Indirect Estimation of Persistent Inward Currents in Patients with Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects motor neurons and leads to muscle atrophy and eventually to death. Motor neurons in ALS disease are hyperexcitable and may die from excitotoxicity. About 10% of ALS cases are familial and linked to mutations in several genes. Mice with mutations in superoxide dismutase 1 (SOD1) remain the best currently available model to investigate ALS. It has been shown that motor neurons of SOD1 mice become intrinsically hyperexcitable at several stages of the disease. Persistent inwards currents (PIC), large voltage-activated currents amplifying motor neuron response to synaptic inputs, have been investigated in these mice and found enhanced. This study investigates for the first time whether PIC are enhanced in ALS human patients compared to healthy subjects, and whether PIC can contribute to motor neuron excitability and subsequent cell death in patients suffering from ALS. A non-invasive method was used to estimate PIC in human, based on paired motor unit recordings and the measure of firing frequencies. PIC are proportional to the difference between the firing frequency of a control unit at the recruitment of a test unit and firing frequency of the control unit at decruitment of this same test unit (ΔF). This ΔF is obtained following two paradigms: one where the subject voluntarily recruit the test unit by increasing its level of contraction, the other one where the test motor unit is recruited via reflex loop activated by muscle tendon vibration. We estimated PIC in four muscles: the tibialis anterior, the quadriceps, the extensor carpi radialis and the triceps brachii. We found in healthy subjects that ΔF varied greatly from one muscle to the other. Preliminary results show that PIC tend to be enhanced in patients, but the difference was not large enough to make it significant. However, the level of contraction was significantly higher in the patients, and the hypothesis that this was due to an increase in the potentials amplitude must be checked.
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INTRODUCTION

1. Overview

Amyotrophic Lateral Sclerosis (ALS), also known as Charcot’s disease from the French neurobiologist who described it for the first time in 1869 or Lou Gehrig’s disease from the famous baseball player, is a progressive neurodegenerative disease. In ALS, motor neurons gradually degenerate and die, which causes the weakness of muscles and atrophy. Eventually, all muscles under voluntary control are concerned. When the muscles in the diaphragm are reached, the individual cannot breathe without ventilatory support. Most people die within three to five years after diagnosis from respiratory failure.

There is currently only one treatment called riluzole, but it extends life for a few months only and it doesn’t improve the living conditions. The mechanisms behind the degeneration of motor neurons are not well understood. This is why it is so important to investigate and to better understand the mechanisms of ALS in order to develop new therapeutic approaches.

Even though the mechanisms behind riluzole are not well identified, it has been shown that it interferes with motor neuron excitability. Persistent inward currents (PIC) - large voltage-activated currents amplifying motor neuron response to synaptic inputs – also interfere with motor neuron excitability and several studies suggest that they might be involved.

2. Purpose and objectives

First, the estimation of PIC in motor neurons supplying upper and lower limb muscles in healthy subject muscles will be performed in order to compare one muscle to another. This has never been studied before.

Then, the main objective of this study is to investigate the mechanisms behind the increased excitability of motor neurons and to see whether participants with ALS display increased PIC compared to healthy subjects. The force of contraction was also investigated during the protocol.

A hypothesis is that amplified PIC might occur in ALS patients, contributing to motor neuron hyperexcitability. This has never been investigated in patients suffering from ALS so far.
BACKGROUND
1. Amyotrophic lateral sclerosis

1.1 Initial forms of disease

The disease can begin according to three forms. The forms can develop simultaneously, but in every case, the disease progresses towards a complete form where all of these forms coexist.

The spinal form is the most common with 70% of cases [Vucic et al. 2014, Kiernan et al. 2011]. It encompasses the superior motor neuron syndrome, which is characterized by muscle spasticity, abnormal deep tendon reflex and the Babinski and/or Hoffman reflex. Inferior motor neuron syndrome, characterized by muscle weakness, fasciculations and amyotrophy in the upper or/and lower limb [Vucic et al. 2014, Kiernan et al. 2011, Pradat et al. 2010]. The first affected muscles are at the extremities (hand, foot; distal muscles) and then the disease progresses to proximal muscles (arm, leg, trunk).

The bulbar and pseudo bulbar forms represent 25% of initial cases. While weakness of throat, mouth and tongue muscles lead to language impairment, change of voice, hyper-salivation and difficulty to swallow in the bulbar form, the pseudo-bulbar form is characterized with an emotional hypersensivity, with sudden crying and laughter [Vucic et al. 2014, Kiernan et al. 2011, Pradat et al. 2010].

The axial and respiratory form represents 5% of cases. This form targets the neck, trunk and respiratory muscles.

All the initial forms of ALS can evolve to respiratory trouble at the final stage of the disease [Kiernan and al. 2011, Pradat and al. 2010].

1.2 Epidemiology

ALS incidence in the world is 2/100 000 and had a prevalence of 4-5/100 000 [Riva and al. 2016]. Three locations of very high incidence (50 to 100 times higher than the mean value) have been found around the globe, all in the Pacific Ocean: New Guinea, Guam and Kii Peninsula.

