Development of an Automated System for Ex Vivo Measuring the Neuro Muscular Junction Functionality

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1 RESEARCH PROBLEM

The present work is part of a research project, which aims to characterize the nerve-muscle interaction in Amyotrophic Lateral Sclerosis (ALS), a fatal neuromuscular disease associated with motor neuron degeneration, muscle atrophy and paralysis. The loss of connection between muscle and nerve is a crucial biological mechanism severely impaired in ALS. In this context, to investigate the alterations in the communication between muscle fibers and motor neurons, we started studying the neuro muscular junction (NMJ) from a functional point of view. The capability of measuring the NMJ functionality can therefore give essential information on its physio-pathological conditions. Therefore, this study may be useful to discriminate damages of the different motor unit components in neuromuscular diseases and, in long-term, to design more appropriate therapeutic approaches.

In particular, the result that we expect to achieve is to better clarify which element of the motor unit is initially affected by this disease. In fact, the recently proposed "dying-back" hypothesis supports the idea that the damages begin in the NMJ and then spread towards the motor neuron body, in opposition to the more traditional idea according to which the muscle is only a secondary target of the disease.

2 OUTLINE OF OBJECTIVES

In the described context, the aim of my PhD project is to characterize the functionality of the communication between muscle and nerve in pathological mouse models, by developing new automated experimental methodologies.

The measurement of NMJ functionality is obtained by comparing muscle contractile response elicited by nerve stimulation (indirect), with the response of the same specimen to membrane stimulation (direct). Since this latter stimulation bypasses the neuronal signalling, any difference between the two contractile responses may be related to NMJ alterations. To date, I started working with Soleus muscle-nerve specimens of healthy Wild Type mice, to develop an experimental system for studying NMJ functionality of ex vivo muscle-nerve preparations. After that I'm approaching the study of a ALS mouse model. In the future years, I will approach the realization of new experimental systems for testing NMJ functionality in isotonic conditions and investigate directly *in vivo* the muscle behavior. In all the systems We aim to develop we will pay special attention to the accuracy and the repeatability of the experimental procedures. These metrological qualities will counteract the negative effects due to the high variability that usually arises when working with biological tissues.

3 STATE OF THE ART

To better define the alterations in the coupling between motor neuron conduction and muscle contraction we started evaluating the physio-pathologic properties of both skeletal muscle and NMJ, by stimulating the muscle directly and indirectly. In particular, we studied standard muscle contractile properties, as previously described by us and by other research groups (Brooks SV and Faulkner JA, 1988; Del Prete et al, 2008). On the other hand, although the NMJ functionality technique has been extensively used on rats (Aldrich et al., 1986; Van Lunteren and Moyer, 2004), only a few works have been attempted on pathological mouse models: Personius et al. measured the diaphragm NMJ functionality of adult dystrophic mdx mice (Personius and Sawyer, 2006), while Lee et al. (Lee et al., 2011) and Ling et al. (Ling et al., 2009) measured the NMJ properties respectively in

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Soleus and EDL of an animal model of spinal muscle atrophy at a few days after birth. This literature has been taken as a starting point, but these studies are based on animal models of different ages and sizes or different muscles, if compared with ours. For this reason it was not possible to employ the stimulation parameters as proposed.

The pathological model we decided to study at first is the $SOD1^{G93A}$ mice (Gurney et al., 1994), one of the most studied animal model of ALS (Turner and Talbot, 2008). However, its skeletal muscles contractile properties have been poorly investigated and the NMJ functionality at all. Previous studies reported a deficit in the generation of maximum force in hind limbs muscles of $SOD1^{G93A}$ mice, when compared to wild type littermates (Hegedus et al., 2008; Derave et al., 2003). Some authors detected a reduced number of motor neurons associated with disruption of the neuromuscular junction (Ngo et al., 2012; Fischer et al, 2003). Temporal analysis of axon and NMJ degeneration in transgenic mice indicate that motorneuron pathology begins distally from the synaptic area, and then proceeds towards soma in a retrograde dying back manner (Luc Dupuis and Jean-Philippe Loeffler, 2009).

4 METHODOLOGY

To the proposed aims, I have developed a system to measure, ex vivo, the muscle contractile response due to indirect stimulation trough the nerve and the muscle contractile response due to direct membrane stimulation. The proposed system is fully automated, through the use of a custom-made software, that allows to precisely control the experiment, making all the tests repeatable and accurate.

