Transient increase in recurrent inhibition in amyotrophic lateral sclerosis as a putative protection from neurodegeneration

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TRANSIENT INCREASE IN RECURRENT INHIBITION IN AMYOTROPHIC LATERAL SCLEROSIS AS A PUTATIVE PROTECTION FROM NEURODEGENERATION

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Short title: Renshaw cell adaptation in ALS

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ABSTRACT

Aim: Adaptive mechanisms in spinal circuits are likely involved in homeostatic responses to maintain motor output in amyotrophic lateral sclerosis. Given the role of Renshaw cells in regulating the motoneuron input/output gain, we investigated the modulation of heteronymous recurrent inhibition.

Methods: Electrical stimulations were used to activate recurrent collaterals resulting in the Hoffmann reflex depression. Inhibitions from soleus motor axons to quadriceps motoneurons, and *vice versa*, were tested in 38 patients and matched group of 42 controls.

Results: Compared to controls, the mean depression of quadriceps reflex was larger in patients while that of soleus was smaller suggesting that heteronymous recurrent inhibition was enhanced in quadriceps but reduced in soleus. The modulation of recurrent inhibition was linked to the size of maximal direct motor response and lower limb dysfunctions suggesting a significant relationship with the integrity of the target motoneuron pool and functional abilities. No significant link was found between the integrity of motor axons activating Renshaw cells and the level of inhibition. Enhanced inhibition was particularly observed in patients within the first year after symptom onset and with slow progression of lower limb dysfunctions. Normal or reduced inhibitions were mainly observed in patients with motor weakness first in lower limbs and greater dysfunctions in lower limbs.

Conclusion: We provide the first evidence for enhanced recurrent inhibition and speculate that Renshaw cells might have transient protective role on motoneuron by counteracting hyperexcitability at early stages. Several mechanisms likely participate including cortical influence on Renshaw cell and reinnervation by slow motoneurons.

Key words: H-reflex, Interneurons, Motoneurons, Renshaw cells, Spinal cord, Spinal excitability

1 | INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the third most frequent neurodegenerative disorder, characterized by a focal motor onset, which most often manifests in distal part of upper or lower limbs[1] and rapidly spreads to other body regions. Cognitive dysfunctions are also reported in ∼50 % of cases[2]. ALS has a relative low prevalence (~6/100 000)[3] partly explained by its rapid progression (respiratory failure leading to death within the 2-5 years after the disease onset) and the limited effects of current therapy (riluzole prolonging the lifespan to only 3-6 months). About 90 % of cases are sporadic, with heterogenous phenotype, but ALS hallmark is the progressive motor neuron loss, including bulbar and spinal motoneurons and pyramidal cells in the primary motor cortex, commonly termed as lower (LMNs) and upper motor neurons (UMNs), respectively[4]. Research has mostly dealt with motor neuron dysfunctions which have been reported in presymptomatic ALS mouse models and human patients at both levels[5–11], but non-cell autonomous pathogenic mechanisms have also been reported, involving glial cells and interneurons[12–14]. To date, most of the studies focused on glial cells and their interaction with motor neurons[12] and much less is known on interneurons. However, it is commonly admitted that excitation/inhibition balance shifts towards excitation, leading to hyperexcitability involved in neurodegeneration[15], but studies primarily focused on the motor cortex[13] and much less is known on the spinal cord and its pathophysiological plasticity. To better understand the mechanisms underlying ALS progression and open new avenues for therapies, it is now crucial to consider the complex organization of the spinal circuitry and its interaction with supraspinal structures[14,16].

Post mortem analyses in humans have evidenced that degeneration in the spinal grey matter likely occurs first in the ventral horn (LMNs and interneurons) and, subsequently, in the intermediate zone (interneurons); an hypothesis further supported by white matter analysis and the progressive degeneration of propriospinal fibers[17,18]. However, it has not been possible to dissociate degeneration of LMNs and interneurons in the ventral horn[19]. In presymptomatic ALS

mouse models, specific subsets of glycinergic inhibitory interneurons in the intermediate zone and ventral horn degenerate before LMNs[20–22], and it has been evidenced that alteration of glycinergic interneurons in the ventral horn is not consecutive to LMN degeneration[23]. More recently, it has been shown that LMNs innervating fast-type motor units, which are among the first LMNs to degenerate in ALS[24–28], receive more glycinergic inhibitory synaptic inputs from V1 interneurons compared to more resistant LMNs innervating slow motor units[29]. Furthermore, loss of inhibitory synapses from V1 interneurons on fast LMNs precedes LMN degeneration and causes locomotor dysfunctions[29]. V1 interneurons include group Ia interneurons mediating reciprocal inhibition between antagonists (glycinergic, located in intermediate zone and ventral horn), non-reciprocal group Ib interneurons mediating autogenic inhibition (also termed as Ib interneurons; mostly glycinergic but with some GABAergic synapses, same location as Ia interneurons), and Renshaw cells mediating recurrent inhibition (glycinergic and GABAergic, located in the ventral horn)[16,30–33].

Renshaw cells have been particularly explored in ALS, liable to their activation by recurrent collaterals from LMNs. Studies in both mouse models[34,35] and humans[36,37] revealed that recurrent inhibition is particularly depressed in ALS, likely due to cell loss or reduced inhibitory action of Renshaw cells secondary to a possible decreased excitation from cholinergic interneurons[35,38]. The depression of recurrent inhibition reported in patients has raised questions[39] *i)* for methodological and physiological reasons, given that the reduced silent period after mixed nerve stimulation reported in ALS[40], is not specific to Renshaw cell activity and the targeted LMNs (innervating intrinsic hand muscles) have no recurrent collaterals[41,42], and *ii)* for pathophysiological reasons, since initial wasting mostly occur in muscles innervated by LMNs without recurrent collaterals[39,41,42]. These led Mazzocchio and Rossi[39] to suggest that Renshaw cell impairment is not a general feature of ALS. Altered connectivity between Renshaw cells and LMNs is indeed unlikely involved in the *primum movens* of the human form of ALS, but maladaptive mechanisms at this level might contribute to disease spread and progression[16]. More convincing results in patients were obtained by testing

Hoffmann reflex (H-reflex)[36,43], using the particular technique of H', enabling to evaluate the level of homonymous recurrent inhibition (inhibition produced by recurrent collaterals from the target LMNs)[44]. However, the technique has not been properly implemented in ALS patients[39] and the results have to be interpreted with caution when the LMN pool is already affected[44]. Moreover, the H' technique is not sufficient to test the hypothesis that altered recurrent inhibition might contribute to disease progression. Testing heteronymous recurrent inhibition between different LMN pools[42,45–47], with or without clinical signs of degeneration, has additional value to further investigate the modulation of recurrent inhibition in humans and its putative role along the disease course.

The present study was thus designed to further investigate the modulation of recurrent inhibition in patients with ALS by testing heteronymous recurrent inhibition between soleus and quadriceps LMNs[46,47]. For this, we have examined the H-reflex evoked in the electromyogram (EMG) of vastus lateralis (VL) head of quadriceps by femoral nerve (FN) stimulation, and its modulation after stimulation of the posterior tibial nerve (PTN, activating soleus motor axons) at the optimal interstimulus intervals (ISIs) for producing VL H-reflex depression due to recurrent inhibition in quadriceps LMNs[44]. Experiments were performed in patients with ALS without clinical signs of motor degeneration in proximal muscles (quadriceps) but with or without distal muscle weakness, and in age and gender-matched group of healthy controls. In a subgroup of participants, we also tested the inhibition in soleus LMNs produced by activating quadriceps motor axons, by testing the modulation of H-reflex in soleus EMG after FN stimulation, at the optimal ISIs for eliciting recurrent inhibition in soleus LMNs[44]. Lastly, we studied the link between the modifications of recurrent inhibition and the patient clinical and electrophysiological profiles.

2 | RESULTS

2.1 | Recurrent inhibition from soleus to quadriceps LMNs

The first experiment consisted in testing the recurrent inhibition from soleus motor axons to

quadriceps LMNs (Fig. 1A). Figures 1B and C illustrate the mean VL EMG recordings without (FN stimuli delivered alone; test H-reflex) and with conditioning PTN stimuli (combined PTN and FN stimuli delivered at 15, 20 and 25-ms ISIs[44,45]; conditioned H-reflexes) in 1 control (Fig. 1B) and 1 patient (Fig. 1C). In both participants, the amplitude of H-reflex was smaller on combined stimuli. Figures 1D and E show the resulting mean level of inhibition in quadriceps LMNs plotted against the ISI between conditioning PTN and test FN stimuli. In both participants, PTN stimuli reduced significantly VL H-reflex between 30 and 60 % of its mean test size at the 3 ISIs investigated. Paired *t* test was performed at each ISI, in each participant: i) control: 15-ms ISI, $p = 0.02$; 20-ms ISI, $p = 0.007$; 25-ms ISI, $p = 0.005$, and ii) ALS: 15-ms ISI, $p = 0.0003$; 20- and 25-ms ISIs, $p < 0.0001$. Figures 1 F and G show the mean amplitude of the maximal direct motor response (Mmax) in VL and soleus EMG in the same control (Fig. 1F) and the same patient (Fig. 1G). Mmax in quadriceps was ~1 mV in both participants (0.85 ± 0.08 *vs.* 1.22 ± 0.01 mV in the control and the patient, respectively) while that in soleus was smaller in the patient (1.20 ± 0.01 mV) compared to the control (3.58 ± 0.04 mV). Lastly, the test H-reflex in VL EMG was of similar size in both participants, reaching on average 9.7 ± 7.7 % of Mmax (in VL EMG) in the control and 10.3 ± 4.8 % in the patient (patient #25 in Table 1).

