



HAL
open science

Absence of hyperexcitability of spinal motoneurons in patients with amyotrophic lateral sclerosis

Veronique Marchand-Pauvert, Iseline Peyre, Alexandra Lackmy-Vallée, Giorgia Querin, Peter Bede, Lucette Lacomblez, Rabab Debs, Pierre-François Pradat, Véronique Marchand-Pauvert

► To cite this version:

Veronique Marchand-Pauvert, Iseline Peyre, Alexandra Lackmy-Vallée, Giorgia Querin, Peter Bede, et al.. Absence of hyperexcitability of spinal motoneurons in patients with amyotrophic lateral sclerosis. *The Journal of Physiology*, 2019, 597 (22), pp.5445-5467. 10.1113/JP278117 . hal-02378881

HAL Id: hal-02378881

<https://hal.sorbonne-universite.fr/hal-02378881>

Submitted on 30 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Absence of Hyperexcitability of Spinal Motoneurons in Patients With Amyotrophic Lateral Sclerosis

Véronique MARCHAND-PAUVERT¹, Iseline PEYRE¹, Alexandra LACKMY-VALLEE¹, Giorgia QUERIN^{1,2}, Peter BEDE^{1,2,3}, Lucette LACOMBLEZ², Rabab DEBS² and Pierre-François PRADAT^{1,2}

1- Sorbonne Université, Inserm, CNRS, Laboratoire d'Imagerie Biomédicale, LIB, F-75006, Paris, France

2- Neurologie, AP-HP, Hôpital Pitié-Salpêtrière, F-75013, Paris, France

3- Computational Neuroimaging Group, Trinity College Dublin, Ireland

Running title: Motoneuron excitability in ALS

Key words: EMG, Motor units, Motor Neuron Disease, Humans

Corresponding author:

Véronique Marchand-Pauvert

Laboratoire d'Imagerie Biomédicale (LIB) - Centre de Recherche des Cordeliers

15 rue de l'École de Médecine - 75006 Paris, France

veronique.marchand-pauvert@inserm.fr

Table of contents: Neuroscience

Key point summary:

- ALS motoneurons become hypoexcitable with disease progression in experimental models, raising questions about the neural hyperexcitability supported by clinical observations.
- A variant of the ΔF method, based on motor unit discharge frequency modulations during recruitment and derecruitment, has been developed to investigate the motoneuron capacity to self-sustained discharge in patients.
- The modulation of motor unit firing rate during ramp contraction and vibration-induced recruitment are modified in ALS, suggesting lower motoneuron capacity to self-sustained discharge, a sign of hypoexcitability.
- ΔF -D decreases with functional impairment and its reduction is more pronounced in fast progressors.
- In patients with ALS, motoneurons exhibit hypoexcitability, which increases with disease progression.

ABSTRACT

Experimental models have primarily revealed spinal motoneuron hypoexcitability in ALS, which is contentious considering the role of glutamate-induced excitotoxicity in neurodegeneration and clinical features rather supporting hyperexcitability. This phenomenon was evaluated in human patients by investigating changes in motor unit firing during contraction and relaxation. Twenty-two ALS patients with subtle motor deficits and 28 controls performed tonic contractions of extensor carpi radialis, triceps brachialis, tibialis anterior and quadriceps, to isolate a low threshold unit (U1) in EMG. Subsequently, they performed a stronger contraction or tendon vibration was delivered, to recruit higher threshold unit (U2) for 10 sec. before they relaxed progressively. EMG and motor unit potential analyses suggest altered neuromuscular function in all muscles, including those with normal strength (MRC score at 5). During the preconditioning tonic phase, U1 discharge frequency did not differ significantly between groups. During recruitment, the increase in U1 frequency ($\Delta F-R$) was comparable between groups both during contraction and tendon vibration. During derecruitment, the decrease in U1 frequency ($\Delta F-D$) was reduced in ALS whatever the recruitment mode, particularly for $\Delta F-R < 8$ Hz in the upper limbs, consistent with the muscle weakness profile of the group. $\Delta F-D$ was associated with functional disability (ALSFRS-r) and its reduction was more pronounced in patients with more rapid disease progression rate. This *in vivo* study has demonstrated reduced motoneuron capacity for self-sustained discharge, and further supports that motoneurons are normo- to hypoexcitable in ALS patients, similarly to the observations in experimental models.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common and devastating motor neuron disease of middle-aged adults, characterised by the degeneration of upper and lower motor neurons (UMN and LMN, respectively). The progressive and selective loss of pyramidal cells (UMN) in the motor cortex and of bulbar and spinal motoneurons (LMN) leads to progressive paralysis and death. Mutations identified in familial forms (< 10 % cases) have been also reported in less than 10 % sporadic forms (Millecamps *et al.*, 2012). Several putative aetiological factors have been proposed, including oxidative stress, intracellular protein aggregation, mitochondrial dysfunction, impaired axonal transport, inflammation and glutamate excitotoxicity, but the unifying pathogenesis of ALS remains notoriously elusive (Rothstein, 2009; Morgan & Orrell, 2016).

Glutamate-induced excitotoxicity and enhanced neuronal discharge (hyperexcitability) contributes to increased calcium influx which, coupled with the reduced buffering capacity of neurons and astrocytes, results in oxidative stress and subsequent selective motor neuron loss (Bogaert *et al.*, 2010; Fogarty, 2018). This hypothesis is further supported by the fact that riluzole (an anti-glutamatergic agent) and edaravone (antioxidant) are the only drugs known to date that significantly slow down disease progression (Bensimon *et al.*, 1994; Abe *et al.*, 2017; Dharmadasa & Kiernan, 2018; Bhandari *et al.*, 2018). At a spinal level, several studies have been undertaken to investigate the intrinsic excitability of motoneurons and its link to excitotoxicity and motor neuron degeneration. Reports of excitability are inconsistent in superoxide dismutase 1 (SOD1) mutant mice, ranging from hyperexcitability in embryos (Pieri *et al.*, 2003; Kuo *et al.*, 2005; Martin *et al.*, 2013) to hypoexcitability in presymptomatic adults (Delestrée *et al.*, 2014). A recent study has reported that the most sensitive motoneurons to degeneration (innervating fast motor units) are hypoexcitable while the most resilient motoneurons (innervating slow motor units) exhibit normal excitability (Martínez-Silva *et al.*, 2018). Studies of human motoneurons derived from induced pluripotent stem cells (iPSCs) have confirmed similar stages in excitability (Wainger *et al.*, 2014; Devlin *et al.*, 2015; Naujock *et al.*, 2016). Taken together, these studies indicate that vulnerable motoneurons are not hyperexcitable. This raises of number of important questions on: i) the link between hyperexcitability,

excitotoxicity and degeneration, ii) the transferability of observations from animal models to humans, and iii) the physiological interpretation of iPSC-derived motoneuron experiments, *i.e.* in motoneurons disconnected from the motor network. Furthermore, while motoneurons or astrocytes derived from sporadic ALS iPSCs exhibit similar characteristics to those derived from familial forms (Burkhardt *et al.*, 2013; Qian *et al.*, 2017), to our knowledge, no electrophysiological investigations have been reported in sporadic forms. Because mutations account for less than 20 % ALS cases, and given the disappointing results of therapeutic translation (from mice to humans), there is a possibility that hypoexcitability is a specific feature of familial forms and animal models.

This *in vivo* study has been specifically designed to address these questions in patients with sporadic form of ALS. We settled on a variant of the non-invasive method of paired motor unit recordings that has been developed in humans to investigate the ability of spinal motoneurons to maintain self-sustained discharge (Kiehn & Eken, 1997; Gorassini *et al.*, 1998, 2002a). This method is based on the frequency of occurrence of an isolated single motor unit potential (control unit U1) on the electromyogram (EMG), and the modulations of the motor unit firing rate produced by transient tendon vibration or during increase contraction force that lead to recruit a higher-threshold motor unit (test unit U2). The discharge frequency of U1 is used to estimate the common synaptic drive to motoneuron pool, and its modulations during recruitment are used to estimate the changes in firing threshold and ability for self-sustained discharge of spinal motoneurons. The difference in U1 frequency between recruitment and derecruitment of U2 (ΔF) is between 1 and 8 Hz (Gorassini *et al.*, 1998, 2002a, 2002b, 2004; Mottram *et al.*, 2009; Vandenberg & Kalmar, 2014; Wilson *et al.*, 2015). The slower U1 frequency at U2 derecruitment, compared to recruitment, indicates that U2 motoneuron firing threshold is lowered and its firing can be maintained despite the lower synaptic drive, reflecting the non-linear motoneuron properties. It has been proposed, and confirmed in animal studies (Bennett *et al.*, 2001), that ΔF are related to sodium and calcium-induced persistent inward currents (PICs), which are known to enhance motoneuron response to synaptic inputs and to produce self-sustained motoneuron discharge (Heckmann *et al.*, 2005; Powers & Heckman, 2015, 2017). However, another group has proposed that ΔF may depend on other motoneuron

intrinsic properties (spike-threshold accommodation and spike-frequency adaptation) and on the level of tonic inhibition (Revill & Fuglevand, 2011, 2017).

Paired motor unit recordings, allowing for ΔF evaluation, is currently the only non-invasive methodology to estimate the intrinsic motoneuron excitability in humans *in vivo*. We have implemented it, to assess patients with ALS, and we used both ramp contractions and tendon vibrations, to investigate the modulations of discharge frequency of a low-threshold U1 motor unit during the recruitment and derecruitment of an higher-threshold U2 motor unit. We hypothesised that ΔF may be smaller in patients compared to age and sex-matched controls if spinal motoneurons were hypoexcitable in ALS.

METHODS

Ethical approval

The study conformed to the standards set by the latest revision of the Declaration of Helsinki. The procedures were approved by Inserm (clinical research sponsor; protocol C14-21) and have obtained the authorizations of the national French ethics committees (CPP n° 16-15 - Ile de France VI; ANSM 150154B-31; RCB 2014-A01240-47). The study has also been registered on ClinicalTrials.gov PRS (NCT 02429492). Before inclusion into the protocol, all the subjects have provided written informed consent.

Subject groups

The experiments were performed on 22 patients with ALS (5 females; mean age in years \pm standard deviation = 61.6 ± 10.7 ; range 39-78) and 28 controls (4 females; 62.8 ± 8.2 ; range 45-77). The inclusion criteria for ALS patients were 1) *probable* or *definite* ALS according to the El Escorial criteria (Brooks *et al.*, 2000a) (Brooks *et al.*, 2000b), 2) mild motor deficits or preferably no clinical signs of motor deficits with normal clinical EMG examination in the triceps brachii (TB) and in quadriceps, to allow for comparison between proximal (less affected) and distal muscles (more affected than proximal ones), and 3) the absence of peripheral neuropathy. Only two patients had a familial form of ALS (P7: C9ORF72; P22: SOD1). The 20 sporadic cases were screened and tested negative for the 4 most common ALS-causing mutations (SOD1, FUS, C9ORF72 and TDP43; DNA extraction was performed by Genethon, Evry, France; DNA analysis was carried out at the

University of Tours, France). Table 1 summarises the main clinical features of the cohort, including i) the disease duration in months from symptom onset, ii) the revised ALS functional rating scale (ALSFRS-r) scores, which measures disability in activities of daily living (Cedarbaum *et al.*, 1999), iii) the progression rates indicating ALSFRS-r decline per month, iv) the UMN scores (see Methods) for upper (UL) and lower limbs (LL), v) UMN vs. LMN predominance or a classical ALS (with both UMN and LMN affection), and vi) the muscle strength evaluated by manual muscle testing and rated using the cumulative Medical Research Council (MRC) scores. Patients with comorbid neurological conditions were not included. The inclusion criteria for controls included the absence of prior or current neurological illness.

Insert Table 1 near here

Protocols

The participants were comfortably seated in a reclining chair. Their forearms and hands rested on the arms of the chair, their shoulders at 20° abduction, the elbows at 120° extension, and their wrists in neutral position (hand in pronation). The investigated leg rested on a device fixed to the chair, with the hip semi-flexed at 110° extension, the knee at 120-130° extension, and the foot rested on a platform forming an angle with leg of 120°. During the recordings, the subjects were asked to perform isolated contraction of the muscle of interest and the movements were free from any constraint: i) wrist extension when studying extensor carpi radialis (ECR), ii) elbow extension when studying TB, iii) ankle dorsiflexion when studying tibialis anterior (TA), and iv) knee extension when studying quadriceps. Under these conditions, the risk of co-contracting the antagonistic muscles was greatly reduced. The 4 muscles were systematically investigated 1 by 1 in each participant, during the same experimental session.