4.1 Experimental Setup

The muscle to be tested is excised with its intact innervation and vertically mounted in an oxygenated (95% O_2 and 5% CO_2) and temperature controlled chamber (30°C), containing a bicarbonate-buffered solution at pH 7.4. One end of the muscle is linked to a fixed clamp and the other end is connected to the lever-arm of an Aurora Scientific Instruments Inc. (ASI) 300B actuator/transducer. The apparatus allows to stimulate the muscle both directly and indirectly. For direct stimulation, two platinum electrodes are located 2 mm from each side of the muscle and the electrical stimuli are current pulses of 300 mA generated by an ASI 701C stimulator. For indirect stimulation, the nerve is sucked into a suction electrode (A-M Systems Inc.) and supramaximal pulses were delivered to the nerve by an ASI 701B stimulator.

4.2 Protocol

We have developed a protocol that allows to study in Soleus muscles several parameters of the isometric muscular contraction and of the NMJ functionality, proposed in the literature, in a single test. Indeed, Personius et al. applied the fatigue protocols at two frequencies on different specimens and van Lunteren et al. studied the intratetanic fatigue, using separate repetitive stimulations, delivered or directly on the muscle or through the nerve.

The protocol is composed of four parts. Initially, the muscle is stimulated with 4 single pulses to measure twitch force (F_{tw}) and kinetic parameters, namely time to peak (TTP), half relaxation time (1/2RT) and maximum value of force time derivative (dF/dt), for both membrane and nerve stimulations, as shown in figure 1.

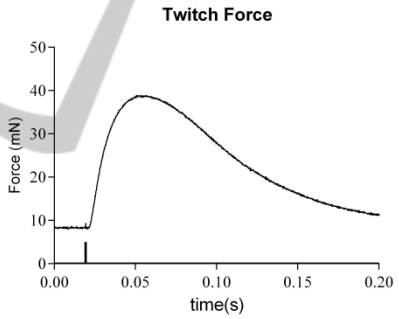

Figure 1: Twitch force (Ftw), time to peak (TTP), half relaxation time (1/2RT) and maximum value of force time derivative (dF/dt) can be measured with single pulse stimulation.

After that, 8 shuffled stimulations in the range between 20 Hz, which is the lowest summation frequency, and 80 Hz, the Soleus tetanic frequency, are used to measure the force-frequency curves for both muscle and nerve stimulations. Finally, the NMJ functionality parameters, namely Neurotransmission Failure (NF) and Intratetanic Fatigue (IF), are measured with two isometric fatigue paradigms. These paradigms are made up of 14 stimulations via the nerve and 1 stimulation on the membrane, to induce a specific stress in the NMJ. The first paradigm is based on pulse trains delivered at a physiological firing frequency of 35 Hz while the second is composed of pulse trains delivered at the tetanic frequency. We employed

0.8 s pulse trains, with a rest period of 1.2 s between each of them, to stimulate the soleus, differently from Personius et al. that used 0.33s pulse train to stimulate the diaphragm membrane and the phrenic nerve, with a rest period of 0.67s after each train. This differences are due to the different fiber composition of the two muscles. This sequence is repeated 20 times, for a total paradigm of 10 min.

The Neurotransmission Failure parameter compares the force decrease due to the nerve conduction to that due to muscle contraction during the paradigm.

$$NF = \frac{F - MF}{1 - MF}\%$$

where F is the force decrease after nerve stimulation and MF is the force decrease after membrane stimulation .

Intratetanic Fatigue represents the force drop which can occur within the same tetanic contraction, in case of stimulation via the nerve.

$$IF = \frac{F_{LP}}{F_{MAX}}\%$$

where F_{LP} is the force at the last pulse and F_{MAX} is the maximum value of force within the single train of pulses (see figure 2).

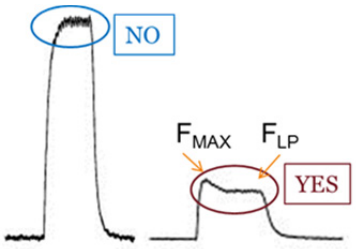

Figure 2: Intratetanic Fatigue is the force decrease within the same pulse train. From van Lunteren et al., 2004.

4.3 Software

The experimental setup is controlled by a Windows PC and a custom-made software in LabView 2011. The interface between the computer and the equipment is managed by the National Instruments data acquisition board NI PCI Express 6353. This choice allows to have a high flexibility in the management of the experiments. The software is used to control synchronously and automatically the pulse stimulators and the actuator/transducer. In particular, all the stimulation parameters are manageable by means of simple text files provided to the software as input.

During the experiment the software automatically changes the acquisition frequency setting it to 20 kHz in case of stimulation with single pulses, and to 1 kHz during the remaining stimulations. This choice is due to the fact that the temporal parameters measured during a single pulse stimulation are of the order of tens of milliseconds, and an accuracy of 50µs is therefore needed to point out any significant difference. During stimulations at frequencies higher than the single pulse, no temporal parameters are calculated, therefore is sufficient to acquire the data at 1 kHz to correctly sample the signals of interest avoiding an overloading of the system.