Figure 1 near here

For a reliable comparison of conditioned H-reflexes, the most important is to ensure that the test size of H-reflex (normalized to Mmax) and the peripheral volley in motor axons were comparable between groups[44,46,48]. In the full group of participants (42 controls *vs*. 38 patients), Mmax in quadriceps was similar in controls and patients (Kolmogorov-Smirnov test, $p = 0.94$; Cohen's d[49,50] = 0.1) while Mmax in soleus was significantly smaller in patients than in controls (Kolmogorov-Smirnov test, $p = 0.0246$; d = 0.6; Fig. 2A); none of the outliers in Figure 2A were detected as significant using the interquantile range (IQR) method. Despite this difference in soleus Mmax, the intensity of conditioning PTN stimuli, which was adjusted at the threshold intensity for Mmax, was not statistically different between groups: 60.4 ± 21.6 *vs.* 65.8 ± 23.9 mA in controls and

patients, respectively (Student *t* test, $p = 0.29$; d = 0.2). A particular care was taken to adjust the intensity of the test FN stimuli so as to produce a stable H-reflex in VL EMG and within a range that its amplitude has limited effect on the level of inhibition (*i.e.*, ~25 % of Mmax as revealed for soleus H-reflex[48]). However, the mean size of VL H-reflex in the control group was 12.9 ± 11.9 % of Mmax. This result is consistent with previous studies[51–53]: H-reflex in VL EMG is indeed generally much smaller than in soleus, hardly reaching 10-20 % of Mmax. In the patient group, the test FN stimuli were adjusted the same way as in controls but the resulting mean H-reflex in VL EMG was significantly larger than in controls, reaching 22.3 ± 15.3 % of Mmax (no significant outlier; Kolmogorov-Smirnov test, $p = 0.0038$; d = 0.7; Fig. 2B).

Figure 2 near here

The level of recurrent inhibition was first compared between groups at similar ISIs and Figure 2C shows the data distribution in both groups (no significant outlier). The levels of recurrent inhibition were compared at the 3 ISIs (repeated measures) between groups (controls *vs*. ALS) by building a linear mixed model including the subjects as random effect and fixed effects were group (controls *vs*. ALS), ISI (15 *vs*. 20 *vs*. 25 ms), amplitude of conditioning Mmax in soleus and of test H-reflex in VL. The adjusted R^2 was 0.84 and variance analysis revealed a significant influence of ISI ($p < 0.0001$; $f^{2\ 54} = 0.49$). The other regressors in the model had no significant influence: group ($p = 0.1176$; $f^2 = 0.04$), conditioning Mmax ($p = 0.2335$; $f^2 = 0$) and test H-reflex ($p = 0.593$; $f^2 = 0$). Interestingly, the inhibition least mean square (*i.e.*, the best unbiased estimates of the marginal means of the model) was stronger in ALS than in controls (Fig. 2D), and a significant interaction was found between ISI and group ($p = 0.0394$; $f^2 = 0.04$). *Post-hoc* multiple comparisons revealed a significant difference between groups at ISI 25 ms (Student test, $p = 0.0269$; d = 0.4); at shorter intervals, the difference did not reach the statistically significant level (15-ms ISI: $p = 0.5441$, d = 0; 20-ms ISI: $p = 0.1225$, d = 0.3). These results suggest that the inhibition from soleus motor axons to quadriceps LMNs was larger in patients with ALS than in controls and this was particularly true at ISI

25 ms.

According to previous studies[44,45], the 3 ISIs are within the range for optimal ISIs for recurrent inhibition from soleus motor axons to quadriceps LMNs. They have been tested systematically in each participant to ensure inhibition would occur at least once among the 3 ISIs. Interindividual variability regarding participants' height and conduction velocity indeed influence the ISI (*e.g.*, inhibition can manifest at shorter interval in small participants compared to tall ones). Indeed, the inhibition has not manifested at all the 3 ISIs in all participants: i) 15-ms ISI: 27 (significant in 19)/42 controls *vs*. 23 (significant in 16)/38 patients, ii) 20-ms ISI: 35 (significant in 24)/42 controls *vs*. 33 (significant in 26)/38 patients, and iii) 25-ms ISI: 35 (significant in 24)/42 controls *vs*. 33 (significant in 27)/38 patients. As we could expect, the optimal ISI for inhibition was not the same in each participant. Therefore, we identified the ISI at which the level of recurrent inhibition was the strongest in each participant. Maximal recurrent inhibition was indeed observed at ISI 15 ms in 6 controls and 3 patients, at ISI 20 ms in 19 controls and 9 patients, and at ISI 25 ms in 15 controls and 24 patients. Consistently with Figure 2C, maximal inhibition was thus mainly observed at the 20- and 25-ms ISIs in both groups. Student *t* test was performed to compare maximal inhibition between the 2 groups and it was found significantly greater in patients with ALS than in controls ($p = 0.0282$; d = 0.4; Fig. 2E). This result further confirms that recurrent inhibition in quadriceps LMNs was significantly increased in ALS group.

2.2 | Recurrent inhibition from quadriceps to soleus LMNs, and reciprocally

In the second half of the participants included in the study, we performed a second experiment which consisted in testing recurrent inhibition from quadriceps motor axons to soleus LMNs (Fig. 3A)[44,45]. Accordingly, experiment 1 (inhibition from soleus to quadriceps; see 2.1) and experiment 2 (inhibition from quadriceps to soleus) were successively performed during the same experimental session in a subgroup of participants (17 controls *vs*. 17 patients).

Figure 3 near here

Figure 3B shows the distribution of Mmax amplitude in VL and soleus EMG in the subgroup of participants performing the 2 experiments (no significant outliers). The difference between controls and patients was not statistically significant (Kolmogorov-Smirnov test, p = 0.7344 [d = 0.5] and 0.9539 [d = 0.3] for quadriceps and soleus, respectively). As observed in the full group of participants, H-reflex in VL EMG was significantly larger in patients than in controls (Welch ANOVA, p 0.0034; d = 1.1; Fig. 3C). Similarly, H-reflex in soleus EMG was significantly larger in patients (Student *t* test, p = 0.0042; d = 1.1; no significant outliers; Fig. 3C). Lastly, the intensities of conditioning stimuli, adjusted at the threshold intensity for Mmax, were not statistically different between groups: *i*) in experiment 1 (PTN-induced depression of VL H-reflex), the mean intensity of conditioning PTN stimuli was 57.6 ± 31.4 *vs*. 65.3 ± 29.6 mA in controls and patients, respectively (Kolmogorov-Smirnov test, p = 0.45; d = 0.2) and *ii*) in experiment 2 (FN-induced depression of soleus H-reflex), the mean intensity of conditioning FN stimuli was 69.1 ± 29.8 *vs*. 78.8 ± 17.09 mA in controls and patients, respectively (Student *t* test, p = 0.17; d = 0.5).

Results of experiments 1 (inhibition from soleus to quadriceps) are illustrated in Figure 3D (no significant outlier) and the levels of inhibition at the 3 ISIs were compared as in 2.1. The adjusted R^2 of the linear mixed model was 0.88 and only ISI had a significant influence on the level of inhibition ($p < 0.0001$; $f^2 = 0.23$). The other regressors had no significant influence: group (p = 0.9783, $f^2 = 0$), conditioning Mmax in soleus (p = 0.091, $f^2 = 0$) and test H reflex in VL (p = 0.8021, $f^2 = 0$). Contrariwise to the full group, the recurrent inhibition in quadriceps did not increase in the subgroup of patients: Figure 3E shows that the inhibition least mean square was comparable between groups and the interaction between ISI and group was not significant (p = 0.1735; $f^2 = 0$). Multiple *post hoc* comparisons were thus limited to ISI, showing a significantly greater inhibition at ISIs 20 and 25 ms, compared to ISI 15 ms (whatever the group): *i)* 15 vs. 20 ms: $p < 0.0001$, d = 0.5, *ii)* 15 vs. 25 ms: $p <$ 0.0001, d = 0.7 and *iii)* 20 vs. 25 ms: p = 0.2036, d = 0.1.

Results of experiment 2 (inhibition from quadriceps to soleus) are illustrated in Figure 3G and,

as for experiment 1, a linear mixed model was built to compare the level of inhibition at the 3 ISIs tested. The adjusted R^2 was 0.97 and ISI had a significant influence on the level of inhibition (p = 0.0102; f^2 = 0). The other regressors had no significant influence: group (p = 0.0999, f^2 = 0), conditioning Mmax in VL (p = 0.5758, f^2 = 0) and test H reflex in soleus (p = 0.2529, f^2 = 0). Figure 3H shows that the inhibition least mean square was lower in patients compared to controls, and the interaction between ISI and group was significant (p = 0.0275; f^2 = 0). However, multiple *post hoc* comparisons did not reveal any significant difference between groups tested at the same ISIs: *i)* ISI 5 ms: p = 0.1367, d = 0.4, *ii)* ISI 10 ms: p = 0.1438, d = 0.4 and *iii)* ISI 15 ms: p = 0.0515, d = 0.7. The only significant difference between groups was found at ISI 5 ms in controls *vs*. ISI 15 ms in ALS (inhibition being less in the latter; p = 0.0475, d = 0.7).

In each participant and each experimental paradigm, we retained for further analysis the maximal amount of inhibition observed at the 3 ISIs tested. In experiment 1 (inhibition in quadriceps LMNs), the inhibition was maximal at ISI 15 ms in 3 controls and 2 patients, at ISI 20 ms in 7 controls and 4 patients and at ISI 25 ms in 7 controls and 11 patients. In experiment 2 (inhibition in soleus LMNs), the maximal inhibition was observed at ISI 5 ms in 8 controls and 9 patients, at ISI 10 ms in 5 controls and 6 patients and at ISI 15 ms in 4 controls and 2 patients. Then, we compared the maximal amount of inhibition in both LMN pools. We did not find any significant difference between controls and patients when comparing the inhibition produced in quadriceps LMNs (experiment 1; Student t test, p = 0.8923, d = 0; Fig. 3F) but we confirmed that the inhibition in soleus LMNs was significantly depressed in patients (experiment 2; Kolmogorov-Smirnov test, p = 0.0463, d = 0.5; Fig. 3I).

Figure 4 near here

To determine whether the modulation of recurrent inhibition in patients was different between LMN pools (quadriceps *vs*. soleus), independent from the fact that heteronymous recurrent inhibition between both motor nuclei is stronger in soleus than in quadriceps LMNs *per*

se[44], we calculated the 95 % of confidence interval (CI95) of the mean level of maximal recurrent inhibition in the control group of which the lower and upper cut-offs were respectively 17.5 and 31.6 % of the mean test H-reflex for quadriceps (n = 42 controls) and 52.8 and 79.5 % of the mean test H-reflex for soleus (n= 17 controls). Figure 4A shows the proportion of patients with reduced (below CI95), normal (within CI95) or increased (above CI95) maximal recurrent inhibition as a % of the total number of patients (n = 38 patients for quadriceps and 17 for soleus). In quadriceps LMNs, a much greater proportion of patients exhibited an increase in the level of recurrent inhibition compared to controls: in 50 % of the patients the inhibition was above the CI95 upper limit, 21.1 % had inhibition within CI95 and 28.9 %, below the CI95 lower limit. In soleus LMNs, the recurrent inhibition was almost equally distributed between values within CI95 (52.9 % of the patients) or below its lower limit (47.1 %); we did not find any patient with recurrent inhibition above the CI95 upper limit. Chi^2 test revealed that the modulation of recurrent inhibition in the patient group was significantly different between quadriceps and soleus ($p = 0.0022$). It is important to notice that for quadriceps, we found the same repartition in the subgroup of 17 patients in whom recurrent inhibition was tested in both LMN pools: 53 % of the patients had inhibition in quadriceps above the CI95 upper limit, 23.5 % within the CI95 and 23.5 % below the lower limit (Chi^2, $p = 0.0012$).