1.3 Risk factors

Genetics

ALS can be either sporadic (90% of cases) or hereditary (10% of cases) [Siddique & Ajroud-Driss 2011]. Mutations in at least 10 genes and 4 chromosomal loci are linked to the familial forms of disease. Among them, the mutation in the superoxide dismutase 1 (SOD1) represents 20% of familial forms. The SOD1 mouse model remains the best model to study motor neuron degeneration in ALS.
A few differences have been noticed between these two forms. First, the initial symptoms appear between 60 and 85 years old for the sporadic form, while the symptoms of the familial form appear earlier, between 47 and 52 years old [Kiernan et al. 2011]. Moreover, there are more men affected by the sporadic form than women (ratio between 1.3 and 1.5), but not in the familial case where men are as affected as women.

**Environment factors**

Exposure to chemicals compounds, pesticides, metals, bacteria, virus, electromagnetic fields and electric shock might contribute to developing the disease.

**Lifestyle**

Cancer, diabetes and neural inflammation might contribute to the development of ALS. The role of tobacco is controversial but some state it might be a risk factor for women only. Antioxidant rich diet might decrease the risk of contracting ALS. Clinical observations tend to show that high level sport athlete seem to be more concerned by the disease.

1.5 Therapeutic approaches

Riluzole (2-Amino-6-(trifluoromethoxy)benzothiazole) was originally marketed in the 1950s as a muscle relaxant and in the 1980s as an anticonvulsant and neuroprotective drug. It remains the only Food and Drug Administration approved treatment for ALS since 1995. It prolongs median survival by about 2 to 3 months in patients with amyotrophic lateral sclerosis, but it doesn’t improve living conditions [Miller et al. 2012].

Even though mechanisms behind this drug are not very-well known, it is acknowledged that riluzole interferes with glutamate processes (decrease in glutamate release and desensitization of post-synaptic receptors) and decrease the intrinsic and extrinsic motor neuron excitability [Bellingham et al. 2013]. Indeed an increase in ALS motor neuron excitability is one of the causes of their degeneration.

2. Excitability of motor neuron

2.1 Definition

The excitability of motor neurons depends on both extrinsic and intrinsic properties. An increase in excitability can thus result from a hyperexcitability (intrinsic) or hyperexcitation (extrinsic). Hyperexcitability is an enhancement of the response evoked by a synaptic input. It results from a change in electric membrane properties. Hyperexcitation is defined as an imbalance between excitatory and inhibitory inputs from neurons and interneurons.
2.2 Excitotoxicity

The increase in excitability of motor neurons can lead to a calcium-dependent event cascade that leads to its degeneration. This is called excitotoxicity, which is one of the causes of motor neuron death in ALS. Excitotoxicity can happen through an increase in calcium influx, a saturation of calcium storage capacity or buffer capacity by mitochondria or endoplasmic reticulum [Ilieva et al. 2009].

2.3 Excitability in ALS motor neurons

Intrinsic properties of motor neurons are related to their electric membrane properties that can be either passive (at rest) or active (following synaptic inputs). These properties have been investigated in ALS motor neurons.

It has been shown that passive properties responsible for the resting potential are not modified in ex and in vivo model of embryonic SOD1 mice [Kuo et al. 2005], neonatal [Van Zundert et al. 2008], presymptomatic adult [Delestrée et al. 2014], and also in human ALS motor neuron derived from stem cells [Wainger et al. 2014].

Active properties are responsible for the characteristics of action potential such as the rheobase (minimal current to trigger an action potential), the amplitude and duration of the action potential, the amplitude and duration of afterhyperpolarization (AHP) and the gain of the graph representing the firing frequency over the injected current (F-I). Studies suggest an increase in active intrinsic excitability in ALS motor neurons. Indeed, it has been shown in SOD1 mice: a reduction of the rheobase [Kuo et al. 2005], no change of the amplitude and duration of the action potentials and AHP [Kuo et al. 2005, Van Zundert et al. 2008], an increase of the firing rate [Van Zundert et al. 2008] and the F-I gain [Kuo et al. 2005, Van Zundert et al. 2008]. Similar results have been found in in human ALS motor neuron derived from stem cells [Wainger et al. 2014].

Consequently, the increased excitability in ALS motor neurons is at least due to a change in active intrinsic properties and may also be due to extrinsic properties with an imbalance of nerve impulses.

3. Persistent inward currents

Persistent inward currents highly interfere with motor neuron intrinsic excitability.
3.1 Definition

Swindt and Crill demonstrated that motor neurons are not simple integrators and fire cells. When depolarizing slowly the motor neuron membrane, the current over voltage graph shows a curve that has a “N” pattern (figure 1) which indicates that ions channels have opened and that less current is necessary to depolarize the motor neuron [Schwindt & Crill 1977; Alaburda et al. 2002]. This shows the presence of PIC, large voltage activated currents amplifying motor neuron response to synaptic inputs. Motor neuron membrane can then be in two different stable states for a same imposed current. This membrane bistability is seen in the current over voltage curve, where for a definite range of current, two values are possible for the membrane voltage.

