Investigating the Synaptic Physiology of Renshaw Cells and Their Influence on Motoneuron Degeneration

by

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Koç University

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This thesis is dedicated to those who suffer from neurological and movement disorders

ABSTRACT

Among the circuits of interneurons within the spinal cord, the Renshaw's inhibitory circuit is known to directly feedback on alpha-motoneurons. Inhibitory Renshaw cells (RCs) are located in the ventral horn of the spinal cord. Their unique features such as receiving direct cholinergic input from alpha-motoneurons and their ability for burst-firing provide a suitable model for studying circuits of the spinal cord. Although RC circuitry has been investigated for more than 70 years, its exact function has yet to be discovered due to the limitation of techniques. Therefore, the aim of this thesis was to develop a reliable method to investigate the characteristics of recurrent inhibition in healthy people and pathophysiology of RCs in patients who suffer from amyotrophic lateral sclerosis (ALS).

After optimizing the stimulation parameters of the motor axons in a reproducible manner, we recorded single motor units and analyzed the individual sets of action potentials using probability as well as frequency-based analysis. For this purpose, the largest motor axons in the tibial nerve were electrically stimulated to activate RCs antidromically and the smallest motor units innervating soleus muscle were recorded using intramuscular electromyography in healthy people and ALS patients.

The frequency methods indicated that the duration of the recurrent inhibition was between 30 to 55 ms in healthy people depending on the background firing rate of single motor units which were inversely proportional. Moreover, we found an evidence to support a longer inhibition on smaller diameter alpha-motoneurons compared to larger ones. On the other hand, the efficiency of the RCs was extremely reduced in the lumbar-affected ALS patients, i.e. the recurrent inhibition duration was about 11 ms, one-third to one-fifth of the healthy people.

These findings indicate a high correlation with the controlled animal studies, and that this method can be used as a reliable tool to shed more light on the functions and dysfunctions of the RC system in humans. Moreover, the outcome from this thesis provides evidence about the involvement of last-order interneurons, e.g. RCs, in the development of movement disorders, such as ALS.

ÖZETÇE

Hareket mekanizmasının temelinde α-motor nöronlar olmasına rağmen, ara nöronlar motor çıktıyı şekillendirmektedir. Ara nöronlarda oluşabilecek fonksiyon bozukluğu α-motor nöronların dengesini tamamen değiştirip kısa ve uzun vadede amyotrofik lateral skleroz (ALS) gibi nörolojik hastalıklara yol açabilmektedir. Omurilikteki bu ara nöronların oluşturduğu devreler arasında Renshaw'un inhibisyon devresinin α-motor nöronlarla doğrudan geri besleme ilişkisi içinde olduğu bilinmektedir. Yarım asır önce ortaya konan bu inhibitör Renshaw hücreleri omuriliğin ön boynuzunda α-motor nöronlara çok yakın bir şekilde konuşlanmıştır. Bu hücrelerin en önemli özelliklerinden biri de yüksek frekanslı ateşleme yapabilmeleridir. Renshaw hücreleri üzerinde yıllar boyu yapılan araştırmalara rağmen, teknik kısıtlılıklar sebebiyle bu hücrelerin nörolojik hastalıklardaki rolü teoriden öteye gidememiştir. Bu nedenle, hedefimiz Renshaw hücrelerinin etkin şekilde araştırılabileceği bir teknik geliştirmek ve karakteristik özelliklerini sağlıklı insanlarda ve patofizyolojik özelliklerini ise ALS hastalarında çalışarak bu devrenin hareket mekanizmasındaki önemini anlamaktır.

Renshaw hücrelerini araştırabilmek için öncelikle tibial sinirdeki motor aksonların uyarılma parametreleri optimize edilmiştir. Bunun ardından, kalın motor aksonlar elektrikle uyarılmış ve antidromik olarak Renshaw hücreleri aktive edilmiştir. İnhibisyon etkisinin incelenebilmesi amacıyla soleus kası içerisine yerleştirilen iğne elektrotlar ile kişilerin kendi oluşturduğu kas aksiyon potansiyelleri ve Renshaw hücrelerinin oluşturduğu sinaptik potansiyeller kayıt edilmiştir. Bu yöntem hem sağlıklı insanlarda hem de ALS hastalarında uygulanmıştır.

Elde edilen sonuçlar ışığında, Renshaw hücrelerinin oluşturduğu inhibisiyonun 30-55 ms sürdüğü gösterilmiş ve bu sürenin kas aksiyon potansiyeli atım frekansıyla

ters ilişkili olduğu bulunmuştur. Ayrıca Renshaw hücrelerinin inhibisyonu ince motor nöronlarda daha etkin bir şekilde gözlenirken, kalın motor nöronlarda bu inhibisyon süresi oldukça kısadır. Bacak kasları etkilenmiş ALS hastalarında ise inhibisyon süresi çok büyük bir düşüş göstermekte, normal insanlarda elde edilen sürenin yaklaşık üçte biri ile beşte biri seviyesi arasında kalmaktadır.

Bu çalışma, Renshaw hücreleri sinaptik potansiyellerini insanlarda ilk kez detaylı olarak ortaya koymuştur. Ayrıca geliştirilen yöntem sayesinde Renshaw hücrelerinin diğer özelliklerinin de gelecekte incelenebilmesine olanak sağlanmıştır. Öte yandan bacakları etkilenmiş ALS hastalarında elde edilen veriler göstermiştir ki, Renshaw hücreleri ALS gibi hareket hastalıklarının oluşumunda/gelişiminde rol oynamaktadır.

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NOMENCLATURE

MN: Motoneuron

CNS: Central Nervous System

S-type: Slow-Twitch Type I

FR-type: Fatigue-Resistant Type IIa

FF-type: Fast-Fatiguing Type IIb

GABA: Gamma Aminobutyric Acid

RC: Renshaw Cell

P-AD: Post-Activation Depression

ISI: Interstimulus Interval

IPSP: Inhibitory Postsynaptic Potential

H-reflex: Hoffmann Reflex

M-response: Direct Motor Response

SMU: Single Motor Unit

EMG: Electromyography

SEMG: Surface Electromyography

ALS: Amyotrophic Lateral Sclerosis

MVC: Maximum Voluntary Contraction

Mmax: Maximum Direct Motor Response

SD: Standard Deviation

MUNIX: Motor Unit Number Index

MUSIX: Motor Unit Size Index

SIP: Surface Interference Pattern

S_a: Half-wave Rectified Surface Electromyography Area

M_a: Half-wave Rectified Maximum Direct Motor Response Area

S_p: Half-wave Rectified Surface Electromyography Power

M_p: Half-wave Rectified Maximum Direct Motor Response Power

ICMUC: Ideal Case Motor Unit Count

PSTH: Peristimulus Time Histogram

PSF: Peristimulus Frequencygram

CUSUM: Cumulative Sum

MMU: Multi Motor Unit

CV: Coefficient of Variation

H1: Conditioning H-reflex

H': Test H-reflex

MT: Motor Threshold

NMDA: N-methyl D-aspartate

EPSP: Excitatory Postsynaptic Potential

SOD1: Superoxide Dismutase-1

TARDBP: TAR DNA Binding Protein

FUS: Fusion in Sarcoma

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic Acid

EAAT: Excitatory Amino Acid Transporter

Chapter 1

INTRODUCTION

1.1 Spinal control of movement

Although higher centers, e.g. motor cortex, are the determinants for executing the movements, spinal motoneurons (MNs) are the ones that actualize the movement by conveying the information to the muscles. Motor systems provide reflexive and voluntary movements with high speed and specified manner thanks to its functional organization from higher centers, namely motor cortex and brain stem, to lower spinal cord.

After delivery of the environmental inputs by sensory neurons to central nervous system (CNS), spinal interneurons process these inputs and provide it to the MNs mono/polysynaptically that can produce reflexive or rhythmic response, or these inputs can regulate voluntary activities of MNs. In addition to sensory inputs, descending systems can also modulate the interneurons and MNs to control movements since the MNs are the final common pathway for all motor responses, as Sherrington says.

1.1.1 Spinal motoneurons

Peripheral systems, such as muscle receptors of spindles, feed the spinal cord with the muscle condition. Spindles are innervated with two different types of MNs: gamma and beta MNs. Although gamma MNs innervate the spindle endings exclusively to keep it responsive regularly, beta MNs not only innervate intrafusal fibers within spindles but also extrafusal fibers (**Fig 1.1**). Therefore, gamma MN innervation to spindle called as fusimotor innervation [1] and beta MN innervation as skeleto-fusimotor innervation [2]. Both MN groups have common supraspinal inputs especially during voluntary movement. Moreover,

due to the importance of muscle spindles in voluntary contraction, beta MNs might be crucial in small muscle groups whose muscle spindles are close to the motor point. On the other hand, gamma MNs indirectly facilitate another group of MN – alpha MNs – by activating the muscle spindles that excite alpha MNs which innervate extrafusal muscle fibers.

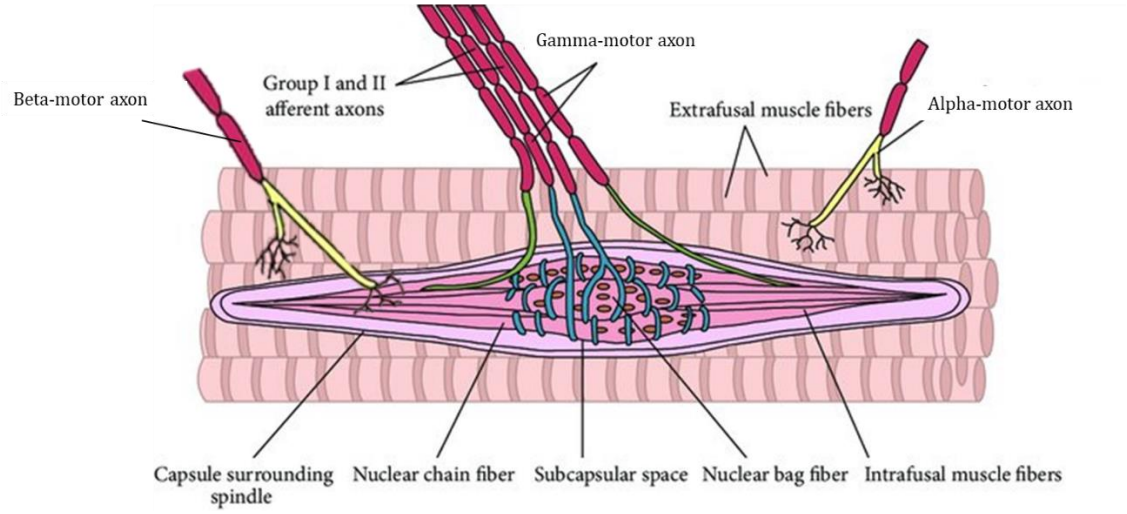

Fig 1.1 *The organization of the muscle spindle receptor and their innervation. The figure was modified from Bieganska [3].*

Spinal alpha MNs, whose soma and dendrites are located at the ventral horn of the gray matter, are regulated by numerous peripheral, interneuronal and supraspinal inputs (**Fig 1.2**). Alpha MNs in the ventral horn are organized by their location to innervate limbs and axial muscles. Alpha MNs that excite distal-limb muscles are located at anterolateral gray matter (lateral column) and the ones that innervate proximal-axial muscles constitute medial column [4, 5], both send axons to the skeletal muscles to actuate movement. One alpha motor axon innervates many skeletal muscle fibers, together they compose the smallest contractile unit, called the motor unit.

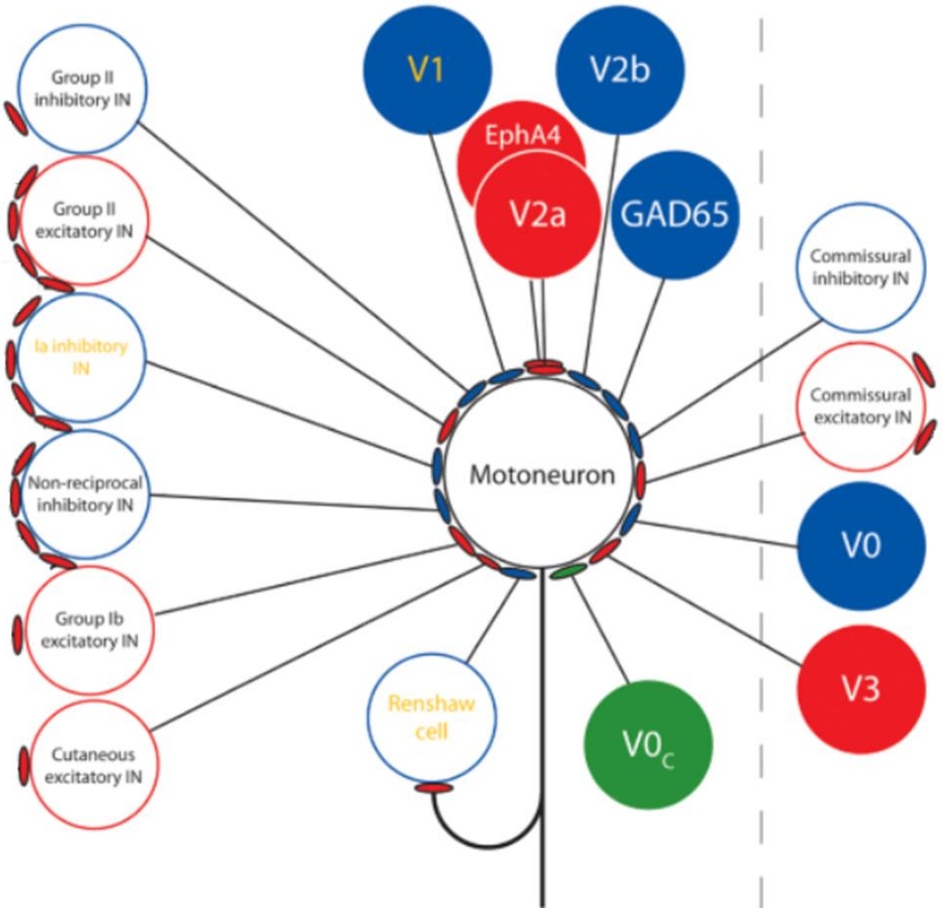

Fig 1.2 *Some neuronal inputs converge on alpha MNs in the spinal cord. The figure was modified from Brownstone [6]. Abbreviations; IN: interneuron, GAD: glutamic acid decarboxylase.*

MNs (***MN: alpha MN*** from this sentence on, unless otherwise stated) are further classified according to the skeletal muscle fiber type innervated (**Fig 1.3**). Smaller size MNs innervate a few muscle fibers and generate slow twitch are called S-type (also referred type I) motor units. They are non-fatiguing and produce relatively lower force. Intermediate sized MNs that innervate more muscle fibers are fatigue resistant and generate fast twitch referred

to as FR-type (type IIa) motor units. Lastly, the largest MNs innervating even more muscle fibers are fast-fatigable and produce highest force called FF-type (type IIb) motor units [7]. S-type units have the lowest threshold for voluntary recruitment, therefore, minimum voluntary drive activates the S-type units first. In contrast, peripheral electrical stimulation activates the thickest ones easily, therefore, FF-type units selectively.

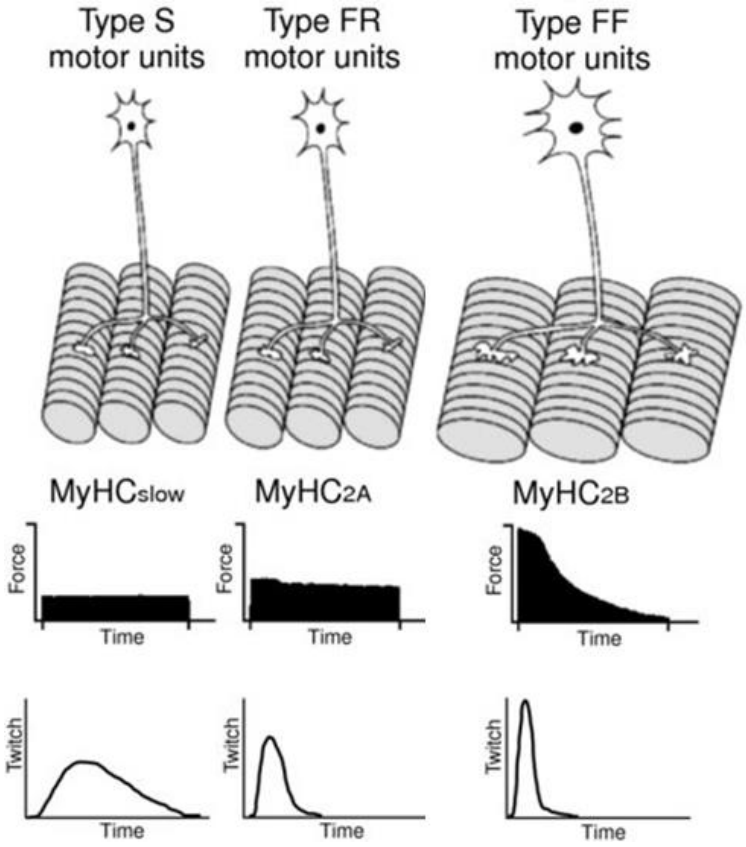

Fig 1.3 *Physiological properties of the different sized motor units. The figure was modified from Harro [8].*

1.1.2 Spinal interneurons

There are four major class of interneurons in the spinal cord at the ventral gray matter (**Fig 1.4**) [9];

- V0: these are commissural gamma aminobutyric acidergic (GABA)/glutamatergic interneurons in the embryonic term but assumed to mediate disynaptic crossed inhibition. They express Evx1/2.
- V1: these interneurons are identified by their Engrailed-1 expression. They inhibit their targets using GABA and glycine neurotransmitters. Adult subgroups of V1 interneurons are Renshaw Cells (RCs; ***the cell of interest in this thesis***) that mediate recurrent inhibition and Ia inhibitory interneurons that mediate reciprocal inhibition facilitate flexor-extensor alternation.
- V2: they are identified by Chx10 and Gata3 expression and this group has both inhibitory and excitatory interneurons. V2a (excitatory) and V2b interneurons are major classes within V2 group.
- V3: these glutamatergic excitatory interneurons express Sim1. They send projections to contralateral Ia inhibitory interneurons, MNs and RCs.

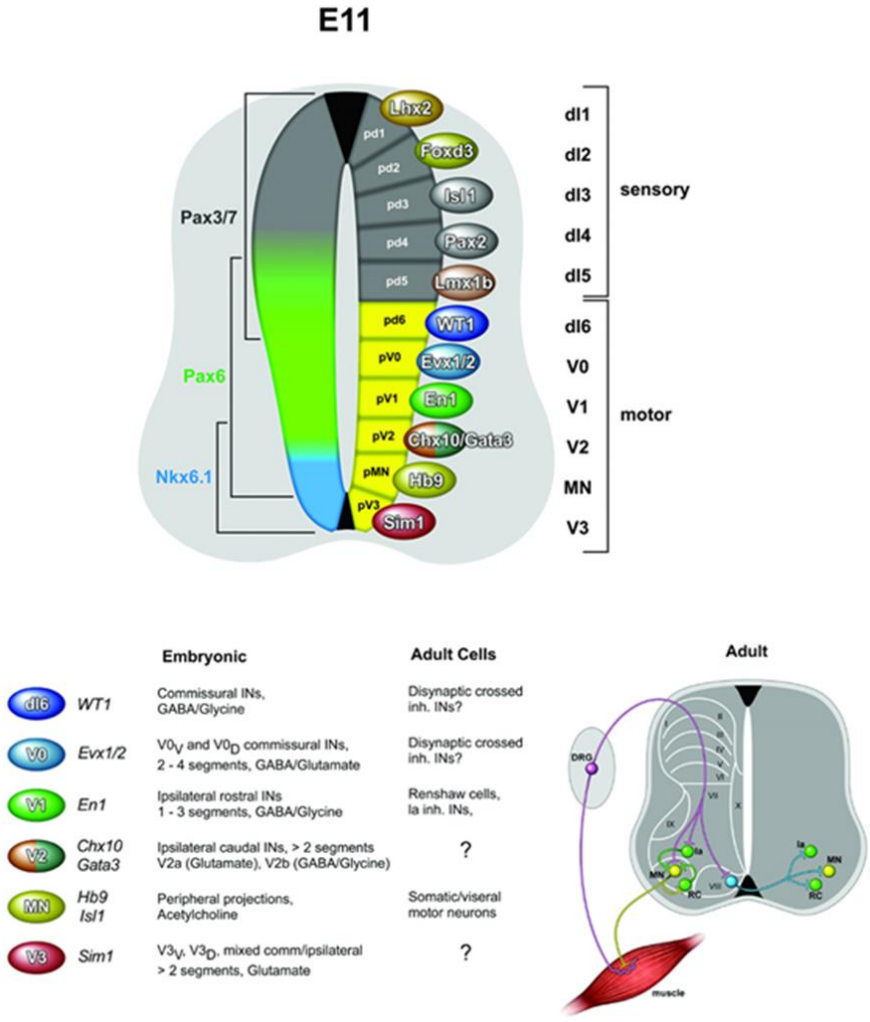

Fig 1.4 *Spinal interneurons located at ventral and dorsal region, identified by various transcription factors in embryonic age of 11. This figure was modified from Goulding [9].*

Spinal interneurons play an integral role in the coordination of movement in part by responding to somatosensory inputs from the environment. These somatosensory inputs are important not only for short-term responses to environmental disturbances but also for the long-term adaptation necessary for motor functional recovery following injury or disease [10]. The spinal cord contains neuronal circuits (**Fig 1.5**) that produce basic locomotor output and integrate sensory inputs arising during locomotion by MNs [11]. Therefore, understanding the spinal interneurons allow us to manipulate the movement towards recovery from disorders.

