subtracted from each of the bin values all along the analysis period. Then the residual values left in each bin were cumulatively summed to obtain the CUSUM trace (Ellaway 1978). Maximum deviation from the mean at the prestimulus region was taken as the maximal background CUSUM change and was used to build the symmetrical 'error box' (Figure 1A horizontal broken lines around CUSUM). The size of this error box was then used to pinpoint significant synaptic activities. Usage of error box approach to pinpoint genuine poststimulus events was validated in brain slice experiments (Türker & Powers, 2003).

In CUSUM traces, if the amplitude of the post stimulus deflection was larger than the error box, it was accepted as a significant event. The latency of this event was determined as the first turning time point of the significant deflection. The only exception to this rule is the determination of latency of inhibitions in PSF records. In this case latency was calculated as the last consistent firing of the unit (time of termination of motor unit firing) (Türker & Powers, 2005). The duration of any significant event, on the other hand, was found by calculating the horizontal distance between the latency and the next turning point in the CUSUM record (Figure 1A-D).

***Experimental procedure for paired H-reflex technique*:** Similar SEMG preparation configuration for soleus muscle as in M-only technique was used. Anode and cathode were fixed in place using a tight knee pad while the tibial nerve was stimulated with pulses of 1 ms width. Exact location for the cathode that induced H-reflex only was obtained at midpoint of the popliteal fossa instead of slightly lateral of the midpoint as in M-only technique (Ozyurt

et al., 2018). Interval between conditioning (H1) and test stimuli (H′) was chosen to be 5, 10, 15, 20, 25 and 30 ms (Bussel & Pierrot-Deseilligny, 1977). H1 was determined as 35% of M_{max} and followed by supramaximal stimulus that induced H′. Each conditioning-test interval protocol was repeated 5 times and before each protocol H1 was induced to be compared with H′ at that particular protocol. Each paired pulse and H1 determination step was separated by at least 10 seconds to diminish the effect of post-activation depression (Hultborn *et al.*, 1996). Each protocol was applied in a random order. Lastly, peak-to-peak amplitude of the H′ was calculated and expressed as percentage of peak-to-peak amplitude of H1. After normalization of H′ to H1, results were pooled.

For the analysis of the significance of the changes in latency and duration of RI with changing stimulus intensity and background frequency, we used one-way ANOVA using Tukey correction for multiple comparisons. Linear correlation was employed to reveal interactions between several variables and duration of RI. The level of significance was set at $p<0.05$.

Results

We have recorded 54 SMUs from 12 subjects. For each SMU, the latency and duration of the inhibition were calculated, after confirming the distribution of the discharge rate of the unit using interval histogram **(Figure 1A).**

For every SMU, the duration and the latency of the inhibition estimated using 3 different analysis methods. The mean duration of the RI was found to be 30.8 ± 7.2 ms, 16.1 ± 5.8 ms, and 14.4 ± 3.5 ms when estimated using PSF, PSTH, and MMU, respectively **(Figure 2A)**. Although RI duration between PSTH and MMU was not significant ($p=0.2400$), duration estimation using PSF was significantly longer than both PSTH ($p<0.0001$) and MMU ($p<0.0001$).

The RI latency was 37.7 ± 2.3 ms, 37.7 ± 2.4 ms, and 39.9 ± 2.3 ms when assessed from PSF, PSTH, and MMU, respectively. The inhibition latency in averaged MMU was significantly longer than that in PSF ($p<0.0001$) and PSTH ($p<0.0001$), while there was no significant difference in latencies recorded using PSF and PSTH ($p=0.9683$) **(Figure 2B)**. The duration of RI calculated from PSF-CUSUM showed no correlation with the increasing stimulus intensity ($p=0.3195$) **(Figure 2C)**.

Paired H-reflex technique, most used protocol to investigate RI, was also employed to evaluate RI in two additional volunteer subjects. Average of 10 experiments from 2 subjects is presented in **Figure 2D**. Using paired H-reflex technique investigating intervals over 30 ms was not practical as test stimulus should be delivered before the conditioning stimulus arrives at the muscle. Therefore, it is not possible to assess the duration of RI using this method. As conditioning-test interval was increased, a significant rise in H′ amplitude was noted ($p<0.05$) indicating that the duration of RI was at least 30 ms long. The amplitude of H′ was 13.2 ± 2.57 % of H1 when conditioning-test interval was 5 ms. H′ increased in amplitude to 29.4 ± 5.48 % and to 24.9 ± 5.70 % of H1 as conditioning-test interval changed to 25 and 30 ms, respectively.

The latency of RI is longer than H-reflex

The latency of RI was also compared with the H-reflex latency using PSTH-CUSUM. For this reason, the H-reflex was evoked in 7 subjects and the difference between the H-reflex latency and RI latency was investigated using paired t-test within the subjects **(Figure 3)**. The average H-reflex latency of SMUs was found as 35.7 ± 1.2 ms which is significantly shorter than RI latency of these subjects that was 37.4 ± 1.8 ms ($p=0.0074$).

The RI duration is shorter on high threshold SMUs

A total of 27 different SMUs whose force recruitment threshold could be identified, were investigated (**Figure 4)**. A significant negative correlation was found between the duration of RI and force recruitment thresholds of units ($p<0.001$) (**Figure 4A)**. However, there was no correlation between the duration and background discharge rate of the units (**Figure 4B**), revealing that the observed change in duration was related to different size of motor units and not due to differences in background discharge rates ($p=0.7184$). This finding provides evidence suggesting that recurrent IPSP lasts longer in smaller sized MNs compared to larger ones (**Figure 4C**).

Stimulus evoked twitch produces *long latency* synaptic potentials

Since M-responses, especially larger ones, induce detectable twitches in soleus muscle which in turn can activate tendon organs in homonymous muscle, we examined the relationship between twitch profile and secondary excitation using PSF-CUSUM **(Figure 5)**. Electrical stimulation of the thickest motor axons produces M-response as the earliest activity and it causes muscle to contract after an electromechanical delay of around 15 ms. Concomitantly with the muscle contraction, antidromic activity of the thickest motor axons excite RCs via axon collaterals and their inhibitory volley arrives at smaller MNs to reduce their firing rate momentarily.

A multicomponent inhibition and excitation series has been proposed in **Figure 5A**. Hypothesized mechanism behind this complex system is schematized in **Figure 5B**. We observed a large twitch in isometric force recordings especially when M-response was also large. M-response induces muscle contraction hence activating tendon organs to send their inhibitory volleys to MNs via Ib afferents. Since RI lasts around 40 ms, the M-response induced Ib inhibition may arrive at MNs during the tail of the RI IPSP. These summed inhibitory volleys can relax the contracting muscle and by doing so activate stretch sensitive muscle spindles. Spindle activation, after a further electromechanical delay, initiates homonymous muscle contraction via increased discharge rate of MNs.

The duration of RI changes with the background discharge rate

Background discharge rates of SMUs have been noted to affect the duration of RI **(Figure 6)**. As muscle twitch can affect the duration of RI **(see Figure 5)**, we only included the recordings which were free of detectable muscle twitches. Stimulation of 29 units out of 54 did not produce any detectable twitch. For these 29 units, linear regression between inhibition duration and discharge rate indicated significant inverse relationship ($p<0.001$). A duration of 53.55 ms was calculated by extrapolating the background discharge rate to a silent MN **(Figure 6)**.