Table 1 near here

2.3 | Relationship between the modulation of recurrent inhibition, electrophysiological and clinical features

The clinical features of the patient group are detailed in Table 1. In most patients, the first clinical symptoms have manifested in upper (UL, 44.7 %) or lower limbs (LL, 36.8 %) and only 7/38 patients have had bulbar signs at first (18.4 %), which corresponds to the classical repartition observed in ALS[1]. The mean disease duration (time from symptom onset) was 21.7 ± 16.4 months, ranging between 5 and 72 months, median being 15.5 months. The mean score to revised ALS functional rating scale (ALSFRS-r), which measures disability in activities of daily living[55], was 40.0 ±

4.5 (ranging between 24 and 47, median at 40). We also calculated a sub-score for lower limb functions, including walking and climbing stairs (maximal score being 8 indicated in bold caps in Table 1), which was on average 5.4 ± 2.0 (ranging between 2 and 8, median at 6). No patients had non-invasive ventilation nor gastrostomy. The mean progression rate, indicating ALSFRS-r decline per month, was 0.5 ± 0.5 points/month (between 0.1 and 2.4, median at 0.4). Based on UMN and LMN scores[56,57], we identified a greater proportion of patients with predominant UMN signs (60.5 %) than with predominant LMN signs (13.1 %) or mixed form (equal score for UMN and LMN signs, 26.4 %). The muscle strength on the investigated limb, evaluated by manual muscle testing and rated using the cumulative Medical Research Council (MRC) scale, was normal (scored 5) in quadriceps in all patients according to inclusion criteria, and was depressed in only 2 patients in soleus (scored 3 in patient #2 and 2 in patient #35, Table 1); 13/38 patients had lower strength in tibialis anterior (TA) and/or in extensor hallucis longus (EHL; in bold caps in Table 1). Lastly, almost all patients were under riluzole therapy except 6 of them.

Table 2 near here

Table 2 summarises the electrophysiological profile of the patient group regarding quadriceps and soleus. To smooth the intrinsic differences between quadriceps and soleus (electrophysiological measures being systematically smaller in quadriceps than in soleus), we calculated the CI95s for each measure in the control group, to evaluate their modulation in patients. We thus estimated that the test H-reflex in the patient groups was particularly enhanced in both LMN pools: 63.2 and 70.6 % of the patients had larger H-reflexes in VL and soleus EMG, respectively, compared to controls. While quadriceps H-reflex could be smaller in 21.0 % of the patients, we did not find any patient with soleus H-reflex below the lower CI95 limit. Chi^2 test revealed a significantly different distribution of the results between LMN pools (p = 0.0297). Then, we ran the same test in the subgroup of patients performing the 2 experiments (n = 17 for quadriceps instead of 38), and while the repartition was quite similar as in the full group (17.6 % below the lower limit, 17.7 % within the

CI95 and 64.7 % above the upper limits; to be compared with results for quadriceps H-reflex in Table 2), Chi^2 test was not anymore significant ($p = 0.095$). This result suggests that test H-reflex was larger in both LMN pools in patients, compared to controls, with a tendency to be more enhanced in soleus than in quadriceps.

The evaluation of Mmax, also termed as maximal compound muscle action potential (CMAP), is included in routine electrodiagnosis for ALS and is used as a biomarker of muscle denervation (LMN loss)[58,59]. Moreover, its size is used to normalize the test H-reflex and to monitor the consistency of conditioning stimuli. Therefore, we also calculated the proportion of patients exhibiting significant depression of Mmax in VL and soleus EMG, according to CI95 in the control group. Table 2 indicates that a greater proportion of patients had reduced Mmax in soleus (73.7 %) than in quadriceps (39.5 %) and Chi^2 test indicated that the repartition was significantly different between LMN pools ($p = 0.0026$) suggesting that Mmax in soleus was more depressed than in quadriceps. We also ran the test in the subgroup of 17 patients performing the 2 experiments and we found the same results: 41.2 % of the patients with depressed Mmax in quadriceps *vs*. 76.5 % of for soleus (Chi^2, $p = 0.0365$).

The last part of the statistical analysis consisted in determining the possible link between the patient profile and their modulation of recurrent inhibition (according to CI95 in controls) in the two distinct target LMN pools (quadriceps *vs*. soleus). The parameters included in their clinical and electrophysiological profiles were: the site of onset (lower limbs *vs*. other), the disease duration (2 classes: ≤ 1 year *vs*. > 1 year), the total and sub-score (lower limb functions) to ALSFRS-r (2 classes according to the median scores in the group: ≥ 40 or < 40 for total score and ≥ 6 or < 6 for sub-score), the progression (based on the total ALSFRS-r score; 2 classes according to the median score in the group: slow *vs*. fast), the progression of lower limb dysfunctions (LL-progression; based on the ALSFRS-r sub-score for lower limb functions; 2 classes according to the median score in the group: slow *vs*. fast), the predominant form (UMN *vs*. LMN *vs*. mixed form), riluzole intake (yes *vs*. no), the

size of conditioning and test Mmax (according to CI95 in controls; 2 classes: not depressed *vs*. depressed), and of H-reflex in the test and conditioning muscle (according to CI95 in controls; 3 classes: > upper limit *vs*. within CI95 *vs*. < lower limit).

The first step consisted in analysing the relationship between the modulation of recurrent inhibition in patients (according to CI95 in controls) and each parameter, individually (analyse of contingency tables and of the correspondences between the modulations of recurrent inhibition and the modalities of each parameter). The results are summarized in Table 3 in which the clinical and electrophysiological features are listed according to their statistical significance. We only found 3 significant parameters (Chi^2 tests; column 3, Table 3): *i)* the target LMN pool (LMNs inhibited by Renshaw cells): as in 2.3, we found again a significant difference between quadriceps and soleus, with more patients exhibiting increased recurrent inhibition in quadriceps and no modulation or depressed inhibition in soleus (results of correspondence analysis in column 4, Table 3), *ii)* the test Mmax: when Mmax in the test muscle (*e.g.*, Mmax in VL EMG when testing soleus-induced inhibition in quadriceps LMNs) was not depressed compared to controls, recurrent inhibition was most often increased, but when Mmax was depressed, the inhibition was more within the same range as controls or depressed, and *iii)* the progression: recurrent inhibition was more within the normal range in fast progressors. The influence of the other factors did not reach the statistically significant level. However, the correspondence analysis revealed interesting associations between measures for further analysis and Discussion (column 4, Table 3): *i)* inhibition was most often increased or not modified in patients with predominant UMN or mixed form, *ii)* the size of H-reflex in the test and conditioning muscles and that of Mmax in the conditioning muscle (*i.e.*, the motor axons we stimulated to activate Renshaw cells; Figs. 1A and 3A) were not at all associated with the modulation of recurrent inhibition, *iii)* inhibition was mostly increased in patients with mild or no motor dysfunction in lower limbs, and not modified or decreased in those with greater lower limb disabilities (ALSFRS-r sub-score), *iv)* inhibition was most often increased in patients with slow LL-

progression but normal or decreased in those with faster LL-progression, *v)* the patients with first symptoms in lower limbs exhibited more often depressed inhibition, and at last *vi)* enhanced recurrent inhibition was particularly observed in patients within the first year after the first symptoms.

In the second step, we thus performed a multiple correspondence analysis (MCA) to evaluate the relative links between clinical and electrophysiological features and the modulation of recurrent inhibition. First, we performed MCA using the significant parameters in Table 3 (target LMN pool, test Mmax and progression). The projection of each modality in a 2-dimension (2-D) plot is illustrated in Figure 4B, and their partial contribution to inertia (*e.g.*, strength of the links between variables) in each dimension, in Figure 4C. Dimension 1 (X-axis; Chi^2, $p < 0.01$) represents 71.1 % of the deviation from independence between variables (inertia). The target LMN pool and the modification of test Mmax size particularly contribute to dimension 1 (Fig. 4C). Dimension 2 (Y-axis; Chi^2, $p > 0.99$) represents 28.9 % of the total inertia, and the progression (according to the total ALSFRS-r score) particularly contributes to it (Fig. 4C). These results indicate that the modulation of recurrent inhibition was significantly influenced by the origin of the target LMN pool (test muscle) and the size of the test Mmax, and the 2-D plot indicates a strong link between an increase in recurrent inhibition and the absence of depression of test Mmax, in quadriceps in particular. Concerning the progression, the plot distinguishes slow from fast progressors but there is no significant link with the other modalities and the modulation of recurrent inhibition; the corresponding symbol (open triangles) are close to 0 in dimension 1 and dimension 2 did not reach the statistically significant level ($p > 0.99$).

Lastly, we performed another MCA taking into account the results of the correspondence analysis as well. We thus tested whether the modulation of recurrent inhibition was influenced by the target LMN pool, the test Mmax size, the predominance of UMN signs or at least the equal involvement of both UMNs and LMNs (yes [UMN + mixed forms] *vs*. no [LMN form]), the LL-

progression (slow *vs*. fast), the site of onset (lower limbs *vs.* others), the duration (1st year *vs.* > 1 year) and their respective links (Figs. 4DE). Figure 4D shows the 2D-plot with *i)* significant dimension 1 representing the most part of the total inertia (78.3 %; Chi^2, $p < 0.001$), and *ii)* dimension 2 representing only 21.7 % (Chi^2, $p > 0.99$). Here again, we found that the target LMN pool and the test Mmax particularly contribute to dimension 1 together with LL-progression but to a much lesser extent; onset site and duration also contribute to dimension 1 but to an even smaller extent than LL-progression (Fig. 4E). The predominance of LMN signs (N in Fig. 4E) particularly contributes to dimension 2; the corresponding symbol is outside the plot in Figure 4D given the scale used for illustration (coordinates = 0.004 in dimension 1 and 0.66 in dimension 2). This result indicates the predominance of LMN form has no significant link with the modulation of recurrent inhibition ($p > 0.99$), nor the fact that patients exhibit predominant UMN or mixed form. Even if the corresponding symbol indeed appears in Fig. 4D (filled, right-orientated triangle 'yes'), it is positioned in the middle of dimension 1, almost at equal distant from the 3 modalities of modulation of recurrent inhibition. The repartition of the other modalities on either side of dimension 1 indicates more links on the left part of Figure 4D, between the enhanced recurrent inhibition and, from the strongest to the weakest link, normal test Mmax, quadriceps, slow LL-progressors within the 1st year after the first symptoms which did not manifest in lower limbs (filled circle 'Other' in Fig. 4D). The items on the right part of Figure 4D are more spaced, indicating less links between unchanged or depressed recurrent inhibition and the patient features, except the close link between the depression of recurrent inhibition and the fact that the first symptoms have manifested in lower limbs (filled circle 'LL' in Fig. 4D), suggesting that the inhibition was more depressed in patients with first symptoms in lower limbs.