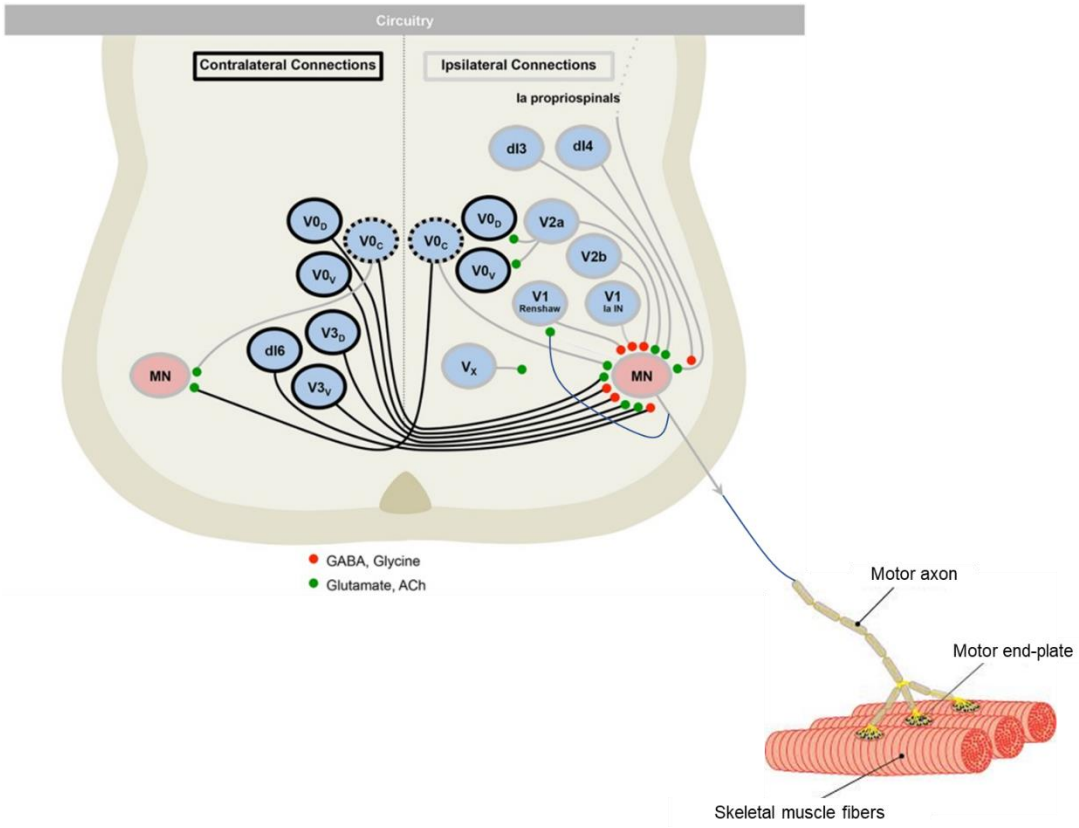

Fig. 1.5 *Spinal interneurons that regulate motor output. Contralateral and ipsilateral projections on MNs control movement and modify sensory inputs. Spinal last-order*

interneurons that directly provide input to MNs are some of the key elements to produce locomotion. The figure was modified from Lu [12].

1.2 Inhibitory mechanisms

Inhibitory interneurons are distributed throughout the spinal cord to control movement. They can regulate the motor output directly/indirectly, converge the various inputs on motor systems, are responsible for fine tuning of the contraction and regulate the alternation of the antagonist motor pool activation such as in locomotion. Inhibitory interneurons exert their inhibition either at the presynaptic endings or on the postsynaptic regions.

1.2.1 Presynaptic inhibition

Presynaptic inhibition is mediated at the presynaptic region and reduce the readily available vesicles, thus, reducing the effectiveness of the synapses (**Fig 1.6**). One of the presynaptic inhibition types is the classical one which is the GABAergic inhibition mostly observed in presynaptic endings of afferent fibers. The duration of the classical presynaptic inhibition occurring at Ia afferent terminals is a few hundred milliseconds and accompanied with dorsal root potential and does not affect the descending systems [13, 14].

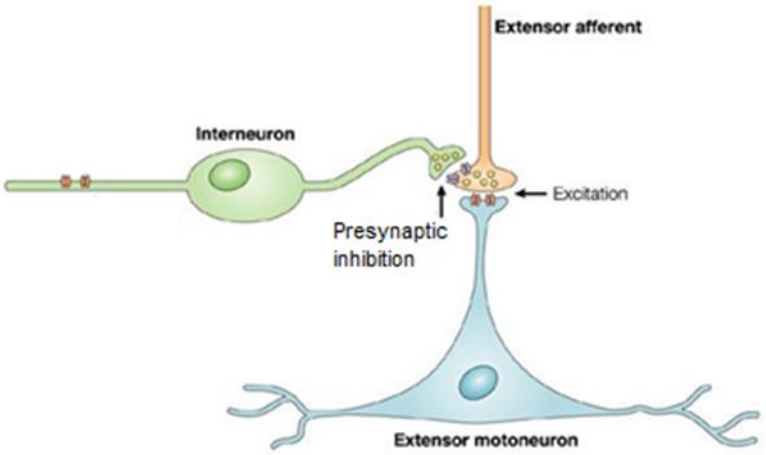

Fig. 1.6 *Spinal presynaptic inhibition acting on the extensor afferent endings before the MNs. The figure was modified from Engelman [15].*

Classical GABAergic inhibition is not the only type of presynaptic modulatory mechanism. One type of presynaptic inhibition with a very long duration is post-activation depression (P-AD) [16]. Short interstimulus intervals (ISIs), e.g. 1-2 secs, used to stimulate of Ia fibers induce P-AD, induce significant reductions in the amplitude of the following responses. In contrast to the classical type of presynaptic inhibition, P-AD is a homosynaptic form of inhibition, can last as long as 8 seconds and is not accompanied by a dorsal root potential [14, 16, 17]. In addition to electrical stimulation with short ISIs [18], various means of Ia fiber activation such as passive lengthening of the muscle [19], tendon tap [20] or transcutaneous spinal stimulation [21] can result in P-AD. Several explanations have been put forward and suggested that P-AD might be caused by neurotransmitter depletion of Ia terminals [20, 22] or by recurrent inhibitory systems [23]. Although there is no consensus regarding the mechanism underlying P-AD, it has been concluded that P-AD is not a postsynaptic phenomenon but occurs at the presynaptic level [14, 19] (**Fig 1.7**).

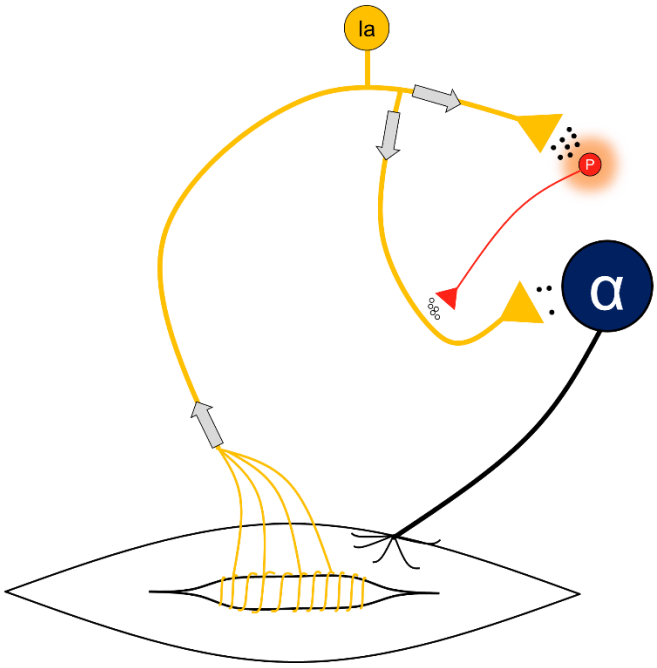

Fig. 1.7 *Diagram of one of the proposed mechanisms underlying P-AD. One of the hypotheses is that the first electrical stimulus of primary afferents evokes a synchronous discharge which acts as an "ON" signal and generates a plateau potential in plateau (P) interneuron. This activation shunts the activity on spindle primary afferent and/or MN dendrites for several secs. Modified from Özyurt et al (unpublished).*

1.2.2 Recurrent inhibition

In addition to presynaptic inhibition, many inhibitory interneurons directly inhibit the target neuron on its soma or dendrite by generating inhibitory postsynaptic potential (IPSP). Particularly in the spinal cord, majority of the inhibitory neurons regulate the movement by targeting MNs or other circuit elements. Many circuitries in the spinal cord control the movement via feedback loops. One of the well described and highly distributed loops is the recurrent mechanisms, especially the recurrent inhibitory systems. Interneuron-mediated

recurrent inhibition either shape the input arising from the periphery or control the output of the local networks' own activity by stabilizing their discharges [24]. Since the recurrent inhibition is widely distributed throughout the CNS, the physiological significance of the recurrent inhibitory elements has been studied at distinct levels. For instance, at the brain level, the recurrent inhibition in the thalamic reticular nucleus arrange the synchrony to tune the spontaneous firing which may lead to undesired seizures [25]. On the other hand, in the spinal cord level, MNs functioning in the locomotion uniquely receive recurrent inhibition upon their own excitation through axon collaterals on RCs which are one of the most studied recurrent inhibitory interneurons [26, 27].

1.2.2.1 Recurrent inhibition in supraspinal centers

Recurrent inhibition plays a key role in modulating the output of a neuron. Some of the brain and other supraspinal centers, therefore, exhibit recurrent inhibition to control their "own" output to the other supra/spinal locations in the CNS [28]. To date, several regions in the supraspinal levels have been shown to have recurrent inhibition. A canonical cortical circuit exists in the neocortex which includes two populations of excitatory neurons in cortical layers and one population of GABAergic inhibitory neurons that are recurring. Excitatory populations recurrently inhibit themselves and modulate the cortical excitability [29]. Another part which contains recurrent inhibition is basal ganglia at different organization levels, such as in the subthalamic nucleus, internal segment, substantia nigra pars reticulata and within striatum [30].

Moreover, hippocampus, also involves recurrent inhibitory systems where some hippocampal afferents activate axo-axonic cells which play a functional role in the cortical circuits. It has been proposed that high-intensity electrical stimulation evokes IPSPs which are strong enough to diminish depolarization induced firing in axo-axonic cells. This IPSP

is an example of a feedforward mechanism, the recurrent inhibition which has GABAergic background [31]. Principal neurons in the thalamus relay environmental data to the cortex and local inhibitory neurons regulate this transmission via recurrent inhibition mechanism, such as the intrageniculate interneurons that inhibit the principal cells [32]. In addition, thalamic reticular nucleus involves GABAergic interneurons which are activated by the thalamic neurons and inhibit themselves in a recurrent manner [33]. Late inspiratory neurons in the brainstem control the excitation of the inspiratory ramp [34] and Purkinje cells in the cerebellum inhibit each other recurrently [35]. Commonly, neurons that are highly excited are controlled by recurrent mechanisms in supraspinal centers of the CNS. Although there are various different levels of centers that exhibit the recurrent inhibition, the one highly studied and well-known is located at the spinal cord, namely the recurrent inhibition of MNs by RCs.

1.2.2.2 Recurrent inhibition in spinal cord

1.2.2.2.1 Renshaw cells

Recurrent inhibitory RCs, located at the ventral part of the lamina VII close to MNs [36], receive the largest amount of motor collaterals from the FF-type motor units to inhibit the S-type motor units (**Fig 1.8**), as well as they inhibit the same MN that excite them [37, 38]. Although RCs were first thought to be restricted to the lumbar spinal cord, other segments were also found to have recurrent inhibition by RCs. Moreover, proximal muscles were suggested to exhibit recurrent inhibition while distal muscles, such as intrinsic hand muscles, do not have associated RCs [39-41]. Moreover, an activated RC as effective as to inhibit a MN by shunting its excitation [42].

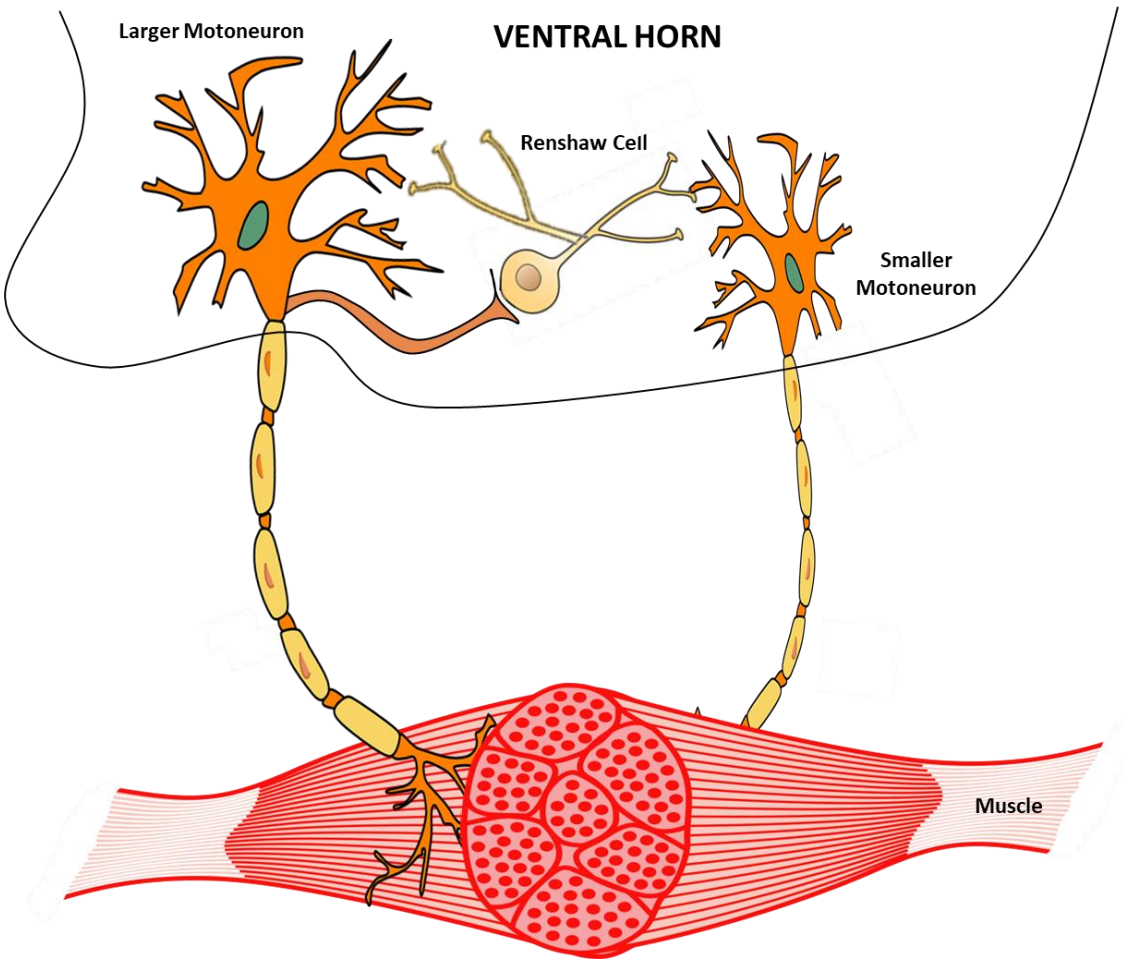

Fig. 1.8 *Wiring of the RCs in ventral horn that mediate recurrent inhibition. The major findings suggest a strong recurrent inhibition arises from larger MN firing on smaller MNs.*

There are many types of interneurons in the spinal cord, however, RCs are the only ones that receive input directly via collaterals of the MNs, and RCs, in turn, make many inhibitory contacts with the dendrites of the MNs mediated by GABA or glycine [27, 43-45]. Moreover, in addition to acetylcholine, activation of RCs is also mediated by several other neurotransmitters, such as aspartate and glutamate [26, 46, 47].

On the other hand, findings from the adult cat provided that synaptic contacts on RCs consist of large gephyrin postsynaptic proteins which indicate the strict control of RCs by inhibitory systems [48]. These inhibitory contacts are distributed to block powerful excitatory effects on RCs [49] via GABA and glycine [50, 51]. RCs gets activated by primary afferents only until postnatal day 15 in mice, but after that is decreased [52]. Additionally, RCs are capable of firing action potentials in very high frequencies [53]. Due to the maintenance of the firing in high rates which is dependent on calcium at vesicle release, RCs produce high amount of calcium buffering protein "calbindin" which is widely used to identify RCs [50].

1.2.2.2.2 Wiring of the Renshaw cells

In addition to their connections in the spinal segment, RCs also receive descending inputs. Earlier slice studies and paired Hoffmann reflex (H-reflex) method (will be discussed in *1.2.2.3*) performed indirect measurements of recurrent inhibition have suggested only a weak modulatory effect. These studies were limited by the lack of observed MN responses to inputs from single RC and their inability to measure animal/human behavior. A recent study showed that the recurrent inhibition by RCs is remarkably effective, in that a single action potential from one RC is sufficient to silence a MN. These results underlined a need for a reevaluation of the role of RCs [42, 54].

RCs are regulated by many supraspinal and local systems including; primary afferents until postnatal day of 15 [52], group III-IV afferents [55], other RCs [56], alpha MNs [27], some regions and tracts [57-63]. On the other hand; RCs, in turn, inhibit many centers and some interneurons such as Ia inhibitory interneurons [64], alpha MNs, gamma MNs [65], some ascending tracts [66]. Detailed connections of RCs (inputs and outputs) are illustrated in **Fig 1.9**.

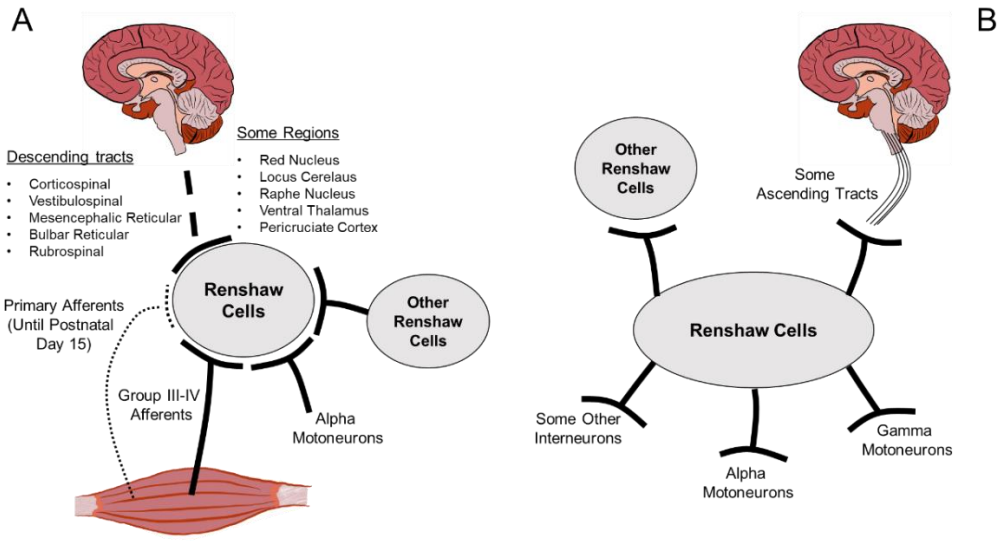

Fig 1.9 *Summary of RC connections. A) Inputs on RCs and B) Outputs from RCs.*

1.2.2.3 Methods to study spinal recurrent inhibition in humans

Although the majority of the experiments were performed on the animals, the extensive connections of RCs were also investigated in awake human subjects. The latency, duration, and strength of recurrent inhibition are the essential parameters to be investigated. To find those 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 [67] or in deafferented patients which was detected by the direct motor response (M-response) [68]. This method was based on stimulating the thickest motor fibers while recording inhibition on the smallest ones. Because the fact that electrical stimulation activates the thicker fibers while lowest voluntary contraction activates the thinner MNs, it is promising to measure the effect of activation of large MNs on small MNs without any conflict of occupying the same axons (see **Fig 1.8**: large MN to be stimulated to activate RCs and small MN to be recorded to detect the recurrent inhibition). Studying recurrent inhibition on single

motor units (SMUs) has a lot of advantages, e.g. more controlled variables, specificity and minimum contaminants such as primary afferent discharges. However, stimulation of only-motor axons has been a challenge due to limitations in reproducibility. Because, the nerve to be studied is mixed, primary afferents are typically thicker than motor fibers within the nerve and electrical stimulation activates the thickest axons first [69, 70] (**Fig 1.10**). Due to these issues, it is complicated to stimulate the motor axons without primary afferents.

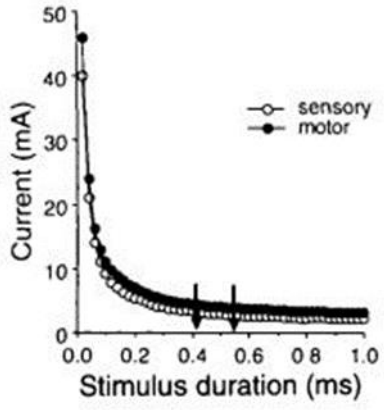

Fig 1.10 *Current-stimulus duration curve of the motor and sensory axons. The figure was modified from Mogyoros [69].*

In contrast, another widely used protocol is the double electrical stimulation of the primary afferent fibers of human (**Fig 1.11)** [71]. A pair of electrical stimulation, i.e. average intensity conditioning stimulus followed by a maximum intensity test stimulation, is applied through the same cathode and the reduction in the H-reflex amplitude is investigated by surface electromyography (SEMG) (**Fig 1.11A and Fig 1.11B**). Using this technique, extensive connections of recurrent inhibition (**Fig 1.9**) in humans have been revealed both in the aspect of inputs on (**Fig 1.9A**) and outputs from RCs (**Fig 1.9B**). Although this method was used commonly to investigate the recurrent inhibition in different conditions [61, 72],

several variables, including P-AD [16], limit the reliability of this technique for investigating the RCs in humans.