Inhibition was recorded only if M-response was present

To make sure that we have stimulated only the motor axons but not afferent fibers, we investigated RI in 3 different stimulus intensities according to motor threshold (MT): one just below MT (0.9x of MT, 0% M_{max}) and two suprathreshold intensities: one slightly higher

than the MT (2% of M_{max}) and one even stronger (10% of M_{max}). The results were evaluated using PSTH-CUSUM and M-responses in SEMG **(Figure 7)**. We found that the stimulus intensity just below MT did not produce an inhibitory response on the SMUs **(Figure 7A)**. However, as soon as the stimulus intensity reached the MT (2% of M_{max}), inhibition appeared (**Figure 7B**) and inhibition increased further at 10% M_{max} (**Figure 7C**). Thus, we confirmed that the inhibition we observed was likely to be due to antidromic motor axon stimulation.

The observed inhibition was neither cutaneous nor reciprocal

To test whether the inhibition we observed could result from stimulation of cutaneous afferents, we stimulated the same sensory dermatome but away from our usual stimulation point using similar stimulus intensities in 2 subjects. For this, we measured the distance between our usual stimulating point and the upper pelvic bone. Without changing the anode location and stimulus intensity, we moved the cathode proximally to one-third of that distance in the same dermatome. The data then were analyzed using PSTH-CUSUM (to detect inhibition) and SEMG (to find M-response and H-reflex). We found that the new stimulation arrangement induced no detectable response **(Figure 8A)**.

To test the possibility of activating Ia and Ib afferents, we have tested their possible effect on the antagonist muscle, tibialis anterior. For this, during our usual stimulation of the tibial nerve at the popliteal fossa, we have also recorded SEMG from tibialis anterior muscle. We have found that there was no noticeable response in averaged tibialis anterior SEMG during period of inhibition in the soleus muscle **(Figure 8B, 8C, 8D)**. The lack of stimulus-induced activity in tibialis anterior muscle may indicate that these afferent fibres were not activated to a level to induce noticeable effects as we found no reciprocal excitation via Ib afferents and no reciprocal inhibition via Ia afferents.

Discussion

Although most of the knowledge about the duration and distribution of RCs comes from animal experiments, a reliable tool to investigate RCs in human is a necessity to be used in research and in clinics. Moreover, with respect to the possibility of a defining role for RI, human experiments are more promising, since here intact MNs are studied in their physiological environment. To understand the functioning of the RCs in human, we

optimized and improved the precision of a previously used technique which involves electrical stimulation of the largest motor axons in the tibial nerve and recording from the voluntarily-activated low threshold SMU from the soleus muscle (Kudina & Pantseva, 1988) to determine stimulus-locked inhibition. Here we discuss 5 original findings;

- Characteristics of RI using three different methods
- Effect of discharge rate on RI duration
- Presumed RI duration on silent MN
- Distribution of RI to different sized MNs
- Reliability of M-only stimulation

Characteristics of RI using three different methods

We have investigated the influence of motor unit discharge rate and stimulus intensity on the latency and duration of RI estimated using three different methods. We have shown that the stimulus amplitude does not influence the duration of the RI. However, the duration of the RI depends on the background discharge rate.

Latency: The latency of RI did not seem to be connected with any of the controlled variables. The best method for estimating inhibition latency was PSTH-CUSUM as it deflected down immediately following a reduction in spike counts. The RI latency estimated from PSTH-CUSUM did not differ from that of the estimation from PSF, but the latency determined from MMU-CUSUM was longer. This observation can be explained by the fact that whereas PSTH is made up of spikes each of which only covers a narrow bin width (0.1 ms), the MMU is the rectified averaged raw data from the intramuscular electrode. Effectively, the MMU is a low pass filtered version of several motor unit action potentials and hence timing information (similar to the SEMG records) in MMU data are not reliable for latency measurements.

The latency of the RI has been studied to understand its multi-synaptic component which is mostly compared to the H-reflex. Stimulation of the thickest primary afferents (Ia afferents) produces the H-reflex monosynaptically. The latency of this reflex upon stimulation from the popliteal fossa varies between 30-35 ms for the soleus muscle depending on the height of the

subject (Hoffmann, 1910; Pierrot-Deseilligny & Mazevet, 2000). We found the H-reflex latency to be slightly above this value, around 35 ms that could be due to our recording region which was the distal part of the soleus muscle. The RI latency in our experiments, on the other hand, has been found to be around 37 ms (2 ms longer than the latency of the H-reflex) which is in accordance with another single unit study on the soleus muscle (Kudina & Pantseva, 1988). The delay of RI compared to that of H-reflex is due to relatively lower conduction velocity in large motor axons compared with primary afferents. This time delay may also be due to an additional synapse/s in the RC circuitry and to conduction delay in the RC which has been found to be between 1.1 ms and 1.8 ms in animals (Callister & Graham, 2010).

While it is possible to pinpoint the latency of RI in our experiments, the paired H-reflex technique could not identify latency of RI as it relied upon interstimulus intervals which could not be shorter than 5ms due to conduction dispersion in axons. With this limitation, RI latency was claimed to be 6ms longer than the H-reflex latency by Bussel and Pierrot-Deseilligny (1977) which falls within the range of spike silence (a gap in PSTH from 2 to 17 ms relative to the H-reflex latency) in our M-only stimulation method.

Duration: The best method for estimating the duration of the inhibition, on the other hand, was the PSF-CUSUM as it indicated the end of reduction in discharge rate of the unit very clearly (Türker & Powers, 2005). Due to the stimulus induced synchronization and poor estimation of the tail of the PSPs in PSTH-CUSUM, the duration measured in PSTH-CUSUM would be effectively contaminated (Türker & Cheng, 1994; Türker & Powers, 2005).

In our experiments, we found significantly longer RI duration than that estimated using classical methods (Bussel & Pierrot-Deseilligny, 1977; Hultborn & Pierrot-Deseilligny, 1979; Rossi *et al.*, 2003). This is due to the fact that the conditioning-test interval that was used in the classical method could not be increased over 30ms as the test stimulus should be delivered before the conditioning stimulus arrives at the muscle. Therefore, it is not possible to assess the duration of RI using the classical paired H-reflex method.

Also, this duration was dependent on the background discharge rate of the SMU. The longer lasting duration may originate from differences in the conduction velocity of stimulated motor axons which might generate dissipated IPSPs on MNs. Also, variability in the RI

duration on different sized MNs (Mazzocchio & Rossi, 1997) could be due to variable number of inputs arising from RCs or intrinsic properties of larger MNs such as shorter AHP duration and higher firing rate capability (Matthews, 1996). However, the duration determined using the PSF method was still shorter compared with the range of 45-50 ms determined using direct recordings from RCs upon stimulation of ventral roots in animal studies (Renshaw, 1941; Eccles *et al.*, 1954; Ellaway & Murphy, 1981; Callister & Graham, 2010). Nevertheless, the discharge of RCs may occasionally continue for up to 100 ms (Wilson, 1959). This prolonged activity may be due to the association of slow component receptors, especially N-methyl D-aspartate (NMDA) receptors on RCs in addition to faster nicotinic receptor activity. Slow but longer lasting activation of RCs by NMDA receptors can increase the effective IPSP by increasing the range of temporal summation (Alvarez *et al.*, 2013; Lamotte d'Incamps & Bhumbra, 2017). However, especially in anaesthetized animals, the descending inputs are less effective which may alter the RI duration due to reduced cortical influence (Mazzocchio & Rossi, 2010). This may explain why in our study such long RI durations were not observed.