![Figure 1: PIC in motor neurons](image)

**Figure 1: PIC in motor neurons**
A: Response of the membrane current to a ramp voltage in a motor neuron
B: Current over voltage graph from data in A
The arrow “onset” shows the activation of PIC and “offset” the deactivation of PIC [adapted from Lee & Heckman 1999]

As the input of motor neurons is current and the output is the frequency rate, the activation of PIC increases the gain of the input-output function, as seen in figure 2. The hysteresis shows that PIC stay activated below the level of synaptic input that activated them. Consequently, the intrinsic motor neuron excitability is increased and the amount of synaptic current needed to recruit the motor neuron is reduced.
Figure 2: Effect of PIC on a motor neuron F-I function

$p$ corresponds to the primary range before the activation of PIC, $s$ corresponds to the secondary range during the activation of PIC, and $t$ to the tertiary range after full activation of PIC [taken from Li and al. 2004]

Also, this lasting activation of PIC causes depolarizations called "plateau potentials", and allow motor neurons to respond to brief synaptic input with prolonged firing activity, even after cessation of the input (figure 3) [Gorassini and al 1998]. Without PIC, the test unit would cease firing after the end of the input.

Figure 3: Plateau potential generated by PIC

The blue trace is taken while the cell was held hyperpolarized and indicates the time course of the applied synaptic input; the red trace was taken at more depolarized levels where the PIC can be activated. Lower panel: the synaptic input activates a strong persistent inward current when the cell is voltage clamped at a level where spikes are generated in unclamped
conditions. *Middle panel:* When spikes are blocked in current clamp, the PIC generates a sustained plateau potential. *Upper panel:* When the cell is allowed to fire normally, the persistent inward current drives self-sustained firing of the motor neuron [adapted from University of Colorado 2004]

An inhibitory synaptic input hyperpolarizes the membrane potential and deactivates the PIC.

### 3.2 Types of persistent inward currents

There are two kinds of PIC: the calcium PIC and the sodium PIC.

**Calcium PIC**

It has been shown in turtle motor neurons that PIC are partly due to type “L” calcium channel [Hounsgaard & Mintz 1988]. They were then similarly described in mouse motor neurons [Carlin et al. 2000a; b], guinea pig motor neuron [Hsiao et al. 1998], and rat motor neuron [Li et al. 2004]. L type calcium PIC can be activated at different levels of voltage (subthreshold, threshold and suprathreshold).

**Sodium PIC**

On the contrary sodium PIC are activated with a subthreshold potential [ElBasiouny 2010]. Sodium PIC were described in rat [Li & Bennett 2003] and guinea pig [Hsiao et al. 1998]. Sodium PIC are activated instantaneously while calcium PIC have a low constant of activation [Li et al. 2004].

### 3.3 Neuromodulation of persistent inward currents

It was shown that PIC are the result of endogenous neuromodulators that act via metabotropic receptors [Heckman et al. 2009]. Contrary to ionotropic receptors, metabotropic receptors do not directly open an ion channel when binding with neurotransmitters. Instead, their binding initiate a cascade (more often beginning with a G protein- in this case the metabotropic receptor is a G protein coupled receptor), that can lead to channel opening or other cellular effects. Since opening channels by metabotropic receptors involves activating a number of molecules in turn, channels associated with these receptors take longer to open than ionotropic do, and they are thus not involved in mechanisms that require quick responses. The activation of metabotropic receptors lasts from seconds to minutes, which is much longer than the activation of ionotropic receptors that lasts only a few milliseconds. Concerned neuromodulators are mostly serotonin and norepinephrine that are released by axons originating in the brainstem. Contrary to classical synaptic transmission where a pre synaptic neuron sends neurotransmitters to a post synaptic neuron, neuromodulation is a process by which a neuron influences a group of neurons with neuromodulators. Indeed, the neuromodulators are not either reabsorbed by the pre synaptic neuron or broken down like it is the case with neurotransmitters in classical neural transmission. Neuromodulators end up in the cerebrospinal fluid where they can reach by diffusion other neurons. The facilitation of PIC in motor
neurons occurs via 5HT2 receptors and NE alpha 1 receptors [Heckman et al. 2005]. The level of monoaminergic drive controls the amplitude of PIC. Indeed, the range of enhancement, two- to six-folds, is entirely dependent on the level of monoaminergic input to the cord [Lee & Heckman 2000].

![Figure 4: Influence of the level of monoaminergic drive on PIC](image)

Effective synaptic current as a function of voltage in four different cells. Synaptic currents were obtained during a linearly rising voltage ramp. Unlike voltage clamp conventions, excitatory current is positive (upward). Low and high threshold motor neurons refer to the level of their rheobases. The intrinsic I-V function of the cell was subtracted to only reveal the influence of the PIC on the effective synaptic current [adapted from Heckman et al. 2005]

It is important to notice on the figure 4 that PIC are activated in a much more polarized level in low threshold motor neurons compared to high threshold motor neurons.