Recordings. EMG activities were recorded using single-use electrodes. Bipolar surface electrodes (foam electrodes with solid gel, 2-cm apart; FIAB, Florence, Italy) were secured on the skin, over the muscle belly of ECR, TB, TA or quadriceps. Intramuscular recordings were performed using paired hook wire electrodes (40-cm polytetrafluoroethylene -PTFE- insulated stainless steel wire, 0.08-mm diameter, 40G) threaded through a hypodermic needle. In the needle, the tips of wires were positioned so that 2 mm of one wire and 5 mm of the second wire protruded from

the needle. The first wire was stripped 2 mm, while the second wire was insulated 3 mm and stripped 2 mm. The protruding ends were bent at 180° (SGM d.o.o., Split, Croatia). The needle was inserted in the ECR, TB, TA or quadriceps to implant the fine wires, and the needle was subsequently removed. Surface electrodes and fine wires were plugged to wifi connectors that transmitted the signals to an EMG zero wire system (Cometa Srl, Milan, Italy). EMG activities were amplified and filtered (x 5,000 and 10-500-Hz bandpass for surface EMG; x 1,000 and 10-1000-Hz bandpass for intramuscular EMG) before being digitally stored on a personal computer (2-kHz sampling rate; Power 1401 controlled by Signal 6, CED, Cambridge, UK). For the quadriceps, we placed the surface and fine wires on different heads: the surface electrodes were placed on VL to save place for the fine wire that we inserted in VM, another head of quadriceps most often used for intramuscular recording (less painful than VL). All controls were investigated on their dominant side (Oldfield, 1971), except one participant who sustained an orthopaedic injury on the dominant leg. Similarly, most of the patients were tested on their dominant side. The non-dominant side was only evaluated if the dominant side was too affected by muscle weakness. Consequently, 3 patients were tested on their non-dominant side: 2 on their dominant upper limb and non-dominant lower limb and the other way round for the last remaining patient.

Contraction Protocol. The 4 muscles (ECR, TB, TA and quadriceps) were tested separately but on the same day, during the same session. To avoid any discomfort during strong contractions, the mean level of surface EMG produced during the maximal voluntary tonic contraction (MVC) was evaluated before implanting the fine wires. When the fine wires were inserted, the subjects were asked to perform and maintain a weak tonic contraction for 10-15 sec., to generate EMG activity allowing the evaluation of one motor unit potential (U1, control unit). During the recordings, the intramuscular EMG signal was transmitted to an external threshold system, with low and high amplitude threshold levels adjusted manually (visual checked on oscilloscope): when a unit potential was between these 2 levels, the system generated a TTL signal that triggered a sound. This system was used to monitor the EMG activity online, so that no more than 2 units triggered a TTL signal during this tonic phase. When the unit discharge was stable, the subjects were asked to increase their level of voluntary contraction so that higher threshold

motor units were activated, but within a range of EMG activity which allowed the extraction of a second motor unit potential (U2, test unit). They were asked to maintain the contraction for ~10 sec. before they relaxed the muscle progressively so that U2 and then U1 did not discharge anymore (derecruitment; Fig. 1A). Five trials were performed in each muscle, with a few minutes (> 2-3 min.) of rest between each trial.

Insert Figure 1 near here

Vibration Protocol. The vibration protocol was performed during the same session as the contraction protocol. It also started with a steady tonic contraction for 10-15 sec. before the muscle tendon was vibrated for 5 sec. at 100 Hz (Vibrasens system, TECHNO Concept, Mane, France). At the end of the vibration, the subjects were asked to maintain the contraction during 5 extra sec. before they relaxed the muscle progressively (Figs. 1BC). The subjects were asked not to resist the movement produced by the tendon vibration and not to modify their effort, especially after vibration. However, some participants had a tendency to increase their contraction force at the end of vibration. This was specifically monitored using surface EMG, and the data were not saved for subsequent analyses. Five trials were performed in each muscle, with few minutes (> 2-3 min.) of rest between each trial.

The 2 protocols were performed the same day, during the same experimental session. During both, standardised instructions were given by the operator, *i.e.* when to contract and to relax, and the subjects received biofeedback: EMG signals were displayed on an oscilloscope and audio feedback was also provided (triggered by motor unit potentials).

Data Analyses

Clinical parameters

Disease progression was calculated as follows:

$$Progression = \frac{48 - Score\ to\ ALSFRr}{disease\ duration\ in\ months}$$

and the UMN score (derived from Simon *et al.*, 2015):

$$UMN\ score =$$

reflex scores (0 to 4) + Babinski or Hoffmann sign (0 or 1) + Ashworth \geq 3 (0 or 1)

i) reflex scores: tendon reflexes in soleus and quadriceps (to calculate the UMN score for the lower limbs) or in ECR and TB (to calculate the UMN score for the upper limbs), score 0 = normal or absent, 1 = present in wasted muscle, 2 = brisk;
ii) Babinski (lower limbs) or Hoffmann sign (upper limbs), score 0 = absent, 1 = present, and iii) Ashworth score \geq 3 (in upper or lower limbs): if quotation was $<$ 3 to the modified Ashworth scale, score = 0; if quotation was \geq 3 (*i.e.* with high possibility of muscle clonus), score = 1.

Electrophysiological parameters

Surface EMG. The surface EMG recorded during MVC was rectified and the running mean was calculated over a 200-ms period, to evaluate the mean level. Then, the surface EMGs developed during the contraction and vibration protocols were expressed as a % the mean EMG level recorded during MVC (Figs. 1A,B). The profile of the mean rectified surface EMG (% mean EMG level at MVC) was used to evaluate the duration of rise, plateau and relaxation phases of the contraction ramp (Fig. 1A). Then, the proportion of EMG increase and decrease during rising and declining phases, respectively, divided by their duration, was used to estimate the corresponding velocities.

Intramuscular EMG. The intramuscular EMG was decomposed using Spike2 (CED, Cambridge, UK) to extract single motor unit potentials (Fig. 1); spike sorting was visually verified after the automatic procedure. For the contraction protocol, motor unit pairs were retained for further analysis only if U2 was recruited at least 2 sec. after U1 frequency started to increase, at the beginning of the ramp (maximal PICs activation; Udina *et al.*, 2010). Less motor unit pairs were analysed in the vibration protocol, compared to the contraction protocol for 2 reasons (154 vs. 265, respectively; Tables 2 and 3). Firstly, the tendon vibration was not efficient enough to recruit higher threshold units in some subjects, irrespective of the subject groups; Chi² analysis did not reveal any significant differences between controls and ALS, regardless of the tested muscle ($P = 0.62$: 3 controls vs. 3 ALS for ECR, 6 vs. 5 for TB, 5 vs. 2 for TA and 10 vs. 13 for quadriceps). Secondly, in 84 motor unit pairs, U2 disappeared at the end of the vibration, like U2.2 in Figure 1C (passive derecruitment), and it did not appear anymore in the intramuscular EMG

recording during the 5 extra sec. after the end of vibration, before the subject voluntarily relaxed. We compared the occurrence of passive derecruitment in controls and ALS, and we did not find any significant differences between the groups, irrespective of the investigated muscle (Chi^2 , $P = 0.08$; 12 pairs in controls vs. 11 in ALS for ECR, 3 vs. 10 for TB, 20 vs. 16 for TA and 9 vs. 3 for quadriceps). Since our objective was to evaluate the motoneuron ability to maintain self-sustained firing after synaptic activation, we only retained the motor unit pairs with U2 derecruited only when the subjects voluntarily relaxed (active derecruitment) for group analysis (U2 in Fig. 1B and U2.1 in Fig. 1C). Finally, the unit pairs analysed in each protocol did not include the same units (U1 and U2); a specific pair was only sampled once for each protocol. Based on these criteria, a total of 583 motor units (U1 + U2) and 419 pairs were systematically analysed. Tables 2 and 3 indicate the number of unit pairs collected in each investigated muscle, in the contraction (Table 2) and vibration protocol (Table 3). Next to the number of unit pairs, the number of subjects is indicated between brackets. We obtained data in both upper and lower limbs in all subjects, at least in 1 of the 2 muscles investigated at each level.

Insert Table 2 near here

Insert Table 3 near here

Motor unit analyses. The amplitude and duration of motor unit potentials were estimated based on the templates extracted from intramuscular EMGs using Spike2. Their recruitment thresholds were estimated using the mean surface EMG, which was expressed as a % mean EMG level at MVC. The discharge rate (Hz) was estimated using the smoothed average of the instantaneous frequency and the 5th order polynomial fitting available in Spike2 (Figs. 1AB). The frequency of U1 was estimated when the unit discharge was stable during the weak tonic contraction (f-U1 tonic), and before it increased by either: i) increasing voluntary effort during the contraction protocol (Fig. 1A), or ii) applying a tendon vibration in the vibration protocol (Figs. 1BC). Then, the mean frequencies of U1 and U2 were calculated when both units were activated together. The increase of U1 frequency during the recruitment ($\Delta F-R$) was measured as the difference in the firing rate of U1 when U2 was recruited during the rising phase of the contraction, which

corresponded to the maximum firing rate of U1 ($f\text{-U1-max}$; Fig. 1A), and the firing rate of U1 during the prior tonic phase of the contraction ($f\text{-U1 tonic}$): $\Delta F\text{-R} = f\text{-U1 max} - f\text{-U1 tonic}$ (Fig. 1A). During the derecruitment of U2, ΔF was calculated as in previous studies, and corresponds to the difference between the frequency of U1 when U2 was recruited and derecruited: $\Delta F\text{-D} = f\text{-U1 recruitment} - f\text{-U1 derecruitment}$; Fig. 1B).

EMG and contraction force analysis

In a subgroup of 7 ALS patients and 12 controls, we investigated the relationship between the mean level of surface EMG and the contraction force. The subjects were asked to perform a tonic wrist extension (ECR) or a tonic foot dorsiflexion (TA) at various force levels that were measured using wireless handheld dynamometer muscle tester (MicroFet2TM; Hoggan Scientific LLC, Salt Lake City, UT, USA). The surface EMG was collected and the running mean was calculated over a 200-ms period, as in the protocols (see above). Then, the mean EMG level was plotted against the force level in each subject and we compared the slope of the linear regression between groups using unpaired t test.

Statistics

Data analyses were performed taking into account all the motor unit pairs collected in the 2 protocols because motoneurons can manifest different sensitivities to PICs (Cotel *et al.*, 2009; Wilson *et al.*, 2015), and they can be differentially altered in ALS (Nijssen *et al.*, 2017; Martínez-Silva *et al.*, 2018). Moreover, we also compared $\Delta F\text{-D}$ between subjects (controls vs. patients with ALS); the maximal $\Delta F\text{-D}$ observed in each muscle in each individual subject was considered when several motor unit pairs were collected.

Statistical analyses were performed with SigmaPlot 13.0 (Systat Software Inc., San Jose, CA, USA), and XLStat 21.1.2 (Addinsoft, Paris, France). The significance level α was fixed at 0.05 and the results were considered statistically significant only if $P < 0.05$. Mean values are indicated ± 1 standard deviation (normal distribution), and median values are indicated with Q1 and Q3 quartiles (respectively, 25th and 75th percentile) between brackets (non-normal distribution).

Homoscedasticity (Levene median test) and normality (Shapiro-Wilk test) were first verified to allow parametric analyses (analysis of variance, ANOVA; analysis of

covariance, ANCOVA; unpaired *t* test). Alternatively, non-parametric methods were used (Mann-Whitney U test, ANOVA on ranks), and *post hoc* multiple pairwise comparisons were performed with Dunn's method. Correlations were tested using Spearman test, multiple regression analysis and partial least square (PLS) regression in case of collinearity between variables (including quantitative and qualitative predictor factors); the latter included a principal component analysis (PCA). Influence of several factors were tested, including: i) subject groups (controls vs. ALS), ii) muscle groups (ECR vs. TB vs. TA vs. quadriceps), iii) muscle subgroups according to their level of action (upper vs. lower limbs), their functional role (flexors vs. extensors) and the gradient (distal vs. proximal), iv) experimental protocols (contraction vs. vibration), v) motor unit size, and vi) subject age.

Chi² analyses were performed to compare the data distribution: i) MRC scores in the different muscles (score = 5 vs. score ≤ 4), ii) number of subjects in each group without recruitment during the vibration protocol (controls vs. ALS), and iii) number of motor unit pairs with passive derecruitment in each subject group (controls vs. ALS; see above). Fisher exact test was used to compare the number of patients without clinical motor vs. those with similar muscles weakness in upper and lower limbs vs. those with more weakness in lower limbs or in upper limbs.

Lastly, the relationship between $\Delta F-R$ and $\Delta F-D$ in the patient group was tested taking into account the patient phenotype characterised on clinical features including ALSFRS-r score, disease duration, progression rate, MRC score, UMN score and ALS form (predominant UMN/LMN/Both). We also tested the association with the duration of motor unit potentials and their activation threshold.

For clarity, the statistical tests and the parameters included in each test are specifically indicated in Results.

RESULTS

Motor unit characteristics

Insert Figure 2 near here

The amplitude and duration of motor unit potentials were measured and Spearman correlation analysis revealed a significant positive relationship between both metrics ($R = 0.09$, $P < 0.05$; Fig. 2A). Then, one-way ANOVA on ranks was used

to compare motor unit amplitude and duration between controls and patients with ALS. Figures 2AB show the data distribution in each group, for each investigated muscle. The difference between the median amplitudes was found significant ($P < 0.001$) and *post hoc* analyses revealed significantly larger motor unit potentials in ALS, compared to controls, only in distal muscles (Dunn method, $P < 0.001$ in ECR and 0.05 in TA); the difference between the study groups was not significant in proximal muscles ($P = 1$ for TB and quadriceps; Fig. 2A; see also Fig. 1 showing larger quadriceps motor units in one patient in B, compared to one control in A). Differences in motor unit duration were also significant (one-way ANOVA on ranks, $P < 0.001$) and the *post hoc* analyses revealed that the motor unit potentials had a significantly longer duration in ALS, compared to controls, in all muscles (Dunn method, $P < 0.001$ for ECR, TB and quadriceps, and $P < 0.05$ for TA; Fig. 2B).