In addition, due to tendon organ activation which could prolong inhibition duration as proposed in **Figure 5**, we did not take twitch-produced intensities into account to evaluate RI duration in order to eliminate the effect of Ib inhibition.

Discharge rate effect and presumed RI duration

In addition to the receptors (depending on the GABA and/or glycine receptors) underlying the IPSP duration, the state of the MNs in terms of excitability should also be considered for the duration of RI. Larger excitatory drives on MNs make them easily excitable, more importantly, increase their discharge rate. The MNs that fire at high discharge rates are highly excitable and hence less likely to respond IPSPs. Current study and that of Kudina and Pantseva (1988) showed that even in the same type of MNs, faster discharge resulted in shorter duration of RI. This shortened duration with increasing in discharge rate is even valid for gamma-MNs. Less pronounced RI on gamma-MNs due to their higher discharge rate up to 80 Hz has been shown (Ellaway & Murphy, 1981). Along the same line of claims, it has been shown that Renshaw IPSP durations are shorter in firing MNs than those recorded at rest (Obeidat *et al.*, 2014). In our study, that is exactly what we have observed, the higher the discharge rate of the SMU the shorter the RI duration. Besides, since the RI duration is estimated indirectly and since it is affected by the discharge rate of the unit, we decided to

suggest that correct duration of RI in a MN at rest can be estimated by linear extrapolation from discharge rate data. Similar linear relationship was proposed by Ellaway and Murphy (1981). We have performed this extrapolation in 29 units and estimated the RI duration at around 55 ms. This is an important step towards understanding synaptic potential profiles in human MNs as they have to be estimated indirectly using SMUs or the entire motor pool in SEMG.

Since the threshold of the largest Ia afferents and thickest motor axons are similar, our M-only stimulation is likely to initiate action potentials in a few Ia fibers though they may not generate a recordable H-reflex (Figure 5 dashed arrow 1). Stimulation of Ia afferent fibers can escalate the MN excitability and may slightly increase the discharge rate of the unit. This may lead to an underestimation of RI. Although we found no correlation between inhibition duration and amount of motor axons stimulated, majority of the RI was evoked using low stimulus intensities generating M-response around 7.6 % M_{max}. Therefore, we aimed to minimize the number of stimulated Ia fibers by using low intensity stimulation.

Distribution of RI to different sized MNs

Motor units physiologically differ in size (Burke *et al.*, 1973). Since we used soleus muscle motor units that were recruited at low contraction levels, our units were mostly low-threshold ones (Gollnick *et al.*, 1974). We were also careful to use low contraction levels to keep the discharge rate regular but low (de Luca *et al.*, 1982; Piotrkiewicz & Türker, 2017). Since discharge rate was one of the variables that had a significant impact on the indirectly estimated duration of recurrent IPSPs, we wanted to make sure that units fired at their optimum rates. The SMUs that require higher contraction levels to recruit were found to be inhibited by RCs as well but with much shorter duration even though these units fired at similar discharge rates as the lower threshold units (**Figure 4**). The proposed findings support the study of Friedman *et al.* (1981) and Hultborn *et al.* (1988) who suggested Renshaw IPSP is larger on smaller MNs. Therefore, we suggest that the lower duration of RI on higher threshold units might be explained by this relatively weaker RC inputs on larger units.

Reliability of the methodology

Majority of the knowledge on human RCs connections, physiology and their involvement in diseases was gathered from the experiments using paired H-reflex technique. Double stimulation of the primary afferent fibers not only activates the RCs through MN collaterals

but also allows the lagging stimulus to pass freely on MNs without any collision of ortho- and antidromic activation (Pierrot-Deseilligny & Bussel, 1975). Although this technique provided valuable information for decades, there are several drawbacks that might directly affect the presumed RI. For instance, such a high-intensity stimulus used in paired H-reflex technique is likely to evoke autogenic Ib inhibition. Since latencies of both inhibitions are very close to each other, they would have merged together (Delwaide & Oliver, 1988). In addition, primary afferent endings in such frequent stimulation are susceptible to presynaptic inhibition (Hultborn *et al.*, 1996). Those variables and outcomes might lead to misinterpretation of RI when paired H-reflex is employed.

M-only stimulation applied in our study, on the other hand, was shown to be highly reliable to investigate the RI with minimum contamination of other reflex pathways. We showed that the recorded inhibitory effect was neither reciprocal nor cutaneous inhibition **(see Figure 8)**. In the autogenic inhibition aspect, the voluntary activation of the soleus muscle minimize the possibility of the Ib inhibition since it is mostly reduced when voluntary contraction takes place (Lafleur *et al.*, 1992). In addition, recorded inhibition in this study was so much longer than that obtained for Ib inhibition alone (Delwaide & Oliver, 1988). Moreover, to decide if we have stimulated the Ib fibers that may contaminate RI, we checked if any longer latency responses were obtained in the antagonist muscle. Tendon organ activation or direct Ib afferent stimulation results in excitatory influence on antagonist muscle, tibialis anterior in this study, via oligosynaptic pathways (Pierrot-Deseilligny *et al.*, 1981; Katz *et al.*, 1991). As we electrically stimulate the mixed nerve and if some of the stimulated fibers were Ib afferents, then we could expect synchronous reciprocal excitation in tibialis anterior muscle. However, we did not observe any excitation several milliseconds later than H-reflex latency in tibialis anterior muscle **(see Figure 8D)**. Therefore, it can be proposed that Ib fibers did not effectively contaminate our results.

The extrapolation technique allowed us to deduce the synaptic potential as it develops in a silent MN. This technique provided information about how an IPSP influences a silent MN (**see Figure 6**). Hence, it may also shed light onto why longer duration of RI was obtained in anaesthetized animals where MNs were mostly silent.

In conclusion, we report an extensive investigation of RI in firing MNs in conscious human volunteers. The duration of RI has been shown to be inversely proportional to the background firing rate of the MNs. The stimulation of afferent fibers with higher intensity produced

stronger inhibition. RI was distributed differently onto different sized MNs. The optimized technique, parameters, and reported findings can be applied in clinics, particularly for further elucidating the role of RI in MN diseases such as ALS.

Limitations of the study

The proposed study is only applicable to RI input from larger to smaller MNs. Since the lowest supra threshold intensity electrical stimulus induces action potentials in the thickest fibers in a mixed nerve, it is possible to stimulate low-threshold motor axons along with low-threshold sensory axons (e.g. Types Ia and Ib fibres). This means that the results may represent net effects of all excitatory and inhibitory PSP activity rather than pure RI especially when large stimulus intensities are used. To minimize these unwanted effects, we have used low stimulus intensities and also tested for possible effects of significant contamination by examining their effects in agonist and antagonist muscles (Figures 7 and 8). These cautionary experiments showed that other fibre activity did not effectively contaminate our results. Therefore, we can propose that the dominant mechanism in the net inhibitory effect originates from the Renshaw circuitry.