The software so designed and realized is extremely flexible and functional. In fact, it is able to perform all the main tests to define the mechanical characteristics of muscle tissue and NMJ functionality. This versatility has been extremely useful when carrying out the preliminary tests aimed at determining the optimal stimulation parameters, such as the durations of the pulses, the stimulation frequencies and the waiting times. On the other hand, during the trial it is possible to perform different protocols in a simple way.

5 EXPECTED OUTCOME

The proposed protocol allows the measurement of the NMJ functionality in Soleus muscle of any mouse model, by testing isometric contraction. We expect to be able to characterize the NMJ functionality of other muscle types by the application of this protocol with only minor modifications.

5.1 Future Plans

To perform a functional characterization of the muscle-nerve preparations that better represents the muscle and NMJ *in vivo* behavior, I am confident of being able to develop a similar methodology to study the muscle isotonic contraction. To do this, a tool which allows the suction electrode for nerve stimulation to follow the muscle shortening will be designed and realized. I am also going to develop a in vivo/in situ methodology that can allow to study different muscles or muscular groups to give information on the nerve conduction in addition to NMJ and muscle functionality. Based on this latter technique, the long-term goal is to study a possible improvement of muscle functionality following stimulation training protocols.

6 STAGE OF THE RESEARCH

6.1 Setting Stimulation Parameters

To determine the optimal pulse parameters to stimulate the sciatic nerve I started performing preliminary tests on Soleus muscle-nerve preparations of four months-old Wild Type mice. At first, I checked the current intensity necessary to elicit the nerve stimulation: this was found to be between 5 mA and 10 mA. The lower limit is the first current intensity that causes the maximum twitch contraction force. Increasing the current sent through the suction electrode beyond 10 mA it generates a current field so intense as to induce also the direct stimulation of the muscle membrane through the solution. Event that should absolutely be avoided.

Once set the optimal current value, I looked for the relation between the width of single pulses and the twitch specific force. To this purpose 10 Wild Type Soleus specimens were stimulated with single pulses of widths between 0.2 ms and 2 ms, the range most used in the literature. There was no significant variation of specific force varying the pulses length. However, in correspondence of 1.4 ms duration the forces showed the highest value, as shown in figure 3.

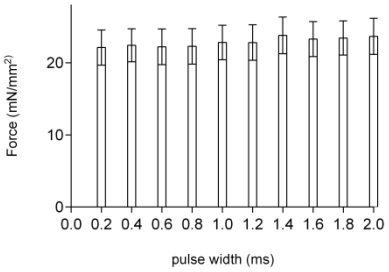

Figure 3: Twitch specific forces developed stimulating via the nerve with pulses of width between 0.2 and 2 ms.

The choice has been also supported by tests of tetanic stimulation in which trains of pulses of 1.4 ms caused a developed force on average higher than the other widths.

Once we have determined the two parameters of the stimulation pulse, we moved to verify the validity of the method. In a healthy specimen is expected that direct and indirect stimulations bring the muscle to contract in the same manner and develop the same forces. In fact, as shown in figure 4, the single pulse tests showed the same twitch force and kinetic contraction parameters in both cases of stimulation: the direct and the indirect one.

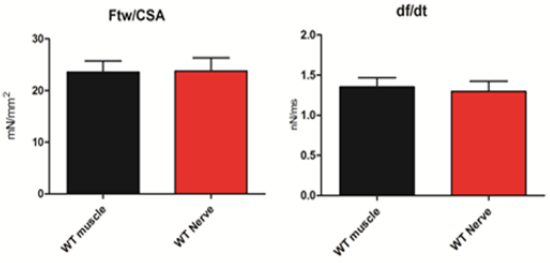

Figure 4: Twitch force and df/dt of WT Soleus stimulated directly and indirectly.

The force-frequency relations are also comparable in both cases of stimulation (see figure 5).

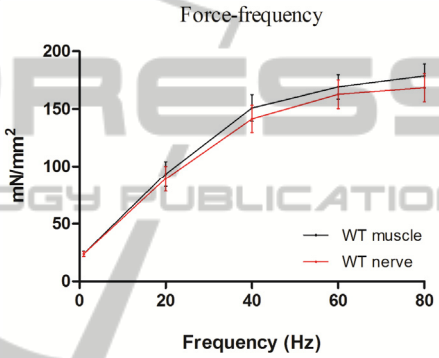

Figure 5: Force-frequency curves from WT Soleus directly and indirectly stimulated.

The fatigue paradigm is derived from Personius et al., however the Soleus stimulation parameters were modified accordingly to its fiber composition and to the standard membrane stimulation parameters. Since the Soleus muscle is composed by a higher percentage of slow fibers than the diaphragm, it needs a longer stimulation time to develop the maximum force. This stimulation time is estimated to be 0.8 s and 1 s is necessary to the muscle relaxation. Therefore, the fatigue paradigms were set as follows: one 0.8 s pulse train delivered directly on the membrane, followed by fourteen 0.8 s pulse trains delivered through the nerve, with a rest period of 1.2 s after each pulse train. By repeating this series of stimulations for 20 times, as for the diaphragm, each paradigm takes 10 minutes.