While the motor unit potentials were similarly altered in all muscles (Fig. 2BC), especially in their duration, the clinical evaluation revealed that more patients in the group had altered MRC score in ECR (< 5 ; 36.4 %), compared to other muscles (TB 22.7 %, TA, 27.3 % and none in quadriceps; Chi², $P < 0.05$; Table 1).

Insert Figure 3 near here

The recruitment thresholds of U1 and U2 were estimated using the mean surface EMG. Figure 3A illustrates the mean level of rectified surface EMG in controls and ALS, collected during MVC of each investigated muscles. Two-way ANOVA did not reveal any significant difference between the subject groups (controls vs. ALS; $P = 0.46$) and no interaction between the 2 factors (subject groups x muscles; $P = 0.36$), suggesting that the mean EMG levels during MVC were similar in controls and ALS, irrespective of the sampled muscle groups. Accordingly, the mean surface EMG during MVC was used to normalise the mean surface EMG during U1 and U2 recruitment to compare their thresholds (% EMG during MVC). Tables 2 and 3 show that motor unit thresholds were higher in ALS compared to controls (one-way ANOVA on ranks, $P < 0.001$ for units collected in both contraction and vibration protocols; compare EMG activity in one control and one patient in Fig. 1AB); the results of *post hoc* analyses (Dunn method) for multiple pairwise comparisons are indicated by asterisks in the tables.

Given that large motor unit potentials in patients likely contribute to a greater

extent to the mean rectified surface EMG than in controls, this might interfere with the linear relationship between contraction force and EMG (Milner-Brown & Stein, 1975), especially at low level of tonic contraction. Therefore, we compared the relationship between the contraction force and the mean surface EMG in a subgroup of patients and controls, in ECR and TA. Figure 3B shows that the mean rectified surface EMG linearly increased with the contraction force in one control and one patient. A significant linear regression was found in all subjects ($P < 0.001$) with $0.6 < R^2 < 0.9$. However, Figure 3C shows that the mean slope was significantly greater in ALS compared to controls, in both muscles (unpaired t test, $P < 0.05$). This result indicates that for a similar force, the mean level of surface EMG was larger in ALS than in controls (also compare the Y-axis in the 2 individuals illustrated in Fig. 3B).

Criteria allowing ΔF -D measurements to estimate PICs

Several parameters have been first checked to allow the use of ΔF during derecruitment (ΔF -D) to estimate PICs. Firstly, we ensured that the ramp conditions were comparable in controls and patients with ALS. During the contraction protocol, the subjects were asked to increase and maintain their contraction force to activate U2, and finally to relax progressively. Although the instructions and biofeedback were similar for both groups, we tested whether the ramp conditions were comparable. Firstly, we compared the duration of rise, plateau and decline of the mean rectified surface EMG during ramp contraction (Fig. 1A), and one-way ANOVA on ranks revealed no differences between groups, irrespective of the muscles tested ($P > 0.2$; Table 4). We also compared the velocities of the rising and relaxation phases, and multiple regression analyses revealed no significant influence of subject and muscle groups (subject groups: $P = 0.34$ and 0.85 for rise and relaxation, respectively; muscle groups: $P = 0.16$ and 0.65). This suggests that the speed of the contraction changes during the ramp were likely comparable in controls and ALS, irrespective of studied muscles (mean speed \pm SD = 1.65 ± 1.90 % change/ms in controls vs. 1.36 ± 2.48 in ALS during rise and 0.72 ± 1.98 vs. 0.70 ± 1.61 during relaxation). These results confirm that the ramp characteristics were similar in controls and patients with ALS, and that the conditions for ΔF estimation and PICs activation were comparable between subject groups (Lee & Heckman, 1998; Revill & Fuglevand, 2011).

Insert Table 4 near here

Secondly, we checked whether U1 and U2 frequencies were correlated to ensure that both received a common synaptic drive to validate the use of U1 frequency changes during U2 recruitment (ΔF), to estimate the synaptic drive to motoneuron pool and PICs (Gorassini *et al.*, 2002a). The mean discharge frequencies of U1 and U2 for each pair were estimated and significant multiple regression analysis (adjusted $R^2 = 0.76$, $P < 0.001$) revealed a positive relationship between both frequencies (slope = 0.77, $P < 0.001$), without any significant difference between subject groups (controls vs. ALS; slope = 0.10, $P = 0.53$) and protocols (contraction vs. vibration; slope = 0.02, $P = 0.89$). Accordingly, the scatter plots in Figures 4A and B show similar linear regressions between U1 and U2 frequencies in controls and patients with ALS, whatever the protocol (contraction vs. vibration, respectively Figs. 4A and B). The multiple regression analysis also revealed a significant influence of the muscle groups (ECR vs. TB vs. TA vs. quadriceps; slope = 0.40, $P < 0.001$). We therefore compared the ratio between U1 and U2 frequencies between muscle groups. Significant one way ANOVA on ranks ($P < 0.001$) allowed *post hoc* pairwise multiple comparisons (Dunn Method), and we found that U1 and U2 fired at a very similar rates in controls and ALS, irrespective of the muscle groups (median ratio between 1.1 and 1.2, taking into account all muscles). This result supports that the changes in U1 frequency can be used to evaluate the synaptic drive, common to U1 and U2, and to validate the estimation of ΔF (Gorassini *et al.*, 2002a).

Insert Figure 4 near here

Finally, we measured the time difference between the increase in U1 frequency and the activation of U2 (time recruitment), because it has been shown—that maximal PICs' activation (plateau) was reached when U2 occurred at least 2 sec. after U1 with the classical ΔF method (Udina *et al.*, 2010). Furthermore, we assumed that PICs might not be maximally activated during the 10 sec. tonic contraction prior to the ramp, and they could gradually increase during the recruitment leading to greater force contraction (Heckman *et al.*, 2008; Powers *et al.*, 2008). Indeed, compared to the median ΔF -D observed with recruitment time > 2 sec., we found lower ΔF -D in 80 % the motor unit pairs with time recruitment < 2

sec. (3.15 [1.88-3.78] in controls and 2.60 [2.00-4.40] in ALS; compare with Figs. 4A,B). To our knowledge, no similar investigations were performed during vibration, and our data revealed that the activation of U2 was faster with vibration than during contraction. Therefore, we analysed all the unit pairs from the vibration protocol; not only those with time recruitment ≥ 2 sec., as for the contraction protocol. Significant multiple regression analysis (adjusted $R^2 = 0.24$, $P < 0.001$) revealed no influence of group membership (controls vs. ALS; slope = 0.11, $P = 0.47$) but significant influence of the experimental protocol (contraction vs. vibration; slope = -1,76, $P < 0.001$). This confirms that the time difference was significantly longer during the contraction protocol compared to the vibration protocol (Mann-Whitney U test, $P < 0.001$; Fig. 4D), but crucially there was no difference between subject groups.

Insert Figure 5 near here

Comparisons of ΔF -D

Similarly to previous studies, we first compared the ΔF during the derecruitment of U2 (ΔF -D) between motor unit groups (controls vs. ALS motor unit pairs), taking into account the muscle groups (ECR vs. TB vs. TA vs. quadriceps) and the protocols (contraction vs. vibration). The multiple linear regression was significant (adjusted $R^2 = 0.11$, $P < 0.001$), revealing a significant influence of the motor unit groups (slope = -1.1, $P < 0.001$), and of the muscle groups (slope = 0.47, $P < 0.001$ for both), and no difference between protocols (slope = -0.27, $P = 0.27$). Figures 5A,B show a trend of reduced ΔF -D in motor unit pairs collected in patients with ALS, compared to controls, which was particularly marked in ECR, TB and quadriceps, compared to TA. This trend was observed in both the contraction (Fig. 5A) and vibration protocols (Fig. 5B). However, multiple pairwise comparisons (Dunn method) performed after significant one way ANOVAs on ranks ($P < 0.05$), only revealed a significant difference between controls and ALS, in quadriceps during the contraction protocol (Fig. 5A). Similar analysis was performed taking into account each individual subject in both groups, instead of the unit pairs (the maximal ΔF -D was selected in each individual, for each muscle), and we observed the same results as at the level of motor unit pairs. While the median ΔF -D were lower in patients, compared to controls, no significant differences were identified

in any muscle groups or protocols (Figs. 5CD; one way ANOVA on ranks, $P < 0.001$; multiple pairwise comparisons using Dunn's method, $P > 0.92$).

Insert Figure 6 near here

Both in experimental models and humans, the motoneuron excitability can be different between flexors and extensors (Cotel *et al.*, 2009; Wilson *et al.*, 2015). Therefore, we further investigated the influence of muscle groups taking into account their functional role (flexors vs. extensors). Moreover, because patients could exhibit greater functional impairment in distal muscles, and more muscle weakness in the upper limbs, we also tested the effect of the muscle localisation (upper vs. lower limbs and proximal vs. distal muscles). The multiple regression analysis was significant ($R^2 = 0.11$, $P < 0.001$), and we found again a significant difference between groups (controls vs. ALS; slope = -1,07, $P < 0.001$), and no differences between protocols (contraction vs. vibration; slope = -0.34, $P = 0.18$). Moreover, the regression analysis revealed no influence of the functional groups (flexors vs. extensors; slope = -0.19, $P = 0.67$) nor of the gradient (proximal vs. distal muscles; slope = 0.24, $P = 0.48$). The only significant differences were observed between upper and lower limbs (slope = 1.18, $P < 0.001$). Figure 6A shows the data distribution in each group of motor unit pairs (controls vs. ALS), in upper and lower limbs (data from both protocols were grouped). One way ANOVA on ranks was significant ($P < 0.001$), and *post hoc* multiple pairwise comparisons (Dunn method) revealed that $\Delta F-D$ were significantly reduced in ALS compared to controls, in both the upper ($P < 0.05$) and lower limbs ($P < 0.01$). We also identified smaller $\Delta F-D$ in lower limbs, compared to upper limbs, in both controls ($P < 0.01$) and ALS ($P < 0.01$).

To sum up the $\Delta F-D$ results *per se*: i) $\Delta F-D$ was similar in both protocols while the time to activate U2 was < 2 sec. during vibration, and ii) $\Delta F-D$ was significantly lower in ALS only when evaluating upper limb (ECR + TB) and lower limb muscles (TA + quadriceps) together.

Influence of recruitment conditions on $\Delta F-D$

The motor unit firing rate is used to estimate the synaptic drive to motoneurons in humans, due to its link with the synaptic activity and the influence of the synaptic noise (Segundo *et al.*, 1963; Matthews, 1996; Wienecke *et al.*, 2009). PICs

also contribute to the motor unit firing rate by amplifying the motoneuron response to synaptic inputs. On the other hand, PICs are strongly modulated by monoamines (Heckman *et al.*, 2008), and the total excitatory drive to motoneurons likely influences their level of activation. In line with this, it has been shown in decerebrated cat preparations that ΔF -D linearly increased with the rate modulation of U1 during recruitment (see Fig. 4B and legend of Powers *et al.*, 2008), which likely reflects the “*graded synaptic activation of PICs*” during motor unit recruitment, together with increase rate modulation of U1. Accordingly, we tested the influence of U1 frequency rate and its modulation during recruitment (ΔF -R) on ΔF -D.

In the pre-conditioning phase, the subjects performed a tonic contraction for 10 s. with a steady U1 discharge (Figs. 1AB). The frequency of U1 during this tonic phase (f-U1 tonic) was compared between controls and ALS motor unit pairs, taking into account the muscle groups and the protocols. Significant one-way ANOVA on ranks ($P < 0.001$) allowed *post-hoc* multiple pairwise comparisons (Dunn method). Although the median frequencies were slower in ALS, compared to controls, the pairwise comparisons failed to reveal any significant differences between groups, irrespective of the investigated muscles and the protocols ($P > 0.2$; Tables 2 and 3). Subsequently, we investigated the relationship between f-U1 tonic and its increase during U2 recruitment (ΔF -R), taking into account the subject and muscle groups, and the protocols. Multiple regression analysis (adjusted $R^2 = 0.02$, $P < 0.001$) failed to reveal any significant link between f-U1 tonic and ΔF -R (slope = 0.05, $P = 0.22$), and any other potential effects (subject groups: slope = -0.46, $P = 0.08$; muscle groups: slope = 0.10, $P = 0.42$; protocols: slope = -0.46, $P = 0.11$).