In addition, although this methodology would be very useful to study RI in healthy individuals and in patients with MN loss in an early stage, the application of this technique is limited in the cases with severe MN loss where higher stimulus intensities are needed to activate the RC system. This larger intensity stimulation can not only induce a twitch mediated Ib afferent activation but also Ia spindle primary afferents activation as well. Both activations can lead to misinterpretation of RI. Similarly, when studying RI in patients with hyperreflexia (spastic patients for example), obtaining an M-only response without an H-reflex may not be possible, which will limits the use of the current method for RI study.

Acknowledgement

We acknowledge the subjects who participated in this project. We thank Koç University School of Medicine and Polish Academy of Sciences for supporting this study.

Competing Financial Interests

The authors declare no competing financial interests.

References

Alvarez FJ, Benito-Gonzalez A & Siembab VC. (2013). Principles of interneuron development learned from Renshaw cells and the motoneuron recurrent inhibitory circuit. *Ann N Y Acad Sci* **1279,** 22-31.

Bhumbra GS, Bannatyne BA, Watanabe M, Todd AJ, Maxwell DJ & Beato M. (2014). The Recurrent Case for the Renshaw Cell. *J Neurosci* **34,** 12919.

Burke RE, Levine DN, Tsairis P & Zajac FE. (1973). Physiological types and histochemical profiles in motor units of the cat gastrocnemius. *J Physiol* **234,** 723-748.723.

Bussel B & Pierrot-Deseilligny E. (1977). Inhibition of human motoneurones, probably of Renshaw origin, elicited by an orthodromic motor discharge. *J Physiol* **269,** 319-339.

Callister RJ & Graham BA. (2010). Early history of glycine receptor biology in Mammalian spinal cord circuits. *Front Mol Neurosci* **3,** 13.

Cullheim S & Kellerth JO. (1978). A morphological study of the axons and recurrent axon collaterals of cat alpha-motoneurones supplying different hind-limb muscles. *J Physiol* **281,** 285-299.

Crone C & Nielsen J. (1989). Methodological implications of the post activation depression of the soleus H-reflex in man. *Exp Brain Res* **78,** 28-32.

De Luca CJ, LeFever RS, McCue MP & Xenakis AP. (1982). Behaviour of human motor units in different muscles during linearly varying contractions. *J Physiol* **29,** 113-128.

Delwaide PJ & Oliver E. (1988). Short-latency autogenic inhibition (IB inhibition) in human spasticity. *J Neurol Neurosur Ps* **51,** 1546-1550.

Eccles JC, Fatt P & Koketsu K. (1954). Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. *J Physiol* **126,** 524-562.

Ellaway PH. (1978). Cumulative sum technique and its application to the analysis of peristimulus time histograms. *Electroencephalogr Clin Neurophysiol* **45,** 302-304.

Ellaway PH & Murphy PR. (1981). A comparison of the recurrent inhibition of alpha- and gamma-motoneurones in the cat. *J Physiol* **315,** 43-58.

Friedman WA, Sypert GW, Munson JB & Fleshman JW. (1981). Recurrent inhibition in type-identified motoneurons. *J Neurophysiol* **46,** 1349-1359.

Fung SJ, Pompeiano O & Barnes CD. (1987). Suppression of the recurrent inhibitory pathway in lumbar cord segments during locus coeruleus stimulation in cats. *Brain Res* **402,** 351-354.

Gollnick PD, Sjödin B, Karlsson J, Jansson E & Saltin B. (1974). Human soleus muscle: A comparison of fiber composition and enzyme activities with other leg muscles. *Pflügers Archiv* **348,** 247-255.

Gorassini MA, Norton JA, Nevett-Duchcherer J, Roy FD & Yang JF. (2009). Changes in locomotor muscle activity after treadmill training in subjects with incomplete spinal cord injury. *J Neurophysiol* **101,** 969-979.

Heckman CJ, Johnson M, Mottram C & Schuster J. (2008). Persistent inward currents in spinal motoneurons and their influence on human motoneuron firing patterns. *Neuroscientist* **14,** 264-275.

Hoffmann P. (1910). Beitrag zur Kenntnis der menschlichen Reflexe mit besonderer Berucksichtigung der elektrischen Erscheinungen. *Arch Anat Physiol* **1,** 223–246.

Hultborn H, Jankowska E & Lindström S. (1971). Recurrent inhibition from motor axon collaterals of transmission in the Ia inhibitory pathway to motoneurones. *J Physiol* **215,** 591-612.

Hultborn H, Illert M, Nielsen J, Paul A, Ballegaard M & Wiese H. (1996). On the mechanism of the post-activation depression of the H-reflex in human subjects. *Exp Brain Res* **108,** 450-462.

Hultborn H, Katz R & Mackel R. (1988). Distribution of recurrent inhibition within a motor nucleus. II. Amount of recurrent inhibition in motoneurones to fast and slow units. *Acta Physiol Scand* **134,** 363-374.

Hultborn H & Pierrot-Deseilligny E. (1979). Changes in recurrent inhibition during voluntary soleus contractions in man studied by an H-reflex technique. *J Physiol* **297,** 229-251.

Jankowska E & Hammar I. (2002). Spinal interneurones; how can studies in animals contribute to the understanding of spinal interneuronal systems in man? *Brain Res Brain Res Rev* **40,** 19-28.

Katz R, Penicaud A & Rossi A. (1991). Reciprocal Ia inhibition between elbow flexors and extensors in the human. *J Physiol* **437,** 269-286.

Kudina LP & Pantseva RE. (1988). Recurrent inhibition of firing motoneurones in man. *Electroencephalogr Clin Neurophysiol* **69,** 179-185.

Lafleur J, Zytnicki D, Horcholle-Bossavit G & Jami L. (1992). Depolarization of Ib afferent axons in the cat spinal cord during homonymous muscle contraction. *J Physiol* **445,** 345-354.

Lamotte d'Incamps B & Bhumbra GS. (2017). Segregation of glutamatergic and cholinergic transmission at the mixed motoneuron Renshaw cell synapse. *Sci Rep* **7,** 4037.

Lamotte d'Incamps B, Krejci E & Ascher P. (2012). Mechanisms shaping the slow nicotinic synaptic current at the motoneuron-renshaw cell synapse. *J Neurosci* **32,** 8413-8423.

Mattei B, Schmied A & Vedel JP. (2003). Recurrent inhibition of wrist extensor motoneurones: a single unit study on a deafferented patient. *J Physiol* **549,** 975-984.

Matthews PB. (1996). Relationship of firing intervals of human motor units to the trajectory of post-spike after-hyperpolarization and synaptic noise. *J Physiol* **492,** 597-628.

Mazzocchio R & Rossi A. (1989). Recurrent inhibition in human spinal spasticity. *Ital J Neurol Sci* **10,** 337-347.

Mazzocchio R & Rossi A. (1997). Involvement of spinal recurrent inhibition in spasticity. Further insight into the regulation of Renshaw cell activity. *Brain* **120,** 991-1003.

Mazzocchio R & Rossi A. (2010). Role of Renshaw cells in amyotrophic lateral sclerosis. *Muscle Nerve* **41,** 441-443.