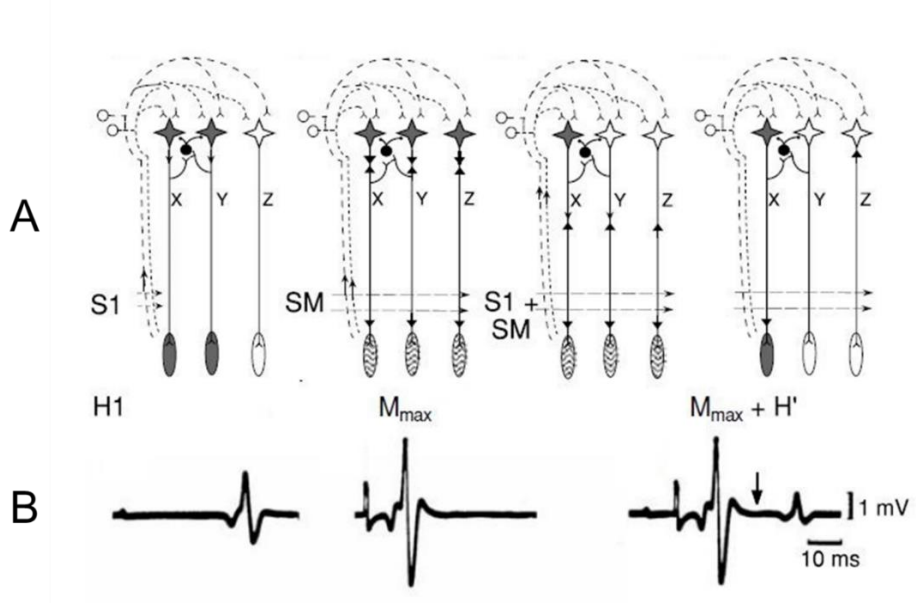

Fig 1.11 *Paired H-reflex technique to study recurrent inhibition. The figure was modified from Pierrot-Deseilligny [73].*

1.2.2.4 Renshaw cells and diseases

The significance of RCs has been studied widely in either developmental or acquired diseases in humans and animal studies. Recent evidence proposed that thoracic spinal cord damage at postnatal day 5 resulted in excitatory deficit and caused an increased number of primary afferent connections on RCs, which normally should decrease after postnatal day 15. This increased primary afferent activation on RCs may result in spasticity [74] and this new synaptic formation propose an important point about compensatory mechanisms to activate recurrent inhibition [75]. Further, patients with a severe MN disease, such as amyotrophic lateral sclerosis (ALS), showed reduced recurrent inhibition [76]. The collateral

connection loss at the early stage of ALS results in impairment of recurrent inhibition due to reduced MN collateral inputs on RCs [77] (*see the Chapter 5 for more details*). Not only MN degeneration leads to damage in recurrent inhibition but also intrinsic changes in RCs might also cause this impairment. A rare but inhibitory interneuron affecting stiff-man syndrome resulted in atrophy in RCs due to antibodies against glutamic acid decarboxylase enzyme, leading to reduced recurrent inhibition [78].

1.3 Aims of the thesis

Given that recurrent inhibition is possibly involved in many neurological disorders, it is a need of a reliable method to investigate the physiology of RCs. Although most of the knowledge comes from animal experiments, we need to increase our understanding of the normal functions of the human nervous system. Since the previously developed methods to study recurrent inhibition in humans rely on many assumptions that could affect the estimation and reproducibility, there is a need of a reliable and precise tool to investigate RCs in health and disease.

Therefore, the aims of this thesis are:

1. To propose optimized stimulation and recording parameters for recurrent inhibition in healthy humans (Chapter 3).
2. To investigate the temporal properties of the recurrent inhibition on firing MNs (Chapter 4).
3. To characterize the recurrent inhibition in various background discharge rates of the SMUs (Chapter 4).
4. To understand the synaptic connectivity of RCs and various sized motor units in soleus muscle (Chapter 4).

5. To investigate alterations in the recurrent inhibition duration in patients with ALS (Chapter 5).
6. To compare the RC function of healthy individuals and ALS patients (Chapter 5).

To understand the functioning of the RC circuit we used a technique that involves electrically stimulating the tibial nerve to activate the largest motor axons and to simultaneously record from one of the smallest motor axons innervate the soleus muscle. This approach showed the existence of the recurrent inhibitory pathway between MNs in human subjects previously but not reproducibly [67]. For more reproducible and precise outcome, while we record SMUs of the soleus muscles using intramuscular EMG electrodes, we optimized thicker motor axon stimulation of human subjects together with analysis of the individual SMU firings using probability as well as frequency-based methods [79, 80].

Chapter 2

MATERIALS AND METHODS

The Human Ethics Committee of Koç University approved the procedure in accordance with the Declaration of Helsinki (2017.124.IRB2.038). All the human experiments were performed in the neurophysiology laboratory of Koç University. Before the experiments, all the subjects filled the informed consent form (please see the **Appendix**). Experiments were performed on the right leg of the healthy subjects and on both legs of ALS patients. Inclusion criteria for experiments performed on healthy people (protocol 1 and 2): no known neuromuscular disease, brain injury or nervous system related disorder, normal body habitus and not regularly using/recently taken (in the last 2 days) medication/drug.

2.1 Equipment

Software Spike2 7.20 was used to perform data acquisition and offline analyses. CED 1902 Quad System MKIII amplifier and CED 3601 Power 1401 MKII DAC were used for recording. Isometric force during dorsiflexion of the right foot was measured using a linear strain gauge. Tip-active Teflon-insulated silver fine-wire electrodes were used as intramuscular EMG electrodes. Details of the equipment used is shown in **Table 2.1**.

Table 2.1 *List of equipment used in this thesis.*

Equipment	Purpose	Specs	Model	Company	Country
Software	Data acquisition	Software	Spike2 – 7.20	CED	UK
Amplifier	Data amplification	Quad system	1902	CED	UK
Converter	Data analog to digital converter	8-channel	Power 1401 MkII	CED	UK
Stimulator	Electrical nerve stimulation	Constant current	DS7A	Digitimer	UK
Transducer	Force recording	Linear to 196 N	LC1205-K020	A&D Co. Ltd.	Japan
Electrode	SEMG recording	Ag/AgCl	50F	Redline	Turkey
Electrode	SMU recording	75 µm core diameter	Medwire Teflon insulated silver fine-wire – 3T	Leico	USA

2.2 General preparation

The subject rested prone on a bed-like platform and was asked to relax his/her leg muscles throughout the experiment, which lasted for about two hours. To record the activity of the soleus muscle, SEMG and intramuscular EMG were used. After the foot was

restrained to a plate that was positioned parallel to the base of the foot, two of the standard SEMG electrodes were placed on lateral part of the soleus muscle after rubbing with sandpaper, cleaning with alcohol and applying electrode gel to reduce the impedance. The electrodes were placed 4 cm apart in proximal-distal plane for optimal signal recognition [81].

Autoclave-sterilized bipolar wire electrodes were placed in the muscle via 25 G surgical needles in between two SEMG electrodes. After insertion, the needle was removed immediately by leaving a pair of fish-hooked fine wires inside the soleus. Both electrodes were stabilized with surgical tape to minimize the movement artifact. Sterile lip clip was used as a ground electrode in all trials for both SEMG and intramuscular EMG [82]. Detailed localization of the recording electrodes was drawn in **Fig 2.1**.

After placement of the recording electrodes, stimulating anode and cathode were placed and fixed with a knee pad. An anode (10 ×12 cm) was placed immediately proximal to the patella and a cathode (3×3 mm) was placed on specified points at the popliteal fossa to stimulate the tibial nerve which innervates the soleus muscle using a constant current stimulator. Stimuli of single square pulses of 1 ms width were delivered in varying interstimulus intervals (see the protocols 1 to 3).

Both SEMG and intramuscular EMG recorded at a sampling frequency of 20,000 Hz. The recordings were filtered with a frequency of 20-10,000 Hz for SEMG and 200-10,000 Hz for intramuscular EMG. Signals were amplified 100 times. The foot was fixed to a force transducer (slight changes in its length is converted to force signals) with the active recording area was contacting the sole of the foot. Force signals were amplified 1,000 times, filtered with DC-100 Hz, and sampled at 2,000 Hz using the same data acquisition system. Before each of the experimental protocol starts, we determined the maximum voluntary contraction (MVC) for soleus and tibialis anterior muscle, maximum M-response and maximum H-

reflex of the subjects. For MVCs, subjects were asked to perform maximal plantar (soleus) or dorsiflexion (tibialis anterior) against the stabilized force transducer with verbal encouragement from one of the researchers. On the other hand, maximum responses was found by increasing the stimulus intensity until no further increment in M-response and until reduction in the amplitude of H-reflex, separately.

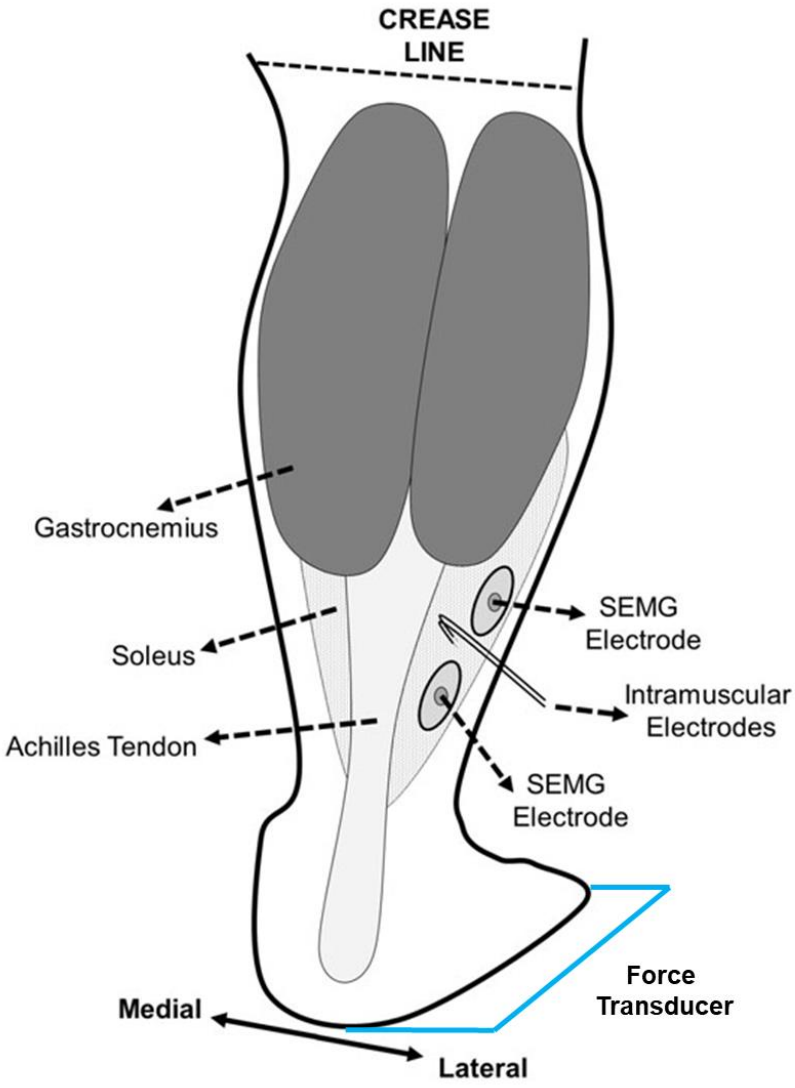

Fig. 2.1 *Recording electrode localization that was used in all the experimental protocols, modified from Özyurt [83]. EMG recordings were performed on the lateral part of the soleus muscle.*

2.3 Experimental protocol 1: Optimization of the stimulus location

We used the following procedure in each of the subject in order to optimize the location of the cathode. Ten participants (8 men and 2 women) participated in this part of the study. After application of the general procedure, two lines were drawn on the popliteal fossa. The lower line (crease line/bending line) was at the bending line of the knee. The upper line (proximal line) was drawn 3 cm proximal the first line, estimated to be close to the branching of the sciatic nerve. The middle of both crease and proximal lines were determined as points zero and were on the hypothetical vertical line originating from the Achilles' tendon (**Fig 2.2A**).

We have then marked four points on both sides of the zero point (midpoint), which were equally separated. The points that were lateral to the midpoint were tagged as positive (+1, +2, +3, +4) and the points that were medial to the midpoint were tagged as negative (-1, -2, -3, -4). In total, 18 points were marked (9 at the upper line and 9 at the lower line). The minimum stimulus current that induced a clear H-reflex on the intramuscular EMG at the midpoint of the crease line was classified as the threshold stimulus intensity. For experimental protocol, we used 20% (1.2xT) above the threshold level to allow both increase and decrease. The order of the points to be stimulated were determined randomly.

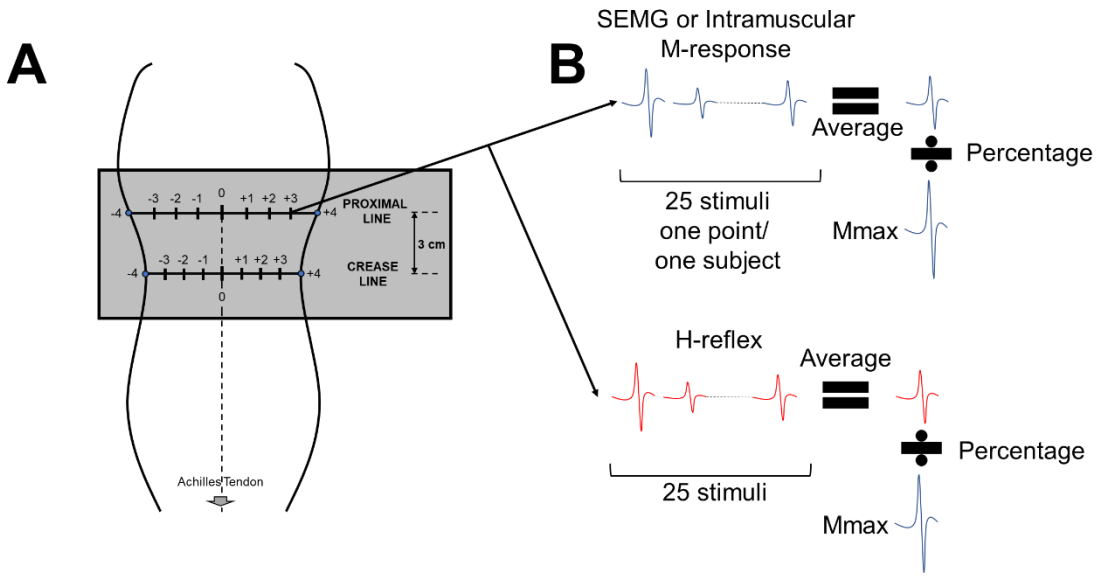

Fig. 2.2 *The location of the defined points drawn on the popliteal fossa and analysis of the responses. A) The crease line and the proximal line that were separated by 3 cm. Along each line there are 9 points. The midpoint of each line (point 0) was determined with reference to the Achilles' tendon. Points that are lateral with respect to the midpoint are specified with a plus sign (+), whereas points that are medial with respect to the midpoint are specified with a negative sign (−). B) Analysis of the responses, separately for M-response and H-reflex The figure was modified from Özyurt [83].*

Twenty-five stimuli (1 ms pulse width), were applied to each of the marked points on both lines separately. This was repeated for all subjects using inter-stimulus interval between 0.8 and 1.2 seconds. The size of the H-reflex and the M-response were determined as the peak-to-peak amplitude of the unrectified EMG records. Since the stimulation activates a fixed number of motor axons in each trial, the resultant M-response can be used as a reliable tool for standardizing these responses. Hence, each recording was standardized according to the maximal M-response for that trial.

We, then, averaged responses of 25 stimuli for each point for both M-response and H-reflex separately for SEMG and intramuscular EMG. Finally, each normalized coordinate of 10 subjects was pooled and presented as a percentage (**Fig 2.2B**). Among those averaged values, A two-way ANOVA test was used to determine the significance of individual points in each line and their interactions. Multiple comparisons were corrected using Tukey test. The level of significance was selected as $p < 0.05$.

2.4 Experimental protocol 2: Recurrent inhibition in healthy people

After determination of the key point to evoke M-only response without any H-reflex (shown in Chapter 3), stimuli with a 1 ms of pulse width and 1-2 seconds of interstimulus interval were delivered at the optimum location in all 12 subjects (7 males and 5 females; age 18-35 years). Simultaneously, tibialis anterior SEMG was also recorded to see its activity and rearrange the stimulation location/intensity to avoid any cross-talk during the tibial nerve stimulation. After that, the maximum M-response (Mmax) was found by increasing the stimulus intensity above supramaximal level until no increment in M-response was observed. Then, subjects were asked to perform slight plantar flexion to produce steadily firing single motor units. Also, visual feedback was provided to subjects. While single motor units were activated, M-only stimulation was delivered to the tibial nerve. At least two different single motor units in size and shape or firing rate were obtained from each subject. The stimulus intensity determined as a percentage of the averaged M-response in SEMG compared to the Mmax. The experiments were designed to use the stimulus intensity between 0% Mmax (just below the motor threshold) to 20% Mmax. The defined stimulus intensities were randomly applied, and the number of M-only stimuli delivered was ranging between 250 and 1,000 (mean: 454 ± 144.6 (standard deviation: SD)) during periods when participants were continuously recruiting SMUs.

2.5 Experimental protocol 3: Recurrent inhibition in ALS patients

Completely similar experimental protocol as in healthy people was followed. Instead of using stimulus intensity up to 20% of Mmax, we selected M-only stimulus intensity between 0% and 5% of Mmax, as patients had loss of larger MN and higher intensity stimuli found to evoke H-reflex in our pilot studies. There were 2 groups of ALS patients; i) lumbar onset/affected and ii) other part of the body onset/no sign of motor loss in leg (nonlumbar affected). For each of the patients, we repeated the experimental protocol for both legs of the patients from who we recorded 10 SMUs and 12 SMUs from lumbar-affected (6 male and 1 female) and nonlumbar affected (8 male) patients, respectively. After determination of the key point to stimulate the tibial nerve and placement of recording electrodes to soleus muscle, we delivered around 205 ± 170 stimuli to the tibial nerve.

In order to determine the severity of MN loss in the soleus muscle, we calculated motor unit number index (MUNIX) and motor unit size index (MUSIX) in ALS patients and some of the healthy people [84, 85]. MUNIX was calculated using surface interference pattern (SIP) of the SEMG and Mmax (**Fig 2.3**). After determination of the Mmax and MVC, we asked subjects to contract their soleus muscle in incremental/decremental order at 8 equal levels; between 100% MVC and 12.5% MVC. Then, negative peaks of the SEMG signals were taken (half-wave rectified) and its area (S_a and M_a) and power as area square (S_p and M_p) was calculated for 300 ms epoch from voluntary contraction and Mmax (normalized to 300 ms). Then, ideal case motor unit count (ICMUC) was calculated as shown in **Fig 2.3** (part 2). ICMUC is the motor unit count in an ideal case in which the individual motor unit potentials are identical for all of the motor units without superimposition. After that, a nonlinear regression was applied between ICMUC and SIP as shown in **Fig 2.3** (part 3). From the regression equation, SIP of 20 mV.ms was used to find MUNIX. This SIP value shows a low contraction level which evokes motor units with small amplitude and low

background firing rates which prevents most of the superimposition of the individual motor unit potentials, as described in the ICMUC (as ideal case). MUSIX, on the other hand, was simply calculated as dividing the negative peak-to-peak value of Mmax (absolute value) by MUNIX [84, 85].

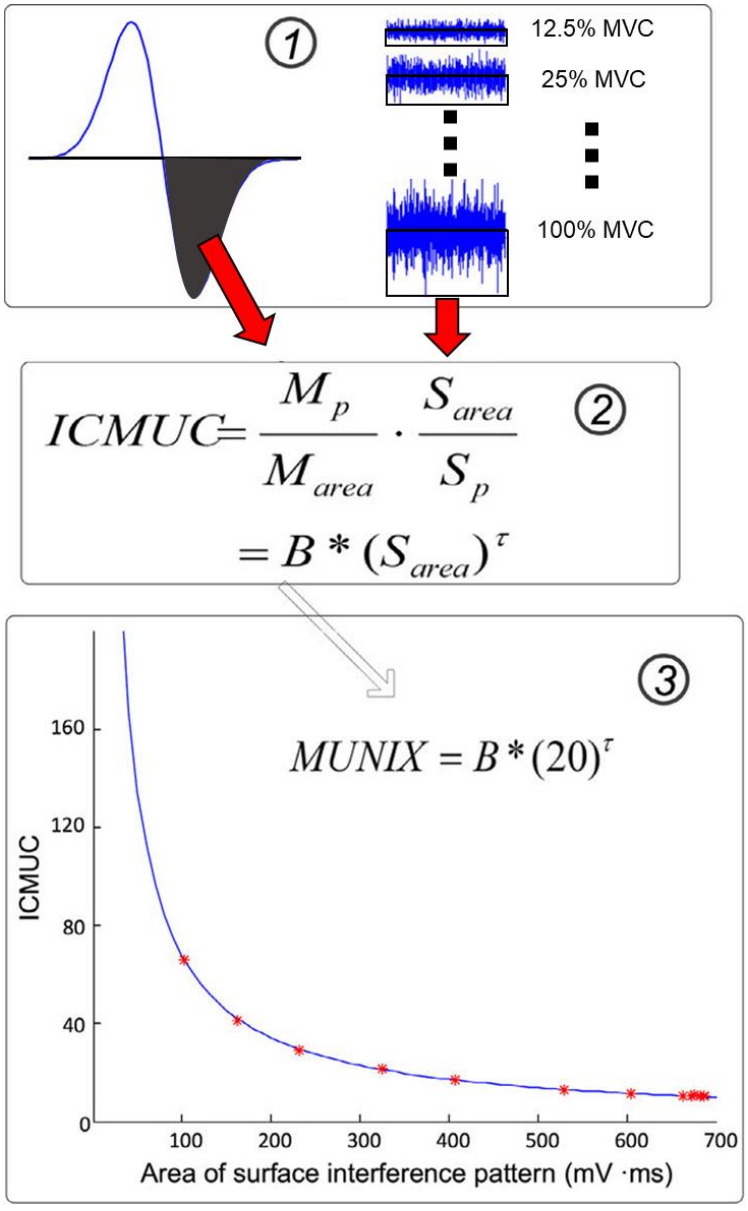

Fig. 2.3 *Steps of calculation of the MUNIX. (1) Recording of the Mmax and various levels of voluntary contraction. (2) Calculation of the ICMUC. (3) Calculation of the MUNIX from the graph of ICMUC vs SIP. Adapted from Li [86].*

2.6 Analysis

While peristimulus time histogram (PSTH) is a histogram indicating the timing of spikes against the stimulus, peristimulus frequencygram (PSF) is made up of superimposition of the instantaneous discharge rates of a selected unit around the time of the stimulus and indicates profile of synaptic potential [87]. To build PSF and PSTH, electrical activity from the intramuscular fine wire electrodes was displayed and a shape of an individual motor unit action potential defined as a template in the Spike2 program. During the experiment and during off-line analysis, any spike whose shape matched this pre-established template, generated acceptance pulses in the program. The acceptance pulses from the discriminated units were used to construct PSTHs and PSFs around the time of stimulation. PSTH and PSF cumulative sums (CUSUMs) [88] were then constructed from data. SMU firing probability (PSTH) and SMU discharge rate (PSF) responses were compared with SEMG responses and rectified-average intramuscular EMG responses, referred as multi motor unit (MMU).