3 | DISCUSSION

This study has thus shown that heteronymous recurrent inhibition between lumbar LMNs is modified in ALS, being particularly increased in quadriceps LMNs in the present cohort but not

reciprocally in soleus ones where inhibition was unchanged or decreased (compared to matched control group). The modulations of recurrent inhibition were particularly linked to the size of the test Mmax: the inhibition was particularly enhanced when the test Mmax was within the control range, or it decreased to similar or lower levels than controls when the test Mmax was depressed (see Supplemental Material 1AB: the inhibition increased with the size of test Mmax in both LMN pools). On the contrary, the conditioning Mmax had no influence. These results suggest that the level of recurrent inhibition likely depends on the integrity of the target LMN pool (test Mmax) but not on the integrity of the motor axons activating Renshaw cells (conditioning Mmax). Lastly, the modulation of recurrent inhibition was linked to the level of lower limb dysfunctions and their progression: the inhibition was enhanced in patients within the first year after the first symptoms, when onset site was not in lower limbs, and when the progression of lower limb disabilities was slow (see Supplemental Material 1CD: the inhibition decreased with LL-progression in both LMN pools but this was more pronounced in quadriceps LMNs). On the contrary, the inhibition was particularly decreased in patients with first symptoms in lower limbs. These results suggest that the level of recurrent inhibition likely depend on the integrity of the target LMNs and is associated to the peripheral denervation of the corresponding muscles and the resulting functional disabilities.

3.1 | Methodological considerations

Heteronymous recurrent inhibition at both cervical and lumbar spinal levels has first been described in humans by investigating the modulation of single motor unit discharge after peripheral nerve stimulation. It has been shown that the depression observed in the resulting post-stimulus time histograms always appeared and increased with the conditioning motor discharge (*i.e.*, the size of the H-reflex and/or of the M response elicited in the conditioning EMG), was independent of the conditioning stimulus intensity *per se* (a characteristic further supported by the present study since the intensity of conditioning was similar in both groups and we found different modulations), and had short latency and long duration[42,46]. Then, heteronymous recurrent inhibition has been

assessed by testing the modulation of H-reflex, of on-going EMG activity and of motor evoked potential, and its origin was confirmed using L-acetyl carnitine known to potentiate recurrent inhibition[41,44,45].

To limit the duration of the experiments for the comfort of the participants (we also tested other spinal pathways in the same participants), we only tested 3 ISIs at which recurrent inhibition was found maximal in previous studies[60,61]. Indeed, the range of ISIs tested for each motor nucleus corresponds to optimal ISIs for investigating Renshaw cell activity: i) it excludes the first 10 ms of central delay during which disynaptic non-reciprocal goup I inhibition (also termed as group Ib inhibition) manifests and ii) it was limited to inhibition purely spinal in origin (< 12 ms duration; excluding longer ISIs likely contaminated by transcortical effects)[44,62]. Moreover, we found the conditioned H-reflexes in soleus EMG more depressed than those in VL EMG. Similarly, it has been previously reported that heteronymous recurrent inhibition between both nuclei is stronger in soleus LMNs than that in quadriceps ones[44], and it is interesting to note that this might also be the case when comparing homonymous recurrent inhibition using the H' technique[41].

The depression of conditioned H-reflex assessed using the H' technique (homonymous paradigm) is partly due to the post-spike after hyperpolarization (AHP)[41,63,64], which is shortened in patients with mild motor dysfunction and increased again with motor deficit progression[65]. There is thus a possibility that the increase of H' reflex in ALS[36,43], interpreted as a result of depressed recurrent inhibition, might be partly due to reduced AHP. The great advantage of the heteronymous paradigms we used in the present study is that the depression of conditioned H-reflex is not contaminated by AHP (conditioned and test stimuli not applied to the same nerve; inhibition produced in post-stimulus time histograms, even without preceding monosynaptic group Ia excitation; inhibition independent of the strength of preceding monosynaptic group Ia excitation)[44,47]. Additionally, modification in AHP cannot explain the enhanced inhibition in quadriceps LMNs nor its depression with progressive lower limb dysfunctions.

Other spinal inhibitory mechanisms could have contributed to the H-reflex depression: i) group Ib inhibition but this is unlikely at the ISIs we tested (see above), and ii) presynaptic inhibition of group Ia terminals. In the experimental paradigms we used, presynaptic inhibition is assessed at shorter ISIs than those we tested. Indeed, presynaptic inhibition between soleus and quadriceps is estimated based on the modulation of heteronymous group Ia facilitation of H-reflex (which precedes the long lasting inhibition[44,60]) *i.e.*, about 5 ms ISI when testing conditioning PTN on VL H-reflex and about -5 ms ISI when testing conditioning FN on soleus H-reflex[66,67]. These ISIs are shorter than those for D1 method we used in Howells et al. 2020 (10 to 30-ms ISIs)[68] *i)* due to the difference in the peripheral afferent conduction time between PTN and FN, while this time is similar between PTN and fibular nerve in the D1 method, and *ii*) because presynaptic inhibition between soleus and quadriceps is estimated based on the modulation of heteronymous facilitation of H-reflex (adding central processing) while the direct modification of H-reflex size by the conditioning is investigated in the D1 method. Moreover, it has been previously shown that the inhibition reported in the present study is evoked only when a motor volley is produced in the conditioning nerve, independently of the intensity of the conditioning stimuli, which does not match the characteristics of presynaptic inhibition for which the threshold intensity of the conditioning volley is below the motor threshold[44–47,60]. Lastly, it has been shown that presynaptic inhibition is depressed in ALS[68–71]. Similarly, in our cohort of patients, we found presynaptic inhibition depressed, and we also found the group Ib inhibition unchanged compared to controls (unpublished data). Accordingly, the depression of conditioned H-reflexes in the present study was mostly due to Renshaw cell activity and its modification in ALS, to modulation of recurrent inhibition.

3.2 | Basics on Renshaw cells and recurrent inhibition

The distribution of recurrent inhibition has been particularly well described in humans and has been found closely linked to that of monosynaptic group Ia excitation of LMNs, as reported in animal models (cats, baboons). However, the projections are less diffuse in human upper limbs

compared to cat forelimbs and, on the contrary, more diffuse in lower limbs compared to hindlimbs, likely due to the development of the bipedal stance and gait, and the parallel release of the upper limbs from their locomotor functions in humans[42,44,46,47,72]. Renshaw cells are indeed activated by axon collaterals from LMNs of different motor pools and they project onto homonymous and synergistic (heteronymous) LMNs in the same or adjacent spinal segments; they also project onto gamma LMNs (different Renshaw cells from those projecting onto alpha LMNs) and group Ia reciprocal interneurons[16,44,45,73,74]. Beside their excitation by LMN discharge, Renshaw cells also receive polysynaptic excitation and inhibition from flexor reflex afferents (FRA)[73]. Moreover, transcranial magnetic stimulation over the primary motor cortex has been shown to reduce the level of recurrent inhibition likely due to cortical suppression of a tonic excitatory drive from the reticular formation rather than a direct corticospinal inhibition of Renshaw cells[75,76]. The same way, recurrent inhibition is primarily depressed during voluntary contractions, likely to reinforce reciprocal inhibition between antagonists and assist muscle synergies during movement[77]. However, during a weak tonic contraction, recurrent inhibition has been found increased[77], suggesting a more complex organization and control of Renshaw cell activity. The functional role of recurrent inhibition has been debated for a long time and is still discussed. However, it is commonly accepted that it participates in the control of muscle synergies during movement[44] and likely mostly serves as a variable gain regulator of the spinal motor output[78]. Accordingly, it has recently been proposed that adaptative mechanisms at the level of Renshaw cells would take part in the homeostatic response to maintain force output as long as possible during the course of ALS. Then, a gradual unbalanced activity in local micro-circuitries linking different LMN pools would overwhelm the initial homeostatic response and contribute to excitotoxicity participating in LMN degeneration and disease progression[16].

3.3 | Modulations in ALS and adaptive mechanisms

Glycinergic inhibition mediated by ventral horn interneurons has been found particularly

altered in mouse models of ALS and interneurons start to degenerate before LMNs; the authors have speculated on the involvement of Renshaw cells[29,35]. However, none of these studies focused on Renshaw cells in particular, which mediate both glycinergic and GABAergic recurrent inhibitions to LMNs[31,32]. More recently, specific alteration of V1 interneurons has been reported[29] but Renshaw cells constitute only a small part of V1 interneuron pool (9 %); the rest being associated to proprioceptive interneurons, including group Ia interneurons[79]. To our knowledge, only one study has assessed specifically Renshaw cells and modulation of recurrent inhibition. This study has evidenced an early (presymptomatic) downregulation of vesicular acethylcholine transporters in recurrent collaterals and of cholinergic receptors, associated to major structural abnormalities of motor axon synapses. However, the authors also reported a transient sprouting of Renshaw cells to remaining LMNs. The synaptic disorganization between LMNs and Renshaw cells was followed by retraction of motor collaterals but it was not clearly accompanied by any degeneration of Renshaw cells which, for the most part, survived until the late stages. They concluded that the alteration of LMN recurrent inhibition in ALS is likely due to synaptic pathology and not to interneuron cell death[34].

In ALS patients, it has been proposed that recurrent inhibition is reduced but this assumption relies on misinterpreted results based on mixed nerve silent period in LMNs without recurrent collaterals[40] and H' technique contaminated by AHP[36,43,44]. Accordingly, the possible alteration of recurrent inhibition and the implication of Renshaw cells in ALS has been quite rightly questioned[34]. However, a recent study has shown that the inhibitory period in peristimulus frequencygram (PSF) of single motor units is shortened in lumbar-affected ALS patients but unchanged in nonlumbar-affected ones[37]. The authors, who developed the method, have argued that this inhibition is mostly due to recurrent inhibition of soleus LMNs produced by stimulation of PTN in the lateral part of the popliteal fossa, which primarily produces M response in soleus EMG[80,81]. They discussed the possible involvement of other inhibitory mechanisms involving group I afferents from proprioceptors, and

argued on their minimal involvement. Their results in ALS patients are consistent with our observations that the inhibition can be within the normal range or decreased according to the level of lower limb disabilities. Accordingly, Ozyurt et al.[37] and the present study allow to reconsider the possible role of Renshaw cells in ALS.