Mazzocchio R, Rossi A & Rothwell JC. (1994). Depression of Renshaw recurrent inhibition by activation of corticospinal fibres in human upper and lower limb. *J Physiol* **481,** 487-498.

McCurdy ML & Hamm TM. (1994). Topography of recurrent inhibitory postsynaptic potentials between individual motoneurons in the cat. *J Neurophysiol* **72,** 214-226.

Moore NJ, Bhumbra GS & Foster JD. (2015). Synaptic Connectivity between Renshaw Cells and Motoneurons in the Recurrent Inhibitory Circuit of the Spinal Cord. *J Neurosci* **35,** 13673-13686.

Obeidat AZ, Nardelli P, Powers RK & Cope TC. (2014). Modulation of motoneuron firing by recurrent inhibition in the adult rat in vivo. *J Neurophysiol* **112,** 2302-2315.

Ozyurt MG, Shabsog M, Dursun M & Türker KS. (2018). Optimal location for eliciting the tibial H- reflex and motor response. *Muscle Nerve*. doi: 10.1002/mus.26308.

Panizza M, Nilsson J, Roth BJ, Rothwell J & Hallett M. (1994). The time constants of motor and sensory peripheral nerve fibers measured with the method of latent addition. *Electroencephalogr Clin Neurophysiol* **93,** 147-154.

Pierrot-Deseilligny E & Bussel B. (1975). Evidence for recurrent inhibition by motoneurons in human subjects. *Brain Res* **88,** 105-108.

Pierrot-Deseilligny E & Mazevet D. (2000). The monosynaptic reflex: a tool to investigate motor control in humans. Interest and limits. *Neurophysiol Clin* **30,** 67-80.

Pierrot-Deseilligny E, Morin C, Bergego C & Tankov N. (1981). Pattern of group I fibre projections from ankle flexor and extensor muscles in man. *Exp Brain Res* **42,** 337-350.

Piotrkiewicz M & Türker KS. (2017). Onion Skin or Common Drive? *Front Cell Neurosci* **11,** 2.

Raynor EM & Shefner JM. (1994). Recurrent inhibition is decreased in patients with amyotrophic lateral sclerosis. *Neurology* **44,** 2148-2153.

Renshaw B. (1941). Influence of discharge of motoneurons upon excitation of neighboring motoneurons. *J Neurophysiol* **4,** 167-183.

Rossi A & Mazzocchio R. (1991). Presence of homonymous recurrent inhibition in motoneurones supplying different lower limb muscles in humans. *Exp Brain Res* **84,** 367-373.

Rossi A, Mazzocchio R & Decchi B. (2003). Effect of chemically activated fine muscle afferents on spinal recurrent inhibition in humans. *Clin Neurophysiol* **114,** 279-287.

Rossi A, Mazzocchio R & Scarpini C. (1987). Evidence for Renshaw cell-motoneuron decoupling during tonic vestibular stimulation in man. *Exp Neurol* **98,** 1-12.

Tucker KJ & Türker KS. (2005). A new method to estimate signal cancellation in the human maximal M-wave. *J Neurosci Methods* **149,** 31-41.

Türker KS & Cheng HB. (1994). Motor-unit firing frequency can be used for the estimation of synaptic potentials in human motoneurones. *J Neurosci Methods* **53,** 225-234.

Türker KS, Miles TS & Le HT. (1988). The lip-clip: a simple, low-impedance ground electrode for use in human electrophysiology. *Brain Res Bull* **21,** 139-141.

Türker KS & Powers RK. (1999). Effects of large excitatory and inhibitory inputs on motoneuron discharge rate and probability. *J Neurophysiol* **82,** 829-840.

Türker KS & Powers RK. (2003). Estimation of postsynaptic potentials in rat hypoglossal motoneurones: insights for human work. *J Physiol* **551,** 419-431.

Türker KS & Powers RK. (2005). Black box revisited: a technique for estimating postsynaptic potentials in neurons. *Trends Neurosci* **28,** 379-386.

Türker KS, Yang J & Brodin P. (1997). Conditions for excitatory or inhibitory masseteric reflexes elicited by tooth pressure in man. *Arch Oral Biol* **42,** 121-128.

Wilson VJ. (1959). Recurrent facilitation of spinal reflexes. *J Gen Physiol* **42,** 703-713.