For sustained contraction, intramuscular EMG analysis involved identification of SMU potentials using Spike2 algorithms. Analysis of each SMU involved extraction of a defined time period from around each stimulus (-250 ms to +250 ms) followed by construction of a PSTH and PSF. Both PSF and PSTH were built with 0.1 ms binwidths.

CUSUMs for PSTH graphs were obtained to make any subtle but significant changes in bin counts detectable. Similarly, CUSUMs for PSF were calculated to pinpoint the subtle changes in discharge rate that were not immediately visible. Significant event in the CUSUM was categorized if the poststimulus deflection was bigger than the prestimulus error box [89,

90]. The latency of the event in the CUSUM was defined as the time of the turning point that initiated the significant event. The duration of the significant event was the horizontal distance between the latency and the next turning point (**Fig 2.4**).

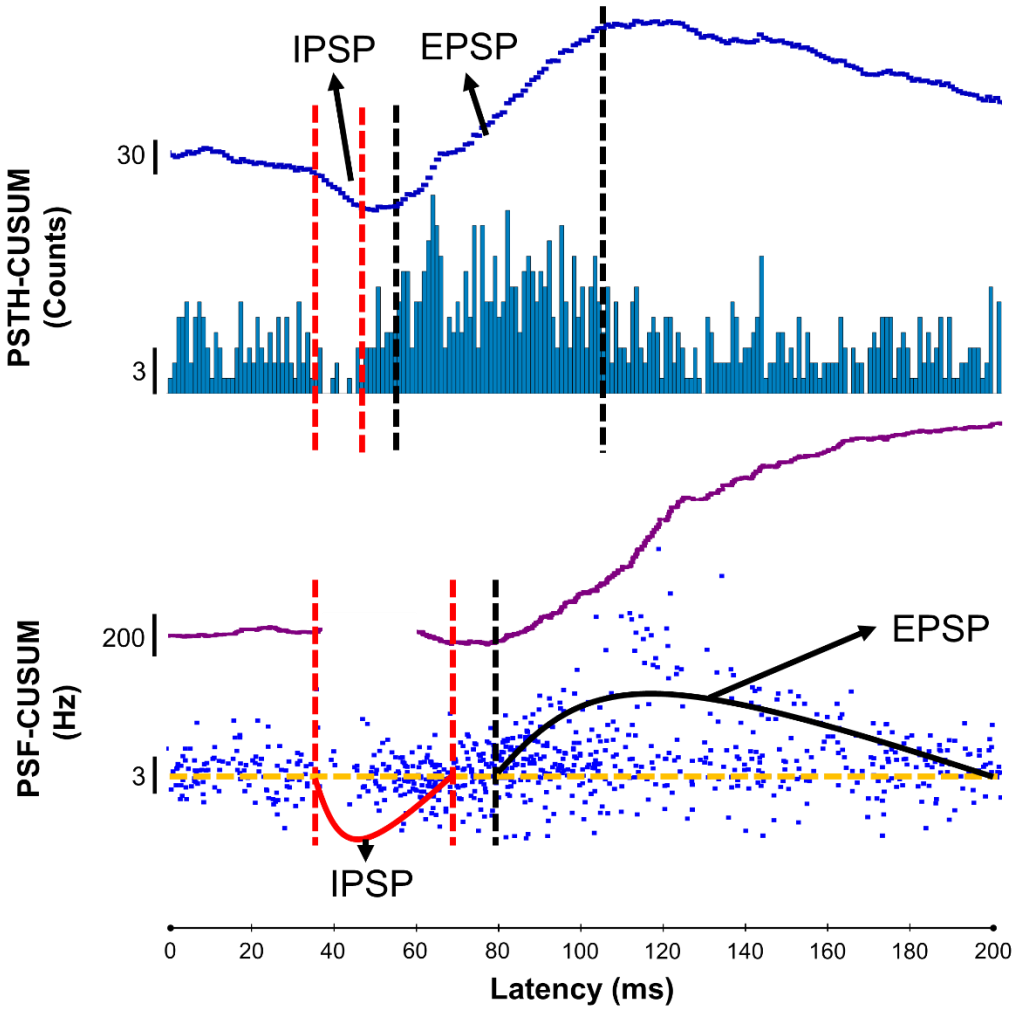

Fig. 2.4 *A sample analysis to pinpoint onset and end of the IPSP and EPSP. Figure on the top is PSTH and graph below is PSF. Lines just above the both graphs are CUSUM. The figure is modified from Uysal [91].*

Basically, CUSUM and CUSUM related analyses were performed in the following steps. First, the prestimulus average bin value in microvolts (for SEMG), in number of events (for PSTH), and in discharge rates (for PSF) was determined (**Fig 2.5; yellow dashed line in PSF-prestimulus is not shown**). Then the prestimulus average bin value was subtracted from each of the bin values in the entire analysis period (usually -250 to +250 ms period). Then the residual values left in each bin were integrated to obtain CUSUM. If this residual value is more than the average, then the CUSUM increases while it reduces if the residual value (negative value) is less than the average. Maximum deflection in the prestimulus CUSUM was then used to build an error box which was then used to determine the significant poststimulus deflections [89, 90]. Any post stimulus deflection that was larger than the largest prestimulus deflection and appears before the reaction time to this stimulus is considered as a genuine/significant response to stimulus [90]. If such significant deflections are up-going they are classified as 'excitation' (**Fig 2.5; EPSP between black vertical dashed lines**) and if they are down-going as 'inhibition' (**Fig 2.5; IPSP between red dashed lines**). This is a conservative but accurate method for pinpointing genuine poststimulus deflections that underlie stimulus-induced changes in the MN activity [92].

Also, percent coefficient of variation (%CV) was calculated as 100*(SD/MEAN) of the SMU firings obtained in the 250 ms prestimulus region of the PSF, for each of the units in order to determine the variation of the SMU firings.

Latency difference between recurrent inhibition and H-reflex was investigated using t-test. For determination of the significance of recurrent inhibition in latency and duration with changing stimulus intensity and background frequency individually, we used ordinary one-way ANOVA using Tukey correction for multiple comparisons. The correlation between variables was tested using linear regression. The level of significance was selected as $p < 0.05$.

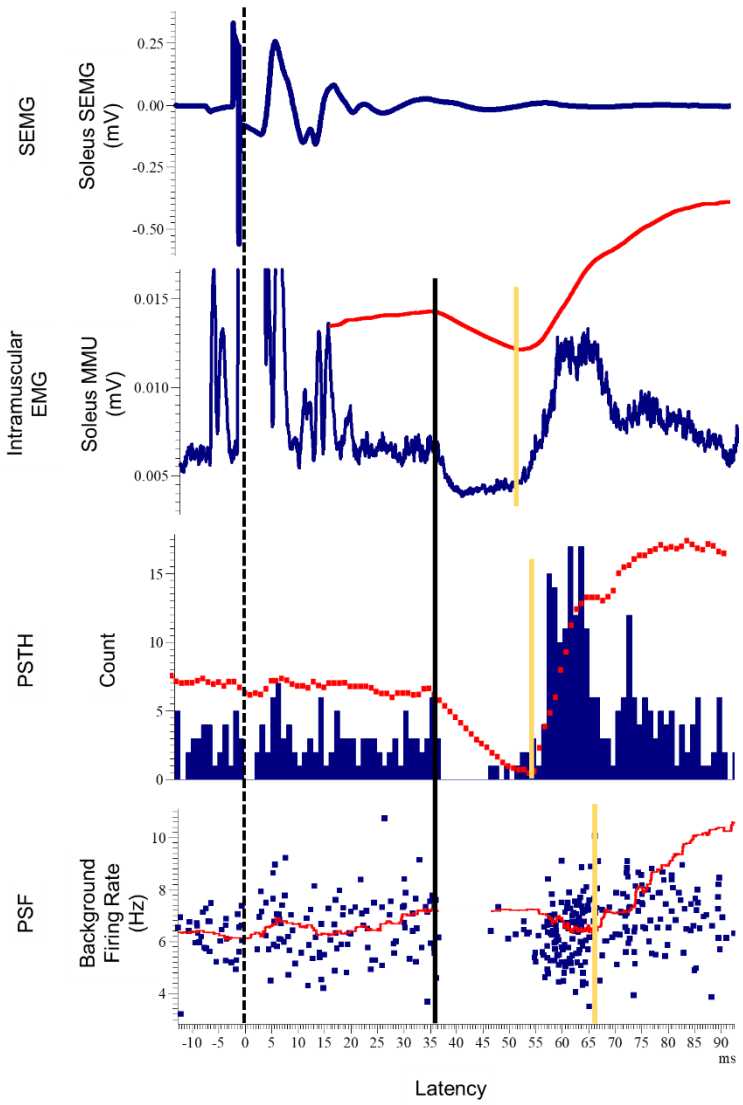

Fig. 2.5 *A sample analysis used in this thesis. Blue graphs are the data produced from the raw data. Red lines are CUSUMs of the analysis tools. Black vertical dashed line is the stimulation time point. Black vertical line is the onset of the inhibition and golden lines are the points where the IPSP ends. For this sample, the number of stimuli delivered was 494 and background firing rate was 6.25 ± 1.33 Hz.*

Chapter 3

OPTIMIZATION OF THE MOTOR AXON STIMULATION

3.1 Introduction

Using electrophysiological tools, sensory and MN circuitries as well as their connections with interneurons can be extracted from the CNS. The electrical equivalent of the monosynaptic spindle reflex, the H-reflex, is one of the most convenient tools to extract information about those circuitries [93]. However, obtaining a pure M-response without the H-reflex, is tricky since Ia fibers are thicker than motor axons, therefore, easier to be stimulated with electricity [69]. The electrical stimulation technique involves delivering an electrical pulse to a mixed nerve at a specific location using a specific stimulus intensity. Since more than one nerve can be activated through the popliteal fossa (common peroneal and tibial nerves: [94, 95]) exact position of the stimulation electrodes needs to be standardized. Using SEMG of the muscle of interest can be contaminated by cross-talk from another active muscle/s. We aimed to optimize stimulation location at the popliteal fossa in order to selectively stimulate the motor axons only for studying the recurrent inhibition from soleus muscle, using both SEMG and intramuscular EMG to reduce the cross-talk.

Therefore, specific aims of this experimental protocol are;

1. To identify various electrophysiological recording site on the popliteal fossa.
2. Determination of the H-reflex and M-response sizes at each stimulation point.
3. Locating the optimal position to evoke M-response without H-reflex contamination to study recurrent inhibition.

3.2 Results

3.2.1 H-reflex

Intramuscular EMG: The highest amplitude recorded was 73% of the Mmax at the midpoint of the bending line which showed significantly larger amplitude compared to the midpoint of the proximal line ($p<0.0001$). The reflex amplitude at the midpoint in the bending line was twice as that of point +1 ($p=0.0365$). Nonetheless, these points, 0 and +1 at the bending line, was larger than the other values recorded (**Fig 3.1, figures on the middle left**). On the other hand, in the proximal line, 0, +1, and +2 were also reliable points which evoked H-reflex, yet, none of them was significantly different from the others. The comparison of the largest values in proximal and bending lines, the midpoint, showed a significantly larger H-reflex at the bending line ($p<0.0001$) (**Fig 3.1, figures on the middle left**).

SEMG: The largest amplitude was the 40% of the Mmax value which was obtained from the midpoint of the bending line (**Fig 3.1, figures on the right**). The midpoint and +1 induced greater response than the other points at the bending line just as in the intramuscular EMG. On the other hand, points 0, +1, +2 and +3 on the proximal line were similar as none of them is significantly different than each other (**Fig 3.1, figures on the right**).

3.2.2 M-response

Intramuscular and surface recorded M-response sizes were well correlated. Points +2 and +3 showed greater amplitudes compared with the other points in both lines. In addition, the highest observable M-response were seen in point +2 at the bending line. Furthermore, occasional signals were detected at the -1 and +4 coordinates at the bending line. In the proximal line, due to high variability in the proximal line, no significant difference was

observed (**Fig 3.1**) (for MMU; +2 vs +3: p=0.3783, +2 vs +4: p=0.2517, for SEMG;-1 vs +2: p=0.2071, +2 vs +4: p=0.4763, all at points of the proximal line).

3.2.3 Ideal positions to evoke pure M-response and H-reflex

In the bending line, it was found that the H-reflex was significantly greater at the midpoint when compared with the M-response at that point (p=0.001). In contrast, there was no such exact position for eliciting the H-reflex in the proximal line. Therefore, ideal location to evoke pure H-reflex was found to be the point 0 of the bending line.

The best locations to evoke M-response with minimum H-reflex contamination were +2 (For MMU; p=0.0028, SEMG; p<0.0001) and +3 in the bending line (For MMU; p=0.0017, SEMG; p<0.0001). Also, points +2 and +3 are almost identical in terms of the amplitude of the M-response (p>0.9999 for both recordings). However, no such convenient position was detected for the proximal line. Therefore, ideal location to evoke a M-only response is either +2 or +3 of the bending line.

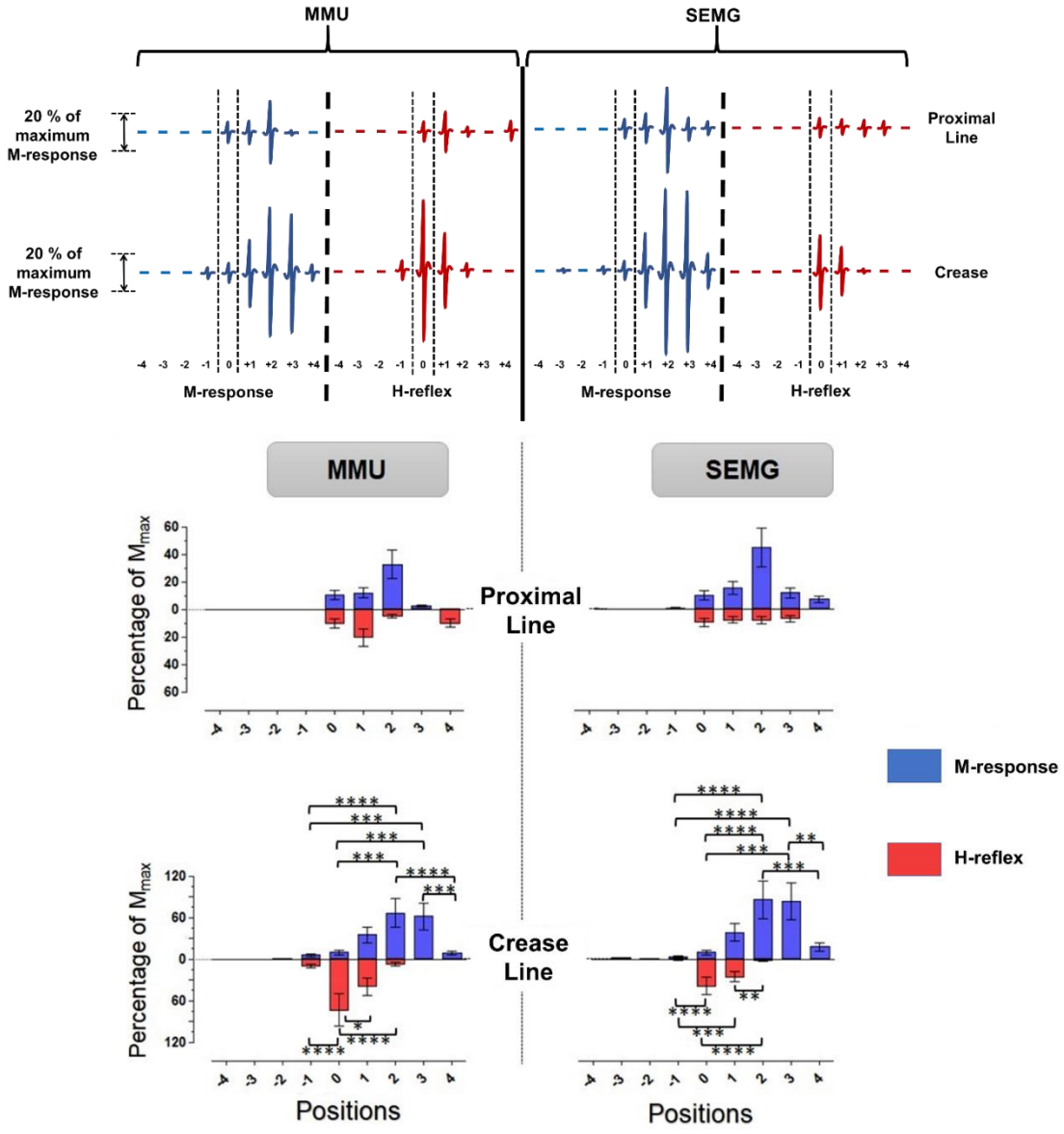

Fig 3.1 *M-response and H-reflex amplitudes of soleus muscle MMU and SEMG recordings for each of the 18 points have been shown as columns and as waveforms. The midpoints and +1 points for each line were the main points for obtaining H-reflex. Also, lateral points evoked significantly larger M-responses but not at point +4. Proximal line, on*

*the other hand, showed no significant advantage to be used. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars are standard errors of the means (for better representation).*

3.3 Discussion

Some difficulties may arise when attempting to evoke pure H-reflex or M-responses in the triceps surae muscles via electrically stimulating the sciatic nerve branches. In this experiment, we attempted to localize the optimal positions for stimulating the tibial nerve in the popliteal fossa while using the bending line, proximal line, and their midpoints as frames of reference. Although the pathways of the nerves are expected to slightly vary between individuals, the results turned out to be strikingly similar.

Majority of the protocols in the literature are based on specific techniques to decide the optimal location for electrical nerve stimulation using physiological tools. Although almost all protocols diverge from the similar EMG assisted techniques, none of those has put forward the details of the cathode positioning as we proposed. The widely used ones involve the EMG recording of the responses [96-99] or twitch recording [100, 101]. These protocols involve the placement of the recording electrodes on the surface of a muscle and/or force transducer to the innervated limb for twitch observation. Twitch protocol involves the force transducer recording and the observation of the ankle positions to decide the best stimulation point. The best position for EMG assisted protocol, on the other hand, is decided depending on the purpose of the study. The various parts of the popliteal fossa region are stimulated with a feedback from the EMG recording (either as H-reflex or M-response). The location is decided as the point where the minimal intensity evokes an observable response or as the position where changing the angle of the knee does not alter the recruitment curve for M-response [102]. Using both protocols in parallel is advantageous as it purely shows M-response and H-reflex with the twitch of the foot and relatively easy to be applied.

However, the biggest problem in both protocols is the cross-talk issue. When a region is stimulated, the other nerves around which may include the antagonist muscles' nerve can also be stimulated and may result in nonspecific twitch and H-reflexes or M-responses, especially at higher stimulus intensities [103]. Therefore, using intramuscular EMG, multi-differential SEMG electrodes [104] and/or specific positioning of the cathode such as in our study might minimize the cross-talk as well as assistance to obtain the desired means of response. Hence, we aimed to suggest a protocol for optimal localization of the cathode to provide goal dependent electrical nerve stimulation.

We put forward two major findings in this study: Firstly, we found the ideal electrode position for obtaining sole H-reflex with minimum M-response contamination. Secondly, we found the ideal electrode position for eliciting M-response without much contamination by the H-reflex.

Ideal position for the sole H-reflex response: This was found to be the midpoint of the bending line. In the literature, to induce the H-reflex either bipolar stimulation where both anode and cathode placed on the popliteal fossa [105] or monopolar stimulation where anode fixed just above patella and cathode placed on the nerve in popliteal fossa [106] were used. However, the exact positioning of the cathode has not been introduced in either case.

Ideal position for the sole M-response: Selective stimulation of the motor fibers was the second major finding in this study. The more lateral points of the bending line, especially the +2 and +3 coordinates, displayed larger M-response with minimal H-reflex contamination. However, since the marked points at lateral are close to the common peroneal nerve, using relatively medial point such as +2 instead of +3, can be desirable to limit the possibility of common peroneal nerve stimulation. ***As both points evoked almost the same responses, +2 can be defined as the best location for sole M-response to be used in recurrent inhibition studies***.

Most studies briefly described the positioning of the cathode on the popliteal fossa [20] while others give even less information [21, 107]. Generally, the stimulation points to selectively excite the motor or Ia axons are indicated as the point which evokes an observable muscle response with the minimum current intensity [96, 102]. Although several protocols have been put forward, none of those discuss the exact positioning or standardization as we propose in this study. Therefore, our detailed description of the cathode localization on the popliteal fossa could be useful for stimulating the motor or Ia sensory fibers with minimum cross-talk possibility.