Furthermore, we investigated a possible link between ΔF -R and ΔF -D, taking into account the subject groups, the protocols, the size of motor units and the muscle groups according to their functional role, and their localisation (as performed when comparing ΔF -D alone). The multiple regression analysis was significant (adjusted $R^2 = 0.37$, $P < 0.001$), revealing a significant increase of ΔF -D together with ΔF -R (slope = 0.39, $P < 0.001$), which was significantly different between subject groups (controls vs. ALS; slope = -0.44, $P < 0.05$). On the other hand, there was no significant influence of the experimental protocols (contraction vs. vibration; slope = -0.15, $P = 0.50$), nor of motor unit size (slope = 0.09, $P = 0.40$), nor of muscle

functional role (flexors vs. extensors; slope = -0.26, $P = 0.50$) and gradient (distal vs. proximal; slope = 0.43, $P = 0.14$). Regarding the influence of muscle groups, we found a significant influence only when taking into account their level of action (upper vs. lower limbs: slope = 0.75, $P < 0.01$). Figures 6BC show that $\Delta F-D$ increased with $\Delta F-R$ in both subjects groups, at both upper and lower limb levels. However, for similar $\Delta F-R$, the $\Delta F-D$ were smaller in ALS compared to controls, especially at low-to-medium levels of $\Delta F-R$: < 8 Hz in upper limbs and < 6 Hz in lower limbs. Furthermore, in controls, for similar $\Delta F-R$, $\Delta F-D$ were smaller in the lower limbs than in upper limbs. A similar difference was observed in ALS for low level of $\Delta F-R$ (< 6 Hz), but to a smaller extent than in controls.

We also tested the effect of smoothed U1 rate modulation on $\Delta F-D$, as in Powers *et al.*, 2008 ($\Delta F-R$ /time of increase), and we found the same results.

Finally, we investigated if the relationship between $\Delta F-R$ and $\Delta F-D$ changed with age. ANCOVA further confirmed the influence of $\Delta F-R$ on $\Delta F-D$ ($P < 0.001$) and the difference between subjects groups (interaction $\Delta F-R \times$ subject groups, $P < 0.001$). The analysis did not reveal any significant age effect on $\Delta F-D$ (age factor alone $P = 0.92$; interaction age \times subject groups, $P = 0.99$).

These results further confirm that $\Delta F-D$ were significantly smaller in patients with ALS, compared to controls, irrespective of age. This was particular true for low-to-medium increases ($< 6-8$ Hz) of U1 frequency necessary to activate U2 ($\Delta F-R$). Furthermore, our analyses indicate that the $\Delta F-D$ reductions in ALS were more marked in the upper limbs up to 8-Hz $\Delta F-R$, than in the lower limbs in which the $\Delta F-D$ reduction was observed up to 6-Hz $\Delta F-R$ (*i.e.* at a lower level than in the upper limbs).

Correlations with clinical measures

Given the limited number of patients with familial ALS, we did not perform any statistical analysis in this cohort (P7 and P22; Table 1). However, the median value of $\Delta F-D$ in the lower limbs of these 2 patients corresponded to the median value observed in the entire group (2.5 [1.7-4.1]) while the median $\Delta F-D$ in the upper limbs was among the 30 % largest $\Delta F-D$ observed in the group (4.7 [3.5-5.7]; compare with box plots in Fig. 6A). Interestingly, 1 of these 2 patients (P7) was more disabled in the lower limbs than in the upper limbs (Table 1). In the entire group of patients, we found 9 patients with normal MRC score (= 5) in the 4

investigated muscles, 1 with comparable disability in the upper and lower limbs (-2 points in total MRC for both upper and lower limbs), 3 with motor deficits restricted to the lower limbs, and 9 with upper limb impairment only (Fischer exact test, $P < 0.05$). This result indicates that we investigated more patients without clinical deficit in the 4 investigated muscles or with upper limb motor deficit, which might explain why we observed more altered $\Delta F\text{-R}/\Delta F\text{-D}$ relationship in the upper limbs compared to the lower limbs (Fig. 6BC). One way ANCOVA was performed to further investigate this possibility. While the degree and level (upper vs. lower limbs) of motor disability (according to MRC scores) had no significant influence on $\Delta F\text{-D}$ *per se* (weak upper limb vs. weak lower limb vs. equally weak vs. normal; $P = 0.66$), we found a significant interaction between the distribution of muscle weakness and $\Delta F\text{-R}$ on $\Delta F\text{-D}$ ($P < 0.05$). This finding further supports that the $\Delta F\text{-R}/\Delta F\text{-D}$ relationship changed significantly according to the level of motor deficit.

Insert Figure 7 near here

PCA and PLS-regression analyses were undertaken to further investigate the possible link between clinical phenotype and $\Delta F\text{-D}$, taking $\Delta F\text{-R}$ into account. The load plot in Figure 7 represents the impact of $\Delta F\text{-D}$, $\Delta F\text{-R}$ and the most commonly used clinical parameters in ALS, on excitability (t1) and phenotype (t2). The best predictors for excitability (parameters with the strongest absolute coefficient contributing to t1 component) includes $\Delta F\text{-D}$, $\Delta F\text{-R}$, ALSFRS-r and progression rate. PLS-regression analysis was undertaken to investigate the influence of each parameter on $\Delta F\text{-D}$, and a significant link was identified between $\Delta F\text{-D}$ (dependent variable in blue) and $\Delta F\text{-R}$, ALSFRS-r and progression rate (significant predictors in red; $P < 0.05$). Conversely, we did not find any significant link with MRC score (likely due to score at 5 in most unit pairs), with UMN score (combined score for pyramidal syndrome, hyperreflexia and spasticity), disease duration and the ALS motor phenotype (predominant UMN/LMN/both). The results of PLS-regression further confirm the considerable influence of $\Delta F\text{-R}$ on $\Delta F\text{-D}$. Furthermore, this analysis indicates that $\Delta F\text{-D}$ decreases with ALSFRS-r, suggesting that motoneuron excitability decreases with functional loss. Moreover, the negative coefficient for progression rate indicates that $\Delta F\text{-D}$ is greater in patients with slow progression

rates, compared to those with faster progression, which suggests that motoneuron excitability is more depressed in fast progressors.

Lastly, we investigated the possible link between the $\Delta F\text{-R}/\Delta F\text{-D}$ interaction and motor unit characteristics (amplitude, duration and threshold). The multiple regression analysis was significant (adjusted $R^2 = 0.49$, $P < 0.001$) and revealed a significant positive correlation between $\Delta F\text{-D}$ and $\Delta F\text{-R}$ (slope = 0.55, $P < 0.001$). This analysis also revealed that the amplitude (slope = -0.03; $P = 0.82$) and threshold (slope = -0.01; $P = 0.71$) had no significant effect on $\Delta F\text{-D}$, while duration significantly increased with $\Delta F\text{-D}$ decline (slope = -0.08; $P < 0.001$). This result indicates that smaller $\Delta F\text{-D}$ accompanied longer duration motor unit potential, which is a sign of peripheral denervation. We found no link with amplitude and activation threshold, which is consistent with EMG amplitudes as well.

DISCUSSION

While similar levels of EMG activity were recorded during MVC in controls and ALS, the characteristics of the first motor units activated during contraction, including U1 and U2, were different in ALS: i) the peak-to-peak amplitude of motor unit potentials was larger, especially in the most affected muscles in the group (ECR, TA), ii) their duration was significantly increased irrespective of the muscle groups assessed, and iii) the unit thresholds (% EMG at MVC) were significantly higher. The novel finding of this study is the alteration of U1 frequency modulation during voluntary (contraction protocol) or reflex-induced contractions (vibration protocol), and active relaxation. Irrespective of the recruitment mode, the increase in U1 frequency during recruitment ($\Delta F\text{-R}$) was similar between subject groups. On the other hand, the decrease in U1 frequency during relaxation ($\Delta F\text{-D}$) was smaller in patients than in controls, especially for $\Delta F\text{-R} < 6\text{-}8$ Hz. Finally, $\Delta F\text{-D}$ was found more depressed in the upper limbs, matching the muscle weakness profile of the patient group, and smaller in patients with more functional disability (ALSF_{RS-r}), more rapid disease progression rate, and with longer duration of motor unit potential. These results suggest that the motoneuron ability for self-sustained discharge are reduced in patients with ALS, which indicates that motoneurons become hypoexcitable with disease progression.

EMG and motor unit characteristics in ALS

Numerous previous studies have sought to identify specific and sensitive metrics to track motoneuron pathophysiology and degeneration in ALS (de Carvalho & Swash, 2016). It is well established that the motor unit amplitude is increased in ALS as well as the duration, and the latter is particularly sensitive (de Carvalho *et al.*, 2014). In the present study, the investigated muscles were not or only mildly affected, and the mean surface EMG during MVC was comparable in controls and ALS. However, the motor unit potentials and their duration in particular, were modified which further confirms that the duration of motor unit potential is very sensitive to early neuromuscular alterations in ALS. Changes in motor unit characteristics have been attributed to early reinnervation by slow motor units. These units exhibit axon sprouting abilities that likely contribute to their resilience to degeneration, compared to fast fatigable motor units that are very sensitive to denervation and degeneration in ALS (Frey *et al.*, 2000; Schaefer *et al.*, 2005; Pun *et al.*, 2006; De Winter *et al.*, 2006; Hegedus *et al.*, 2007).

Given the linear relationship between the contraction force and the EMG activity (Milner-Brown & Stein, 1975), the mean level of surface EMG (% mean EMG at MVC) was used to estimate the level of motor unit recruitment (threshold), as we performed in a previous study (Marchand-Pauvert *et al.*, 2000). In patients with ALS, the relationship between force and EMG was found similar to controls, and the slope of the linear fit only tended to be smaller in patients (Jahanmiri-Nezhad *et al.*, 2014). Here, we found a significantly steeper slope in patients. The discrepancy with the previous study (Jahanmiri-Nezhad *et al.*, 2014) likely stems from the fact that the sampled muscles in our study were less affected. Jahanmiri-Nezhad *et al.* studied hand muscles, and the EMG activity during MVC was smaller in patients than in controls. Here, we studied more proximal muscles with mild or no over weakness, and EMG during MVC was comparable in the 2 groups. Our result indicates that for a similar force level in controls, an higher EMG level was developed in patients. Furthermore, while the tonic contraction was very weak (as reported by both controls and patients during the experiments) and a comparable intramuscular EMG profile in both groups (not more than 3-5 motor units to allow a reliable EMG decomposition), a greater mean surface EMG activity was developed in patients than in controls. The large potentials of resilient low-threshold motor

units in ALS have contributed to the mean level of rectified surface EMG activity, to a greater extent than in controls. The difference between groups is more marked during a weak tonic contraction, compared to MVC, because the large motor unit potentials due to high threshold motor units are not activated in controls at low levels of contraction force. Therefore, the estimation of motor unit threshold based on EMG was biased in ALS, due to the peripheral reinnervation. Given the similitude of intramuscular EMG profiles obtained during comparable effort in both groups and the changes in the relationship between EMG and contraction force in ALS, we assume that the contraction forces during protocols were likely within the same range in controls and patients. However, in future studies using this protocol, we strongly advise to combine systematically surface and intramuscular EMG recordings together with quantitative force quotation, especially in patients with supposed denervation like in ALS. Our results also suggest that fewer motor units likely participated in EMG activity during MVC in ALS than in controls. Alternatively, different electro-mechanical coupling may also explain a greater force produced at similar EMG levels in ALS compared to controls. Indeed, stronger force contraction associated to large motor unit potentials have been reported in some patients but the reverse too, without any clear clinical explanations (Schmied *et al.*, 1999).

All these results support that combined force, surface EMG and motor unit analyses are required for a reliable and comprehensive characterisation of the neuromuscular functions in ALS (de Carvalho & Swash, 2016). These functions were likely altered in the 4 muscles we tested in our group of patients, despite most of them appearing clinically normal. Furthermore, our results confirm that the slope of linear regression between contraction force and surface EMG is likely a very sensitive measure of muscle reinnervation and support its putative role as a biomarker of motoneuron degeneration (Jahanmiri-Nezhad *et al.*, 2014).

Changes in unit discharge frequency during recruitment and derecruitment in ALS

On average, the U1 firing rate during the weak tonic contraction (f-U1 tonic) was not significantly different from controls. However, there was a trend to slower firing rate in ALS, as reported in a previous study (de Carvalho *et al.*, 2012). The absence of differences between the subject groups can be attributed to the great overlap in unit frequencies in patients and controls (Tables 2 & 3). Our results

further confirm that the interest of motor unit firing rate during tonic contraction to assess spinal motoneuron affection in ALS is limited (Vucic, 2012).

Irrespective of its firing rate during the weak tonic contraction (f -U1), the increase in U1 frequency during U2 recruitment (ΔF -R) was within the same range in controls and patients in all investigated muscles during both protocols. This result suggests that the level of motoneuron activity during the tonic phase, at least within this range, had no influence on the response of low-threshold motoneurons to increased synaptic drive, whatever its origin. Indeed, the synaptic inputs for motor unit recruitment during the contraction protocol were mainly due to increased descending inflow and, to some extent, to increased muscle spindle afferent inputs (Vallbo, 1970; Burke *et al.*, 1978). During tendon vibration, the increased synaptic drive to motoneuron pool was mainly of peripheral origin, mostly mediated by muscle spindle afferents (Macefield, 2005). However, it is well established that during tendon vibration, increased voluntary contraction can occur and, while this was monitored during recording (see vibration protocol in Methods), we cannot rule out the possible contribution of descending inputs during vibration. According to the Henneman's size principle (Henneman & Mendell, 1981), those inputs are equally distributed to motoneuron pool, especially during voluntary tonic contraction (Nielsen *et al.*, 1999). Our results suggest that there is likely no disruption of the size principle of motoneuron recruitment in ALS, as reported in animal models of reinnervation (Cope & Clark, 1993; Gordon *et al.*, 2004).