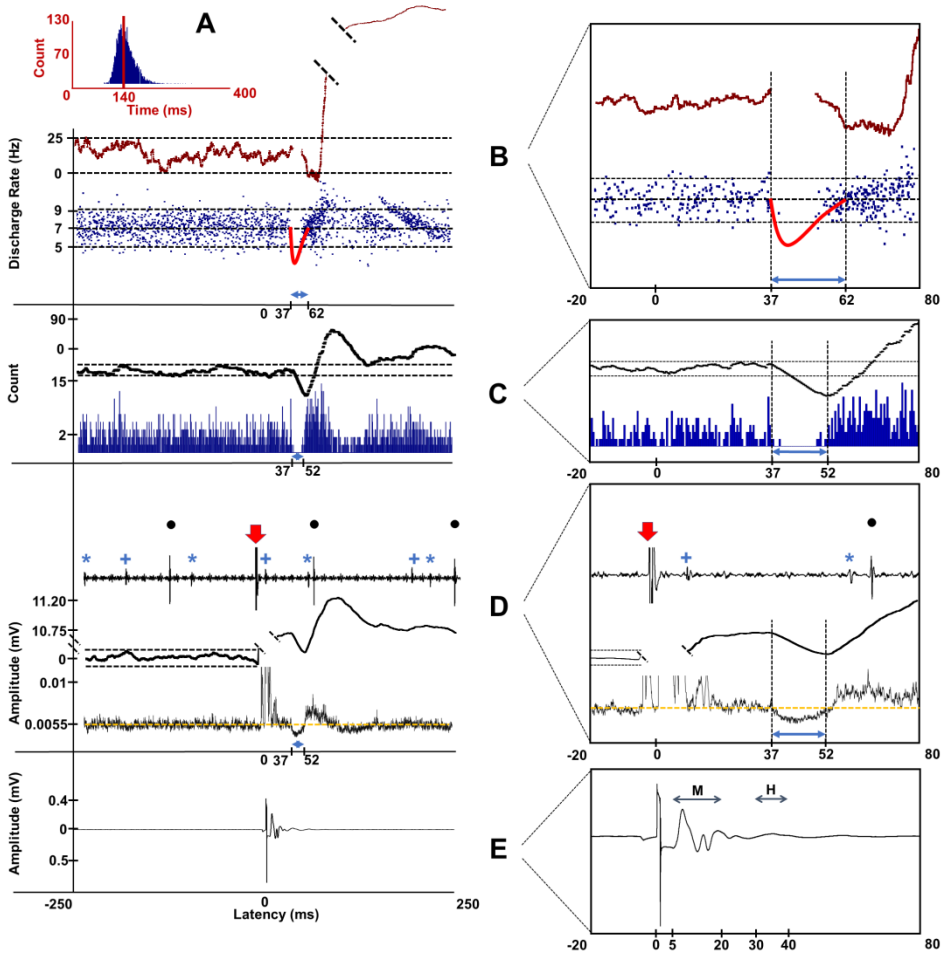

Figure 1. *Analysis of RI from a sample unit. (A) Interval histogram showing the distribution of the interspike intervals of the unit. The red vertical line shows the mean value (around 140 ms) which gives the mean discharge rate of 7.14 Hz. (B) PSF and its CUSUM providing the discharge rate of the unit around the time of the stimulus. The expanded figure on the right shows a closer view of the PSF and its CUSUM. Solid red trace represents imaginary IPSP profile drawn according to PSF CUSUM. Exact amplitude of this IPSP could not be determined as spikes cannot fire during this strong hyperpolarization stage of the IPSP. Therefore the amplitudes of the RI in this and subsequent figures are only imaginary. (C) PSTH and its CUSUM indicate the firing probability of the unit. The horizontal dashed line shows the error box. The figure on the right shows a closer view of the PSTH and its CUSUM. (D) Individual units, MMU and MMU CUSUM show the response of all recorded units using the intramuscular EMG. Intramuscular recording was full-wave rectified and averaged. Yellow line shows the mean background level activity. Black dots above the larger amplitude units are accepted and used in PSF and PSTH while blue asterisk and plus signs are some other SMUs that are not involved in PSF and PSTH but involved in MMU. The figure on the right shows the closer view of the SMUs, MMU and its CUSUM. Red arrows indicate stimulus artifacts. (E) Averaged-SEMG recording. The figure on the right is the closer view of the SEMG recording. The area represented with the indicator "M" is the M-response region and "H" is region where H-reflex would have appeared if the stimulus excited a large number of Ia fibres. The number of stimuli in this unit was 533.*

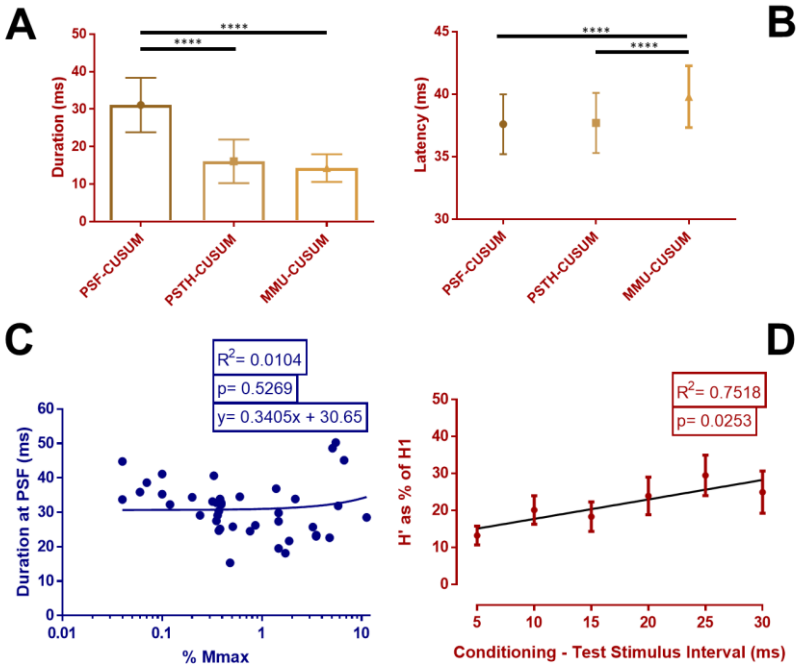

Figure 2. *Overview of the RI properties. Top panel: the mean duration (A) and latency (B) of all 54 units that were obtained using different analysis methods. Here, the longest duration was obtained at PSF-CUSUM while the longest latency was recorded in MMU-CUSUM. Error bars show standard deviation. **** means p<0.0001. Bottom panel: the effect of the stimulus strength on the duration of inhibition (C) determined using PSF-CUSUM. Note insignificant regression in (C) and which was plotted using a logarithmic abscissa. (D) RI by paired H-reflex protocol was assessed using various conditioning (H1) and test (H') stimulus intervals. Error bars represent standard errors.*

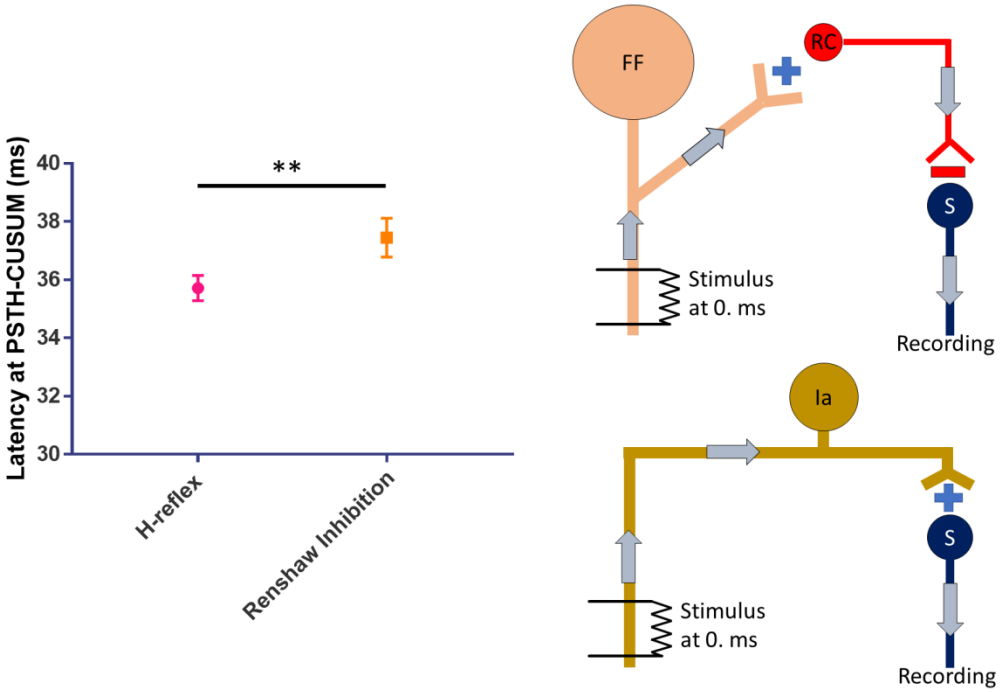

Figure 3. *The latency difference between the RI and H-reflex together with their proposed circuitries are summarized. Significantly longer latency of RI compared to H-reflex is shown in the graph on the left, while the wiring diagram of RI and H-reflex is illustrated in the figures on the right. Top right figure schematizes the larger MN (FF) stimulation and consecutive RI on smaller MN (S) by RC. Bottom right figure shows the primary afferent (Ia) stimulation to evoke H-reflex. Error bars represent the standard deviation, ** means p<0.01.*