![Figure 5: Frequency-current relations for high, medium and low monoaminergic drive](image)

[adapted from Heckman et al. 2005]
The onset of PIC is seen when the slope of the linear function increases. PIC are progressively activated during this secondary range of firing rates. Full activation of PIC is seen when the slope of the linear function changes again and decreases compared to the secondary range, corresponding to a tertiary range. For high monoaminergic drive, no primary range is seen as the level of activation of PIC is around threshold for firing [Heckman et al. 2005].

The low monoaminergic level probably corresponds to a sleeping state while the medium level corresponds to a moderate motor activity and the high level to a state of arousal occurring during situations of emergencies [Heckman et al. 2005].

3.4 Hypothesis about persistent inward currents in amyotrophic lateral sclerosis

Several arguments can lead to think that PIC are enhanced in ALS patients.

Embryonic motor neuron cells from SOD1 mice showed an increase in the excitability of type F (fast) motor neurons via an increase of sodium PIC [Kuo et al. 2005]. Moreover, an increase of calcium PIC has been shown in vivo in pre symptomatic adult SOD1 mice [Delestrée et al. 2014]. The increase of calcium PIC was also shown in neonatal mice.

Our hypothesis is that mechanisms observed in the SOD1 mice are the same in human suffering from ALS. PIC have never been investigated in human ALS before.

Furthermore, ALS patients have a defect of serotonin compared to healthy subjects [Dentel et al. 2013]. As serotonin is one of the neuromodulators of PIC, it is likely that a change of serotonin will impact the amount of activated PIC.

Our main objective was thus to estimate PIC in ALS patients and to compare them with healthy subjects.

4. Indirect estimation of persistent inward currents in humans

In human, it is not possible to measure directly PIC with intracellular recordings. Instead, the firing rate of motor units is used.

4.1 Motor unit

A motor unit is composed of one motor neuron innervating several muscle fibers. Each individual muscle fiber is connected to only one motor neuron. When the motor neuron produces action potentials, all the muscles fibers innervated by this motor neuron contract simultaneously.
The motor units are classified in four types, regarding their resistance to fatigue and their speed of contraction:

- Fast fatigable (FF): high force, fast contraction speed but fatigue in a few seconds
- Fast fatigue resistant (FR): intermediate force, fatigue resistant, fast contraction speed and resistant to fatigue
- Fast intermediate (FI): intermediate between FF and FR
- Slow (S): low force, slower contraction speed, highly fatigue resistant

The force of contraction is controlled by the number of activated motor units. The Henneman’s size principle states that motor units are recruited following a special order that depends on their size, from small and slow to large and fast motor units. Technically this means that first S type motor units are recruited, followed by FI, then FR and finally FF motor units.

![Two motor units](image)

**Figure 6: Two motor units**

Two motor neurons (cell bodies in red in the spinal cord) innervate several muscle fibers in the muscle. All muscle fibers from MU1 (respectively MU2) are recruited simultaneously, but the MU1 and MU2 muscle fibers are not recruited at the same level of synaptic drive (different thresholds of motor neurons).

### 4.2 Use of the firing rate $\Delta F$

The firing rate of motor units is used as an estimate of the synaptic drive it receives. The activation of two motor units with different thresholds is needed. If two motor units receive a common synaptic input, the firing rate of the lower threshold motor unit reflects also the level of input to the higher threshold motor unit.

The test unit is recruited and decruited at intensity levels of $I_r$ and $I_d$. The difference between $I_r$ and $I_d$ is equal to the amount of PIC activated in the test unit. The test unit is recruited after PIC have been fully activated in the control unit in order to be in the tertiary range in figure 5. Indeed, after full activation of PIC, the relation between intensity and firing frequency is linear. This leads to PIC in the test unit being directly proportional to the difference of firing rates of the control unit at recruitment and decruitment of the test unit. This difference $\Delta F$ gives an estimate of the level of PIC activated in the test motor unit (figure).
\[ \text{PIC} = \text{Ir} - \text{Id} = K^*(F1-F2) = K^* \Delta F \]

This method using $\Delta F$ is known as the paired motor unit technique.

**Figure 7: F-I function for the test and the control unit**

A: F-I function of the low threshold unit  
B: F-I function of the high threshold unit

$\text{Ir}$ represents the intensity needed to recruit the high threshold motor unit and $F1$ the corresponding firing rate of the low threshold unit; $\text{Id}$ represents the intensity at which the high threshold is recruited and $F2$ the corresponding firing rate of the low threshold unit [adapted from Li et al. 2004]

### 4.3 $\Delta F$ values in the literature

The measure of $\Delta F$ was only used on the tibialis anterior (TA; ankle dorsiflexor) and soleus (ankle plantarflexor) before. Gorassini et al. found a value of on average 3.9 Hz in the TA and 3.1 Hz in the soleus in healthy subjects [Gorassini et al. 2002a].