The variant of the ΔF method we developed for this study revealed that decreased U1 frequency during U2 derecruitment (ΔF -D, only named ΔF in previous studies) correlates to ΔF -R. It has been shown that ΔF -D mostly depends on PICs, and can be used as an estimate of their activation by excitatory synaptic inputs (Heckmann *et al.*, 2005; Powers & Heckman, 2015, 2017). However, given the relationship between synaptic inputs to motoneurons, PICs' activation and the motor unit firing (Segundo *et al.*, 1963; Matthews, 1996; Wienecke *et al.*, 2009; Powers *et al.*, 2012; Powers & Heckman, 2015, 2017), a correlation between ΔF -R and ΔF -D would be expected. This hypothesis has been explored in cat motoneurons and a significant linear relationship has been reported between the increase of U1 frequency during recruitment and ΔF -D. "*Because both firing rate and PIC activation are potentially modulated by the net excitatory synaptic input,*

this correlation might reflect graded synaptic activation of PIC. In other words, when a motoneuron is first recruited at a given level of excitatory synaptic input, the channels contributing to the total PIC are not maximally activated—further increases in net excitatory input are needed to fully activate the channels” (Powers *et al.*, 2008). To our knowledge, this is the first time that the interaction between $\Delta F-R$ and $\Delta F-D$ was investigated when studying paired motor units in humans. Moreover, our study highlights the interest of studying U1 frequency during tonic contraction and its increase during recruitment to provide reliable comparisons of $\Delta F-D$. Indeed, comparing $\Delta F-D$ *per se* failed to reveal any significant differences between controls and patients; we only identified a trend to reduced $\Delta F-D$ in ALS. Comparing the relationship between $\Delta F-R$ and $\Delta F-D$ in both groups revealed lower $\Delta F-D$ in patients than in controls, especially for $\Delta F-R < 6-8$ Hz, *i.e.* for low-to-medium level of U1 increase firing rate reflecting the increase in synaptic drive. This result suggests that descending and peripheral inputs were likely to be more efficient to elicit PICs and self-sustained motoneuron discharge in controls than in ALS patients. This was particularly true at low-to-medium level of synaptic drive; the difference between study groups becoming less significant at higher levels (greater $\Delta F-R$). It is interesting to note here that the maximal level of $\Delta F-R$ for altered $\Delta F-D$ was higher in the upper limbs (< 8 Hz) than in the lower limbs (< 6 Hz). This suggests that the depressed efficacy of synaptic drive was more marked in the upper limbs, which matches the distribution of muscle weakness within the patient groups (more patients with muscle weakness in ECR + TB, compared to TA + quadriceps). In turn, this suggests that the alteration between of $\Delta F-R/\Delta F-D$ relationship is likely to increase with muscle weakness.

Experimental considerations

A previous simulation study has shown that $\Delta F-D$ were larger during slow speed ramps (rise and decline phases of the ramp) and when the plateau lasted longer (Revill & Fuglevand, 2011). To our knowledge, fast ramps without plateau (triangle contraction) were mainly used in human experiments (Gorassini *et al.*, 1998, 2002a, 2002b, 2004; Mottram *et al.*, 2009; Udina *et al.*, 2010; Vandenberk & Kalmar, 2014; Wilson *et al.*, 2015). However, in a paper comparing the effect of repetitive contractions on $\Delta F-D$, the profile of motor unit firing during short (1 s.) and longer lasting contractions (20 s.) looked quite similar (Gorassini *et al.*, 2002b;

their Fig. 4). Monitored triangle contractions have been mostly used for ΔF -D assessment and PIC estimation in humans, to match the experimental protocols developed in animal models for studying motoneuron bistability, and because it has been established in cat motoneurons that the rate of change of triangular injected current influences the PIC activation, particularly in partially bistable motoneurons (high threshold motoneurons); the effect is lower in low-threshold fully bistable ones. Moreover, in the latter, the speed of voltage change influence PICs especially during the ascending/activation phase, compared to the descending/releasing phase during which the difference in PIC activation during slow and fast speeds was low (Lee & Heckman, 1998). Our study focused particularly on low threshold motoneurons that are likely fully bistable and so, likely less sensitive to the speed of contraction change during the ramp, especially during relaxation. The instructions and biofeedback to subjects (similar in both groups) were based on the intramuscular profile and its usability for reliable spike sorting. We did not impose and monitor the rate of contraction change during the experimental session like in the classical ΔF method, to ensure the feasibility in most of ALS patients investigated. However, the off line analysis did not reveal any significant difference between the velocity estimation of rise and relaxation phases of ramp contraction between groups, irrespective of muscle groups.

Accordingly, we assume that the conditionings were comparable between groups, and could not explain the smaller ΔF -D we observed in ALS. Furthermore, we found ΔF -D within a similar range than in previous studies (Gorassini *et al.*, 1998, 2002a, 2002b, 2004; Mottram *et al.*, 2009; Vandenberg & Kalmar, 2014; Wilson *et al.*, 2015), irrespective of the protocol, which indicates that the methodological variant we developed for the present study was as efficient as the classical method of paired motor unit recordings (using triangle contractions). The limitation of the classical ΔF method in patients with neurological disorders has been previously raised (Powers *et al.*, 2008), and we only found one study in stroke patients (Mottram *et al.*, 2009). When its implementation is possible, the classical ΔF method is of real importance to evaluate motoneuron excitability since it mimics the experimental conditions in animal models. The protocol we developed was easier to perform in patients with ALS, but it is important to monitor online the contraction force and the velocities of contraction changes during ramp in

future studies, to ensure a reliable interindividual comparison.

Pathophysiological interpretations

Clinically, pyramidal signs are observed in about 75 % ALS cases, including spasticity (~ 20 % cases), Babinski signs (~ 50 % cases) and hyperreflexia (70 % cases). These signs tend to remain unchanged over time, although they seem to appear at later stages or even disappear over time in some patients (Álvarez *et al.*, 2018). In our group of patients, at the time of the electrophysiological examination, 77 % exhibited frank pyramidal signs (predominant UMN and balanced UMN/LMN forms; Table 1), with spasticity in 27 %, Babinski or Hoffman signs in 35 %, and frank hyperreflexia in 73 % cases, *i.e.* quite similar to the cohort presented by Álvarez *et al.* 2018. This indicates that our population was representative of a classic ALS population in which hyperreflexia is a common clinical feature.

The smaller ΔF -D observed in patients, irrespective of the protocol (contraction and vibration) when taking into account the ΔF -R, suggest that the motoneuron capacities to repetitive discharge were depressed compared to controls, irrespective of age. It has been previously shown that ΔF -D depends on PICs' activation that enhances motoneuron response to synaptic inputs and induces self-sustained motoneuron discharge (Gorassini *et al.*, 1998; Bennett *et al.*, 2001; Heckmann *et al.*, 2005; Powers & Heckman, 2015, 2017). Monoaminergic centres in the brainstem are known to activate the sodium and calcium channels inducing PICs (Heckman *et al.*, 2008). In line with this, ΔF -D measurements in humans have been shown to be sensitive to monoamines, especially serotonin (D'Amico *et al.*, 2013). In ALS, the brainstem serotonergic neurones are affected (Dupuis *et al.*, 2010; Dentel *et al.*, 2013), which may have repercussion on PICs' activation and the neuromodulation of motoneuron discharge. Spinal cord injury (SCI) studies indicate that motoneuron excitability recover at the chronic stage with the possibility to reactivate PICs by sensory inputs. PICs have been found particularly enhanced in SCI animal models, and the greater reflex responses in SCI patients suggest similar modifications in humans. Therefore, it has been proposed that PICs likely contribute to hyperreflexia, muscles spasms and spasticity in SCI patients (Bennett *et al.*, 2004; Gorassini *et al.*, 2004). ΔF -D have only been studied in stroke patients (Mottram *et al.*, 2009) and, despite the enhanced tonic vibration reflex suggesting larger PICs (McPherson *et al.*, 2008), ΔF -D *per se* were unchanged on the paretic

spastic side. Given the resembling clinical symptoms suggesting spinal hyperexcitability, it has been proposed that the intrinsic motoneuron excitability might be similarly enhanced in ALS (ElBasiouny *et al.*, 2010). Instead, our results indicate that different pathophysiological mechanisms occur in ALS, and the comparison with other pathologies with pyramidal syndrome is not justified. Indeed, if changes in the descending drive, including monoaminergic inputs, would be the main mechanism underlying the change in spinal excitability in ALS, we should have observed similar modifications (regarding the vibration-induced reflex responses and $\Delta F-D$) as those reported after SCI or stroke, which was not the case. Therefore, we assume that the reduction of $\Delta F-D$ in ALS is unlikely to be driven by altered descending inputs alone. On the other hand, PICs are also strongly controlled by post-synaptic inhibition (Johnson & Heckman, 2010; Revill & Fuglevand, 2011; Powers & Heckman, 2017). Cortical hyperexcitability, which is an early feature of ALS (Vucic *et al.*, 2013), is characterized by the imbalance between inhibition and excitation with enhanced facilitation to a greater proportion than depressed inhibition (Van den Bos *et al.*, 2018). Much less is known about spinal excitability. To our knowledge, only recurrent inhibition has been explored at spinal level, and was found reduced in patients (Raynor & Shefner, 1994). Therefore, it appears that post-synaptic inhibitions are likely to be reduced in ALS, which would have the opposite effect on $\Delta F-D$ as those we report. As a consequence, we propose that the reduction of $\Delta F-D$ in patients is likely related to depressed intrinsic excitability of spinal motoneurons, as reported in animal models (Delestrée *et al.*, 2014; Martínez-Silva *et al.*, 2018).

Comparing the ramps we used and the results of the simulation study by Revill & Fuglevand (2011), it seems that $\Delta F-D$ mainly depended on PICs in our experimental conditions, with a small contribution of spike-threshold accommodation and spike-frequency adaptation. Therefore, our results suggest that PICs may be reduced in patients since $\Delta F-D$ were smaller. However, ALS mouse models have revealed larger PICs (Meehan *et al.*, 2010; Quinlan *et al.*, 2011), but it seems that experimental conditions, development stage and type of motoneurons strongly influence the results (ElBasiouny *et al.*, 2010; Delestrée *et al.*, 2014; Leroy *et al.*, 2014; Martínez-Silva *et al.*, 2018). In our experiments, it is possible that larger PICs compensated for a reduced descending and peripheral inputs (Iglesias *et al.*, 2015;

Vaughan *et al.*, 2015; Sangari *et al.*, 2016). As the estimation of PICs based on $\Delta F-D$ is indirect, it is difficult to establish the underlying mechanisms and their modifications in ALS but our results indicate that the motoneuron self-sustained discharge was lower in patients. Alternatively, the extent to which AHP contributes to $\Delta F-D$ has been discussed (Pierrot-Deseilligny & Burke, 2012). It has been shown that AHP was shortened in ALS patients with mild motor dysfunctions and then increased with motor deficits (Piotrkiewicz & Hausmanowa-Petrusewicz, 2011). In line with this observation, the present study has shown a significant link between $\Delta F-D$ depression and functional loss, progression rate, motor unit duration and muscle weakness, suggesting that motoneurons likely become hypoexcitable as the disease progresses. However, it has been shown that synaptic noise and PICs mostly contribute to firing rate of low-threshold motoneurons (Wienecke *et al.*, 2009).

It has been demonstrated in ALS mice models, right before peripheral denervation, that low-threshold motoneurons are normoexcitable, and the higher-threshold motoneurons, hypoexcitable (Martínez-Silva *et al.*, 2018). Here, we did not find any significant influence of the unit threshold on $\Delta F-D$. However, the threshold was estimated with the mean surface EMG, which was confounded by peripheral denervation. We assume that our results were restricted to the low-threshold motoneurons in both groups, due to the limitation of the methodology (weak EMG activity for reliable spike sorting), *i.e.* the motoneurons which resist to ALS, at least at the time of the investigation. Nonetheless, our patients were symptomatic, with signs of peripheral denervation in the investigated muscles despite a normal strength on clinical evaluation in most cases. Thus, there is a possibility that a given portion of the high-threshold motoneuron pool had already died at the time of investigation and that the low-threshold motoneurons, which persist longer, were partly altered. Accordingly, within the resilient motoneuron pool in symptomatic ALS patients, we detected normo- and hypoexcitability, as reported in the presymptomatic models, including resilient motoneurons and those about to die. Thus, we propose that hypoexcitability likely precedes motoneuron degeneration in the human sporadic form, as reported in experimental models, but this hypothesis must be confirmed by longitudinal study, in sporadic forms and in asymptomatic carriers of ALS-causing genetic mutations.

The main finding of this study is that motoneurons in ALS patients exhibited

unaltered responses to increased synaptic drive, but their ability to generate repetitive discharges is lower, whatever the origin of the inputs (descending inflow/peripheral afferents). Our patients were all treated with riluzole, which may have reduced the motoneuron firing due to its inhibitory actions on glutamatergic transmission and sodium currents. However, ΔF -D was particularly altered for ΔF -R < 6-8 Hz and correlated to disease progression rate and functional loss. This suggests that hypoexcitability only manifested in the most affected motoneurons despite the effect riluzole. In addition, it has been shown that riluzole has only a transient effect on brain and peripheral excitability (8 first weeks; (Geevasinga *et al.*, 2016). However, we thought it is necessary to investigate the effect of riluzole using the ΔF method as a biomarker of motoneuron excitability.