Several mechanisms well described in ALS pathophysiology can interfere with Renshaw cell activity and can modulate the level of recurrent inhibition according to the neural network connectivity within the spinal cord and its interaction with supraspinal structures and peripheral afferents. Indeed, indirect electrophysiological techniques in humans do not allow to evaluate the inhibitory post synaptic potentials (IPSPs) produced in LMNs; they only give an estimate of the net motor output resulting from the conditioning-induced activity in the target spinal pathway (*e.g.*, recurrent inhibition in the present study) and the tonic synaptic activity in surrounding neural network.

3.3.1 | Cortico-reticulo-spinal influence

UMN degeneration in ALS manifests in the presymptomatic phase[8,82], which likely reduces the inhibitory cortical influence on Renshaw cell activity and, thus, would likely contribute to enhance recurrent inhibition. Furthermore, degeneration in reticular formation, and alteration of serotoninergic neurons in particular, likely contribute to pyramidal signs in ALS[83] and would thus lead to depressed inhibitory descending influence on Renshaw cells. Recurrent inhibition has previously been tested in other pathologies with pyramidal syndrome[44]: *i)* it has been found increased in stroke and spinal cord injured patients[84,85]; *ii)* in patients with cerebral palsy, the inhibition was found unchanged[86] as in some patients with hereditary spastic paraplegia but, in the latter, inhibition could also be depressed but this was attributed to modification in AHP[87,88], and *iii)* interestingly, it has been found in patients with hyperekplexia that recurrent inhibition is preserved, likely due to its GABAergic components[89]. In the present study, we did not find any significant link between the modulations of recurrent inhibition and UMN sign manifestation likely because most

of the patients have predominant UMN or mixed form of ALS, and different modulations were observed according to the disease duration and the progression of lower limb dysfunctions. Enhanced inhibition was indeed particularly observed at early disease stage *i.e.*, within the first year after the first symptoms, but the inhibition was within the normal range or depressed when the disease was more advanced (depressed test Mmax, link with onset site in lower limbs, functional disability and its progression in lower limbs). Therefore, UMN degeneration and the consecutive changes in the cortico-reticulo-spinal influence on Renshaw cell activity can likely account for the enhanced recurrent inhibition but other mechanisms likely interfere with it, leading to progressive depression of recurrent inhibition.

3.3.2 | Modulation by peripheral afferents

Renshaw cells can receive excitation and inhibition from FRA[73] and it has been shown that recurrent inhibition is particularly reduced by group II afferents[90]. It is commonly admitted that sensory deficits in ALS are secondary mechanisms, occurring at late stages of the disease, and early clinical evidences for sensory defects exclude ALS from diagnosis. However, using spinal diffusion MRI, we evidenced early microstructural alteration in the dorsal columns, correlated with depressed peripheral afferent volleys, in patients without any clinical signs of sensory deficits[91]. These results are in accordance with those in ALS mice exhibiting presymptomatic disorganization of muscles spindles and specific alteration of group Ia and group II muscle afferents, concomitantly with LMN degeneration but developing at a slower rate[92]. The clinical evaluation of sensory deficits is not specific enough to discriminate a specific alteration of muscle spindles and of their resulting afferents inputs, including group II afferents, which likely explains why early sensory deficits in ALS can only be detected using experimental approaches[91]. Depression of muscle spindle group II inputs releases Renshaw cells from peripheral inhibition but this might have little impact at early stages of the disease, when there is no clinical evidence for muscle weakness and LMN alteration, and thus little contribution to enhanced recurrent inhibition. Additionally, this cannot account for the

depression of recurrent inhibition at later stages. Without fully discarding the group II hypothesis, we assume that this might have little role in the modulation of Renshaw cell activity in ALS.

3.3.3 | Influence of LMNs

For obvious reasons, we can expect that LMN dysfunctions and degeneration have strong impact on Renshaw cells. Indeed, a specific loss of their collaterals has been found from ventral horns with extensive loss of LMNs[34]. However, this does not fully match the present results in patients because: *i)* while we found a significant reduction of soleus Mmax (conditioning Mmax) in the full group, the inhibition in quadriceps LMNs was significantly increased, and *ii)* we did not find any significant link with the size of H-reflex and Mmax in the conditioning EMG *i.e.*, with the motor inputs to Renshaw cells, whatever the target LMN pools. On the contrary, we found the modulation of recurrent inhibition closely linked to the test Mmax *i.e.*, with the integrity of the target LMN pool. These results raise questions on the origin of the Renshaw cells mediating heteronymous recurrent inhibition, on the source of their motor axon inputs, on their intrinsic excitability and the repercussion of early LMN dysfunctions.

The *proximity hypothesis* (short projections of Renshaw collaterals), for the diffuse pattern of recurrent inhibition, has been discarded by showing that recurrent inhibition occurs between synergistic LMNs (but not between pure antagonists) whatever their location in the spinal cord (*functional hypothesis*)[45,73]. However, it is not known whether homonymous and heteronymous recurrent inhibitions are mediated by the same Renshaw cells or by different subsets. In the sub-group of participants in whom inhibition was tested reciprocally in the 2 LMN pools, we only found 6/17 patients in whom the recurrent inhibition was modulated the same way in quadriceps and soleus LMNs (decreased in 3 patients and within the control range in the 3 remaining ones). This observation does not help to distinguish between the 2 hypotheses (same interneurons *vs.* different subsets) but if the modulation of recurrent inhibition were due to intrinsic changes of Renshaw cell activity, and if the inhibitions were mediated by the same subsets of interneurons, one would have

595 expected systematic parallel changes in soleus and quadriceps LMNs.

596 The pattern of recurrent inhibition strongly correlates with the distribution of group Ia
597 monosynaptic excitations[45]. If homonymous and heteronymous collaterals converge onto the same
598 Renshaw cells, one would thus expect that the resulting recurrent inhibition would be greater from
599 homonymous motor axons than heteronymous ones. This possibility would explain the link between
600 the modulation of recurrent inhibition and the test Mmax: the global inhibition (from homonymous
601 and heteronymous motor axons) would be particularly depressed when the target (homonymous)
602 LMNs and their motor outputs are particularly altered. However, the rule Ia connections-recurrent
603 inhibition is not exclusive since recurrent inhibition without preceding Ia excitation has also been
604 reported (*extended recurrent inhibition*)[45]. Additionally, it is not possible to argue on the size of H-
605 reflexes and the strength of monosynaptic Ia excitations since the larger H-reflex amplitude in
606 patients, compared to controls, are likely due to a change in presynaptic inhibition of group Ia
607 terminals[69–71,93]. Furthermore, the change in H-reflex size in both muscles does not match the
608 change in recurrent inhibition in their respective LMN pool. However, it is important to keep in mind
609 that while we tested heteronymous recurrent inhibition, its level likely depends on the tonic level
610 of homonymous inhibition, and if the latter was depressed due to degeneration of target LMNs, we
611 could expect less recurrent inhibition.

612 Several alternative mechanisms would also explain the link between the modulation of
613 recurrent inhibition and the test Mmax, and the enhanced inhibition at early stages. I) On one hand,
614 Renshaw cells receive stronger inputs from fast LMNs than from slow ones but, on the other hand,
615 the level of recurrent inhibition (in resting condition) is greater in slow LMNs compared to fast ones
616 (due to intrinsic properties of LMNs)[78]. Since fast LMNs are among the first to degenerate in ALS[24–
617 28], their loss would have a strong impact on Renshaw cell activity and would thus greatly depress
618 the level of recurrent inhibition at early disease stages, which does not correspond to our
619 observations. In addition, we should have observed a link with the conditioning Mmax. Accordingly,

we assume this possibility has little role in the modulation of recurrent inhibition. Moreover, the loss of fast LMNs is accompanied by peripheral reinnervation by resistant slow LMNs (peripheral nerve sprouting)[24–28,94,95], which generates large motor unit potentials in EMG[96]. Therefore, inhibition of slow LMNs would lead to greater depression of H-reflex amplitude (due to suppression of large motor unit potentials) in patients compared to controls, which might account for the enhanced recurrent inhibition in quadriceps. However, we did not find any increase of recurrent inhibition in soleus LMNs. II) Wootz et al.[34] have revealed transient axon sprouting at the level of Renshaw collaterals at early disease stages, projecting onto surviving LMNs. If this result can be transposed to humans, this would contribute to reinforce recurrent inhibition of resilient (slow) LMNs. III) Excitability of LMNs has been found to progress from hyper to hypo-excitability along the course of the disease[5,6,97] and we have shown that LMNs in symptomatic patients with sporadic ALS are normo-to-hypoexcitable (participants are common to the present study)[57]. It has been clearly stated in ALS mice that the equilibrium between opposite effects (excessive activity of the voltage-gated Na^+ and Ca^{2+} channels mediating persistent inward currents [PICs] *vs.* increase in cell size and membrane conductance) is disrupted at the time of peripheral denervation leading to LMN hypo-excitability and death[98]. It would be particularly interesting to investigate the IPSPs from Renshaw cells under these conditions, in different LMN pools (to asses homonymous and heteronymous inhibitions) and at different stages of the disease. Indeed, both intrinsic and extrinsic LMN mechanisms, including Renshaw cells, might contribute to the excessive homeostatic response of LMNs but this has to be further investigated in animal models to open new avenues for therapy[14,16,98].