Another objective of our study is to estimate PIC on healthy subjects and compare them with the literature, and then to compare them with PIC in three other muscles that has never been investigated before: the quadriceps (knee extensor), the triceps brachii (elbow extensor) and the extensor carpi radialis (ECR; wrist extensor).
METHODS
1. Participants

The experiment was conducted in 10 patients and 15 healthy subjects. All gave informed consent and all procedures were approved by the local ethics committees (CPP Ile de France VI – Pitié-Salpêtrière and ANSM). The protocol is identified with ID RCB is 2014 – AO1240-47.

The participants were divided into two groups: the ALS patients and the healthy subjects. The patients were all at an early stage of the disease; most of them have just been diagnosed (approximately around one year after the beginning of symptoms). They generally felt the beginning of the disease with an impaired forearm or leg. They could have weakness in foot/ankle muscles and in hand/wrist muscles but not in arm and thigh muscles which were not clinically affected.

Before the recording, a laterality test [Oldfield 1971] was performed to determine which side (right or left) is dominating. The experiment was then conducted on this side for the healthy subjects. For the patients, the side was chosen not only with the laterality test but also with their level of atrophy. To do so, we relied on the clinical evaluation in which muscles of the limbs were quoted from 1 to 5 (scale developed by the Medical research council –MRC), regarding the muscle strength. If the muscle were quoted similarly, we chose the dominant side. If not, we chose the strongest proximal muscle.

2. Material

We recorded surface and intra muscular electromyograms (EMG) which corresponds to the electrical activity of muscles.

The surface EMG was recorded with the use of surface electrodes (EURO ECG ELECTRODES, FIAB, Florence, Italy) – figure 8 – that were glued to the muscle of interest. They were clipped together with a WIFI electrode (ZeroWire EMG, Aurion Srl, Milan, Italy) and they amplified the signal (x5000) and filtered it between 10 Hz and 500 Hz.

![Surface electrodes clipped together with the WIFI electrode](image)

Figure 8: Surface electrodes clipped together with the WIFI electrode

The needles (Paired Hookwire Electrodes 50mm, SGM\textsuperscript{TM}, Split, Croatia) were used to insert polytetrafluoroethylene insulated stainless steel wire into the muscles, then the wires were connected to the WIFI electrodes (ZeroWire EMG, Aurion Srl, Milan, Italy) – figure 9 – which bandpassed the signal between 10 Hz and 1000 Hz.
The signals passed through a 50/60Hz noise eliminator (HumBug Noise Eliminator, Quest Scientific, Vancouver, Canada).

Then they were transmitted to the computer with the Micro 1401 mkII (CED, Cambridge, England) where they were recorded with a sample rate of 2000 Hz by the software Signal (6.03c x64) (minimal sampled rate required with the Nyquist-Shannon sampling theorem).

The vibrator used was the Vibrasens (Technoconcept, Marseille, France). The vibrator induces a reflex that recruits several motor units.

3. Experimental protocol

We used the paired motor unit technique in four different muscles: two from the superior limb, a proximal (triceps brachii) and a distal (ECR); two from the inferior limb, a proximal (quadriceps) and a distal (TA).

The participants were asked to sit comfortably in an armchair. Once set, they were asked to perform the maximal voluntary contraction (MVC) in order to later calculate the relative force of contraction. They were asked to maintain for a few seconds their maximal contraction in order to see a plateau on the screen. We asked the subjects to perform several times the MVC to make sure we had the same plateau level. The force was calculated from surface EMG that was rectified and averaged over 200 ms. The MVC could have been done with intramuscular electrodes, but for comfort reasons we used the surface electrodes to avoid any pain.

After the MVC was recorded we began the intramuscular recordings. We recorded the intramuscular EMG with a needle in which are hooked PTFE insulated stainless steel wires so that when we take back the needle, the wire stays into the muscle and transmits muscle activity.

The subjects were able to see their level of contraction as well as their intramuscular EMG. They also had auditory feedback generated according to EMG activity to help them maintain a constant level of force. The EMG passed through a window discriminator with upper and lower levels. The EMG activity within these two levels triggered a sound: the greater the number of triggering the higher the sound. We trained the participants a few minutes to perform very low contraction before recording.

The protocol was divided into two parts: the voluntary contraction and the vibration.
Voluntary contraction

In the literature, the subject was asked to perform an isometric torque contraction (figure 10). This way, the subject recruits first the lower threshold motor unit (the control unit) and then a higher threshold motor unit (the test unit).

![Figure 10: Firing rate of two motor units during the voluntary contraction protocol](image)

Force of contraction (top) and firing rate of the control unit (bottom) and the test unit (medium). The test unit is recruited at a higher control unit firing rate than when it is decruited. This difference is $\Delta F$ and gives an estimation of PIC activated in the test unit.