Chapter 4

RECURRENT INHIBITION OF FIRING MOTONEURONS

4.1 Introduction

One of the first identified neuronal circuits with feedback was recurrent inhibition, mediated by a special type of inhibitory interneurons; RCs [26, 27]. Efforts for almost 70 years provided information about RC location at the spinal cord, some molecular properties, neurotransmitters and firing properties, mostly in animal studies. Although some techniques have been developed to study recurrent inhibition in human which mostly provided information about connectome of the RCs within CNS, the most used paired H-reflex technique is based on several assumptions. A useful but not practical technique of stimulating F-type units to activate RCs antidromically lacks reproducibility (M-only technique). To understand the functioning of the RCs in human, we proposed to optimize and improve the precision of the M-only technique in tibial nerve innervated soleus muscle [67].

In this chapter, we aimed:

1. To investigate the outcome of the largest motor axon stimulation on voluntarily recruited smallest SMUs.
2. To determine the latency and duration characteristics of recurrent inhibition.
3. To compare the recurrent inhibition characterized using this optimized technique with previously used protocol.
4. Determining the variability of the recurrent inhibition with several parameters and analysis methods.
5. Finding out the distribution of the recurrent inhibition on different sized MNs.
6. To investigate the reliability of the optimized protocol.

4.2 Results

We recorded from soleus using both SEMG and intramuscular EMG (a total of 54 SMUs) from 12 healthy subjects and stimulated the tibial nerve using +2 location proposed in Chapter 4.

4.2.1 Characteristics of recurrent inhibition

To estimate the duration and latency of recurrent inhibition, we used 3 analysis methods (as described in the Chapter 2): PSF, PSTH and MMU. For all 54 units, the duration and the latency of the inhibition showed differences in different the analysis methods. The mean duration of the recurrent inhibition was found as 30.8 ± 7.2 ms, 16.1 ± 5.8 ms, and 14.4 ± 3.5 ms in PSF, PSTH, and MMU, respectively (**Fig 4.1A**). Although the duration difference between PSTH and MMU was found similar ($p=0.2400$), the PSF showed significantly longer duration than both other methods ($p<0.0001$).

It was recorded that the latency of recurrent inhibition was 37.7 ± 2.3 ms, 37.7 ± 2.4 ms, and 39.9 ± 2.3 ms in PSF, PSTH, and MMU, respectively. The inhibition latency recorded using averaged MMU was found significantly later than both other methods ($p<0.0001$), while there was no significant difference in latency recorded using PSF and PSTH ($p=0.9683$) (**Fig 4.1B**).

Although slight variations in duration among the units have been found (**Fig 4.1C**), linear regression showed that there is no correlation between duration and Mmax, an indicator of the number of motor axons stimulated ($p=0.3195$).

The paired H-reflex technique, comprising the most frequently used protocol for investigating recurrent inhibition, was also employed to evaluate recurrent inhibition in two additional volunteer subjects (**Fig 4.1D**). When the conditioning (H1)-test (H') reflex interval was increased, we detected an increment in H' amplitude ($p=0.0253$). The amplitude

of H' was 13.2 ± 2.57% of H1 for 5 ms of interval. The H' increased in amplitude to 29.4 ± 5.48% and to 24.9 ± 5.70% of H1 as the conditioning test interval was 25 and 30 ms, respectively.

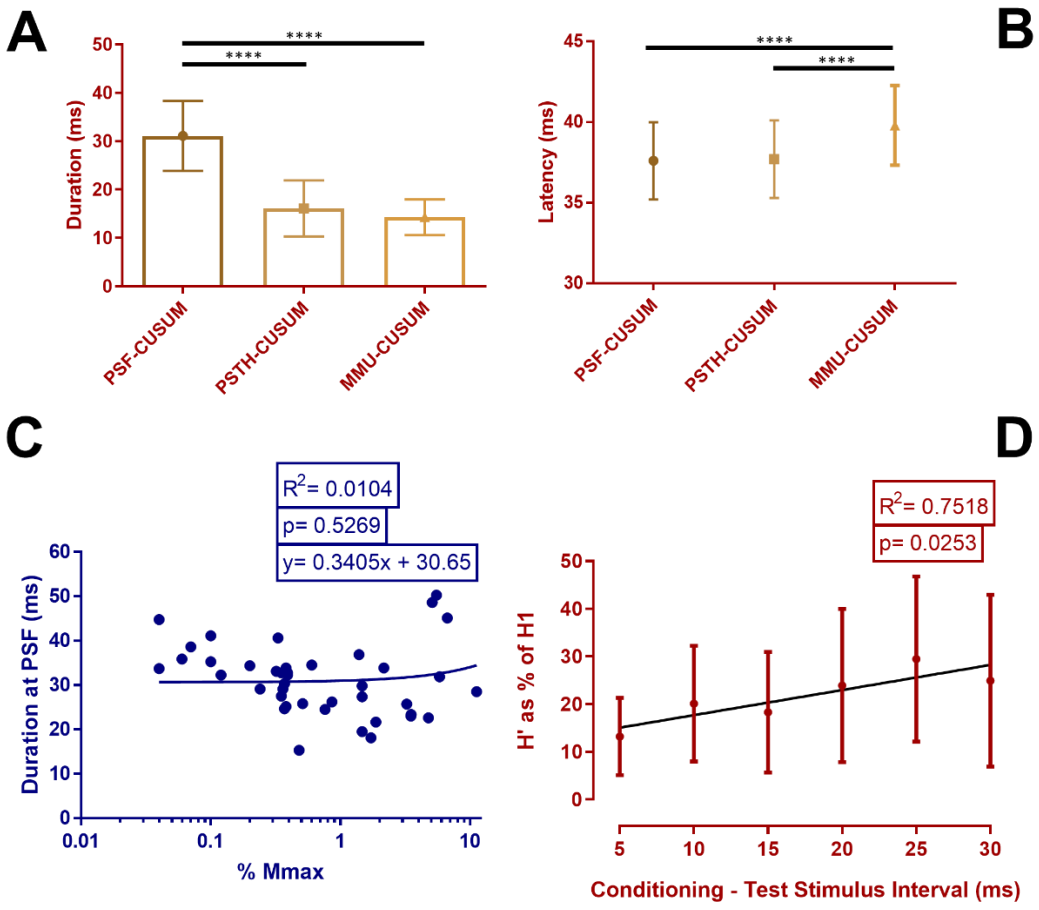

Fig 4.1 *Characteristics of the recurrent inhibition. A) Duration and B) the latency of the inhibition calculated using various analysis tools. C) The relationship between duration and the stimulus intensity. D) Recurrent inhibition calculated by paired H-reflex technique. Error bars are SD. ****p<0.0001.*

4.2.2 Latency of recurrent inhibition versus H-reflex

Using PSTH-CUSUM, we also investigated the central delay of recurrent inhibition due to an additional neuron - RCs - and its synapse, compared to H-reflex (**Fig 4.2**). The average recurrent inhibition latency was found to be 37.4 ± 1.8 ms which was significantly longer than the H-reflex latency (35.7 ± 1.2 ms) of the same subjects (p=0.0074).

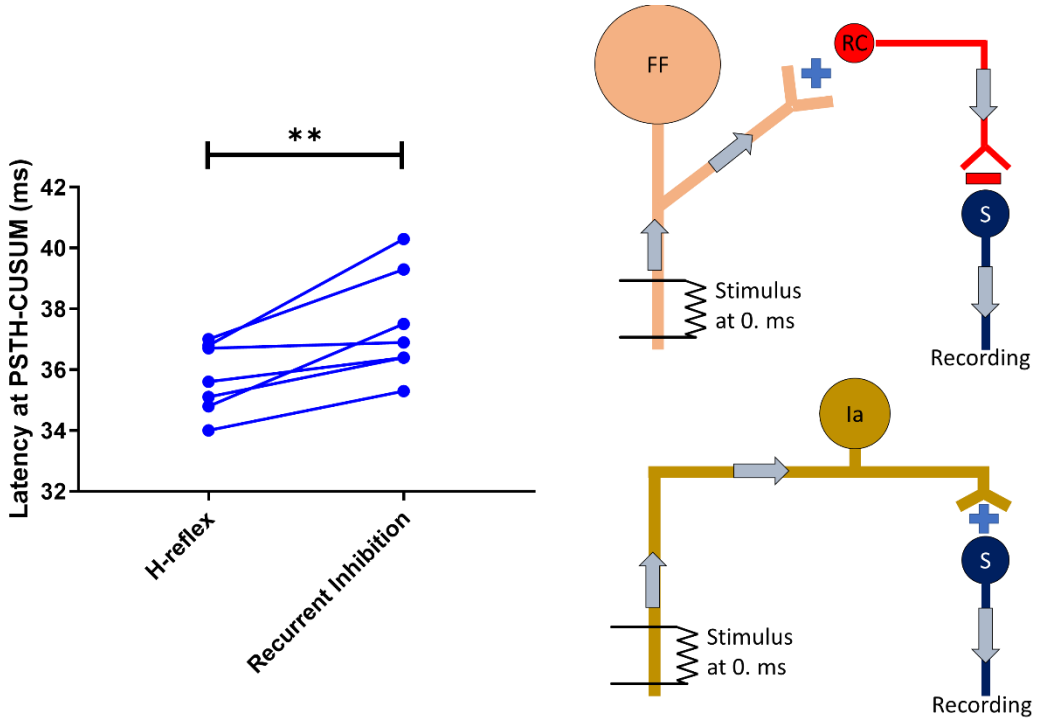

Fig 4.2 *The latency comparison of H-reflex and recurrent inhibition. Figure on the left is the H-reflex and recurrent inhibition latency comparison. The figures on the right are the wiring diagram of the recurrent inhibition (top) and H-reflex (bottom). Error bars are SD. **p<0.01.*

4.2.3 Duration of recurrent inhibition and background firing rate of SMUs

The background firing rate of the motor units has been observed to affect the duration of the inhibition. Since produced twitch might induce later Golgi tendon activation which may alter the recurrent inhibition duration, we investigated the stimulation which did not evoke detectable twitch in the force transducer. Among 54 units recorded, stimulation of 29 units did not produce detectable twitch. Here, linear regression of the 29 units' duration provided information about the time course of the inhibition (**Fig 4.3, linear regression at the figure on the bottom**). The units which have lower background firing rate were found to be inhibited significantly more than those firing faster in rate ($p=0.0076$). Moreover, we proposed a novel approach that suggests that we could have found out the estimated duration of recurrent inhibition when there was no motor unit firing. Therefore, we extrapolated the findings of 29 SMUs to the hypothetical "0 Hz" firing rate which simulate the silent MN. We found the extrapolated duration at silent MN as 53.55 ms (**Fig 4.3**).

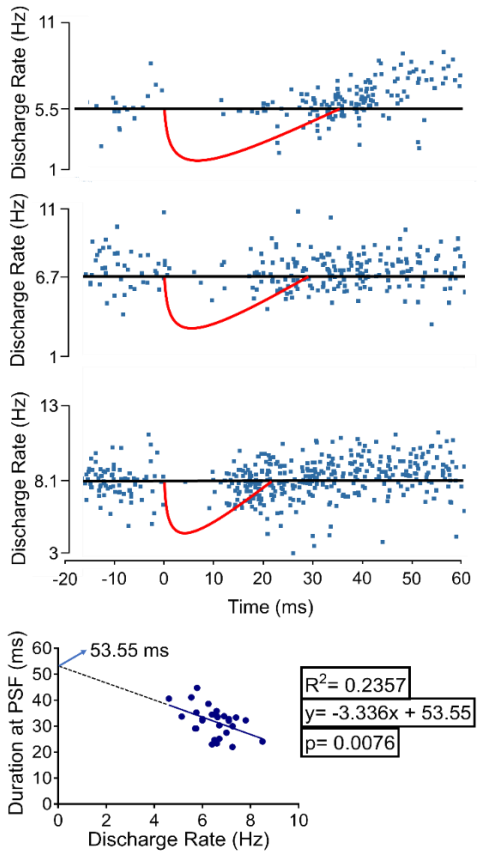

Fig 4.3 *The relationship between the duration of the recurrent inhibition and background discharge rate of SMUs. Red lines are hypothetical IPSP profiles and dashed line in the bottom figure is the extrapolated linear regression line.*

4.2.4 Distribution of recurrent inhibition on various sized motor units

Using force transducer, we could detect the recruitment threshold of 27 SMUs and investigated their recurrent inhibition duration at PSF-CUSUM (**Fig 4.4**). We showed a significantly negative correlation between the duration of the recurrent inhibition and recruitment threshold (**Fig 4.4A**) ($p<0.001$). Since we showed background firing rate of SMUs affect the duration of recurrent inhibition, we also checked whether those 27 SMUs

had different discharge rate. We found no correlation between the discharge rate and duration (p=0.7184), proving that the difference in duration was not due to the background discharge rate (**Fig 4.4B**). Since the largest motor units are recruited with higher common input to the motor pool, we claim that the largest MNs receive lower recurrent inhibition or the same recurrent inhibition is less effective on larger MNs (**Fig 4.4C**).

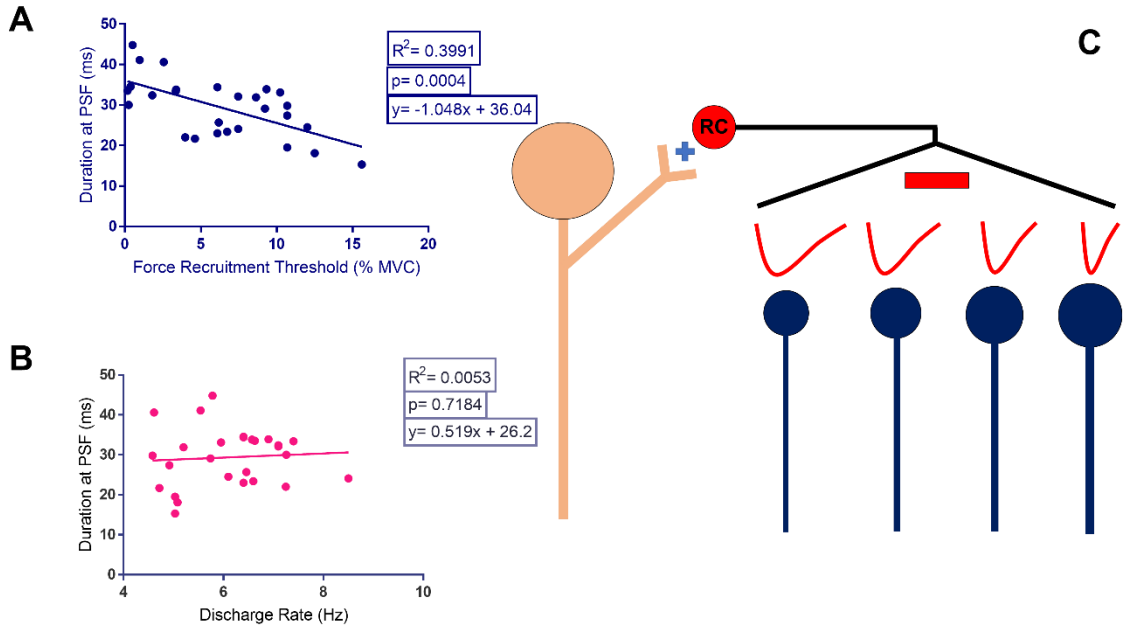

Fig 4.4 *The duration of the recurrent inhibition on different sized motor units determined by their recruitment threshold. A) Duration and motor unit size correlation. B) Duration and background discharge rate of different size motor units. C) Schematic illustrating the recurrent inhibition distribution.*

4.2.5 Motor axon stimulation and recurrent inhibition

We stimulated RCs through collaterals of the MNs which were activated antidromically. To stimulate RCs in that manner, it should be proven that the motor axons

must be stimulated, and the inhibition occurs concomitantly with appeared M-response (**Fig 4.5**). Here, we used 3 different stimulus intensities related to motor threshold (MT) which either did not produce M-response (just below the MT; 0.9 × MT, 0% Mmax) or above the MT; evoked slight (2% of Mmax) and relatively high (10% of Mmax) M-response in the same subject. The inhibitory volleys were investigated using PSTH-CUSUM and M-responses with SEMG. For 0% M-response, we used the stimulus intensity just below the motor threshold (0.9x MT) to observe even if slight decrement in stimulus intensity below MT would eliminate the inhibition. We found that the stimulus intensity just below MT did not produce an inhibitory response on the motor units (**Fig 4.5A**). However, as soon as the stimulus intensity was reached to M-response threshold, inhibition appeared, showed in 2% M-response (**Fig 4.5B**) and further increased in 10% M-response (**Fig 4.5C**). This finding supports that the proposed inhibition was only recorded when M-response was detected in the soleus muscle.

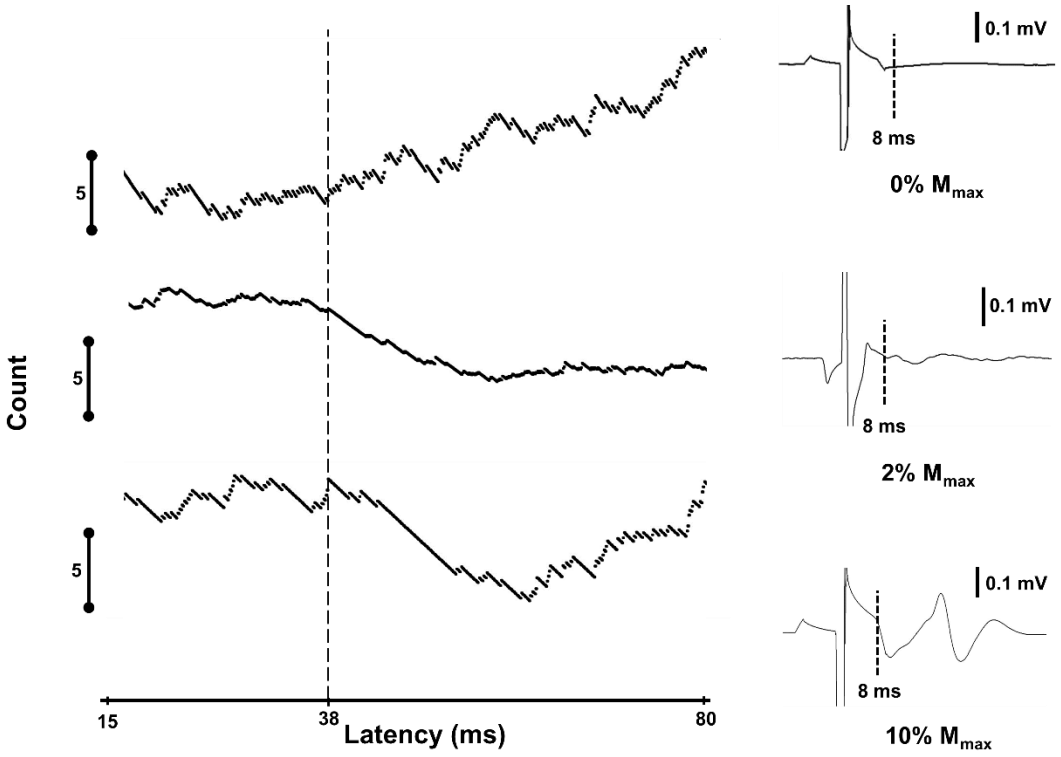

Fig 4.5 *PSTH-CUSUM of the recurrent inhibition in the presence and absence of the M-response.*

4.2.6 Possibility of contamination of the other inhibitory mechanisms

We also tested whether other types of inhibitory mechanisms, i.e. cutaneous and reciprocal inhibition, influenced our protocol (**Fig 4.6**). The measured responses were analyzed using PSTH-CUSUM (to detect inhibition) and SEMG (to find M-response and H-reflex). Therefore, we stimulated the same sensory dermatome with the popliteal fossa to check if the proposed inhibition recorded was due to the cutaneous inhibitory mechanisms. Here, we measured the distance between the stimulating location which was used in M-only experiments and the upper pelvic bone. Then, we moved the cathode to lower one-third of that distance which is in the same dermatome without changing the anode location and

stimulus intensity (this intensity evoked recurrent inhibition when used to stimulate the tibial nerve at the popliteal fossa). We found that stimulating the same sensory dermatome produced neither inhibition nor excitation (**Fig 4.6A**).

Besides, we tested if the corresponding inhibition was due to reciprocal inhibition of the antagonist muscle, tibialis anterior, through Ia inhibitory interneurons. Here, we stimulated soleus motor axons only and recorded single units as well as SEMG from soleus muscle together with SEMG from tibialis anterior muscle. We found that when the recurrent inhibition was detected (**Fig 4.6B**) there was only M-response in the soleus muscle (**Fig 4.6C**) without any activity in the tibialis anterior muscle (**Fig 4.6D**). This result supports that the proposed inhibition was neither reciprocal nor cutaneous inhibition.