3.4 | Pathophysiological role in ALS

The present results suggest that recurrent inhibition is enhanced in LMNs without evidence for peripheral denervation (link with test Mmax), when there was no or only weak muscle weakness, within the first year after symptom onset in particular. This increase was particularly observed in

patients with slow functional progression in the target limbs. On the contrary, the inhibition was within the control range or even decreased when there were electrophysiological and clinical evidences for LMN degeneration in the target motor pool, and particularly in patients with first symptoms in lower limbs. These results are in accordance with Ozyurt et al.[37] and suggest that recurrent inhibition likely transiently increases at early stages of ALS, before decreasing when the target LMNs degenerate. This hypothesis is supported by Wootz et al.[34] who showed a transient sprouting of Renshaw collaterals on resilient (slow) LMNs (with strong recurrent inhibition), which would reinforce recurrent inhibition. Changes along the disease course should be confirmed by longitudinal study. In the present cohort, we had the opportunity to test recurrent inhibition in quadriceps twice in one patient: patient #13 (Table 1) was evaluated 6 and 30 months after the first symptoms, and recurrent inhibition was within the control range during the first evaluation (data retained for the group analysis) but strongly reduced during the second visit (Supplemental material 2A). Between both visits, the patient conditions have worsened (Supplemental material 2B) with depression of Mmax in both VL and soleus EMG, losing 7 points to total ALSFRS-r and 4, to lower limb ALSFRS-r sub-score, MRC score in quadriceps and soleus was still 5 but respectively 1 and 3 in TA and EHL, and patient #13 exhibited a predominant UMN form during the first evaluation but LMN predominant form the next time.

The results in quadriceps clearly indicate that recurrent inhibition can increase at early stage of the disease but likely decreases progressively with time and degeneration of target LMNs. However, similar increase was not revealed in soleus LMNs. Several mechanisms can explain the difference between both motor pools: I) We found again in both controls and patients that recurrent inhibition was greater in soleus than in quadriceps[44]. Since the conditioning stimuli were adjusted so as to produce Mmax in the corresponding EMG, there is a possibility that inhibitions were saturated, and possibly more in soleus LMNs than in quadriceps ones, which makes it difficult the detection of small variations (increase) of recurrent inhibition. However, inhibitions in both LMN

pools were evaluated the same way, and inhibition in quadriceps was likely at saturation too, making this hypothesis less plausible. II) According to MRC scores, the patients exhibited distal muscle weakness, affecting soleus in only 2/38 patients, but TA and/or EHL in 13/38 (Table 1), and in patient #13 we found that reduced recurrent inhibition in quadriceps was accompanied by the development of muscle weakness in TA and EHL (Supplemental material 2B). There is thus a possibility that reduced inhibition from distal LMNs, including TA and EHL, might affect the level of recurrent inhibition but this is unlikely since no recurrent inhibition from pretibial muscles (including TA and EHL; by stimulating the deep peroneal nerve) have been observed in both quadriceps and soleus LMNs[44], and manual muscle testing is not specific for EHL, but also includes intrinsic foot muscles without LMN recurrent collaterals[41]. III) Quadriceps LMN pool is more heterogeneous than soleus, including both fast and slow LMNs while soleus mainly includes slow LMNs. There is thus a possibility that structural reorganisation at both spinal and peripheral levels, and the global homeostatic response, affect more the level of inhibition in quadriceps than in soleus and/or enhanced inhibition in soleus manifests at even earlier (presymptomatic) stages of the disease. Here again, investigating recurrent inhibition in different LMN pools, with different proportions of slow and fast LMNs, and the characteristics of their homeostatic response would be particularly interesting in ALS mice, to determine the possible mechanism at pre- and post-synaptic levels underlying the modulations reported here in patients.

Several mechanisms have been identified in the regulation of the input/output gain across LMN pools, including PICs and recurrent inhibition from Renshaw cells[78], and it has been evidenced that PICs are enhanced in ALS[98]. A theoretical model has allowed to show that recurrent inhibition, and its GABAergic component in particular, is particularly efficient to control PICs and regulate LMN recruitment. It has thus been suggested that modulation of the strength and kinetics of GABAergic currents could provide treatment strategies for uncontrollable spasms[99]. Therefore, we assume that adaptive mechanisms in spinal circuitry involving Renshaw cells and enhanced recurrent inhibition

might be particularly efficient to limit PICs amplification and maintain LMN homeostasis in ALS. Such mechanisms, as long as they are effective, would be protective from neurodegeneration. In line with this, we particularly observed enhanced recurrent inhibition in patients with slow worsening of lower limb functions. Moreover, lithium, which among other effects increases the number of Renshaw cells, had a neuroprotective effect in ALS mouse model[100,101] but its interest in ALS therapy was discarded by the disappointing results of clinical trials[102]. Last important point, distal hand and foot muscles are among the first to manifest clinical symptoms of weakness and wasting in most cases of ALS[1]. LMNs innervating these muscles have no recurrent collaterals[41,42] and, consequently, cannot benefit from any protective role from Renshaw cells. This might participate in their greater sensitivity to ALS. On the other hand, recurrent collaterals are only scarce in the most resistant oculomotor LMN pool[103]. However, this does not rule out the putative protective role of Renshaw cells in ALS and further suggests that several other mechanisms likely participate in the homeostatic response, making it difficult the pharmacological approach to slow down progression.

3.5 | Conclusion and perspectives

The present study provides the first experimental evidence for enhanced activity in spinal circuitry involving Renshaw cells and further confirms that recurrent inhibition is modulated and progressively depressed with LMN degeneration. Our results allow to reconsider the role of recurrent inhibition in ALS and in the LMN homeostatic response, and suggest that Renshaw cells likely have a transient putative protective role on LMNs from neurodegeneration. Several mechanisms likely participate in the adaptive mechanisms, including cortical influence on Renshaw cells and reinnervation by slow LMNs. Accordingly, our study gives strong support to hypotheses recently raised on the role of spinal circuitry organization in the homeostatic response, with enhanced inhibition counteracting PICs amplification, and in the disease progression[14,16] given the diffuse distribution of heteronymous projections supporting muscle synergies and likely participating in the spread of local alterations to other regions, in particular between proximal

muscles[73]. These results in humans cannot help to determine the exact mechanisms underlying those changes at spinal level, due to limited methodological approaches, but encourage further studies, in both animal models and patients in parallel, to deepen the knowledge on spinal network plasticity in ALS, its functional role in homeostatic response to maintain LMN functions and in disease progression.

4 | MATERIALS AND METHODS

4.1 | Ethics

The present study is part of a large study aiming at studying the spinal excitability in patients with ALS (SpinalBioMark-SLA) during which we assessed different spinal circuitries using indirect electrophysiological tools. The full study and the experimental procedures, including those in the present paper, conform to the lasted revision of the Code of Ethics of the World Medical Association (Declaration of Helsinki) and were approved by the ethic committee of INSERM (protocol n°C14-21) and by the national ethical authorities (CPP Ile de France, Paris 6 - Pitié-Salpêtrière, CPP/16-15; RCB 2014-201-A01240-47). It has been registered in a public registry (https://clinicaltrials.gov, NCT02429492). The experiments were performed with the written informed consent of each participant. The data that support the findings of this study are available on request from the corresponding author; they are not publicly available due to ethical restrictions.

4.2 | Participants

Based on dataset from previous studies in the laboratory (mean inhibition in quadriceps and variance), and for minimum difference of 10 % between groups, we estimated that 40 controls and 40 ALS patients had to be included in the present study to obtain a statistical power with an alpha risk (type I error) of 5% and beta risk (type II error) of 10%. Accordingly, 45 patients and 49 controls were included but the experiments could be performed in 38 patients and 42 controls because i) H-reflex could not be produced in VL EMG in 3 patients and 3 controls and hardly evoked making it unusable for the experiments in 3 other patients and 3 other controls, ii) the conditioning stimuli

745 applied to PTN was not selective for soleus in 1 patient, activating pretibial flexors whose spindle
746 afferents produce spinal excitation in quadriceps LMNs and no recurrent inhibition[104], and iii) 1
747 control was too sensitive to electrical stimulation. In both groups, 7 women were tested and the
748 mean age in the patient group was 61.5 ± 9.7 years old (mean ± 1 SD; ranging from 39 to 78), and
749 61.8 ± 9.0 (from 40 to 77) in the control group.

750 The inclusion criteria for controls included the absence of prior or current neurological illness.
751 Those for patients included 1) probable or definite ALS according to the El Escorial criteria[105], 2) no
752 clinical signs of motor deficits with normal clinical EMG examination in quadriceps, 3) absence of
753 peripheral neuropathy, and 4) no comorbid neurological conditions. Patients were screened and
754 tested for the 4 most common ALS-causing mutations (SOD1, FUS, C9orf72 and TDP43; DNA
755 extraction was performed by Genethon, Evry, France; DNA analysis was carried out at the University
756 of Tours, France), and all were negative except 2 (C9orf72 in patient #13 and SOD1 in patient #32;
757 Table 1). Table 1 resumes the main clinical features; MRC scores were those obtained on the
758 investigated side. Patients were explored on their less affected side which explains why some of
759 them had normal MRC score while the onset site was in lower limbs *i.e.*, on the non-investigated
760 side.

761 All participants were indeed tested on one side, preferably the dominant side[106]. When
762 patients had motor deficits in quadriceps on the dominant side, we explored their non-dominant
763 side and, in both groups, the non-dominant side was tested in case of orthopaedic trauma on the
764 dominant side: *i*) right-handers tested on the right (dominant) side: 36 controls *vs*. 26 patients, *ii*)
765 left-handers tested on the left (dominant) side: 4 controls *vs*. 3 patients, *iii*) right-handers tested on
766 the left (non-dominant) side: 1 control *vs*. 8 patients, and *iv*) left-handers tested on the right (non-
767 dominant) side: 1 control *vs*. 1 patient.

768 **4.3 | Materials**

769 EMG activities were recorded using single-use bipolar surface electrodes (sticky foam

electrodes with solid gel; 2-cm apart; FIAB, Florence, Italy) that were secured on the skin, over i) the vastus lateralis (VL) head of the quadriceps femoris, on the antero-lateral part of the thigh, ~15cm above the patella and ii) the soleus, on the posterior part of the leg, ~5cm below the insertion of gastrocnemius muscles. In our experience, H-reflex in quadriceps is larger when recording electrodes are over VL head. However, in the participants in whom there was no H-reflex in VL EMG, we tried unsuccessfully other electrode positions, on rectus femoris and vastus medialis. The signals were amplified and filtered (x 1,000-5,000; 0.1-1kHz bandpass; D360 8-channel Patient Amplifier, Digitimer Ltd, Hertfordshire, UK) before being digitally stored on a personal computer (2-kHz sampling rate; Power 1401 controlled by Signal Software 6.05; CED, Cambridge, UK) for offline analysis.

Percutaneous electrical stimulations (1-ms duration rectangular pulse; DS7A, Digitimer Ltd, Hertfordshire, UK) were applied to the i) FN trough monopolar electrodes: cathode being a 21-cm^2 brass plaque placed on the posterior aspect of the thigh (below the buttock) and anode, a 7-cm^2 brass hemisphere placed in the femoral triangle, and ii) PTN with similar electrodes: the cathode was placed above the patella and the anode, in the medial part of the popliteal fossa. Stimulating electrodes were covered by wet sponge tissue and their positions were checked according to motor response evoked in VL and soleus EMG, respectively, and clinically, by tendon palpation.