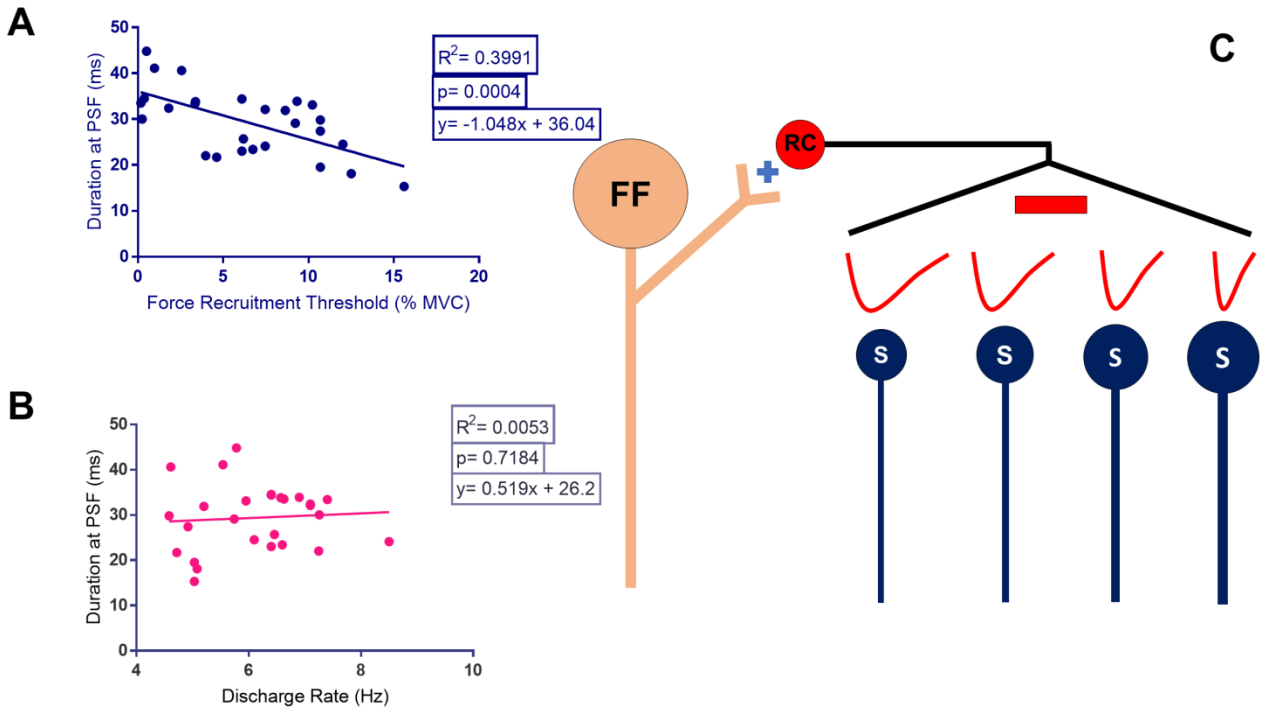

Figure 4. *The effect of various motor unit size on RI duration is shown. (A) Correlation between the RI duration and different force recruitment threshold levels of SMUs. (B) Correlation between the discharge rate and duration of the same SMUs. (C) Schematized wiring diagram of the RI, evoked by stimulation of the largest MNs (FF), on smaller (S) MNs that are graded in size. Solid red traces represent imaginary IPSP profiles. The amplitude of these traces does not represent the amplitude of the Renshaw IPSP*

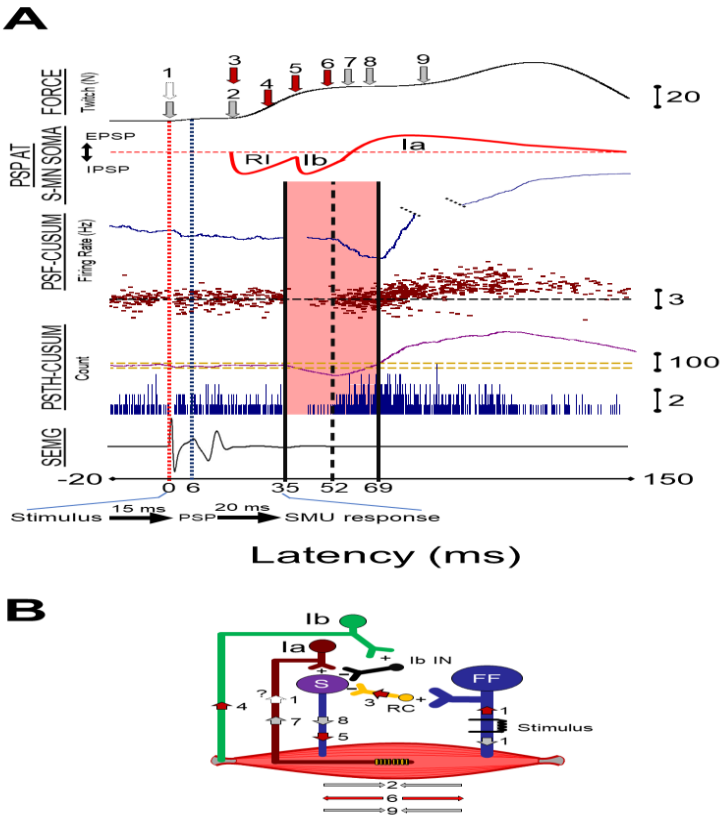

Figure 5. Hypothesized reflex mechanism behind strong M-response stimulation of motor axons. *(A) A sample recording from stimulation of thick motor axons (FF). From top to bottom: force record, schematic of synaptic potential of a small MN (S) at its soma, PSF and its CUSUM, PSTH and its CUSUM and SEMG recording. The stimulation (arrow 1) produced biphasic muscle contraction with a relaxation period in between. It is also possible that a few Ia fibers were activated during the stimulation even though we did not observe an H-reflex. The first twitch (arrow 2) is resulted from M-response induced muscle contraction after an electromechanical delay. An antidromic spike activity arrives at RCs in the spinal cord around the same time period (arrow 3). Since the M-response induces muscle contraction, tendon organs are activated (arrow 4) and S-MNs are inhibited via Ib interneurons (Ib-IN) (arrow 5). Combined recurrent and Ib inhibition cause the muscle to relax (arrow 6). This muscle relaxation may initiate muscle spindle activation (arrow 7) which increases the discharge rate of S-MN (arrow 8) causing muscle contraction again (arrow 9). Compound PSP at S-MN soma panel mimics an imaginary recording from the recruited MN. Since we record from the muscle (SEMG, PSTH and PSF) and this hypothetical recording is occurring at the ventral horn of the spinal cord, there is a time delay due to conduction along the motor axon. Therefore, any voltage change in S-MN arrives at the muscle ~20 ms later. Solid red traces represent imaginary PSP profiles. While the durations of the IPSPs and the EPSP are determined from the PSF CUSUM findings, the amplitudes of the IPSP traces are only our suggestions. (B) Hypothesized mechanism underlying these consecutive responses of the muscle and receptors are shown with corresponding arrows.*