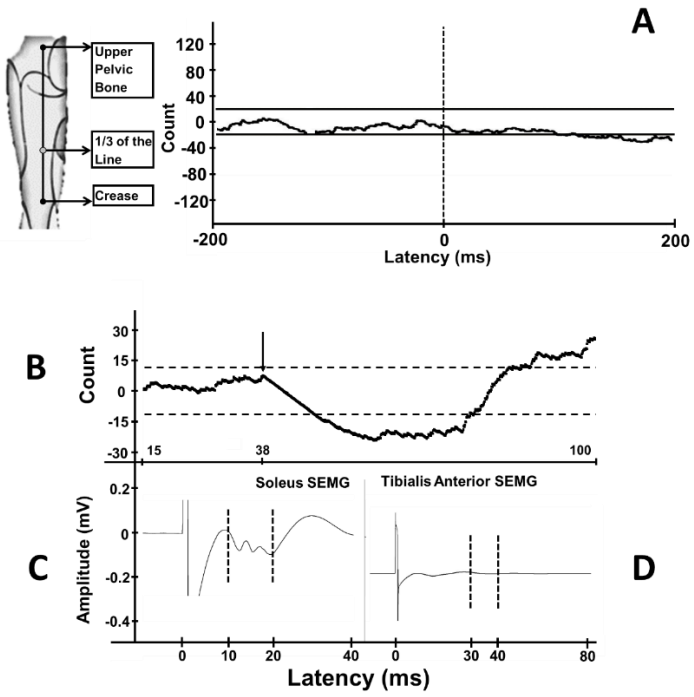

Fig 4.6 *Other possible inhibitory mechanisms that might contaminate our findings. A) Cutaneous inhibition test. B) PSTH-CUSUM of the recurrent inhibition which is present when there is C) M-response only but not D) H-reflex.*

4.3 Discussion

The inhibitory systems directly affect the output of the MNs, therefore, recording from the muscles provide accurate information about the IPSP acting on the MNs. Since the IPSPs on the MNs result in a decrement in the firing rate and since there is no failure rate of the action potential in the neuromuscular junction, the motoneuronal output provides valuable and reliable information about the characteristics of the IPSP. Hence, using the smallest motor unit discharges together with the largest motor axon stimulation, we investigated the characteristics of the recurrent inhibition recorded indirectly from the muscle.

4.3.1 The parameters of recurrent inhibition

The recurrent inhibitory pathway has been recorded directly from RCs or MNs upon stimulation of motor axons in animal studies. It has been proposed that although the majority of the studies indicate the duration of recurrent inhibition between 40 to 50 ms, the discharge of RCs may prolong up to 100 ms, occasionally [108]. This prolonged activity might 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 activation of RCs by NMDA receptors can increase the effective IPSP by increasing the range of temporal summation [109, 110]. However, especially in animal studies, the descending inputs are mostly cut-off and this may affect the actual duration due to reduced cortical influence [72]. Additionally, the duration of the inhibition of MNs might involve other slowly activating, long duration receptors such as GABA receptors. Again, slow component receptor activation prolongs the possibility of the temporal summation which may increase the inhibition on MNs [77]. Hence, antidromic activation of RCs in animal studies might

result in the higher duration of inhibition due to lack of influence of descending inputs and the receptor kinetics both on RCs and their target neurons.

In addition to receptor variability underlying the IPSP duration, the current state of the MNs in terms of excitability should also be considered. Larger excitatory inputs on the MNs make them easily excitable, more importantly, increase their firing probability. Thus, higher firing probability due to sustained excitatory postsynaptic potentials (EPSPs) further increase the firing rate as well as reduce the probability of the silencing of the MN by the inhibition. The higher average rate of action potentials in MNs thus results in apparent shortening of the effect of recurrent inhibition. Our study and Kudina [67] showed that even in the same type of MNs (e.g. slow alpha-MNs), faster discharge resulted in shorter duration of effective recurrent inhibition. Similarly, shorter duration of recurrent inhibition of gamma-MNs was shown which might be due to their higher discharge rate up to 80 Hz [65].

Another parameter, the latency of the recurrent inhibition, has been studied to understand its multi-synaptic component compared to H-reflex. Stimulation of the thickest primary afferents produces H-reflex which is a monosynaptic response. The latency of this reflex varies between 30-35 ms for the soleus muscle depending on the height of the subject, upon stimulation from the popliteal fossa [93, 111]. For the recurrent inhibition, the latency of inhibition has been found to be around 38 ms which was in accordance with another single unit study of the soleus muscle as presented between 35-40 ms [67]. The delay of recurrent inhibition compared to H-reflex in our study was due to; relatively lower thickness of the largest motor axons than primary afferents [112], time delay due to one additional synapse and conduction delay on RCs. In addition, current study investigated the homonymous recurrent inhibition, but the latency might change with the heteronymous recurrent inhibition, such as from soleus to quadriceps due to spatial localization of motor pools and muscles [113]. Reducing the variables such as stimulating directly ventral root and recording

from the RCs might produce more valuable information about the conduction between axon collaterals and RCs in animals which have been found around 1.1 to 1.8 ms [114]. Therefore, focusing on the central conduction would be more useful to understand the multi-synaptic properties of recurrent inhibition in animal studies and difficult to evaluate in human studies involving the peripheral axon stimulation.

4.3.2 Methodological significance

Majority of the experiments in human to investigate RCs connections, physiology and their involvement in diseases has been performed using paired H-reflex technique where different intensity paired electrical stimuli was delivered to the peripheral nerve and reduction in the amplitude of second H-reflex (test reflex), which reduction was attributed to recurrent inhibition, was investigated. 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 [71]. Although this technique provided valuable information, there are several drawbacks that might directly affect the presumed recurrent inhibition. For instance, such high-intensity stimulus used in paired H-reflex technique is likely to favor autogenic Ib inhibition whose latency is almost similar to recurrent inhibition even though Ib inhibition is shorter in duration [115]. In addition, primary afferent endings in such frequent stimulation are susceptible to presynaptic inhibition, therefore, the second H-reflex would already be affected by another inhibitory circuit [14]. Those variables and outcomes might lead misinterpretation of recurrent inhibition.

M-only stimulation as in our study, on the other hand, was shown to be highly reliable to investigate the recurrent inhibition with minimum contamination of other neuromuscular systems. We showed that the recorded inhibition was neither reciprocal nor cutaneous inhibition. In the autogenic inhibition aspect, the longer duration of the recorded inhibition

and the voluntary activation of the soleus muscle minimize the possibility of the Ib inhibition since it is mostly reduced when voluntary contraction takes place [116]. Moreover, the inhibition was recorded only when M-response was recorded and there is almost no possibility of action potential collision or presynaptic inhibition mediated attenuation of proposed recurrent inhibition. Isolated stimulation of motor axons has been thought to be difficult due to higher stimulation threshold of motor fibers compared to primary afferents [117] and reliability of M-only stimulation has been discussed. However, we could able to elicit M-only response with lower intensity stimulations in 12 subjects out of 15. Altogether, M-only stimulation to elicit recurrent inhibition on MNs provided that this technique is reliable, precise and reproducible.

Chapter 5

AMYOTROPHIC LATERAL SCLEROSIS AND RECURRENT INHIBITION

5.1 Introduction

ALS is a neurogenerative disease that is characterized by damage in motor system. Although genetic background of the ALS improved our understanding of the disease progression, 90% of the ALS patients has sporadic background, in total affect about 3 per 100,000 people [118], therefore could be regarded as a rare disorder. Some of the typical clinical versions of ALS involve genetic mutations in superoxide dismutase-1 (SOD1), TAR DNA binding protein (TDP-43 or TARDBP), fusion in sarcoma (FUS) and C9orf72 genes [119, 120], however, the exact pathophysiology of the disease progression has yet to be understood.

Some hypotheses with complex multifactorial and molecular interactions have been put forward as being the cause of the ALS (**Fig 5.1**). Toxic gain of function of SOD1 enzyme may lead to accumulation of the free radicals which eventually damage the cellular structures and disrupts the axonal transport [121-123]. Moreover, dysfunction of ionic pumps and mitochondrial abnormalities may lead to cell death by disrupting intracellular ionic balance and cell structures [124, 125]. Another hypothesis is the glutamate-induced excitotoxicity. Glutamate is the main excitatory neurotransmitter in the CNS that binds to NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) ionotropic receptors. The main mechanism behind excitotoxicity is impairment of excitatory amino acid transporter (EAAT-2) of astrocytes whose malfunction leads to the accumulation of glutamate at the synaptic cleft, in turn, persistent activation of the glutamate receptors. Excessive activation

of NMDA receptor may lead to increase in intracellular calcium ion which in turn activate calcium dependent enzymes that can lead to apoptosis [126, 127].

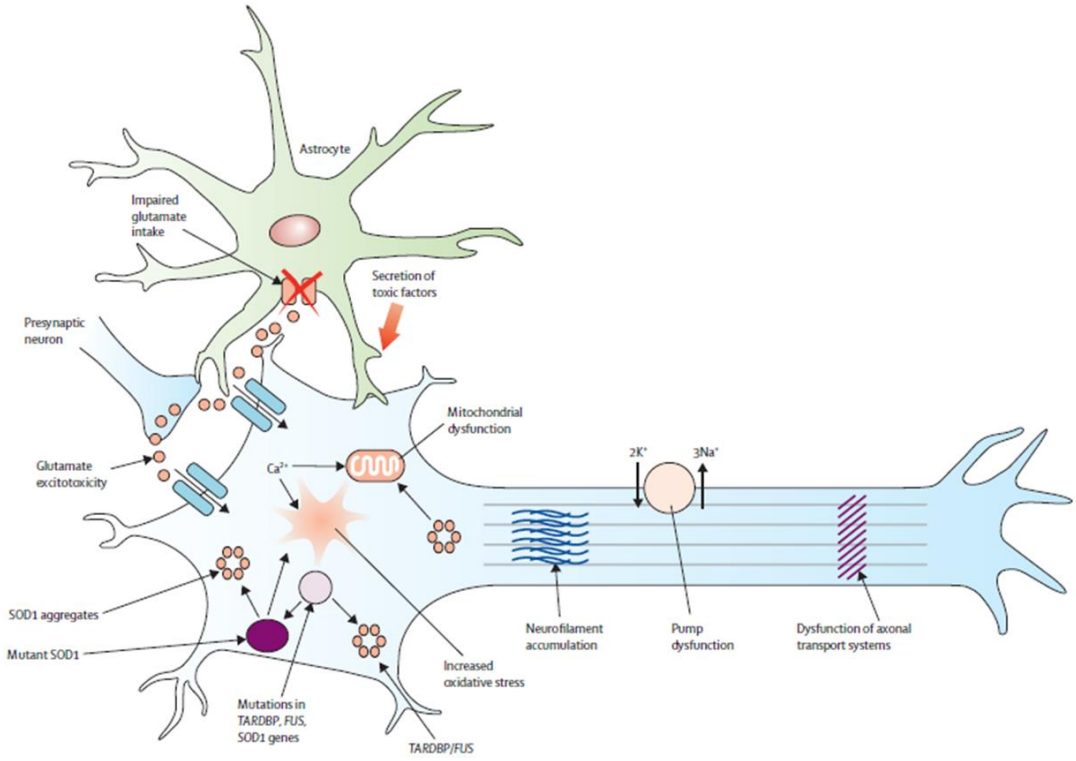

Fig 5.1 *Cellular mechanisms underlying the ALS. Several extracellular (e.g. impaired glutamate removal from the synapse) and intracellular (e.g. mitochondrial dysfunction) were investigated to date. Modified from Kiernan [128].*

In addition to glutamate uptake impairment at synapses, malfunction in inhibitory pathways within CNS may lead to disruption in inhibition-excitation balance of MNs. In many ALS patients fasciculations and involuntary muscle twitch can be observed. The reason for this symptom could be reduction in inhibition converging on MNs, therefore, visible muscle twitches may occur. Moreover, impairment in upper MNs in ALS patients can cause development of spasticity which could further increase excitatory facilitation on

spinal MNs making them vulnerable to excitotoxicity. One of the candidate inhibitory neurons that is closely paired with MNs is the RCs whose dysfunction was proposed to have a role in the impaired muscle control in ALS patients [72].

To understand the functioning of the RC circuit, we used a novel technique that involves electrically stimulating the tibial nerve to activate the largest motor axons and to simultaneously record from one of the smallest axons of the soleus muscle of healthy people. This approach supported the evidence of recurrent inhibitory pathway between MNs in human subjects with a great importance as can be seen in the previous chapter. However, investigating RC in diseased conditions of ALS to understand the possible involvement of the RC in the neurodegenerative diseases is a necessity as very little is known about their role in neurological diseases mostly owing to the technical limitations. Therefore, in this chapter, our specific aims include:

1. To reliably evoke recurrent inhibition in ALS patients.
2. Investigate the recurrent inhibition in 3 groups; (i) healthy people, (ii) ALS patients with lumbar dysfunction (lumbar-affected) and (iii) ALS patients with no lumbar deficit (nonlumbar-affected).
3. Compare the latency and duration of recurrent inhibition in 3 groups.
4. To shed light on the involvement of recurrent inhibition in ALS for a leg muscle.

5.2 Results

5.2.1 Probability analysis for latency of recurrent inhibition in ALS patients

We included 2 groups of ALS patients; lumbar affected and other part of the body onset other than leg muscles (nonlumbar affected). We repeated the experimental protocol for both legs of the patients where we recorded 10 SMUs and 12 SMUs from lumbar-affected

(6 male and 1 female) and nonlumbar affected (8 male) patients, respectively. In order to compare the latency of the inhibition, probability analysis has been found to be the most reliable method, in **Chapter 4**. Therefore, we investigated PSTH-CUSUMs of ALS patients with both lumbar and nonlumbar-affected where we could elicit reliable M-only response, to compare the latency of recurrent inhibition (**Fig 5.2**).

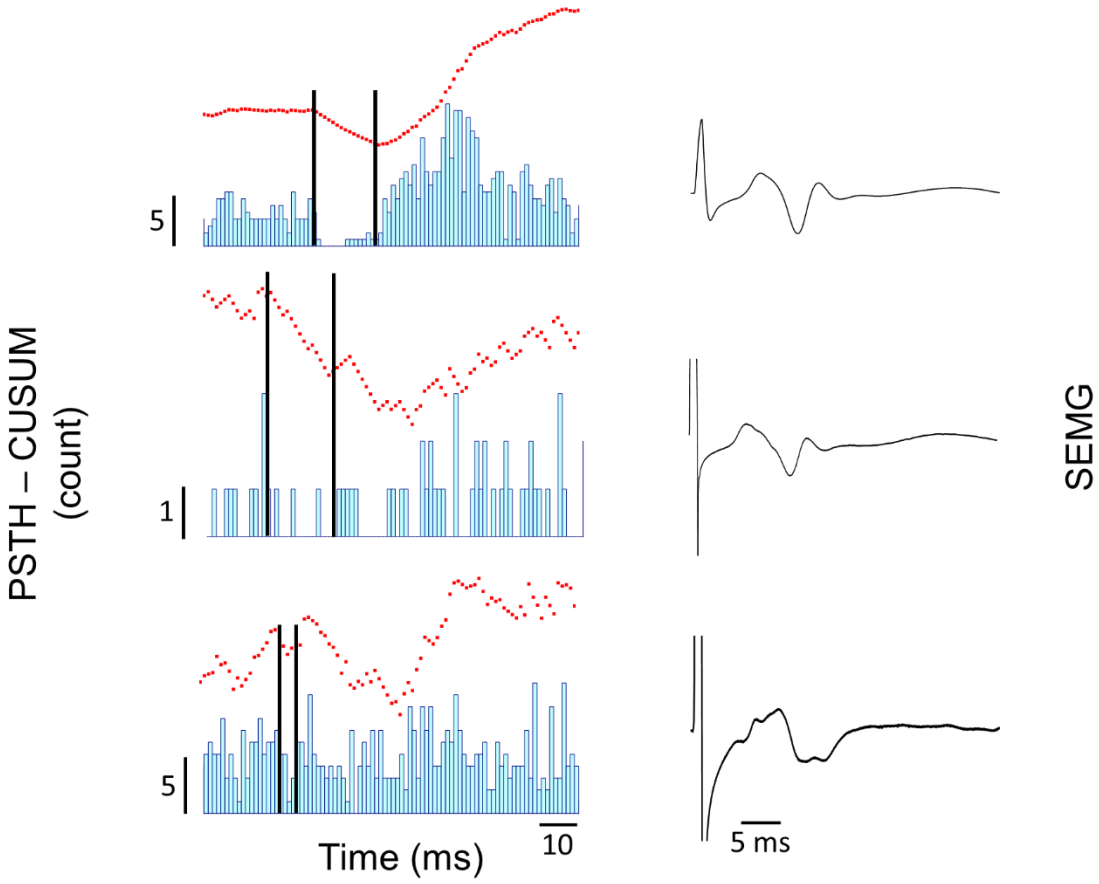

Fig 5.2 *PSTH-CUSUMs of recurrent inhibition in healthy (upper), nonlumbar-affected (middle) and lumbar-affected (bottom) patients. Blue columns are SMU occurrences with 1-ms binwidth. Red dots are CUSUMs developed from PSTH. Black vertical lines are onset*

and end of the inhibition found from CUSUM traces. The time window is between 10 and 100 ms after M-only stimuli detected in SEMG (figures on the right).

5.2.2 Frequency analysis for duration of recurrent inhibition in ALS patients

As the most accurate method, PSF, is useful to show the genuine duration of the IPSP. Given that the spikes could occur within the period of rising phase of the IPSP, PSF can precisely trace the reduced firing rate of the SMUs at this period. Therefore, we used PSF and its average trace to calculate the duration of the inhibition in lumbar and nonlumbar-affected patients (**Fig 5.3**). The comparison of all the methods to calculate the duration was performed in **Chapter 4**.

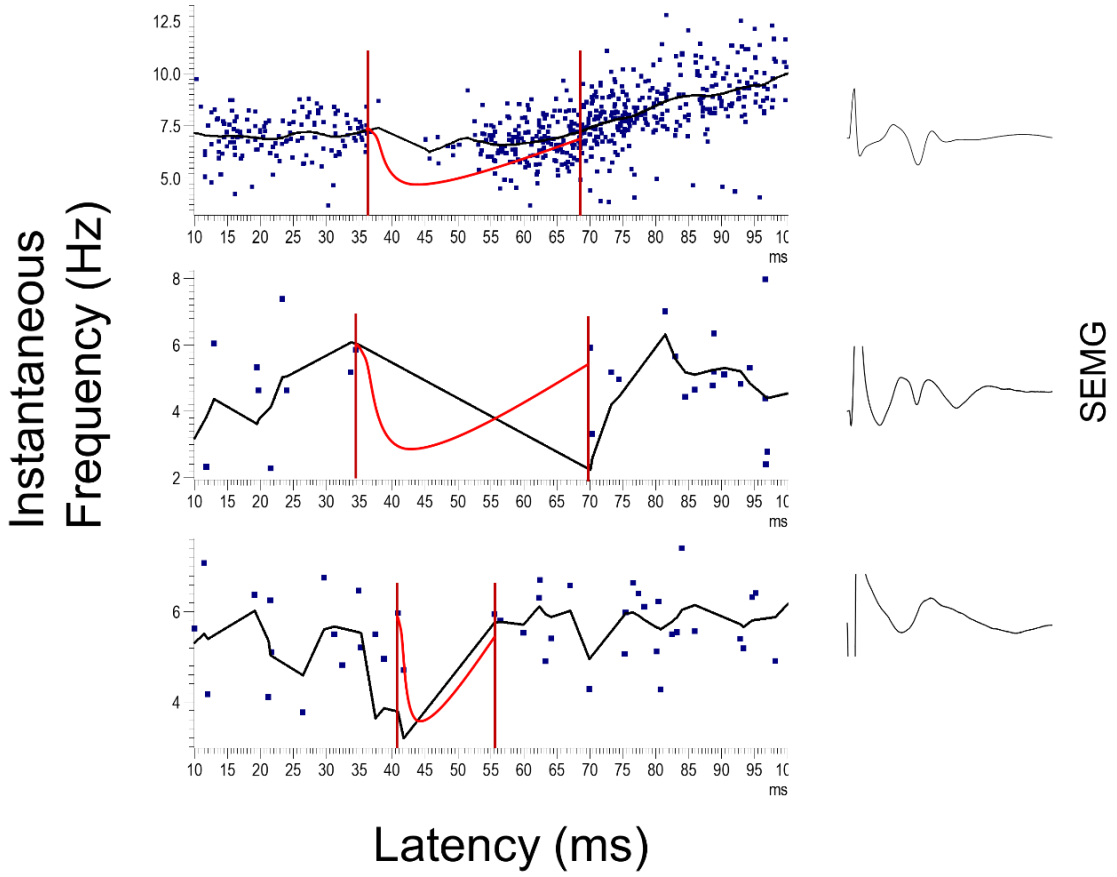

Fig 5.3 *PSF and its average (black line) of recurrent inhibition in healthy (upper), nonlumbar-affected (middle) and lumbar-affected (bottom) people. Blue dots are instantaneous firing rate of the SMUs. Red vertical lines are onset and end of the inhibition. Red parabolic curves are hypothetical IPSP shapes. The time window is between 10 and 100 ms after M-only stimuli detected in SEMG (figures on the right).*

5.2.3 Comparison of the latency and duration characteristics for healthy subjects and ALS patients

To determine the conduction delay, possibly occur due to MN loss, we compared the latency of recurrent inhibition within groups (**Fig 5.4A**). The difference of the latency of the recurrent inhibition between healthy people (37.7 ± 2.4 ms), nonlumbar-affected patients (38.3 ± 3.7 ms) and lumbar-affected patients (37.9 ± 5.1 ms) was not significant (healthy vs nonlumbar: p=0.8115; healthy vs lumbar: p=0.9818; nonlumbar vs lumbar: p=0.9476).

On the other hand, the duration was significantly lower in lumbar-affected condition. Although there was no difference in the average duration of recurrent inhibition in healthy people (30.8 ± 7.2 ms) and nonlumbar-affected patients (31.3 ± 12.4 ms) (p=0.9786), lumbar-affected patients had significantly shorter recurrent inhibition (11.2 ± 2.5 ms) than both healthy (p<0.0001) and nonlumbar-affected (p<0.0001) individuals, despite the lower number of SMUs (**Fig 5.4B).**

The amount of MN estimated, determined by MUNIX, was also found to be significantly less in lumbar-affected patients compared to healthy (p=0.0221) and nonlumbar-affected people (p=0.0412).