We slightly modified this theoretical protocol based on the advices of Monica Gorassini’s team. The subjects were asked to contract their muscle as little as possible until they triggered the firing of the control unit. They should maintain this motor unit for around 10 seconds keeping their level of contraction steady. Then they were asked to contract a bit stronger to trigger a higher threshold motor unit (the test unit). After activation of the test unit, they were asked to maintain their level of contraction to keep the test unit firing for 10 seconds. Finally, they were asked to slowly decrease their level of contraction in order to decruit the test unit without decruiting the control unit, and then at last to decruit the control unit. This was performed 5 times. It was shown that after a motor unit is decruited, less activation is required to rerecruit it within 6 seconds [Gorassini et al. 2002b]. Thus we always waited 1-2 minutes between each data acquisition.
Vibration

It began the same way, with 2 seconds of rest and recruitment of one motor unit (the control unit). But instead of keeping it during approximately 10 seconds and then performs a tougher contraction, the subject was asked to maintain the contraction steady. We applied the vibration on the tendon of the muscle while the subject maintained the contraction, and that recruited through reflex activation other motor units (all potential test units). To decruit the test motor unit, the subject had to slowly decrease its force of contraction (figure 11). This protocol was repeated 5 times also.

![Figure 11: Firing rates of two motor units during the vibration protocol](image)

The vibration induces the same effect as the voluntary contraction. The test unit is recruited at a higher control unit frequency than when it is decruited and the difference ΔF gives an estimation of PIC activated in the test unit.

To ensure complete activation of the PIC in the control unit, we always considered a test unit whose recruitment occurred at least a few seconds with conditioning after the control unit was recruited.
4. Data analysis

The first step consisted in evaluating the MVC. I took the average over a few seconds, preferably on a plateau. I did not take into account the first instantaneous peak when increasing the force contraction because it is due to FF muscles fibers and the force produced decrease quickly after that (figure 12). For instance in the figure below, the peaks can be easily distinguished from the plateaus. The taken value was the average between each couple of cursors.

![Figure 12: Example of data recorded during maximum voluntary contraction. Surface EMG has been rectified and averaged over 200ms. Note the difference between the large peaks due to fast fatigable motor units and the plateaus due to slow motor unit](image)

Then I analyzed the recording data (figure 13) for each acquisition using the software Spike2 (8.07; Cambridge Electronic Design, Cambridge, United Kingdom).
Given that we estimated the force during the maximum voluntary contraction, it was then possible to calculate the percentage of force used to recruit the motor units. To do this I created a virtual channel whose formula was:

\[
\text{virtual channel(\%)} = \frac{\text{mean} - \text{baseline}}{\text{MVC}} \times 100
\]

The baseline was calculated using the mean over a few seconds when no contraction was performed.

Then we wanted to distinguish the recorded motor units from one another. Spike2 software allows us to define a time range as well as an amplitude range (figure 14). Then it can recognize the different patterns whose spikes end between the low and high threshold values. If there are different shapes because several motor units have the same amplitude, Spike2 distinguish them and associate each of them with a code. Sometimes two shapes can represent a same motor unit; in this case we can chose to fuse several shapes. After that it is necessary to review one by one each pattern and to check that it is well classified, and if not to manually reclassify each potential one after the other.
Figure 14: Example of pattern recognition
Here the low threshold was put to 0.27V and the high threshold to 0.75V, which corresponds to the action potential amplitude of the control unit.

After the different motor units have been correctly classified, it is possible to display their firing rate. The firing rate of each discriminated motor unit was calculated as the reciprocal of the interval between each motor unit action potential and the motor unit action potential preceding it (red dots for the control unit, blue dots for the test unit) (figure 15).

Figure 15: Firing rate of the paired motor units
In red, the instantaneous frequency of the control unit
In blue, the instantaneous frequency of the test unit
In each selected file, I recorded the level of force contraction at recruitment of the control unit, recruitment and decruitment of the test unit as well as the control unit firing frequency at recruitment and decruitment of the test unit. In order to limit the random choice of one specific instantaneous frequency value, we fitted the data to a 5th order polynomial in the zone of interest (best outcome in terms of fit).

![Figure 16: Use of a 5th order polynomial to smooth the instantaneous frequency of the control unit](image)

5. **Statistical analysis**

For each couple of motor units, I calculated the mean of the corresponding ΔF.

For the healthy subjects, I compared the means of ΔF between the different muscles to see whether the ΔF depends on the analyzed muscle or not.

Then I performed a two way analysis of variance ANOVA on the mean of all ΔF (patients and healthy subjects) with the factors “muscle” (TA, quadriceps, ECR or Triceps) and “subject” (healthy or patient). We also performed a two way ANOVA on the MVC with the factors “muscle” and “subject in order to determine whether the MVC was statistically different or not between healthy subjects and patients. The software SigmaPlot (12.5) was used.

Then a multiple linear regression was made on SigmaPlot to find the significant factors that impact the contraction level at recruitment of the test unit.

The limited p value was at 0.05 for significance.
RESULTS
1. Tables of collected data

For every muscle and each protocol, I collected and calculated data in a table. There is all in all eight tables, below are the tables for the TA muscle.

### Table 1: Data collected for the contraction protocol in the TA muscle

<table>
<thead>
<tr>
<th>Subject</th>
<th>Healthy subject / Patient</th>
<th>MRC</th>
<th>Duration after diagnosis (month)</th>
<th>MVC (microvolts)</th>
<th>Pair of MU</th>
<th>Force % recruitment U1</th>
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### Table 2: Data collected for the vibration protocol in the TA muscle

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Each subject is designed by a code, and it is indicated how long since they have been diagnosed, the calculated forces at recruitment and decruitment of the motor unit, and the mean of the calculated $\Delta F$.