4.4 | Experimental protocols

The participants were comfortably seated in a reclining armchair, with head support, and the tested leg was positioned in a device fixed to the chair and adaptable so that the hip was semi-flexed (~80°), the knee semi-extended (~130°) and the ankle in semi-plantarflexion (~100°). The skin was first cleaned using exfoliating cream before positioning recording electrodes. All during the experimental protocol, the participants were asked to relax as much as possible and the recordings were performed at rest. Experiment 1 was performed in all the participants and aimed at evaluating the level of recurrent inhibition produced in quadriceps LMNs by activating recurrent collaterals of

soleus motor axons (Fig. 1A). Test stimuli were applied to FN to produce H-reflex in VL EMG and conditioning stimuli, to PTN. First, the maximal amplitude of Mmax was evaluated in VL EMG by testing FN stimuli at different intensities between H-reflex threshold and suprathreshold intensity for Mmax (N = 5 stimuli/intensity; H/M recruitment curve). Then, the intensity of FN-test stimuli was adjusted so as to produce a measurable and stable H-reflex in VL EMG. The intensity of PTN-conditioning stimuli was adjusted at the threshold intensity for evoking Mmax in soleus EMG. The effects of PTN-conditioning stimuli on quadriceps H-reflex were tested at 3 ISIs; the PTN-conditioning stimuli being delivered 15, 20 and 25 ms before the FN-test stimuli *i.e.*, at the optimal ISIs for producing recurrent inhibition in quadriceps LMNs[44,61] (Figs. 1B-E; Fig. 5). Experiment 2 was performed in the second half of each group: 17 ALS patients (2 women; mean age in the group: 62.7 ± 9.5 years old) *vs*. 17 controls (3 women; 60.0 ± 10.8 years old) during the same experimental session as experiment 1. Basically, the experimental procedure followed the reverse design of experiment 1: the test stimuli were applied to PTN and the conditioning to FN, to evaluate the level of recurrent inhibition produced in soleus LMNs by activating quadriceps motor axon recurrent collaterals (Fig. 3A). The intensity of PTN-test stimuli was adjusted to produce H-reflex of ~25% of Mmax in soleus EMG[48], and that of FN-conditioning stimuli, at the threshold intensity for producing Mmax in VL EMG. The ISIs between FN and PTN stimulations were set at 5, 10 and 15 ms *i.e.*, optimal for recurrent inhibition in soleus LMNs[44,60]. In both experiments, one run of acquisition consisted in testing 1 ISI between conditioning and test stimuli, with 20 isolated test stimuli *vs.* 20 combined (conditioning + test) stimuli randomly alternated (0.3-Hz stimulation frequency rate). The size of conditioning Mmax was monitored throughout the experiment to ensure the stability of conditioning stimuli (Fig. 5B).

Figure 5 near here

4.5 | Analysis

The peak-to-peak amplitude of H-reflexes (test EMG; Fig. 5A) and Mmax (conditioning EMG;

Fig. 5B) were evaluated; in Figure 5, VL EMG was the test EMG, and soleus EMG, the conditioning one. For each run of acquisition, we evaluated the mean amplitude of the test H-reflex, which was expressed as a % of the corresponding (test) Mmax for interindividual comparison. H-reflex in VL EMG can be hardly evoked without preceding M response (Figs. 1B, Fig. 5A), and both can overlap making it difficult their distinction especially at intensity producing ~Hmax/2 and above. A particular attention was thus taken to determine the amplitude of quadriceps H-reflex in each participant, according to their own H/M recruitment curve. The amplitude of each conditioned H-reflex was normalized to the mean amplitude of the test H-reflex. The mean difference between the mean test H-reflex and conditioned H-reflexes, expressed as a % of the mean test H-reflex, was calculated to evaluate the level of recurrent inhibition produced in the test LMN pool (Figs. 1DE).

UMN and LMN scores, and their difference (UMN – LMN scores), were calculated to determine whether the patients exhibited predominant UMN (difference > 0), or LMN (difference < 0) or a mixed form (difference = 0) at the time at inclusion:

- UMN score[56,57] = reflex score (0, 1 or 2) + Babinski or Hoffmann sign (0 or 1) + Ashworth ≥ 3 (0 or 1)

Here, the reflex score is based on tendon reflexes in soleus and quadriceps: score is 0 when reflexes were normal or absent, 1, when present in wasted muscle, and 2, when brisk. When Babinski reflex was absent, the score is 0 and 1 when present. If grade from the modified Ashworth scale was < 3, the score is 0, and if the grade was ≥ 3 (*i.e.*, with high possibility of muscle clonus), the score is 1.

- LMN score = atrophy (0 or 1) + fasciculation (0 or 1) + MRC (0, 1 or 2)

Here, when atrophy was absent, the score is 0, and 1 when present. When fasciculations were absent, the score is 0, and 1 when present. Lastly, when MRC grade was 5, the score is 0, when MRC was 4 or 3, the score is 1, lastly if MRC was between 2 and 0, the score is 2.

4.6 | Statistics

Statistical analyses were performed using JMP® Pro 16.0.0 (SAS Institute JMP, Brie Comte

Robert, France). The alpha significance level was fixed at 0.05 and the results were considered statistically significant only if $p < 0.05$. Mean values are indicated ± 1 standard deviation (SD).

Descriptive data in groups of participants are illustrated using box plot charts (Figs. 2 and 3). The lower limit of the box indicates the 25th percentile (1st quartile, Q1), the upper limit, the 75th percentile (3rd quartile, Q3), the continuous line within the box, the median and the cross, the mean. The lines that extend from the box (whiskers) are limited to minimum and maximum data values; values above or below the end of the whiskers are outliers. Homoscedasticity (Levene median test) and normality (Shapiro-Wilk test) were first verified to allow parametric analyses (Student *t* test) to compare electrophysiological parameters and the level of maximal recurrent inhibition between controls and ALS. Alternatively, Welch ANOVA (normal distribution with heterogenous variances) or non-parametric methods were used (Kolmogorov-Smirnov test). Outliers were detected using the inter-quantile range (IQR) method. Linear mixed models were built and ANOVA were performed to test the difference between controls and ALS, taking into account electrophysiological metrics significantly different between groups and that could have influenced the level of recurrent inhibition (ISI, H-reflex and Mmax sizes in test and conditioning EMG*). Post hoc* analyses were performed using Student tests. Effect size was measured using Cohen's d[49,50] when we compared 2 means, and using f^2 [54], when we performed multivariate analysis (linear mixed model). Effect size is very small when d or $f^2 = 0$, small when d = 0.2 and $f^2 \geq 0.15$, medium when d = 0.5 and $f^2 \geq 0.15$ and large when d ≥ 0.8 and $f^2 \geq 0.35$.

Given the intrinsic differences in the size of electrophysiological metrics and the level of recurrent inhibition between quadriceps and soleus[45,46,51–53], we calculated the CI95 in controls and metrics in ALS were classified according to the lower and upper limits of CI95 in controls. Then, Chi^2 tests were performed to compare the LMN pools in ALS. The resulting categorial data were also used to evaluate the link between the modulation of recurrent inhibition in ALS and the patient phenotype including their clinical and electrophysiological features. Chi^2 tests and correspondence

analyses were first performed to evaluate the influence of each parameter individually on the modulation of recurrent inhibition. Then, multiple correspondence analysis (MCA) and Chi^2 tests were undertaken to identify the associations between modalities of clinical and electrophysiological parameters and the level of recurrent inhibition. Similar to other multivariate methods, MCA is a dimension reducing method, representing the data as points in 2 or 3-D space (Figs. 4B and D). For clarity, the statistical tests and the parameters included in each test are specifically indicated in Results.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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TABLES

Table 1: Clinical features

			ALSFRS-r							MRC				
	Site of Onset	Duration	total	sub-score	Progression	LL-progression	UMN score	LMN score	Predominant form	Soleus	TA	EHL	Quadriceps	Riluzole
1	UL	11	45	7	0,27	0,09	2	0	UMN	5	5	5	5	x
2	UL	72	40	4	0,11	0,06	1	2	LMN	**3**	**4**	**3**	5	x
3	UL	15	40	6	0,53	0,13	2	1	UMN	5	5	5	5	x
4	UL	28	40	3	0,29	0,18	4	2	UMN	5	**2**	**3**	5	x
5	LL	15	36	3	0,80	0,33	0	2	UMN	5	5	5	5	-
6	LL	16	44	**8**	0,25	0,00	0	0	Mixed	5	5	5	5	x
7	UL	17	45	**8**	0,18	0,00	0	0	Mixed	5	5	5	5	x
8	LL	5	36	6	2,40	0,40	0	0	Mixed	5	5	5	5	x
9	LL	21	39	4	0,43	0,19	2	1	UMN	5	**3**	5	5	x
10	LL	26	38	3	0,38	0,19	3	1	UMN	5	5	**4**	5	x
11	LL	14	43	4	0,36	0,29	3	2	UMN	5	5	5	5	x
12	UL	9	39	**8**	1,00	0,00	0	0	Mixed	5	5	5	5	-
13*	LL	6	47	7	0,17	0,17	2	2	Mixed	5	5	5	5	-
14	Bulbar	7	44	6	0,57	0,29	3	0	UMN	5	5	5	5	-
15	UL	25	39	**8**	0,36	0,00	0	0	Mixed	5	5	5	5	x
16	UL	16	45	**8**	0,19	0,00	0	0	Mixed	5	5	5	5	-
17	LL	24	41	3	0,29	0,21	0	2	LMN	5	**1**	**0**	5	x
18	Bulbar	23	36	5	0,52	0,13	0	1	LMN	5	5	5	5	x
19	LL	20	42	4	0,30	0,20	4	0	UMN	5	5	5	5	x
20	UL	14	40	6	0,57	0,14	3	1	UMN	5	**4**	5	5	-
21	UL	13	47	**8**	0,08	0,00	2	0	UMN	5	5	5	5	-
22	UL	18	40	7	0,44	0,06	2	0	UMN	5	5	5	5	x
23	LL	7	47	7	0,14	0,14	0	1	LMN	5	**3**	**3**	5	x
24	UL	59	34	5	0,24	0,05	3	1	UMN	5	5	**4**	5	x
25	UL	14	33	7	1,07	0,07	2	0	UMN	5	5	5	5	x
26	UL	22	38	7	0,45	0,05	2	1	UMN	5	5	5	5	x
27	Bulbar	33	39	4	0,27	0,12	0	0	Mixed	5	5	5	5	x
28	Bulbar	14	24	3	1,71	0,36	3	1	UMN	5	5	5	5	x
29	LL	11	34	3	1,27	0,45	3	1	UMN	5	**3**	**4**	5	x
30	Bulbar	48	43	**8**	0,10	0,00	3	0	UMN	5	5	5	5	x
31	LL	14	38	3	0,71	0,36	3	0	UMN	5	**4**	**3**	5	x
32*	LL	12	42	3	0,50	0,42	2	2	Mixed	5	5	5	5	x
33	UL	7	42	**8**	0,86	0,00	3	0	UMN	5	5	5	5	x
34	UL	7	44	**8**	0,57	0,00	3	1	UMN	5	5	5	5	x
35	LL	41	39	2	0,22	0,15	0	4	LMN	**2**	**0**	**0**	5	x
36	Bulbar	63	36	4	0,19	0,06	4	0	UMN	5	5	**3**	5	x
37	UL	46	41	6	0,15	0,04	0	0	Mixed	5	5	5	5	x
38	Bulbar	13	41	3	0,54	0,38	2	1	UMN	5	5	**3**	5	x