Conclusions

The present study did not reveal any evidence for motoneuron hyperexcitability in human sporadic forms of ALS. The results indicate that surviving low-threshold motoneurons are within a range of normo- to hypoexcitability. Moreover, we found that motoneuron hypoexcitability accompanies peripheral denervation, muscle weakness, functional loss, and particularly manifests in patients with rapid progression. These results suggest that motoneuron hypoexcitability likely develop progressively during the course of the disease, and this hypothesis should be further investigated with longitudinal studies, including asymptomatic carriers of ALS-causing mutations. The findings of this *in vivo* human study are consistent with animal models and *in vitro* studies on human iPSC-derived motoneurons, which also revealed motoneuron hypoexcitability in genetic forms of ALS. This notion has been previously regarded as contentious, especially because familial forms of ALS constitute a small proportion of clinical cases and because clinical symptoms seemingly support hyperexcitability (hyperreflexia). Our study confirms motoneuron hypoexcitability and paves the way for further dedicated studies on experimental models, to elucidate the origin of subcellular substrate of motoneuron hypoexcitability, in order to develop novel therapeutic strategies that could be evaluated in human patients using our methodological variant of paired motor unit recordings. In addition, this study highlights the discrepancy between motoneuron excitability changes, reflex responses studied experimentally (Nardone

et al., 2001; Christensen *et al.*, 2003; Simon *et al.*, 2015), and clinical hyperreflexia in ALS. We propose that hyperexcitability classically described in ALS is not related to increased intrinsic excitability of spinal motoneurons but is likely to represent adaptive processes that compensate for motoneuron hypoexcitability, likely involving spinal interneurons that regulate motoneuron excitability (extrinsic excitability). Future studies of spinal pathways, involving interneurons, are therefore of great interest to elucidate the link between the neural network controlling motoneuron excitability and their degeneration.

REFERENCES

- Abe K, Aoki M, Tsuji S, Itoyama Y, et al. & Writing Group; Edaravone (MCI-186) ALS 19 Study Group (2017). Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol* **16**, 505-512.
- Álvarez N, Díez L, Avellaneda C, Serra M & Rubio MÁ (2018). Relevance of the pyramidal syndrome in amyotrophic lateral sclerosis. *Neurol Barc Spain* **33**, 8-12.
- Bennett DJ, Li Y, Harvey PJ & Gorassini M (2001). Evidence for plateau potentials in tail motoneurons of awake chronic spinal rats with spasticity. *J Neurophysiol* **86**, 1972-1982.
- Bennett DJ, Sanelli L, Cooke CL, Harvey PJ & Gorassini MA (2004). Spastic long-lasting reflexes in the awake rat after sacral spinal cord injury. *J Neurophysiol* **91**, 2247-2258.
- Bensimon G, Lacomblez L & Meininger V (1994). A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *N Engl J Med* **330**, 585-591.
- Bhandari R, Kuhad A, Itoyama Y, et al. & Writing Group; Edaravone (MCI-186) ALS 19 Study Group (2018). Edaravone: a new hope for deadly amyotrophic lateral sclerosis. *Drugs Today Barc Spain* **54**, 349-360.
- Bogaert E, d'Ydewalle C & Van Den Bosch L (2010). Amyotrophic lateral sclerosis and excitotoxicity: from pathological mechanism to therapeutic target. *CNS Neurol Disord Drug Targets* **9**, 297-304.
- Brooks BR, Miller RG, Swash M, Munsat TL & World Federation of Neurology Research Group on Motor Neuron Diseases (2000a). El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Mot Neuron Disord Off Publ World Fed Neurol Res Group Mot Neuron Dis* **1**, 293-299.
- Brooks BR, Miller RG, Swash M, Munsat TL & World Federation of Neurology Research Group on Motor Neuron Diseases (2000b). El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Scler Mot Neuron Disord Off Publ World Fed Neurol Res Group Mot Neuron Dis* **1**, 293-299.
- Burke D, Hagbarth KE & Skuse NF (1978). Recruitment order of human spindle endings in isometric voluntary contractions. *J Physiol* **285**, 101-112.
- Burkhardt MF et al. (2013). A cellular model for sporadic ALS using patient-derived induced pluripotent stem cells. *Mol Cell Neurosci* **56**, 355-364.

- de Carvalho M & Swash M (2016). Lower motor neuron dysfunction in ALS. *Clin Neurophysiol Off J Int Fed Clin Neurophysiol* **127**, 2670-2681.
- de Carvalho M, Turkman A & Swash M (2012). Motor unit firing in amyotrophic lateral sclerosis and other upper and lower motor neurone disorders. *Clin Neurophysiol Off J Int Fed Clin Neurophysiol* **123**, 2312-2318.
- de Carvalho M, Turkman A & Swash M (2014). Sensitivity of MUP parameters in detecting change in early ALS. *Clin Neurophysiol Off J Int Fed Clin Neurophysiol* **125**, 166-169.
- Cedarbaum JM, Stambler N, Malta E, Fuller C, Hilt D, Thurmond B & Nakanishi A (1999). The ALSFRS-R: a revised ALS functional rating scale that incorporates assessments of respiratory function. BDNF ALS Study Group (Phase III). *J Neurol Sci* **169**, 13-21.
- Christensen PB, Nielsen JF & Sinkjaer T (2003). Quantification of hyperreflexia in amyotrophic lateral sclerosis (ALS) by the soleus stretch reflex. *Amyotroph Lateral Scler Mot Neuron Disord Off Publ World Fed Neurol Res Group Mot Neuron Dis* **4**, 106-111.
- Cope TC & Clark BD (1993). Motor-unit recruitment in self-reinnervated muscle. *J Neurophysiol* **70**, 1787-1796.
- Cotel F, Antri M, Barthe J-Y & Orsal D (2009). Identified ankle extensor and flexor motoneurons display different firing profiles in the neonatal rat. *J Neurosci Off J Soc Neurosci* **29**, 2748-2753.
- D'Amico JM, Murray KC, Li Y, Chan KM, Finlay MG, Bennett DJ & Gorassini MA (2013). Constitutively active 5-HT₂/α1 receptors facilitate muscle spasms after human spinal cord injury. *J Neurophysiol* **109**, 1473-1484.
- De Winter F, Vo T, Stam FJ, Wisman LAB, Bär PR, Niclou SP, van Muiswinkel FL & Verhaagen J (2006). The expression of the chemorepellent Semaphorin 3A is selectively induced in terminal Schwann cells of a subset of neuromuscular synapses that display limited anatomical plasticity and enhanced vulnerability in motor neuron disease. *Mol Cell Neurosci* **32**, 102-117.
- Delestrée N, Manuel M, Iglesias C, Elbasiouny SM, Heckman CJ & Zytnicki D (2014). Adult spinal motoneurons are not hyperexcitable in a mouse model of inherited amyotrophic lateral sclerosis. *J Physiol* **592**, 1687-1703.
- Dentel C, Palamiuc L, Henriques A, Lannes B, Spreux-Varoquaux O, Gutknecht L, René F, Echaniz-Laguna A, Gonzalez de Aguilar J-L, Lesch KP, Meininger V, Loeffler J-P & Dupuis L (2013). Degeneration of serotonergic neurons in amyotrophic lateral sclerosis: a link to spasticity. *Brain J Neurol* **136**, 483-493.
- Devlin A-C, Burr K, Borooah S, Foster JD, Cleary EM, Geti I, Vallier L, Shaw CE, Chandran S & Miles GB (2015). Human iPSC-derived motoneurons harbouring TARDBP or C9ORF72 ALS mutations are dysfunctional despite maintaining viability. *Nat Commun* **6**, 5999.
- Dharmadasa T & Kiernan MC (2018). Riluzole, disease stage and survival in ALS. *Lancet Neurol* **17**, 385-386.
- Dupuis L, Spreux-Varoquaux O, Bensimon G, Jullien P, Lacomblez L, Salachas F, Bruneteau G, Pradat P-F, Loeffler J-P & Meininger V (2010). Platelet serotonin level predicts survival in amyotrophic lateral sclerosis. *PLoS One* **5**, e13346.
- ElBasiouny SM, Schuster JE & Heckman CJ (2010). Persistent inward currents in spinal motoneurons: important for normal function but potentially harmful after spinal cord injury and in amyotrophic lateral sclerosis. *Clin Neurophysiol Off J Int Fed Clin Neurophysiol* **121**, 1669-1679.
- Fogarty MJ (2018). Driven to decay: Excitability and synaptic abnormalities in

- amyotrophic lateral sclerosis. *Brain Res Bull* **140**, 318-333.
- Frey D, Schneider C, Xu L, Borg J, Spooren W & Caroni P (2000). Early and selective loss of neuromuscular synapse subtypes with low sprouting competence in motoneuron diseases. *J Neurosci Off J Soc Neurosci* **20**, 2534-2542.
- Geevasinga N, Menon P, Ng K, Van Den Bos M, Byth K, Kiernan MC & Vucic S (2016). Riluzole exerts transient modulating effects on cortical and axonal hyperexcitability in ALS. *Amyotroph Lateral Scler Front Degener* **17**, 580-588.
- Gorassini M, Yang JF, Siu M & Bennett DJ (2002a). Intrinsic activation of human motoneurons: possible contribution to motor unit excitation. *J Neurophysiol* **87**, 1850-1858.
- Gorassini M, Yang JF, Siu M & Bennett DJ (2002b). Intrinsic activation of human motoneurons: reduction of motor unit recruitment thresholds by repeated contractions. *J Neurophysiol* **87**, 1859-1866.
- Gorassini MA, Bennett DJ & Yang JF (1998). Self-sustained firing of human motor units. *Neurosci Lett* **247**, 13-16.
- Gorassini MA, Knash ME, Harvey PJ, Bennett DJ & Yang JF (2004). Role of motoneurons in the generation of muscle spasms after spinal cord injury. *Brain J Neurol* **127**, 2247-2258.
- Gordon T, Thomas CK, Munson JB & Stein RB (2004). The resilience of the size principle in the organization of motor unit properties in normal and reinnervated adult skeletal muscles. *Can J Physiol Pharmacol* **82**, 645-661.
- Heckman CJ, Johnson M, Mottram C & Schuster J (2008). Persistent inward currents in spinal motoneurons and their influence on human motoneuron firing patterns. *Neurosci Rev J Bringing Neurobiol Neurol Psychiatry* **14**, 264-275.
- Heckmann CJ, Gorassini MA & Bennett DJ (2005). Persistent inward currents in motoneuron dendrites: implications for motor output. *Muscle Nerve* **31**, 135-156.
- Hegedus J, Putman CT & Gordon T (2007). Time course of preferential motor unit loss in the SOD1 G93A mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis* **28**, 154-164.
- Henneman E & Mendell L (1981). The Nervous System. Motor Control. In *Handbook of Physiology*, pp. 423-407. American Physiological Society.
- Iglesias C, Sangari S, El Mendili M-M, Benali H, Marchand-Pauvert V & Pradat P-F (2015). Electrophysiological and spinal imaging evidences for sensory dysfunction in amyotrophic lateral sclerosis. *BMJ Open* **5**, e007659.
- Jahanmiri-Nezhad F, Hu X, Suresh NL, Rymer WZ & Zhou P (2014). EMG-force relation in the first dorsal interosseous muscle of patients with amyotrophic lateral sclerosis. *NeuroRehabilitation* **35**, 307-314.
- Johnson MD & Heckman CJ (2010). Interactions between focused synaptic inputs and diffuse neuromodulation in the spinal cord. *Ann N Y Acad Sci* **1198**, 35-41.
- Kiehn O & Eken T (1997). Prolonged firing in motor units: evidence of plateau potentials in human motoneurons? *J Neurophysiol* **78**, 3061-3068.
- Kuo JJ, Siddique T, Fu R & Heckman CJ (2005). Increased persistent Na(+) current and its effect on excitability in motoneurons cultured from mutant SOD1 mice. *J Physiol* **563**, 843-854.
- Lee RH & Heckman CJ (1998). Bistability in spinal motoneurons in vivo: systematic variations in persistent inward currents. *J Neurophysiol* **80**, 583-593.
- Leroy F, Lamotte d'Incamps B, Imhoff-Manuel RD & Zytnicki D (2014). Early intrinsic hyperexcitability does not contribute to motoneuron degeneration in amyotrophic lateral sclerosis. *eLife*; DOI: 10.7554/eLife.04046.
- Macefield VG (2005). Physiological characteristics of low-threshold