2. **Persistent inward currents in different muscles in healthy subjects**

PIC have never been investigated in human quadriceps, ECR and triceps before. I have plotted the means of $\Delta F$ calculated for each muscle in healthy subjects with the error bars (figure 17).

![Figure 17: Comparison of $\Delta F$ in different muscles in healthy subjects](image)

A first result is that $\Delta F$ are not similar between different muscles in healthy subjects, the level of PIC activated varies significantly ($p<0.001$) from one muscle to the other.

3. **Persistent inward currents in patients compared to healthy subjects**

The ANOVA on the $\Delta F$ indicated that the subject was not a significant factor ($p=0.255$). On the contrary, the muscle was a significant factor ($p<0.001$), and this is seen with both figures 17 and 18 where $\Delta F$ depends greatly on the analyzed muscle.

Below are the means of $\Delta F$ in patients in comparison with healthy subjects for both the contraction and the vibration protocol.
On average, PIC tend to be slightly higher (0.4 Hz higher) in patients than in healthy subjects, but the difference is not great enough to make it significant. However, we notice that in the TA PIC are clearly higher in patients compared to healthy subjects. We then performed a t-test among the TA muscle only to see whether the difference was significant, and the result is positive, with p=0.004.

4. Maximum voluntary contraction in patients compared to healthy subjects

The ANOVA showed that the muscle was a significant factor for the MVC, as expected. The subject was not a significant factor, which means that there was no statistically difference in the MVC between patients and healthy subjects.

5. Level of contraction at recruitment of the motor units in patients compared to healthy subjects

The multiple linear regression on the level of contraction to recruit the test unit showed that not only the contraction level at recruitment of the control unit was a significant factor (p<0.001) but also that the subject was a significant factor (p<0.001). This is a very interesting result that allows distinction between patients and healthy subjects. The muscle was not a significant factor contributing to the contraction level at recruitment of the test unit (p>0.05).

For each group of subject, we did a linear regression between the two levels of contraction. We found a higher slope for the patients compared to the healthy subjects.
Healthy subjects: Force recruitment Test Unit = 3.12 + 1.44 * Force recruitment Control Unit

Patients: Force recruitment Test Unit = 7.75 + 1.94 * Force recruitment Control Unit

This is illustrated in figure 19.

Figure 19: Linear regression between the levels of contraction of the control unit and the test unit for the patients and the healthy subjects
DISCUSSION
The results in healthy subjects showed the $\Delta F$ cannot be compared between different muscles, while the study in patients did not prove any significant difference between them and the healthy subjects. However a significant difference between patients and healthy subjects was the level of contraction.

1. **Persistent inward currents in healthy subjects**

The literature only had values around 3.9 Hz for the TA and 3.1 Hz for the soleus, based on one study [Gorassini et al. 2002a]. We found lower values for the TA muscle in the healthy subjects (2.3 Hz). A possible explanation for this difference might be the age of the participants. In our study, healthy subjects were around 61 years old to best match with the age of patients while in Gorassini’s study the mean age was 28 years old. PIC might decrease with age; it would be interesting to test this theory in another study on healthy subjects.

Moreover, the results prove that we cannot compare values between different muscles as they vary significantly from one muscle to the other (2.3 Hz for the TA; 3.5 Hz for the quadriceps; 3.3 Hz for the ECR; 5.0 for the triceps). Further studies must then be very cautious when comparing values to make sure they are related.

2. **Persistent inward currents in patients**

As seen in the results, there is no significant difference between patients and healthy subjects regarding the $\Delta F$ values in all muscles. That means that PIC are apparently not increased in ALS patients and that hypothesis is not validated. However this is not true if we only consider the TA muscle where the difference between patients and healthy subjects is significant. Several comments must be made about those results.

First, our patients had mostly a spinal form of ALS, so their affected muscles were mainly TA and/or ECR. Unfortunately it is not clear to know which muscles exactly are the most affected. Indeed, the clinical test evaluate with a grade out of 5, but a loss of force equal to 60% has to be reached to change the grade from 5 to 4. As our patients had mostly just been diagnosed, they were mainly quoted with 5. It seems logical that the TA displays an increased $\Delta F$ compared to the triceps and the quadriceps; however the ECR should too.

Another consideration to take into account is the fact that ALS targets first the large motor neurons before targeting the small ones as well. We only did the experiments on patients that have just been diagnosed. A hypothesis is that their small motor neurons might have not been affected by the disease yet. As we only asked for little contractions, we might have recruited only small motor neurons that have a normal level of activated PIC. To estimate PIC in affected motor neurons it would be then necessary to studied a couple of motor units at higher level of contraction. However, at higher contraction, the EMG is very dense and it not possible to distinguish motor unit patterns from one another. It may be interesting to investigate PIC in patients at later stage of the disease to make sure we are not making measures on healthy motor neurons.
Furthermore, a consideration to take account of is that sodium PIC activate at a subthreshold level, which means that they are not included in the $\Delta F$ measure. Calcium PIC activate at several levels, including suprathreshold. We can thus only make conclusions about those. It is possible that only sodium PIC may be increased in ALS patients while calcium PIC remain at a normal level. In such a case the measured $\Delta F$ would show no difference between healthy subjects and patients.