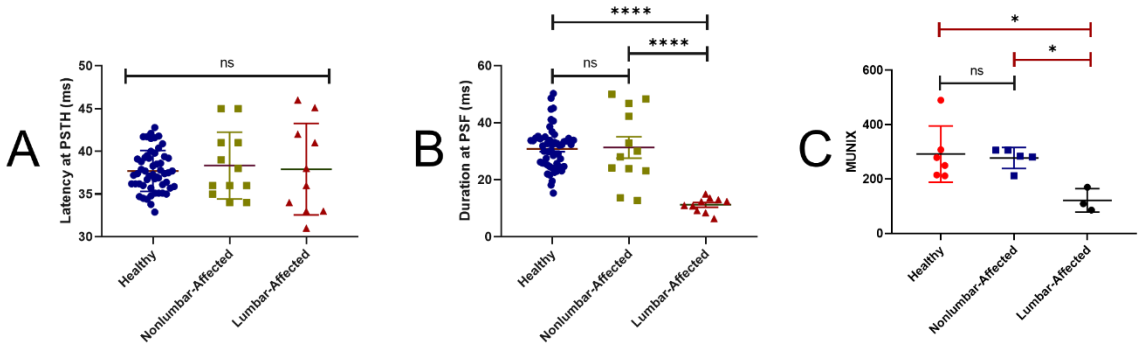

Fig 5.4 *Characteristics of recurrent inhibition in healthy people and ALS patients. A) Latency and B) duration of recurrent inhibition.* $N_{healthy}=54$, $N_{nonlumbar}=12$, $N_{lumbar}=10$ *SMUs. C) MUNIX values for some of the healthy people and patients.* $N_{healthy}=6$, $N_{nonlumbar}=5$, $N_{lumbar}=3$. *Error bars are SD,* $^{ns}p>0.05$, $*p<0.05$, $****p<0.0001$, *one-way ANOVA with Tukey's multiple comparisons test.*

5.2.4 Extrapolation of duration of the recurrent inhibition to silent MN

In **Chapter 4**, we hypothesized that since the duration is inversely proportional with the background discharge rate of SMUs, we extrapolated the duration of 29 twitch-free SMUs to 0 Hz silent MN firing. We found that genuine duration of IPSP in healthy people was 53.55 ms (**Fig 5.5A)**. Despite the insignificant deviation of the slope in ALS patients, we found similar duration in nonlumbar-affected patients as 53.36 ms (**Fig 5.5B)** whilst only 12.98 ms in lumbar-affected patients (**Fig 5.5C)**.

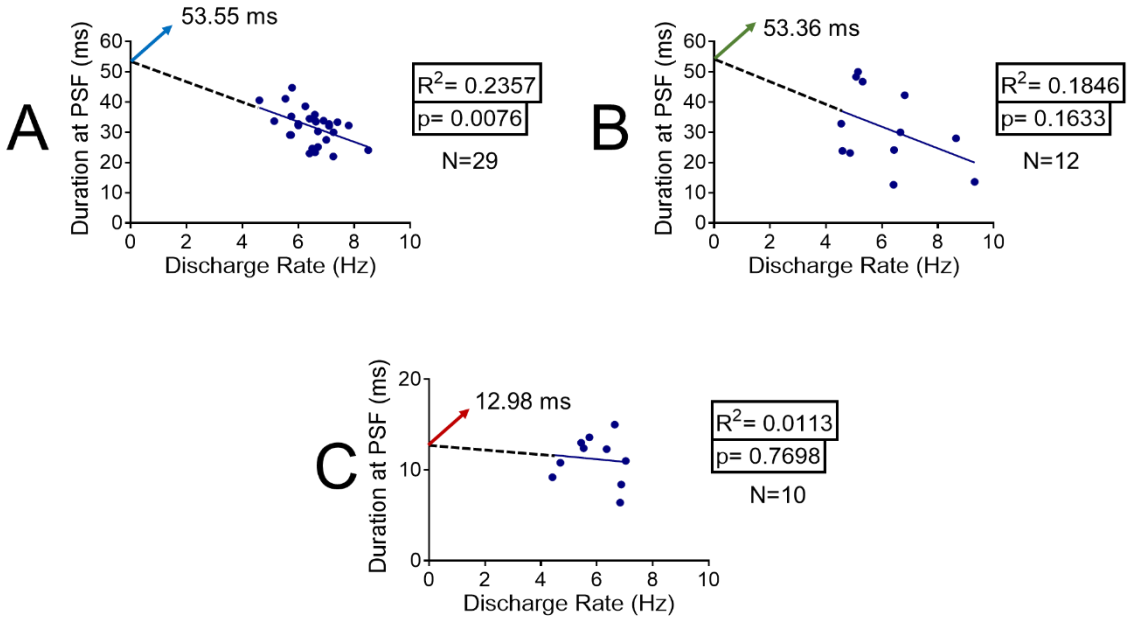

Fig 5.5 *Extrapolation of recurrent inhibition duration to silent MN rate in healthy people and ALS patients. A) Healthy, B) nonlumbar-affected and C) lumbar-affected people.* $N_{healthy}=29$, $N_{nonlumbar}=12$, $N_{lumbar}=10$ *SMUs.*

5.2.5 Firing characteristics of SMUs in ALS patients

In order to be informed about insight of the excitability of the MNs in ALS patients, we investigated the distribution of the MN firing intervals (**Fig 5.6**). We observed similar background discharge rates in ALS patients and healthy people (6.06 ± 1.3 ms vs 6.35 ± 0.9 ms respectively, p=0.3355). The deviation of the SMU firings of ALS patients (1.28± 0.7 Hz) was not significantly higher (p=0.5332) than the healthy individuals (1.19 ± 0.2 Hz).

However, %CV was found to be different for both background firing rate (as in the study of de Carvalho [129]) and deviation in discharge rates. While healthy subjects had a smaller inter-individual firing rate variability (%CV) and its deviation as 14.7% and 20.3%, ALS patients had almost twice as much variation by being 20.7% and 54.4%, respectively.

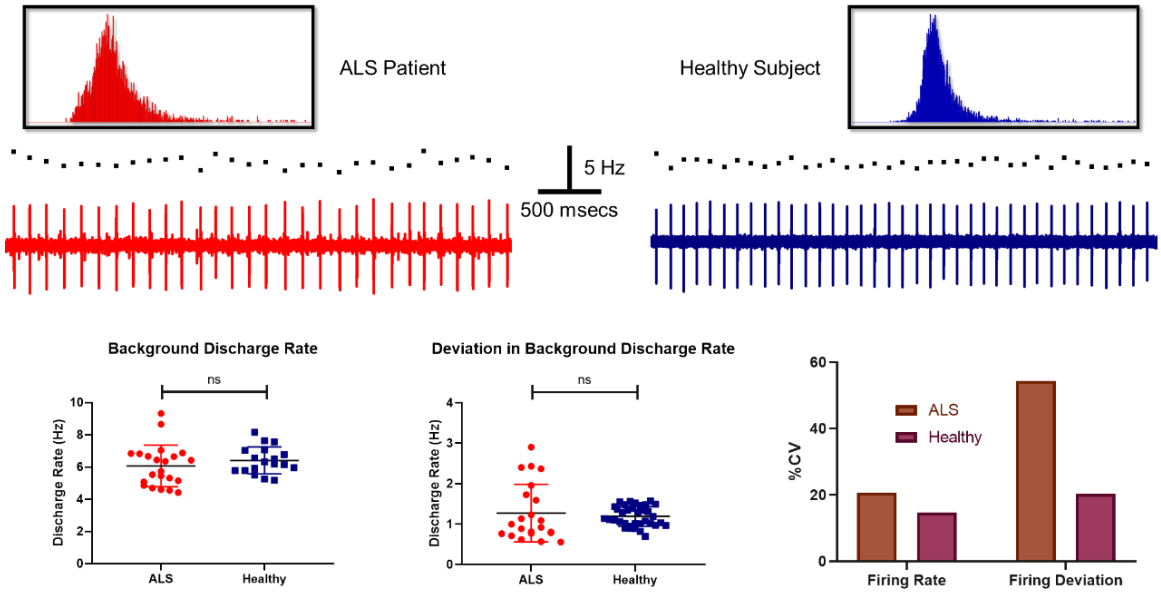

Fig 5.6 *Motor unit firing characteristics of ALS patients and healthy people in terms of discharge rate deviation. Figures on the top-left is the interval histogram of a sample ALS patient whilst on the right is a sample healthy subject. Figures on the bottom is the background discharge rate (left), firing deviation (middle) and their %CV comparisons between SMUs of ALS patients and healthy people.* N_{ALS}*=22 (lumbar + nonlumbar) and* $N_{healthy}$*=35 SMUs,* ^{ns} $p>0.05$.

5.2.6 Reflection of the SMUs to SEMG in ALS patients

We detected a reflection, recording of SMUs in SEMG instantaneously, in some of the subjects whose reflected units were as clear as SMUs recorded using intramuscular EMG (**Fig 5.7**). Although we observe homogenous and mixed signals in SEMG of the healthy subjects (**Fig 5.7A**), ALS patients exhibited multi units or even single units to be detected in the SEMG. A representative figure of lumbar-affected subject (**Fig 5.7B**) indicates a very clear SMU on the SEMG while it was often problematic recording SMUs from the intramuscular EMG electrodes. The reason behind this could be the extensive degeneration

of the SMUs and resultant reinnervation which may lead to a rise in the amplitude of the SMU potential. In some of the subjects who were also lumbar-affected but not as severe as the previous one, also exhibited similar characteristics but the reflected units were rather multi units (**Fig 5.7C**).

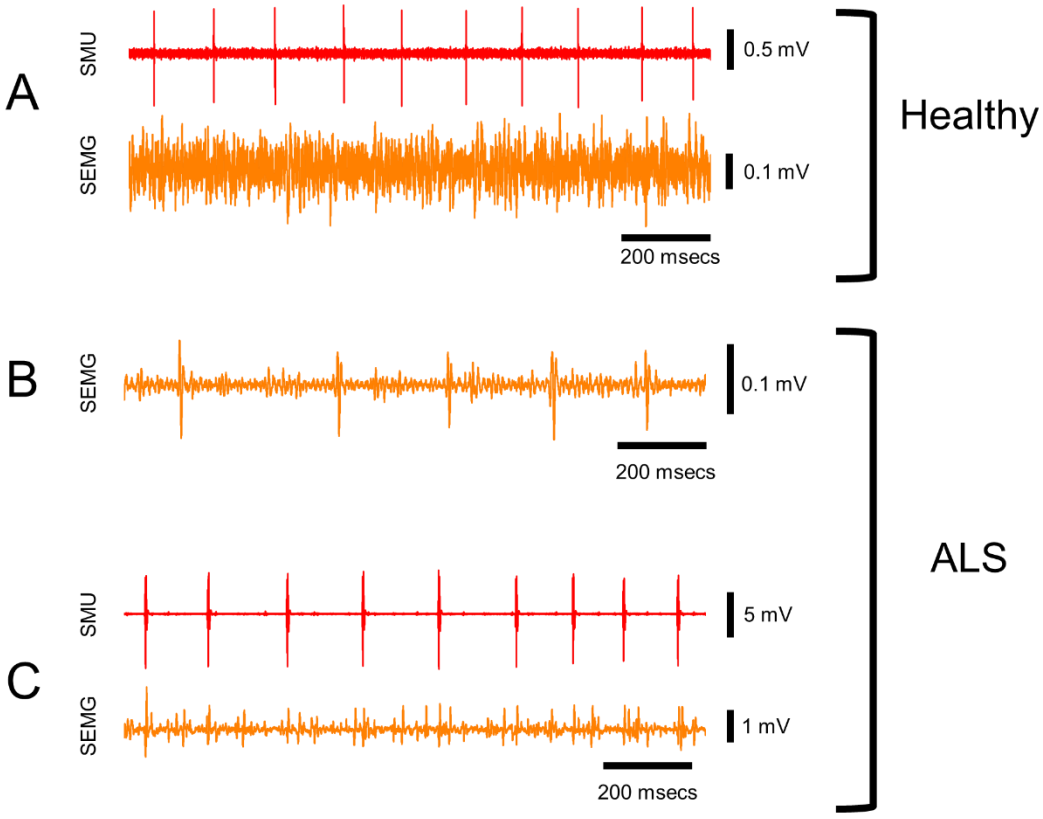

Fig 5.7 *Reflection of the SMU potentials to the SEMG recording. Sample figures of A) Healthy person, B) severe lumbar-affected ALS patient and C) mildly lumbar-affected ALS patients.*

5.3 Discussion

5.3.1 Stimulation of the largest motor axons

In order to evoke recurrent inhibition, we stimulated the thickest motor axons in the tibial nerve whilst recording from the smaller motor units. Although we could evoke M-only response in the majority of the healthy subjects, it was rather difficult to evoke M-only response in lumbar-affected ALS patients as H-reflex contaminated some of the experiments. The reasons behind this issue might be plastic changes in Ia-MN synapse and selective degeneration of the largest motor axons (**Fig 5.8)**.

It is known that MN loss in ALS could lead to spasticity due alternation in the change of MN excitability and spindle afferent inputs, namely increased synaptic contacts of Ia fibers without presynaptic inhibition [128, 130-135]. Normally, we may stimulate different fibers even during M-only response, but the dominant fibers were motor axons. Due to synaptic potential properties, many primary axons should be stimulated to cross the Ia-MN synapse. In healthy subjects, this stimulation cannot evoke H-reflex but due to spasticity evoking H-reflex would be much easier. Therefore, this would be one of the reasons why we had difficulties to evoke M-only response.

Another reason for having difficulties in generating M-only response in patients could be the selective degeneration of the spinal MNs. It has been shown in animal models that fast-fatigable MNs are affected in synchronous manner even before the symptoms of the disease were observed while the slower ones are more resistant to degeneration until the terminal stage [136-139]. Many of the lumbar-affected subjects participated in this study had severe MN loss as can be seen from the MUNIX records. To evoke a minimum amplitude M-response, in this case, we needed to deliver stronger electrical stimuli. This, in turn, resulted in more primary afferents to be stimulated and contaminated our experiments with H-reflex.

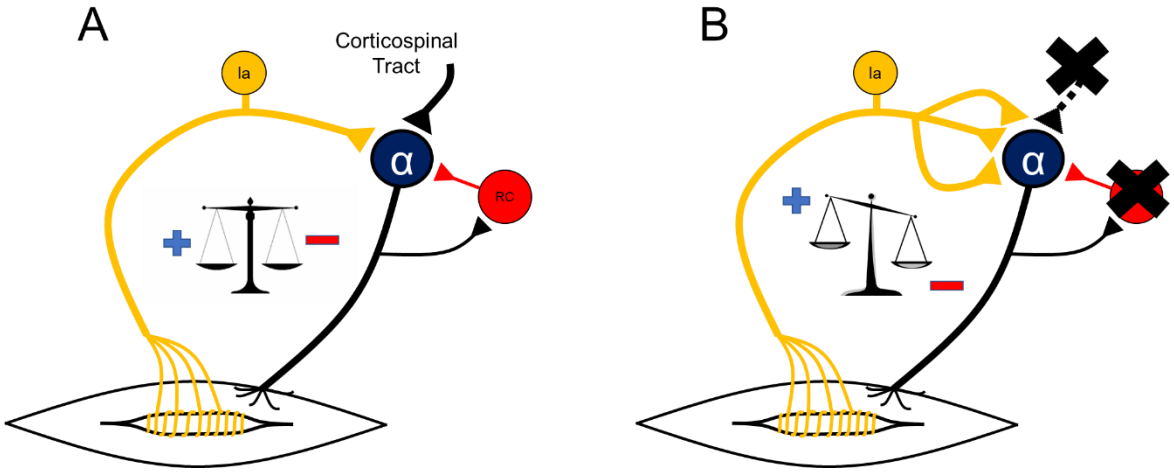

Fig 5.8 *Possible mechanisms of ALS for reduced recurrent inhibition and increased spasticity. A) Healthy subjects in which the excitation and inhibition is balanced. B) ALS patients whose RCs and corticospinal tracts might be damaged, which resulted in spasticity, causing increased excitation and lower inhibition on MNs.*

5.3.2 Reduction in duration of the recurrent inhibition

Previously recurrent inhibition was associated with ALS. On the one hand, animal studies revealed that RCs are impaired in early stages of ALS [77] as well as reduced in strength in human subjects [72]. On the other hand, we investigated the temporal properties of recurrent inhibition in ALS patients with lumbar-affected and unaffected. Our results revealed a significant duration reduction in ALS patients but only in lumbar-affected ones. This may provide an insight about the role of RCs in ALS development in which RC malfunction might be a product of ALS but not its cause as nonlumbar affected ALS patients did not show impaired recurrent inhibition. Despite the fact that duration in lumbar-affected patients was reduced, we did not observe any reduction in patients diagnosed with ALS in other parts of the body other than in the leg muscles.

One hypothesis could be that development of ALS may lead to MN loss in the largest motor pool whose impairment would lead a damage in RCs as they are the major groups to provide input to RCs [37]. If this is the case then we could expect normal recurrent inhibition in subjects who did not show any symptoms in the leg, as we found. However, this situation does not explain that the symptoms do not occur until some portion of the MNs degenerate. In that case, further investigations would need to regression of the duration of the recurrent inhibition with the MUNIX in nonlumbar-affected patients. Since we did not find any correlation, we would reject this hypothesis.

Another explanation is that RCs might be impaired but before MNs were degenerated. In this case, we would expect a decrease in duration of the recurrent inhibition without reduction in MUNIX. Some of the nonlumbar-affected patients exhibited shorter duration, such as around 12 ms, which was similar to lumbar-affected patients (see **Fig 5.4B**). This could be a sign of RC impairment without MN loss as we have never recorded such shorter durations in healthy people. This hypothesis is in line with the muscular fasciculations observed mostly before the observable functional deficits that could be due to reduced inhibition on the MNs. However, to approve this hypothesis we may need further investigation.

5.3.3 Single unit reflection to SEMG

We observed a clear reflection of SMU potentials in lumbar-affected subjects to the SEMG almost as distinct as it was in intramuscular EMG. This could be due to either synchronization of motor units in soleus muscle or due to increased size of motor unit potentials by reinnervation. Fatigue developed or higher level of excitation cause motor units to synchronously fire which can lead to a significant increase in motor unit potentials recorded at SEMG [140]. Since we observed this reflection immediately at the beginning of the experiment, we can claim that if there is a synchronization it would not be due to the

fatigue. Instead, the patients were trying hard to evoke muscle activity which could synchronize the several spared motor units and increase the motor unit potential that could be detected through SEMG.

Another explanation would be the collateral reinnervation of the motor units which could compensate as much as 50% of the motor loss [141, 142]. Due to the reduced number of MNs, motor units may reinnervate the neighbor muscle fibers and produce extremely increased motor unit potential [141, 143]. This increased potential may be revealed in the SEMG easily, therefore, we could observe clear SMU firings.

Chapter 6

CONCLUDING REMARKS AND FUTURE WORKS

6.1 Conclusions and the role of Renshaw cells

Although detailed investigation for almost 80 years shed light on functioning and connections of RCs in the mammalian spinal cord, understanding the exact role of RCs has many gaps to be filled by new sets of experiments. Many studies provided information about functional importance and connections of RCs both in health and disease. In the developmental stage of RCs as studied in mice, it has been shown that although adult RCs lack dorsal root synapses, the functional and wide spread dorsal root connections on RCs have been shown until postnatal day 15 but then starts to decrease and strengthen on MNs [52, 144]. However, not all MNs do synapses with RCs, e.g. recurrent inhibition is lacking in masseter MNs [145] or even synaptic strength of RCs is changing in the same motor pool as shown in this study and Friedman [37].

The extensive connections provided information about the physiology of RCs, thus, the outcome of possible impairment in RCs could be hypothesized and tested in patients but especially in animal models of disease. Although there is lack of consensus about the exact function of RCs, it has been shown that in addition to MNs, both ipsilateral and contralateral networks regulate the discharge of RCs [28, 146]. This substantial control of RCs affects the corticomuscular control but less likely in the isometric contractions elicited by a constant force [147]. However, despite the modulation of the motoneuronal firing, RCs do not diminish the rhythmic motoneuronal bursts completely [6]. For example, oscillations in muscle and tremor have been proposed to be reduced by RCs due to aggregation of the MN activity at certain frequencies [148]. In contrast, recurrent inhibition has been shown to produce synchronous activity in MNs at 30 Hz [149]. Moreover, increased MN

synchronization has been suggested to affect the synaptic activity of recurrent collaterals and this affects the functional output of RCs on their targets [66].

On the other hand, numerous findings and hypotheses have been put forward about the results of disrupted RCs which was investigated in distinct diseases. In fact, some of the studies proposed RC impairment result in a significant functional loss in the presence of certain diseases. Although peripheral nerve injury, especially at the neonatal period, causes recurrent inhibition to be damaged severely [150], most of the knowledge about the malfunction of RCs was provided by neurodegenerative diseases that affect MNs. Due to their tight coupling, any damage occurs on RCs or MNs might result in functional reduction in one another. In addition, findings in ALS patients using paired H-reflex technique showed a reduction in recurrent inhibition and its corticospinal control but decreased recurrent inhibition was suggested to happen at early stages [72, 76, 151]. However, it has been proposed that RCs are not affected at early stages of ALS, instead early expressional changes in the fast MNs, in turn, affected synaptic inputs on RCs by disease have been presented [77, 152]. As a possible outcome of disrupted RCs or other spinal inhibitory interneurons, increased excitation by decreased inhibition on MNs may lead further motoneuronal loss due to excitotoxicity mediated by excessive glutamate activity [45]. Hence, our findings provide information about some of the functions of RCs in locomotion and consequences of the impaired recurrent inhibition due to ALS.

We may classify the functions of RCs under at least 3 subtitles;

1. Facilitation: As we have shown that the duration of the effective recurrent inhibition is longer in the smaller units and it is known that RCs are mostly activated by the largest units, we can conclude that recurrent inhibition may be evolved to facilitate strong contractions of F-type units and reduce the energy

wasting of S-type units during strong contraction, allowing F-units to be activated efficiently, in line with the proposal by Kernell [153].