- mechanoreceptors in joints, muscle and skin in human subjects. *Clin Exp Pharmacol Physiol* **32**, 135-144.
- Marchand-Pauvert V, Mazevet D, Nielsen J, Petersen N & Pierrot-Deseilligny E (2000). Distribution of non-monosynaptic excitation to early and late recruited units in human forearm muscles. *Exp Brain Res* **134**, 274-278.
- Martin E, Cazenave W, Cattaert D & Branchereau P (2013). Embryonic alteration of motoneuronal morphology induces hyperexcitability in the mouse model of amyotrophic lateral sclerosis. *Neurobiol Dis* **54**, 116-126.
- Martínez-Silva M de L, Imhoff-Manuel RD, Sharma A, Heckman CJ, Shneider NA, Roselli F, Zytnicki D & Manuel M (2018). Hypoexcitability precedes denervation in the large fast-contracting motor units in two unrelated mouse models of ALS. *eLife*; DOI: 10.7554/eLife.30955.
- Matthews PB (1996). Relationship of firing intervals of human motor units to the trajectory of post-spike after-hyperpolarization and synaptic noise. *J Physiol* **492** (Pt 2), 597-628.
- McPherson JG, Ellis MD, Heckman CJ & Dewald JPA (2008). Evidence for increased activation of persistent inward currents in individuals with chronic hemiparetic stroke. *J Neurophysiol* **100**, 3236-3243.
- Meehan CF, Moldovan M, Marklund SL, Graffmo KS, Nielsen JB & Hultborn H (2010). Intrinsic properties of lumbar motor neurones in the adult G127insTGGG superoxide dismutase-1 mutant mouse in vivo: evidence for increased persistent inward currents. *Acta Physiol Oxf Engl* **200**, 361-376.
- Millicamps S et al. (2012). Phenotype difference between ALS patients with expanded repeats in C9ORF72 and patients with mutations in other ALS-related genes. *J Med Genet* **49**, 258-263.
- Milner-Brown HS & Stein RB (1975). The relation between the surface electromyogram and muscular force. *J Physiol* **246**, 549-569.
- Morgan S & Orrell RW (2016). Pathogenesis of amyotrophic lateral sclerosis. *Br Med Bull* **119**, 87-98.
- Mottram CJ, Suresh NL, Heckman CJ, Gorassini MA & Rymer WZ (2009). Origins of abnormal excitability in biceps brachii motoneurons of spastic-paretic stroke survivors. *J Neurophysiol* **102**, 2026-2038.
- Nardone A, Galante M, Lucas B & Schieppati M (2001). Stance control is not affected by paresis and reflex hyperexcitability: the case of spastic patients. *J Neurol Neurosurg Psychiatry* **70**, 635-643.
- Naujock M et al. (2016). 4-Aminopyridine Induced Activity Rescues Hypoexcitable Motor Neurons from Amyotrophic Lateral Sclerosis Patient-Derived Induced Pluripotent Stem Cells. *Stem Cells Dayt Ohio* **34**, 1563-1575.
- Nielsen J, Morita H, Baumgarten J, Petersen N & Christensen LO (1999). On the comparability of H-reflexes and MEPs. *Electroencephalogr Clin Neurophysiol Suppl* **51**, 93-101.
- Nijssen J, Comley LH & Hedlund E (2017). Motor neuron vulnerability and resistance in amyotrophic lateral sclerosis. *Acta Neuropathol (Berl)* **133**, 863-885.
- Oldfield RC (1971). The assessment and analysis of handedness: the Edinburgh inventory. *Neuropsychologia* **9**, 97-113.
- Pieri M, Albo F, Gaetti C, Spalloni A, Bengtson CP, Longone P, Cavalcanti S & Zona C (2003). Altered excitability of motor neurons in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Neurosci Lett* **351**, 153-156.
- Pierrot-Deseilligny E & Burke D (2012). *The circuitry of the human spinal cord*, 2nd edn. Cambridge University Press, New York, USA.

- Piotrkiewicz M & Hausmanowa-Petrusewicz I (2011). Motoneuron afterhyperpolarisation duration in amyotrophic lateral sclerosis. *J Physiol* **589**, 2745-2754.
- Powers RK, Elbasiouny SM, Rymer WZ & Heckman CJ (2012). Contribution of intrinsic properties and synaptic inputs to motoneuron discharge patterns: a simulation study. *J Neurophysiol* **107**, 808-823.
- Powers RK & Heckman CJ (2015). Contribution of intrinsic motoneuron properties to discharge hysteresis and its estimation based on paired motor unit recordings: a simulation study. *J Neurophysiol* **114**, 184-198.
- Powers RK & Heckman CJ (2017). Synaptic control of the shape of the motoneuron pool input-output function. *J Neurophysiol* **117**, 1171-1184.
- Powers RK, Nardelli P & Cope TC (2008). Estimation of the contribution of intrinsic currents to motoneuron firing based on paired motoneuron discharge records in the decerebrate cat. *J Neurophysiol* **100**, 292-303.
- Pun S, Santos AF, Saxena S, Xu L & Caroni P (2006). Selective vulnerability and pruning of phasic motoneuron axons in motoneuron disease alleviated by CNTF. *Nat Neurosci* **9**, 408-419.
- Qian K, Huang H, Peterson A, Hu B, Maragakis NJ, Ming G-L, Chen H & Zhang S-C (2017). Sporadic ALS Astrocytes Induce Neuronal Degeneration In Vivo. *Stem Cell Rep* **8**, 843-855.
- Quinlan KA, Schuster JE, Fu R, Siddique T & Heckman CJ (2011). Altered postnatal maturation of electrical properties in spinal motoneurons in a mouse model of amyotrophic lateral sclerosis. *J Physiol* **589**, 2245-2260.
- Raynor EM & Shefner JM (1994). Recurrent inhibition is decreased in patients with amyotrophic lateral sclerosis. *Neurology* **44**, 2148-2153.
- Revill AL & Fuglevand AJ (2011). Effects of persistent inward currents, accommodation, and adaptation on motor unit behavior: a simulation study. *J Neurophysiol* **106**, 1467-1479.
- Revill AL & Fuglevand AJ (2017). Inhibition linearizes firing rate responses in human motor units: implications for the role of persistent inward currents. *J Physiol* **595**, 179-191.
- Rothstein JD (2009). Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. *Ann Neurol* **65 Suppl 1**, S3-9.
- Sangari S, Iglesias C, El Mendili M-M, Benali H, Pradat P-F & Marchand-Pauvert V (2016). Impairment of sensory-motor integration at spinal level in amyotrophic lateral sclerosis. *Clin Neurophysiol Off J Int Fed Clin Neurophysiol* **127**, 1968-1977.
- Schaefer AM, Sanes JR & Lichtman JW (2005). A compensatory subpopulation of motor neurons in a mouse model of amyotrophic lateral sclerosis. *J Comp Neurol* **490**, 209-219.
- Schmied A, Pouget J & Vedel JP (1999). Electromechanical coupling and synchronous firing of single wrist extensor motor units in sporadic amyotrophic lateral sclerosis. *Clin Neurophysiol Off J Int Fed Clin Neurophysiol* **110**, 960-974.
- Segundo JP, Moore GP, Stensaas LJ & Bullock TH (1963). Sensitivity of neurones in aplysia to temporal pattern of arriving impulses. *J Exp Biol* **40**, 643-667.
- Simon NG, Lin CS-Y, Lee M, Howells J, Vucic S, Burke D & Kiernan MC (2015). Segmental motoneuronal dysfunction is a feature of amyotrophic lateral sclerosis. *Clin Neurophysiol Off J Int Fed Clin Neurophysiol* **126**, 828-836.
- Udina E, D'Amico J, Bergquist AJ & Gorassini MA (2010). Amphetamine increases persistent inward currents in human motoneurons estimated from paired motor-

- unit activity. *J Neurophysiol* **103**, 1295-1303.
- Vallbo AB (1970). Discharge patterns in human muscle spindle afferents during isometric voluntary contractions. *Acta Physiol Scand* **80**, 552-566.
- Van den Bos MAJ, Higashihara M, Geevasinga N, Menon P, Kiernan MC & Vucic S (2018). Imbalance of cortical facilitatory and inhibitory circuits underlies hyperexcitability in ALS. *Neurology* **91**, e1669-e1676.
- Vandenberk MS & Kalmar JM (2014). An evaluation of paired motor unit estimates of persistent inward current in human motoneurons. *J Neurophysiol* **111**, 1877-1884.
- Vaughan SK, Kemp Z, Hatzipetros T, Vieira F & Valdez G (2015). Degeneration of proprioceptive sensory nerve endings in mice harboring amyotrophic lateral sclerosis-causing mutations. *J Comp Neurol* **523**, 2477-2494.
- Vucic S (2012). Assessing motor unit firing rates in ALS: A diagnostic and pathophysiological prospect? *Clin Neurophysiol Off J Int Fed Clin Neurophysiol* **123**, 2114-2115.
- Vucic S, Ziemann U, Eisen A, Hallett M & Kiernan MC (2013). Transcranial magnetic stimulation and amyotrophic lateral sclerosis: pathophysiological insights. *J Neurol Neurosurg Psychiatry* **84**, 1161-1170.
- Wainger BJ, Kiskinis E, Mellin C, Wiskow O, Han SSW, Sandoe J, Perez NP, Williams LA, Lee S, Boulting G, Berry JD, Brown RH, Cudkowicz ME, Bean BP, Eggan K & Woolf CJ (2014). Intrinsic membrane hyperexcitability of amyotrophic lateral sclerosis patient-derived motor neurons. *Cell Rep* **7**, 1-11.
- Wienecke J, Zhang M & Hultborn H (2009). A prolongation of the postspike afterhyperpolarization following spike trains can partly explain the lower firing rates at derecruitment than those at recruitment. *J Neurophysiol* **102**, 3698-3710.
- Wilson JM, Thompson CK, Miller LC & Heckman CJ (2015). Intrinsic excitability of human motoneurons in biceps brachii versus triceps brachii. *J Neurophysiol* **113**, 3692-3699.

COMPETITION INTEREST

The authors do not declare any conflict of interest.

AUTHOR CONTRIBUTIONS

VMP conceptualized the study and has developed the protocol. GQ, PB, LL, RD and PFP have selected the patients and performed the clinical evaluation. VMP, IP and ALV have selected the healthy subjects. VMP, IP and ALV performed the experiments and analysed the data. VMP performed the statistics and wrote the draft of the manuscript. All authors participated in finalizing the manuscript.

FUNDING

The study was supported by grants from AFM-Téléthon (AO 2012), ARSLA (Dotation 2013) and by Fondation Thierry Latran (7th call for projects, 2015). GQ

was supported by a grant from Padoue University (Italy).

ACKNOWLEDGEMENTS

The authors wish to express their grateful to Dr. Jessica M. D'Amico for her advices on paired motor unit methodology. Genetic analyses were performed by Dr. Patrick Yourc'h (Dpt of Biochemistry and Molecular Biology, University of Tours, France). Finally, they also thank the Paris ALS referent centre and all the participants.

AUTHOR PROFILE

Dr. Véronique Marchand-Pauvert (PhD, Inserm Research Director, equivalent position as Professor) is neurophysiologist with a specific expertise of the sensory motor system. She is particularly involved in studying the corticospinal connectivity underlying voluntary movements in humans, its plasticity and regulation by sensory feedback, using non-invasive electrophysiological techniques including TMS. She is particularly well-known for her great knowledge of the human spinal circuitry. Since 2014, she leads the neuroscience team in the Laboratory of Biomedical Imaging at the Pitié-Salpêtrière Hospital where she develops research programs coupling neuroimaging and electrophysiology to better understand the ALS pathophysiology and to identify biomarkers.



TABLES

Table 1. Clinical features of the patient group

	Duration	ALSFRS-r	Progression	UMN score			MRC score			
				UL	LL	Form	ECR	TB	TA	Quad
P1	16	45	0.19	0	0	LMN	5	5	5	5
P2	19	42	0.32	0	1	both	5	5	5	5
P3	34	36	0.35	0	4	both	5	5	5	5
P4	24	41	0.29	0	0	LMN	5	5	1	5
P5	25	44	0.16	4	0	both	4	5	5	5
P6	23	36	0.52	0	2	both	5	4	5	5
P7*	30	40	0.27	0	0	LMN	5	5	1	5
P8	19	42	0.32	5	6	UMN	5	5	5	5
P9	14	40	0.57	0	5	both	3	5	4	5
P10	13	47	0.08	4	4	UMN	5	5	5	5
P11	19	40	0.42	0	4	both	5	5	5	5
P12	7	47	0.14	0	0	LMN	5	5	3	5
P13	59	34	0.24	0	5	both	3	4	5	5
P14	13	29	1.46	6	6	UMN	4	5	5	5
P15	14	33	1.07	4	2	UMN	3	4	5	5
P16	22	38	0.45	5	4	UMN	3	4	5	5
P17	33	39	0.27	0	0	LMN	5	5	5	5
P18	14	24	1.71	0	3	both	4	5	5	5
P19	11	34	1.27	4	5	UMN	4	4	3	5
P20	48	43	0.10	4	4	UMN	5	5	5	5
P21	14	38	0.71	0	3	both	5	5	4	5
P22*	12	42	0.50	0	0	LMN	5	5	5	5

Patient number with * indicating familial forms; Duration, time from first symptom (months); ALSFRS-r, score to the revised ALS Functional Rating Scale (48 = normal); Progression (loss of points/month) = (48 - ALSFRS-r score) / duration (months); UMN score for upper limb (UL) and lower limb (LL) taking into account the spasticity

(modified Ashworth scale), clonus, tendon jerk and Babinski or Hoffmann responses (maximum score = 6, increasing with upper motoneuron defect); Form: predominant affection of upper motoneurons (UMN) or of lower motoneurons (LMN) or balanced UM/LMN form (both); MRC, score of muscle strength evaluated by muscle manual testing (from 0 = no force to 5 = normal force) in ECR, TB, TA and Quad (quadriceps).