Finally, my results are only preliminary. Indeed, only 10 patients and 15 healthy subjects have been included so far, whereas at the end of the study they will be 30 in each group. The minimal number of patients is 20 to have conclusive results. Furthermore, the average is 0.4 Hz higher for the patients than for healthy subjects. It is thus not possible to exclude the fact that the $\Delta F$ may be significantly different at the end of the study.

3. Validity of the method

$\Delta F$ has been validated as an accurate estimate of PIC magnitude in chronic spinal rats [Bennett et al. 2001].

However, there are downsides to this method.

First, the fact that PIC are graded (and not all-or-none) may affect the experimental paradigm. Indeed, the PIC measured with this method are only suprathreshold, which means they activate at a higher level of synaptic drive than the one required to release action potentials. There is currently no method to estimate subthreshold PIC in human.

Another limit of this method is the firing rate limitation. Indeed, each motor unit has a rate limitation: when increasing continuously the synaptic drive, the firing rate will also increase until a certain point. This limitation may diminish the $\Delta F$ values obtained.

Spike frequency adaptation (SFA) is a motor neuron property that might contaminate $\Delta F$ values [Vanderberk & Kalmar 2014]. When stimulating the motor neuron with a constant input, the firing frequency will be higher at the beginning before decreasing slowly until it reaches a steady state. This phenomenon is called SFA. If the control unit has not yet reached its steady state before the test unit is recruited, the measure of the control unit firing rate at recruitment of the test unit will be overestimated, and so would be the $\Delta F$. When we conducted the experiments, we tried as much as possible to wait until a steady state was observed in order to limit the effect of SFA on our measures.

4. Level of force contraction

We also investigated the differences in levels of contraction between patients and the healthy subjects.

The MVC was not significantly different between the two groups. However we were surprised to observe that the contraction level at recruitment of the test unit depends significantly on the subject
The linear regression between the levels of contraction at the recruitment of the two motor units is seen in figure 19. The slope for the patients is much higher than for the healthy subjects. However our model is based on the force calculated from the EMG.

A study was conducted about the relation between force and EMG in patients with ALS. They found that the slope of the EMG-Force relation was shallower compared to healthy subjects. However if they excluded the patients at a very advanced stage, the slope was comparable to healthy subjects [Jahanmiri-Nezhad and al. 2014]. That means that with the progression of the disease, the slope decreases.

In our study, we only worked with early stage patients. We can thus imagine that the slope for the early stage patients is higher than for healthy subjects, and then it progressively decreases until it becomes lower. This would be possible with the reinnervation that occurs at the beginning of the disease, and which results with high amplitude potentials [Milner-Brown et al. 1974]. As the force was calculated with the amplitude of the potentials, this would explain why we found a significantly higher force at recruitment of the test unit for the patients compared to healthy subjects. It would be then interesting to look at the amplitudes of the recorded potentials in order to validate this hypothesis. It is also important for the following studies to use a dynamometer in order to measure the force of contraction instead of calculating it from the EMG.
CONCLUSION AND FUTURE WORK

In this study I have presented the preliminary results obtained in 10 ALS patients and 15 healthy subjects. The study will continue until there are 30 people in each group, so that the minimal number of subjects to have conclusive results is more than reached.

We found that PIC vary from one muscle to the other in healthy subjects, it is thus fundamental for further studies to not make a global estimation of PIC but to detail PIC in each concerned muscle.

Even though we did not find any significant difference between ALS patients and healthy subjects in the estimation of PIC, the averaged PIC are slightly higher in patients compared to healthy subjects and the difference is already significant in the TA muscle. We will have to wait until the end of the study to make a proper conclusion about those.

Moreover, we noticed a difference in term of level of contraction calculated through the recorded EMG. The level of contraction to recruit a test unit after the control has been recruited is significantly higher in ALS patients. As the level of contraction was calculated from the surface EMG that was rectified and averaged, this difference is likely to be due to an increase in amplitude of the action potentials in ALS patients. This will be checked before the end of the complete study.

Further protocol will thus include a dynamometer to estimate the force of contraction instead of calculating it from the EMG signal. This is crucial when studying ALS patients as they may display large amplitude potentials that lead to an erroneous level of contraction.
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LIST OF ABBREVIATIONS

AHP: Afterhyperpolarization
ALS: Amyotrophic Lateral Sclerosis
ANOVA: Analysis Of Variance
EMG: Electromyography
MU: Motor Unit
MVC: Maximum Voluntary Contraction
PIC: Persistent Inward Currents
SFA: Spike Frequency Adaptation
ΔF: Difference between the frequencies of the control motor unit at recruitment and decruitment of the test motor unit
BIBLIOGRAPHY