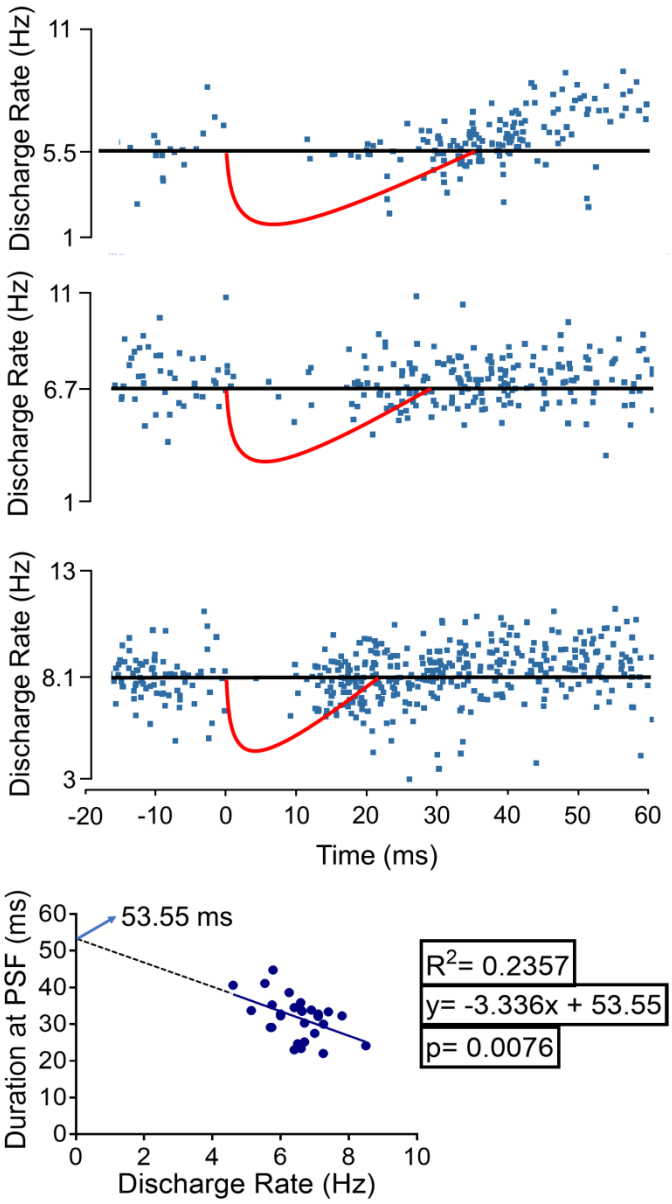

Figure 6. *The effect of background firing rate on duration of RI in single unit recordings. Upper plots are samples with different background rates. Red shapes are hypothetical IPSP with duration estimated from PSF-CUSUM. Abscissa represents time with normalized RI latency to 0 ms. Lower section is the method for extrapolation.*

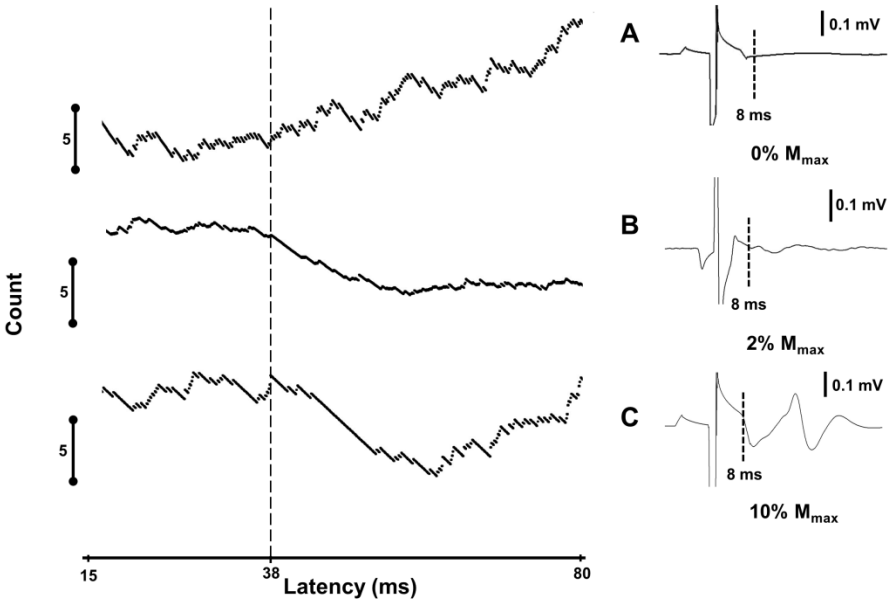

Figure 7. *The effect of M-response on the proposed inhibition: left panel shows PSTH-CUSUMs at three different stimulus intensities and the right panel shows the averaged SEMG traces. The stimulus intensity just below the M-response threshold did not result in an M-response and hence did not produce an inhibition (A), whereas the other two stimulus intensities, 2% (B) and 10% M_{max} (C) resulted in increased inhibition with a latency of around 38 ms.*

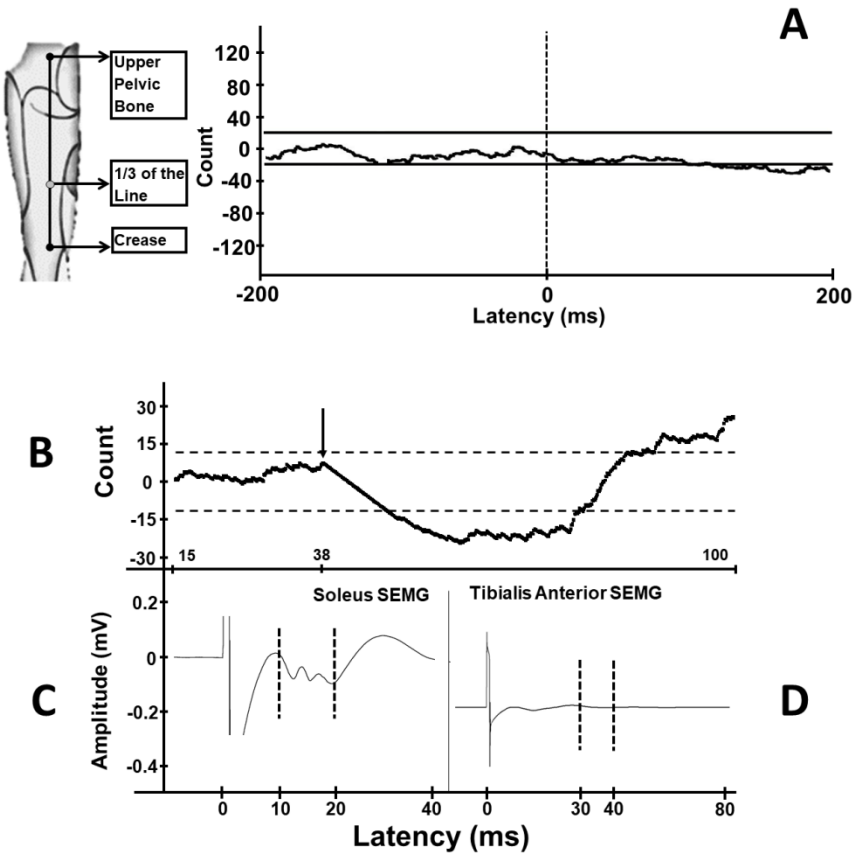

Figure 8. *Possible contaminants for RI.* ***(****A) The effect of cutaneous stimulation within the same dermatome and the same stimulus intensity (stimulation point indicated on the left). The response of a soleus SMU to the cutaneous stimulation indicates absence of any cutaneous effect on this SMU. (B) PSTH-CUSUM of a typical RI in a soleus SMU. The black arrow shows the onset of inhibition. (C) The response of the soleus SEMG to stimuli. The dashed lines show the range of the M-response and (D) no sign of a reflex between dashed lines indicates that there was no stimulus induced activity in tibialis anterior muscle.*