2. Locomotion: Since proximal muscle activation inhibits the distal muscles via RCs (e.g. quadriceps inhibit tibialis anterior), recurrent inhibition may contribute swing phase of locomotion via heteronymous connections.
3. Protection: Fast characteristics and short central delay of recurrent inhibition may protect MNs from overexcitation. Since recurrent IPSP comes to motor pool just after several milliseconds of MN firing (to itself and neighboring MNs) and lasts about 50 ms, RCs may prevent persistent excitatory inputs on MNs which could increase the intracellular calcium level. Although afterhyperpolarization lasts even longer than recurrent inhibition, it does not protect neighboring MNs from overexcitation and does not block calcium influx.

In summary, we report an extensive investigation of recurrent inhibition in firing MNs in awake humans and an accurate manner. The optimized technique, parameters, and reported findings can be applied to clinics, particularly for evaluating the role of recurrent inhibition in diseases such as ALS.

6.2 Possible future works

To find an effective treatment for a disease, both the physiology and the pathophysiology of the affected system must be studied in detail. Despite being one of the first neuronal circuits to be discovered, the exact role of the RCs in motor function has not been elucidated yet. Additionally, the specific role of RCs in neurological disorders has not been the main topic except in a small number of studies in addition to this study [72, 76, 77, 154]. Hence, specific roles of the RCs in both physiological and pathophysiological

conditions in a controlled environment, such as in optogenetically stimulated animals, is necessary.

Optogenetics has the potential to overcome some of the technical shortcomings. This novel groundbreaking technique provides an opportunity to investigate neuronal circuitries in freely behaving animals. Our study is based on electrical stimulation of the F-type units to investigate the recurrent inhibition from F-type units and S-type units. However, optogenetic stimulation of the mixed nerve, selectively activates the thinnest fibers in contrast to electrical stimulation [155]. Therefore, recurrent inhibition between S-type motor units can be studied using optogenetics in transgenic mice.

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Vita

Mustafa Görkem Özyurt was born on the February 11, 1992 in İzmir, Turkey. He received his B.Sc. in Ege University-Bioengineering Department as the top-ranked student. During his B.Sc., he won Erasmus Grant for Internship and worked in Genetics and Molecular Neurobiology Department in Otto-von-Guericke University in Magdeburg, Germany, in 2013 summer for 3 months. In 2014, he was accepted to Ph.D. program in Biomedical Sciences and Engineering at Koç University with B.Sc. degree. His research interest includes investigating the neuronal circuits using reflex pathways, electrical stimulation of the peripheral nerves, transcranial magnetic stimulation of the motor cortex, optogenetic stimulation of the neurons and the response of the single motor units.

Appendix: Sample Informed Consent Form (Turkish)

In addition to the information given/required for participants; age, height and weight is also noted. The form below is converted into the thesis formatting and the document may have formatting differences.

Bilgilendirilmiş Gönüllü Olur Formu

Araştırmanın Adı: İnsan beyninin ve iskelet kasının elektriksel aktivelerinin araştırılması

Protokol no: 2013.066.IRB2.33/11.02.2013

LÜTFEN DİKKATLİCE OKUYUNUZ !!!

Bu çalışmada yer almayı kabul etmeden önce çalışmanın ne amaçla yapılmak istendiğini anlamanız ve kararınızı bu bilgilendirme sonrası özgürce vermeniz gerekmektedir. Size özel hazırlanmış bu bilgilendirmeyi lütfen dikkatlice okuyunuz, sorularınıza açık yanıtlar isteyiniz.

ÇALIŞMANIN AMACI NEDİR?

İnsan iskelet kaslarının santral ve periferal kontrolünün incelenmesi.

KATILMA KOŞULLARI NEDİR?

Bu çalışmaya dâhil edilebilmeniz için anestetik maddelere hassasiyeti olmayan ve herhangi bir nörolojik veya kas bozukluğu olmayan sağlıklı erişkin birey olmanız gerekir.

NASIL BİR UYGULAMA YAPILACAKTIR?

- Çalışma Koç Üniversitesi Tıp Fakültesi Nörofizyoloji laboratuarlarında **veya Koç Üniversitesi Hastanesi Nöroloji laboratuarlarında** yapılacaktır.
- Kas kasılması beyin tarafından kontrol edilir. Bir kasınızı kasmaya karar verdiğinizde beyin sinir hücrelerinden elektriksel sinyal omurilik boyunca motor sinirlerle kasa kadar gönderilerek kasılma sağlanır. Kasılma hareketine reflekslerle ayar ve düzenleme yapılır.

- Katılacağınız deneyde aşağıdaki prosedürler tüm deneyler için aynıdır:
- Deri yüzeyinden yapılacak kayıt için cilt %70 lik alkol ile temizlenecek, deri yüzeyi aşındırıcı jel ile hafifçe ovulacak ve yüzeyel elektrotlar kasın liflerine paralel gelecek şekilde deri üzerine yapıştırılacaktır. Başdaki saçlı deri üzerine elektrotlar yapıştırılırken sadece jel kullanılacaktır.
- Kas içinden yapılacak kayıtlarda, cilt %70 lik alkol ile silinecek ve elektrot telleri kas içine 25G kalınlığında bir enjektör iğnesi yardımı ile yerleştirilecek, bu iğne daha sonra geri çekilip, teller kasın içinde bırakılacaktır ve kasınızı birkaç defa kasmanız istenecektir. Yerleştirilen teller deney sırasında ağrıya neden olmazlar ve deney sonunda da kolayca ağrısız olarak çekilip çıkarılacaktır. Elektrotlar steril ve tek kullanımlıktır.
- Kayıt esnasında sizden karşınızdaki ekrana bakarak istenen derecede kaslarınızı kasmanız istenecektir.
- Çalışmamız baş bölgesinde masseter, digastrik ve temporalis kaslarını; kol bölgesinde biseps, deltoid, thenar ve hipothenar kaslarını ve bacak bölgesinde ise triceps surae ve tibialis anterior kaslarını içerecektir.
- Her bir deneyde sadece bir bölgeniz uyarılacak ve o bölgedeki bir ya da iki kastan kayıt alınacaktır. Uyarılar elektrik ve mekanik olduklarında 1-3 saniye aralıklı olarak yaklaşık 300 adet, lazer olduklarında 10-15 saniye aralıklı 50 kere, transkranyel beyin uyarılarında ise 4-6 saniye aralıklı olarak yaklaşık 100 adet olacaktır.

- **Katılacağınız deneyde aşağıdaki prosedürlerden sadece işaretlenmiş olanlar uygulanacaktır. Uygulanacak prosedürler hakkında gerekli bilgiler aşağıda verilmiştir.**

☐ Transkranial Manyetik Uyarı

- Transkranial manyetik stimülasyon (TMS) beynin ağrısız ve invazif olmayan bir şekilde uyarılarak beyinden kaslara giden yolakların araştırılması için kullanılan bir yöntemdir.
- Katılacağınız bu deneyde motor kortekse verilen manyetik uyarının kas hareketi ve hareketsiz durumlardaki etkileri incelenecektir.
- Uyarı esnasında belli kaslarınızda kasılma veya seğirme hissedebilirsiniz. Uyarılar arası aralık en yakın dört saniye ve tek tek verilen (single pulse) manyetik uyarı ağrısız ve zararsızdır.
- Magnetik koiller klasik sekiz seklinde 15 x 8 x 1 cm ebatlarinda; yuvarlak koil ise 13 cm capli ve 2 cm kalinliktadir.

☐ Mekanik Uyarı

- Katılacağınız bu deneyde kaslara, tendonlara ya da dişlere verilen uyarı ile oluşan refleks yanıtlar ve elektriksel özellikleri incelenecektir.
- Mekanik uyarı esnasında belli kaslarınızda kasılma veya seğirme hissedebilirsiniz. Mekanik uyarı ağrısız ve zararsızdır.
- Eğer mekanik uyarı dişinize verildi ise, deney sırasında uyarı verilen dişinizin çevresine lokal anestetik madde (Diş Hekimlerinin kullandığı anestetik madde ve uygulama yöntemi ile) verilerek geçici bir süre için (2 saat kadar) hissiz hale getirilecektir. Bu aynen diş hekimine gittiğinizde yapılan enjeksiyondur ve hiçbir yan etkisi olmayıp, etkisini iki saat içerisinde tamamen kaybeder. Dişiniz hissiz halde iken deney tekrarlanarak aradaki refleks farkı elde edilecektir.

☐ Lazer Uyarısı

- Katılacağınız bu deneyde lazer uyarı ile oluşan refleks yanıtlar ve elektriksel özellikleri incelenecektir.
- Lazer uygulaması küçük bir alana (yaklaşık 5mm^2) yapılacaktır. Kalıcı bir etkisi olmayıp, hafif rahatsızlık ve sıcaklık hissi verebilir.

■ Elektriksel Uyarı

- Katılacağınız bu deneyde elektrik uyarı ile oluşan refleks yanıtlar ve elektriksel özellikleri incelenecektir.
- Uyarı esnasında belli kaslarınızda kasılma veya seğirme hissedebilirsiniz. 2-100 mA seviyesinde verilen elektriksel uyarılar deride hafif kaşınma hissi ve rahatsızlık oluşturabilir. Bu uyarıların hiçbir kalıcı yan etkileri olmayıp tamamen zararsızdırlar.

☐ Bel – sırt – omuz – boyun manipülasyon (Chiropractic manipülasyonu)

- Eğer chiropractic manipülasyon yapılacak ise bunu bu konunun uzmanı olan Dr. Heidi Haavik (Auckland Üniversitesi, Yeni Zelanda) yapacaktır.
- Bu manipulasyon beş dakika kadar sürecek ve bel-sırt-omuz ve boyun bölgelerinizin kaslarına yapılacak bir masaj gibi hissedilecektir.
- Bu manipülasyonun hiçbir kalıcı yan etkisi yoktur ancak yaşlılarda çok ender de olsa bazı sorunlara yol açabilir.
- Bu sebepten dolayı bu çalışma sadece sıhhatli genç (30 yaş altı) gönüllüleri içerecektir.
- Ayrıca bu çalışmaya katılan gönüllülerin ilişikte verilen chiropractic manipülasyon riskleri yazısını da okuyup imzalamaları gerekmektedir.

SORUMLULUKLARIM NEDİR?

Araştırma ile ilgili olarak nörolojik, dental ya da bir kas hastalığınız olup olmadığı ve anestetik maddelere hassasiyetiniz olup olmadığı konularında sorumlu araştırıcıyı bilgilendirmek sizin sorumluluğunuzdur. Bu koşullara uymadığınız durumlarda araştırıcı sizi uygulama dışı bırakabilme yetkisine sahiptir.

KATILIMCI SAYISI NEDİR?

Araştırmada yer alacak gönüllülerin sayısı 100 kişidir.

KATILIMIM NE KADAR SÜRECEKTİR?

Bu araştırmada yer almanız için öngörülen süre yaklaşık 3 saattir.

ÇALIŞMAYA KATILMA İLE BEKLENEN OLASI YARAR NEDİR?

Bu çalışma araştırma amaçlı olduğu için size herhangi bir yararı olmayacak ve ancak insandaki sinir yolaklarının bulunmasını sağlayacağı için, ileride, kassal ve sinirsel hastalıkların klinik tanı ve sağaltımlarının geliştirilmesi için bilimsel temel oluşturacaktır. Bu yöntem insanda uyarı ile kaslarda ortaya çıkan potansiyeller incelenerek sinir haritalarının (sinir elektrik devrelerinin) ortaya çıkartılması mümkün olabilecek ve yeni yöntem ile bu devreler hatasız olarak yeniden çizilecek ve klinik nörolojide güvenle kullanılması sağlanacaktır.

ÇALIŞMAYA KATILMA İLE BEKLENEN OLASI RİSKLER NEDİR?

- Dişe ya da kaslara uygulanan mekanik ve elektrik uyarının, transkranial manyetik uyarının, lazer uyarının ve deri yüzeyinden yapılan kaydın sağlık açısından hiç bir sakıncası bulunmamaktadır. Kas içinden yapılacak kayıtlar ise, herhangi bir hastalık için yaptırdığınız iğneden (enjeksiyon) farklı değildir ve sağlık açısından hiç bir riski yoktur.
- Ender durumlarda iğne deliğinin yerinde enfeksiyon ya da küçük bir kan pıhtısı olabilir. Olası bir soruna karşı gerekli tedbirler tarafımızdan alınacaktır.
- İğne batırma yerlerinde sterilite kurallarına uyduğumuz için 30 senelik uygulamalarda herhangi bir şekilde cilt enfeksiyonu görülmemiştir. Ancak bu durum bir olasılık olduğu için etik kurul müracaatlarında söylenmektedir. Eğer bir cilt enfeksiyonu ile karşılaşır isek bölgeyi antiseptik (batticon) ile temizleyip üzerine antibiyotik pomat (furacin) sürüp gelişmesini takip edeceğiz. Ayrıca Koç Üniversitesi kampus içerisinde bulunan sağlık merkezindeki doktor ile bu konuyu görüşerek tedavinin devamı için konsültasyon yapacağız.
- Lokal anestezi enjeksiyonu, diş hekiminin size yaptığı enjeksiyondan farksızdır ve etkisi iki saat içerisinde tamamen geçer.
- Lazer kullanılan deneylerde hem araştırmacı, hem de gönüllü lazer ışınlarından gözlerini korumak için özel gözlük kullanacaktır.

Yine de bu konuda danışmak istediğiniz bir konu veya karşılaştığınız bir sorun olur ise, (212) 338 1174 numaralı telefondan Prof. Dr. Kemal Türker ile iletişim kurabilirsiniz.

ÖNCELİKLE AŞAGIDAKİ SORULARI YANITLAYINIZ, EĞER HERHANGİ BİR SORUYA EVET CEVABI VERDİYSENİZ ARAŞTIRMACIYA BİLDİRİNİZ.

GÜVENLİK TARAMASI

Lütfen aşağıdaki sorulara cevap verin.

Herhangi bir nörolojik ya da kas hastalığınız var mı?

☐ Evet
☐ Hayır

Lokal ya da genel anestetik maddelere alerjiniz var mı?

☐ Evet
☐ Hayır

Eğer yukarıdaki sorulardan herhangi birine evet cevabı verdiyseniz lütfen detayları aşağıya belirtiniz.

_____
_____

Son 48 saat içerisinde herhangi bir ilaç kullandınız mı?

☐ Evet
☐ Hayır

Cevabınız evet ise kullandığınız ilaçları belirtiniz

_____
_____

Sürekli kullandığınız bir ilaç var mı?

☐ Evet
☐ Hayır

Cevabınız evet ise kullandığınız ilaçları belirtiniz

_____
_____

Daha önce sara nöbeti, inme, ciddi kafa travması ve buna bağlı ameliyat veya beyinle ilgili başka bir hastalık geçirdiniz mi?

☐ Evet
☐ Hayır

Cevabınız evet ise belirtiniz

_____
_____

Başınızda (ağız içi hariç) şarapnel parçası, cerrahi klip, metal malzeme, vücut içine konulan kardiyak pacemaker, koklear implant, tıbbi pompa veya kalp içi tel gibi cihazlar mevcut mu?

☐ Evet
☐ Hayır

Cevabınız evet ise belirtiniz

_____
_____

Sık veya ciddi baş ağrısı yakınmanız, gebelik veya gebe olma ihtimaliniz veya ailenizde epilepsi hastası var mı?

☐ Evet
☐ Hayır

Cevabınız evet ise belirtiniz

_____
_____

HANGİ KOŞULLARDA ARAŞTIRMA DIŞI BIRAKILABİLİRİM?

İlaç alerjinizin olması, nörolojik veya kas sistemi ile ilişkili herhangi bir hastalığınızın olması durumunda veya çalışma programını aksatmanız nedeniyle doktorunuz sizi çalışmadan çıkarabilir.

Biz deneklerimizin kaslarında güçsüzlük, duyu kaybı, parmak uçlarında karıncalanma gibi belirtilerin olup olmadığını ve ailede bu şekilde kişilerin olup olmadığını sormaktayız. Ayrıca ailede şeker hastalığı, yüksek tansiyon olup olmadığı da sorulacak ve kişinin el ve ayak kasları, yürüyüşü ve dengesine bakılarak kontrol edilecektir. Deneylerimiz herhangi bir şekilde sinirlere zarar verecek bir yöntem içermediği için detaylı anamnez dışında sadece şüpheli gördüğümüz durumlarda sinir iletimi hızı ölçümü yapacağız.

TRANSKRANİAL MANYETİK STİMULASYON (TMS) DENEYLERİNE KATILACAKLAR İÇİN:

TMS' ye bağlı herhangi bir yan etki yaşamışsanız, sara nöbeti, inme geçirmişseniz, ciddi kafa travması ve buna bağlı ameliyat veya beyinle ilgili başka bir hastalık geçirmişseniz, beyin hasarına neden olan bir hastalık geçirmişseniz, başında (ağız içi hariç) şarapnel parçası, cerrahi klip, metal malzeme mevcutsa, vücut içine konulan kardiyak pacemaker,

koklear implant, tıbbi pompa veya kalp içi tel gibi cihazlar mevcutsa, sık veya ciddi baş ağrısı yakınmanız varsa, gebelik veya gebe olma ihtimaliniz varsa, ailede epilepsi hastası varsa çalışmadan çıkartılabilirsiniz.

HERHANGİ BİR ZARARLANMA DURUMUNDA YÜKÜMLÜLÜK/SORUMLULUK KİMDEDİR VE NE YAPILACAKTIR?

Araştırmaya bağlı bir zarar söz konusu olduğunda, bu durumun tedavisi sorumlu araştırıcı tarafından yapılacak, ortaya çıkan masraflar Prof Dr Kemal Türker tarafından karşılanacaktır.

ARAŞTIRMA SÜRESİNCE ÇIKABİLECEK SORUNLAR İÇİN KİMİ ARAMALIYIM?

Uygulama süresi boyunca, zorunlu olarak araştırma dışı ilaç almak durumunda kaldığınızda Sorumlu Araştırıcıyı önceden bilgilendirmek için, araştırma hakkında ek bilgiler almak için ya da çalışma ile ilgili herhangi bir sorun, istenmeyen etki ya da diğer rahatsızlıklarınız için (212) 338 1174 numaralı telefondan Prof. Dr. Kemal Türker ile iletişim kurabilirsiniz.

ÇALIŞMA KAPSAMINDAKİ GİDERLER KARŞILANACAK MIDIR?

Yapılacak her tür tetkik, fizik muayene ve diğer araştırma masrafları size veya güvencesi altında bulunduğunuz resmi ya da özel hiçbir kurum veya kuruluşa ödetilmeyecektir.

ÇALIŞMAYI DESTEKLEYEN KURUM VAR MIDIR?

Şu anda yok ancak ön çalışmalardan sonra destek için müracaat yapılacaktır.

ÇALIŞMAYA KATILMAM NEDENİYLE HERHANGİ BİR ÖDEME YAPILACAK MIDIR?

Yapılacak bu inceleme size veya bağlı bulunduğunuz kuruma hiç bir maddi yük getirmeyeceği gibi, size bu gönüllü katılımınızın karşılığında Prof Dr Kemal Türker'in şahsi kaynaklarından net 50 TL ödenecektir.

ARAŞTIRMAYA KATILMAYI KABUL ETMEMEM VEYA ARAŞTIRMADAN AYRILMAM DURUMUNDA NE YAPMAM GEREKİR?

Bu araştırmada yer almak tamamen sizin isteğinize bağlıdır. Araştırmada yer almayı reddedebilirsiniz ya da herhangi bir aşamada araştırmadan ayrılabilirsiniz; reddetme veya vazgeçme durumunda bile sonraki bakımınız garanti altına alınacaktır. Araştırıcı, uygulanan araştırma şemasının gereklerini yerine getirmemeniz, çalışma programını aksatmanız vb. nedenlerle isteğiniz dışında ancak bilginiz dâhilinde sizi araştırmadan çıkarabilir. Araştırmanın sonuçları bilimsel amaçla kullanılacaktır; çalışmadan çekilmeniz ya da araştırıcı tarafından çıkarılmanız durumunda, sizle ilgili tıbbi veriler de gerekirse bilimsel amaçla kullanılabilecektir.

KATILMAMA İLİŞKİN BİLGİLER KONUSUNDA GİZLİLİK SAĞLANABİLECEK MİDİR?

Size ait tüm tıbbi ve kimlik bilgileriniz gizli tutulacaktır ve araştırma yayınlansa bile kimlik bilgileriniz verilmeyecektir, ancak araştırmanın izleyicileri, yoklama yapanlar, etik kurullar ve resmi makamlar gerektiğinde tıbbi bilgilerinize ulaşabilir. Siz de istediğinizde kendinize ait tıbbi bilgilere ulaşabilirsiniz

ÇALIŞMAYA KATILMA ONAYI

Yukarıda yer alan ve araştırmaya başlanmadan önce gönüllüye verilmesi gereken bilgileri gösteren beş sayfalık metni okudum ve sözlü olarak dinledim. Aklıma gelen tüm soruları araştırıcıya sordum, yazılı ve sözlü olarak bana yapılan tüm açıklamaları ayrıntılarıyla anlamış bulunmaktayım. Çalışmaya katılmayı isteyip istemediğime karar vermem için bana yeterli zaman tanındı. Bu formu imzalamakla yerel yasaların bana sağladığı hakları kaybetmeyeceğimi biliyorum. Araştırmaya gönüllü olarak katıldığımı, istediğim zaman gerekçeli veya gerekçesiz olarak araştırmadan ayrılabileceğimi ve kendi isteğime bakılmaksızın araştırmacı tarafından araştırma dışı bırakılabileceğimi biliyorum. Bu koşullar altında, bana ait tıbbi bilgilerin gözden geçirilmesi, transfer edilmesi ve işlenmesi konusunda araştırma yürütücüsüne yetki veriyor ve söz konusu araştırmaya katılmayı hiçbir zorlama ve baskı olmaksızın büyük bir gönüllülük içerisinde kabul ediyorum.

Bu formun imzalı ve tarihli bir kopyası bana verildi.

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