Some preliminary tests showed that a rest time of 15 min is necessary between the 35 Hz protocol and the 80 Hz one to have repeatable results not affected by muscle fatigue. The rest time that the specimens needed to recover their physiological properties after the first fatigue paradigm was considered adequate when the force generated by the muscle at the

beginning of the second fatigue paradigm was at least 90% of its maximum force.

The application of the fatigue protocol showed that the force developed by nerve stimulation decreases very rapidly while the muscle continues to be able to produce a much greater force as result of direct stimulation, as shown in figure 6.

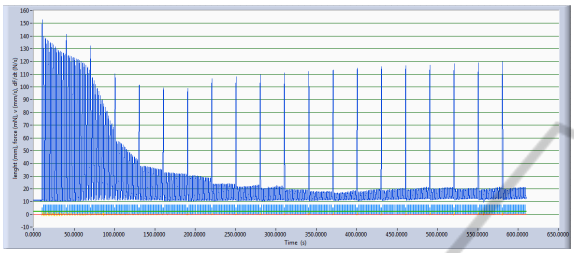

Figure 6: Example of a fatigue paradigm.

The calculation of the Neurotransmission Failure gave results comparable to those existing in the literature for the WT muscles. Likewise was not revealed Intratetanic Fatigue in case of direct stimulation of the muscle. On the other hand, in case of indirect stimulation the IF is similar to that shown by van Lunteren.

6.2 Transgenic Model

Once set the method on the wild type model, to focus our attention on a pathologic model we started studying the Soleus of SOD1^{G93A} mice at the end stage of the disease. At this age, in fact, significant NMJ damages are expected thus allowing to obtain a further confirmation of the validity of the method and to test its sensitivity. The first experimental results showed that transgenic muscles presents a significant slowdown of the kinetic parameters if directly stimulated, and a further slowdown in case of nerve stimulation.

Analysis of force-frequency curves revealed significant differences between WT and transgenic muscles. At the tetanic frequency a decrease of about 20% was reported for SOD1^{G93A} Soleus specific force, in comparison to the controls. Once again, a worsening in the TG Soleus response was reported when stimulated through the nerve. A significant decrease of specific forces was, in fact, measured for all the tested frequencies. The maximum specific force developed in case of nerve stimulation appears halved if compared to the direct stimulation. These results highlight the sensitivity of the method to discriminate even small temporal differences and to separate the components of muscle contraction due to muscle inner damages from the ones due to NMJ conduction defects.

The analysis of the forces measured during the 80 Hz fatigue paradigm pointed out a limitation of the method when calculating intratetanic fatigue at the end of the protocol. In fact, in literature this parameter is measured during repeated nerve stimulations of about 2.5 minutes. We tried to calculate the IF during the entire fatigue protocol and we observed that at the beginning the generated forces are always of the order of tens of mN so it is possible to reliably measure the force decrease within the same pulse train. On the contrary, at the end of the same paradigm, the muscles were exhausted and the contraction forces developed in case of nerve stimulation were near to zero, especially in the transgenic model. In this situation, the sensitivity of the method resulted heavily reduced, and the *IF* values calculated from the seventh minute on, were the forces are about 7 mN, basically expresses noise. For this reason we are evaluating to shorten the paradigm. See figure 7.

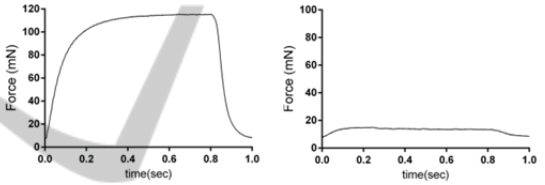

Figure 7: Maximum force developed by the same specimen at the first nerve stimulation end at the last nerve stimulation during the fatigue paradigm.

Because of this reason, I decided to compute NF and IF only in the first seven minutes of stimulation of the 80 Hz fatigue protocol. Results showed higher IF values, starting from the first stimulations, in transgenic muscles stimulated trough the nerve compared to the WT model.

On the contrary the analysis of Neurotransmission Failure did not show any alteration in the transgenic model. A possible explanation of this may be that at the end-stage of the pathology the transgenic skeletal muscles are also severely compromised and can hide the NMJ defects.

In conclusion, the proposed experimental technique allows to determine the NMJ functionality separately from the muscle contractile properties in isolated muscle-nerve preparations of pathological mouse models. Preliminary results obtained from the SOD1^{G93A} model are in accordance with the literature, showing muscle contraction defects and NMJ impairment.

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