Table 2: Characteristics of motor unit pairs collected during the contraction protocol

	ECR		TB		TA		Quadriceps	
	Contr ols	ALS	Contr ols	ALS	Contr ols	ALS	Contr ols	ALS
Number	30 (25)	24 (18)	38 (24)	26 (18)	44 (28)	31 (19)	35 (25)	37 (21)
U1 threshold	1.2 (0.3-2.6)	7.1*** (2.4-14.1)	4.8 (1.1-8.2)	9.6 (2.5-17.8)	2.7 (1.1-5.0)	8.8** (4.3-14.5)	1.3 (0.6-10.0)	7.0 (2.9-11.5)
U2 threshold	4.0 (1.8-6.8)	20.2*** (9.5-62.6)	12.8 (5.3-21.7)	24.0 (15.0-44.8)	8.8 (4.7-15.6)	25.2*** (15.2-39.0)	7.5 (2.8-21.6)	23.4* (9.7-32.3)
f-U1 tonic	8.2 (5.5-11.5)	7.7 (4.7-10.3)	9.7 (6.1-11.3)	6.2 (4.7-10.0)	6.8 (4.1-8.1)	3.9 (3.2-7.8)	6.8 (4.8-9.1)	4.7 (3.9-7.3)
f-U1 recruitment	11.8 (9.5-14.9)	11.5 (8.1-15.1)	13.2 (9.4-17.6)	9.7 (8.0-15.4)	10.0 (7.0-12.1)	7.3 (4.4-11.6)	10.3 (7.1-12.5)	6.7 (5.6-10.4)
f-U1 derecruitment	6.4 (5.3-9.9)	7.6 (6.0-10.4)	7.5 (5.2-9.3)	6.7 (4.7-9.2)	6.8 (4.7-8.4)	4.3 (3.2-6.2)	5.8 (3.8-7.5)	4.7 (3.6-6.4)

Number of motor unit pairs and of subjects between brackets; U1 threshold, level of mean rectified surface EMG (% mean EMG at MVC) when the low threshold control unit U1 appeared in intramuscular EMG; U2 threshold, level of mean rectified surface EMG (% mean EMG at MVC) when the higher threshold test unit U2 appeared in intramuscular EMG during the increase of contraction force level; f-U1 tonic, discharge rate of U1 (Hz) in intramuscular EMG during the tonic phase, before the increase of force contraction level to recruit U2; f-U1 recruitment, discharge rate of U1 (Hz) in intramuscular EMG when U2 was recruited during the increase of force contraction; f-U1 derecruitment, discharge rate of U1 (Hz) when U2 disappeared from intramuscular EMG during the relaxation phase. In each cell: the first row indicates the median values and the second row, the first (Q1) and the third (Q3) quartiles between brackets. ANOVA on ranks and Dunn pairwise comparisons were used to compare the U1 and U2 thresholds between groups: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 3: Characteristics of motor unit pairs collected during the vibration protocol

	ECR		TB		TA		Quadriceps	
	Contr ols	ALS	Contr ols	ALS	Contr ols	ALS	Contr ols	ALS
Number	24 (20)	15 (10)	22 (16)	10 (8)	28 (20)	25 (15)	19 (14)	11 (7)
U1 threshold	1.0 (0.4-2.1)	3.4 (2.2-5.2)	2.5 (1.4-11.0)	2.6 (1.4-13.5)	3.0 (1.6-4.4)	6.9 (4.9-12.6)	1.9 (0.3-4.9)	20.3 (3.1-21.1)
U2 threshold	3.2 (1.8-5.9)	11.0** (8.2-24.0)	10.1 (5.6-22.6)	19.2 (6.8-33.2)	9.2 (5.0-12.8)	17.0 (9.5-33.2)	5.7 (3.1-8.2)	24.9 (7.3-35.9)
f-U1 tonic	8.0 (7.0-10.4)	9.0 (5.4-9.8)	10.0 (8.4-11.2)	5.8 (3.8-7.0)	7.1 (5.7-8.8)	4.2 (3.3-7.1)	8.1 (5.5-10.0)	5.5 (4.4-10.5)
f-U1 recruitment	11.5 (10.4-14.1)	11.5 (9.3-14.9)	11.9 (10.3-14.6)	7.0 (5.8-10.8)	11.0 (8.9-12.2)	6.0 (4.7-10.8)	9.9 (7.6-11.5)	7.2 (5.2-11.6)
f-U1 derecruitment	7.9 (6.3-9.8)	8.9 (7.1-10.2)	7.9 (5.2-10.7)	4.4 (3.2-6.0)	7.0 (5.4-7.8)	4.1 (3.4-6.9)	6.3 (4.3-7.8)	6.0 (3.7-9.4)

Number of motor unit pairs and of subjects between brackets; U1 threshold, level of mean rectified surface EMG (% mean EMG at MVC) when the low threshold control unit U1 appeared in intramuscular EMG; U2 threshold, level of mean rectified surface EMG (% mean EMG at MVC) when the higher threshold test unit U2 appeared in intramuscular EMG during the tendon vibration; f-U1 tonic, discharge rate of U1 (Hz) in intramuscular EMG during the tonic phase, before the tendon vibration; f-U1 recruitment, discharge rate of U1 (Hz) in intramuscular EMG when U2 was recruited by the tendon vibration; f-U1 derecruitment, discharge rate of U1 (Hz) when U2 disappeared from intramuscular EMG during the relaxation phase. In each cell: the first row indicates the median values and second row, the first (Q1) and the third (Q3) quartiles between brackets. ANOVA on ranks and Dunn pairwise comparisons were used to compare the U1 and U2 thresholds between groups: ** $P < 0.01$.

Table 4: Ramp conditions

Muscle	Duration of rise		Duration of plateau		Duration of decline	
	Controls	ALS	Controls	ALS	Controls	ALS
ECR	2.1 (1.2-4.6)	5.2 (3.0-12.2)	10.6 (8.3-15.6)	12.3 (9.3-19.0)	4.1 (2.64-10.7)	2.7 (0.6-5.5)
TB	3.2 (1.9-7.5)	2.9 (1.8-7.1)	9.9 (7.4-12.6)	10.9 (8.1-17.9)	4.7 (1.5-8.3)	3.0 (1.1-6.6)
TA	5.2 (2.6-8.8)	5.1 (2.9-13.4)	9.8 (7.7-12.3)	13.1 (8.8-16.8)	2.5 (1.4-7.3)	4.4 (3.3-5.8)
Quadriceps	3.7 (2.4-6.1)	4.7 (3.2-7.9)	12.0 (9.9-16.2)	12.9 (8.8-16.7)	6.4 (2.0-10.3)	3.8 (2.4-8.3)

Median duration (sec.), and the first (Q1) and the third (Q3) quartiles between brackets, of the increasing force contraction (rise), of maintenance of contraction level with U1 and U2 (plateau) and of the relaxation phase (decline) in controls and patients with ALS. Each line indicate the data for each investigated muscle: ECR, TB, TA and quadriceps.

FIGURES AND LEGENDS

Figure 1. Contraction and vibration protocols. *AB*, From top to bottom: i) running average (1-sec. smoothed) of rectified surface EMG expressed as a percentage of (%) mean rectified EMG produced during maximal voluntary contraction (MVC); ii) intramuscular EMG that was decomposed to extract potentials of low threshold motor unit U1 (control unit) and of an higher threshold motor unit U2 (test unit); iii) instant frequencies (Hz) of U1 and below of U2 superimposed on their smoothed average (1-sec. period; thin line). EMGs were collected in quadriceps (in VL for surface EMG and VM for intramuscular EMG) during the contraction protocol in one control subject (*A*), and in one patient (*B*). Vertical dashed lines indicate when U2 was recruited and derecruited in intramuscular EMG. In *A*, The horizontal dashed lines indicate the U1 frequency during the tonic contraction and when U2 was recruited. In *B*, the horizontal lines indicates the U1 frequency when U2 was recruited and derecruited. In both cases, the difference (ΔF) is represented by the double vertical arrow: the increase in U1 frequency for recruiting U2 is called $\Delta F-R$ (R for recruitment) and the decrease at the derecruitment of U2 is called $\Delta F-D$ (D for derecruitment). Both $\Delta F-R$ and $\Delta F-D$ were calculated for the contraction and the vibration protocols. *C*, From top to bottom: i) intramuscular EMG collected in VM in one control during the vibration protocol; ii) instant frequencies (Hz) of U1 and below of U2.1 and U2.2 both activated by vibration: U2.1 still discharging after vibration, and derecruited during the relaxation (active derecruitment) and, below, U2.2 derecruited at the end of the vibration (passive derecruitment). The black rectangle in *BC* indicate the tendon vibration. Scales were similar in *A* and *B*, but different in *C*.

Figure 2. Motor unit amplitude and duration. *A*, the amplitude of motor unit potentials (mV) is plotted against their duration (ms): each dot represent one motor unit in controls (open circles) and in ALS (grey circles). *BC*, The box plot charts illustrate the data distribution of motor unit amplitude (mV, *A*) and duration (ms, *B*) in controls (white boxes) and in patients with ALS (grey boxes). Motor units were isolated from intramuscular EMG in extensor carpi radialis (ECR), triceps brachii (TB), tibialis anterior (TA) and quadriceps (Quad). The boundary of the box closest to 0 indicates the 25th percentile (Q1), the continuous line within the box marks the median and the dotted line, the mean. The boundary farthest from 0

indicates the 75th percentile (Q3). The whiskers (error bars) above and below indicate the 90th and 10th percentiles, respectively. The open circles represent the 5 % outliers. * $P < 0.05$, *** $P < 0.001$.

Figure 3. Surface EMG and contraction force. A, Bars illustrate the mean rectified surface EMG (mV) recorded during MVC in controls (white columns) and in patients with ALS (grey columns) in extensor carpi radialis (ECR), triceps brachii (TB), tibialis anterior (TA) and quadriceps (Quad). Vertical bars represent the standard deviation. BC, Scatter plots illustrate the relationship between EMG (mV) and contraction force (N) in one control (open circles) and in one patient with ALS (grey circles). The interrupted and continuous lines represent the linear fit in control and ALS, respectively. The slope of the linear regression is indicated at the bottom of each corresponding plot with the coefficient of determination between brackets (R^2). C, bars indicate the mean slope \pm SD (mV/N) in ECR and TA in each group; legend and vertical bars as in A. * $P < 0.05$.

Figure 4. Motor unit frequencies and recruitment timing. AB, The mean frequency of U2 (Hz) is plotted against the mean frequency of U1 (Hz) during the period of co-activation, in the contraction (A) and the vibration protocols (B). Same legend as in Fig. 3BC. The slopes of the linear regressions are indicated in the legend with the R^2 between brackets. CD, Box plots as in Fig. 2BC, illustrating the data distribution of the ratio between U1 and U2 frequencies for extensor carpi radialis (ECR), triceps brachii (TB), tibialis anterior (TA) and quadriceps (Quad; C), and the time difference between U1 and U2 (ms) during contraction- and vibration-induced recruitment (D). *** $P < 0.01$.

Figure 5. Comparisons of $\Delta F-D$. The box plot charts illustrate the difference between the U1 frequencies during the recruitment and derecruitment of U2 ($\Delta F-D$, Hz), in the pool of motor unit pairs investigated during the contraction (A) and the vibration protocols (B), and in the group of subjects during the contraction (C) and the vibration protocols (D). Same legend as in Fig. 2BC. * $P < 0.05$.

Figure 6. Comparison of $\Delta F-D$ between upper and lower limbs. A, The box plot charts illustrate the difference between the U1 frequencies during U2 recruitment and derecruitment ($\Delta F-D$, Hz), in the pool of motor unit pairs investigated in upper and lower limbs, in controls and ALS. Same legend as in Fig. 2BC. * $P < 0.05$, ** $P <$

0.01, *** $P < 0.001$. *BC*, Scatter plots illustrate the relationship between ΔF -R (Hz) and ΔF -D (Hz) in controls (open circle and interrupted line) and in patients with ALS (filled circles and black line), in upper limb (*B*) and lower limb (*C*); each dot represent one motor unit pair. The equation of linear regressions and R^2 are indicated in the legend.

Figure 7. Correlations with clinical features. The loading plot graphs the coefficients of each variable for the first component (t1 “excitability”) vs. the coefficients for the second component (t2 “phenotype”) estimated using PCA: the parameters included in this analysis are commonly used in ALS to characterize the clinical phenotype (clinical scores) and to evaluate the motoneuron excitability (ΔF). Each principal component, examine the magnitude and the direction of coefficients of the original variables: the larger the absolute value of the coefficient, the more important the corresponding variable is in calculating the component. ΔF -D is represented in blue (dependent variable) and the variables (predictors) used for PLS regression analysis included: ΔF -R (red); Progression: progression rate = lost point to ALSFRS-r/month (red); ALSFRS-r: functional score (red); Duration: time since the symptom onset (yellow); MRC: score to MRC scale (purple); Form: ALS forms with predominant lower motoneuron affection (LMN), predominant upper motoneuron affection (UMN) or both (green); UMN score: upper motoneuron score (see Methods for calculation; orange).