Site of onset: location of first symptoms in upper limb (UL), lower limb (LL) or bulbar LMNs (Bulbar); Duration: time since first symptoms (months); ALSFRS-r: total score (maximal score = 48) and sub-score for lower limb functions (walking and climbing stairs; maximal score = 8 indicated in bold); Progression: lost points to ALSFRS-r/month; LL-progression: lost points to ALSFRS-r sub-score for lower limbs (LL)/months; UMN: upper motor neuron score (see Methods); LMN: lower motor neuron score (see Methods); Predominant form in UMN, or LMN, or mixed form, according to the difference between UMN and LMN scores. MRC: muscle strength evaluated on the investigated limb, in soleus, tibialis anterior (TA), extensor hallucis longus (EHL) and quadriceps; Taking riluzole (x) or not (-). * Patients with genetic mutation: C9orf72 in patient #13 and SOD1 in patient #32.

Table 2: Electrophysiological profile of the patient group

		Quadriceps	Soleus
H-reflex	< lower limit	8/38	0/17
		21.0 %	0 %
		6.2 ± 1.0 %	-
	Within CI95	6/38	5/17
		15.8 %	29.4 %
		12.1 ± 2.4 %	20.3 ± 2.6 %
	> upper limit	24/38	12/17
		63.2 %	70.6 %
		30.2 ± 13.8 %	53.8 ± 18.3 %
Mmax	Not depressed	23/38	10/38
		60.5 %	26.3 %
		2.3 ± 1.3 mV	5.4 ± 1.5 mV
	Depressed	15/38	28/38
		39.5 %	73.7 %
		0.8 ± 0.2 mV	2.0 ± 0.9 mV

CI95s of the mean H-reflex and Mmax in quadriceps and soleus were calculated in the group of controls. Row 1 indicates the title of the column corresponding to the results for quadriceps and for soleus. Row 2 indicates when H-reflex was below the CI95 lower limit, row 3, when it was within the CI95 and row 4, when it was above the CI95 upper limit. Row 5 indicates when Mmax was not depressed (within or above the CI95 upper limit) and row 6, when it was depressed (below the CI95 lower limit). In each cell, rows 1 and 2 indicate the corresponding proportion of patients (ratio between the number of patients with results corresponding to the cell and the total number of patients and below, the corresponding %), and row 3 indicates the mean size of the response ± 1 SD (in % of Mmax for H-reflex and in mV for Mmax).

Table 3: Link between modulation of recurrent inhibition and patients features

	r^2	*p* value	Correspondence
Motoneuron pool†	0.15	0.0012	• ↑ RI in quadriceps • ↔/↓ RI in soleus
Test Mmax†	0.09	0.0055	• ↔ Mmax: ↑ RI • ↓ Mmax: ↔/↓ RI
Progression†	0.08	0.0093	• Fast progressors: ↔ RI
Predominant form	0.07	0.0929	• UMN/mixed: ↑/↔ RI
H-reflex in conditioning muscle	0.04	0.5019	• ↑ H-reflex: ↔ RI
ALSFRS-r sub-score (lower limb functions [LL])	0.03	0.1397	• Score ≥ 6: ↑ RI • Score < 6: ↓ RI
LL-Progression	0.02	0.3239	• ↑ RI in slow progressors • ↔/↓ RI in fast progressors
Site of onset	0.02	0.3415	• LL: ↓ RI • Other: ↔/↑ RI
H-reflex in test muscle	0.02	0.7303	None
Conditioning Mmax	0.01	0.4304	• ↔ Mmax: ↔ RI
Total ALSFRS-r	0.005	0.7572	• Score ≥ 40: ↑/↓ RI • Score < 40: ↔ RI
Duration	0.002	0.8579	• 1st year: ↑ RI • > 1 year: ↔/↓ RI
Riluzole	0.002	0.8792	• On riluzole: ↔/↓ RI

Column 1: patient parameters tested to evaluate their relationship with the modulation of recurrent inhibition (RI) expressed according to CI95 in controls. Column 2: r^2 indicating the strength of the regression. Column 3: *p* value (Chi^2 test). Column 4: result of the correspondence analysis: ↑ for increase (value superior to the upper limit of the CI95 in controls), ↓ for decrease (value inferior to the lower limit of the CI95 in controls) and ↔ for value within the CI95 in controls. † $p < 0.01$ and ‡ $p < 0.001$.

LEGENDS TO FIGURES

Figure 1: Recurrent inhibition from soleus to quadriceps. A, Schematic representation of recurrent collaterals of soleus motor axons projecting onto Renshaw cells mediating recurrent inhibition to quadriceps LMNs. Dashed arrows indicate the trajectory of antidromic volley in soleus motor axons after PTN stimulation. Grey line represents group Ia afferent inputs after FN stimulation, mediating

monosynaptic excitation to quadriceps LMNs producing H-reflex in VL EMG. **BC**, Superimposition of mean VL EMG after isolated test FN stimulation (Test, black line) and after combined stimuli (conditioned PTN + test FN stimuli) delivered at 15-ms ISI (grey line), 20-ms ISI (blue line) and 25-ms ISI (red line) in one control (**B**) and one patient (**C**; N = 20 stimuli in each condition). **DE**, The mean inhibition (= the mean difference between the mean test H-reflex and the conditioned H-reflexes in VL EMG, expressed as % of the mean test H-reflex) produced in quadriceps LMNs in the same control (**D**) and the same patient (**E**) as in **BC**, is plotted against the ISI (ms) between PTN (conditioning) and FN (test) stimuli. **FG**, Mean amplitude of Mmax (mV) produced in quadriceps (VL head; left column) and soleus EMG (right column) by FN (test) and PTN (conditioning), respectively, in the same control (white columns; **F**) and the same patient (blue columns; **G**) as illustrated in (**BD**) and (**CE**), respectively. Vertical bars are ± 1 SD. * $p < 0.05$, † $p < 0.01$ and ‡ $p < 0.001$.

Figure 2: Modulation of recurrent inhibition in quadriceps. A, Box plots illustrating the distribution of Mmax amplitude produced in VL (quadriceps, left part) and soleus EMG (right part; mV) in the group of controls (white box and black diamonds; N = 42 participants) and patients with ALS (blue box and black diamonds; N = 38 participants). The lower limit of the box indicates the 25th percentile (1st quartile, Q1), the upper limit, the 75th percentile (3rd quartile, Q3), the continuous line within the box, the median and the cross, the mean. The lines that extend from the box (whiskers) are limited to minimum and maximum data values; values above or below the end of the whiskers are outliers. **B**, Box plots representing the mean amplitude of test H-reflex in VL EMG (% Mmax in VL EMG; same legend as in **A**) in controls (white box on the left) and ALS (blue box on the right). **C**, Box plots (as in ABC) illustrating the distribution of recurrent inhibition (% of mean test H-reflex) in both groups at the ISIs 15, 20 and 25 ms between conditioning PTN and test FN stimuli. **D**, Recurrent inhibition least mean square calculated using the mixed linear model (marginal inhibition conditioned by group, ISI, Mmax in soleus and H test in VL used as fixed effects in the model, and

subject as random effect) in controls (white bar) and patients with ALS (blue bar). Upper and lower crosses indicate respectively the upper and lower limits of the 95 % of confidence interval (CI95), and the interrupted line the CI95. **E**, Distribution of the mean maximal amount of recurrent inhibition (% of mean test H-reflex; box plots as in ABC) in each group. * $p < 0.05$, † $p < 0.01$.

Figure 3: Modulation of recurrent inhibition in quadriceps and soleus. A, Schematic representation of recurrent collaterals of quadriceps motor axons projecting onto Renshaw cells mediating recurrent inhibition in soleus LMNs. Dashed arrows indicate the trajectory of antidromic volley in quadriceps motor axons after FN stimulation. Grey line represents group Ia afferent inputs after PTN stimulation, mediating monosynaptic excitation to soleus LMNs producing H-reflex in soleus EMG. **B**, Box plots (as in Fig. 2) showing the distribution of amplitude of Mmax (mV) produced in quadriceps (VL head; left side) and soleus EMG (right side) by FN and PTN respectively, in the subgroup of controls (n = 17; white boxes and black diamonds) and of patients (n = 17; blue boxes and black diamonds). **C**, Box plots showing the distribution of amplitude of test H-reflex produced by FN stimuli in quadriceps (VL head) and by PTN stimuli in soleus EMG in the control and patient groups (same groups and same legend as in **B**). **D,G**, Box plots (same legend as in **BC**) illustrating the distribution of the level of recurrent inhibition (% of mean test H-reflex) in the control and patient groups in quadriceps LMNs at the ISIs 15, 20 and 25 ms between conditioning PTN and test FN stimuli (**D**) and in soleus LMNs at the ISIs 5, 10 and 15 ms between conditioning FN and test PTN stimuli (**G**). **E,H**, Recurrent inhibition least mean square (as in Fig. 2D) in controls (white bar) and patients with ALS (blue bar). Upper and lower crosses, and the interrupted line delimit the 95 % of confidence interval (CI95; as in Fig. 2D). **F,I**, Box plots (as in **C,D,G**) showing the distribution of maximal amount of recurrent inhibition (% of mean test H-reflex) in each group, in quadriceps and soleus LMNs. * $p < 0.05$, † $p < 0.01$ and ‡ $p < 0.001$.

Figure 4: Link between the modulation of recurrent inhibition and patient features. A, The

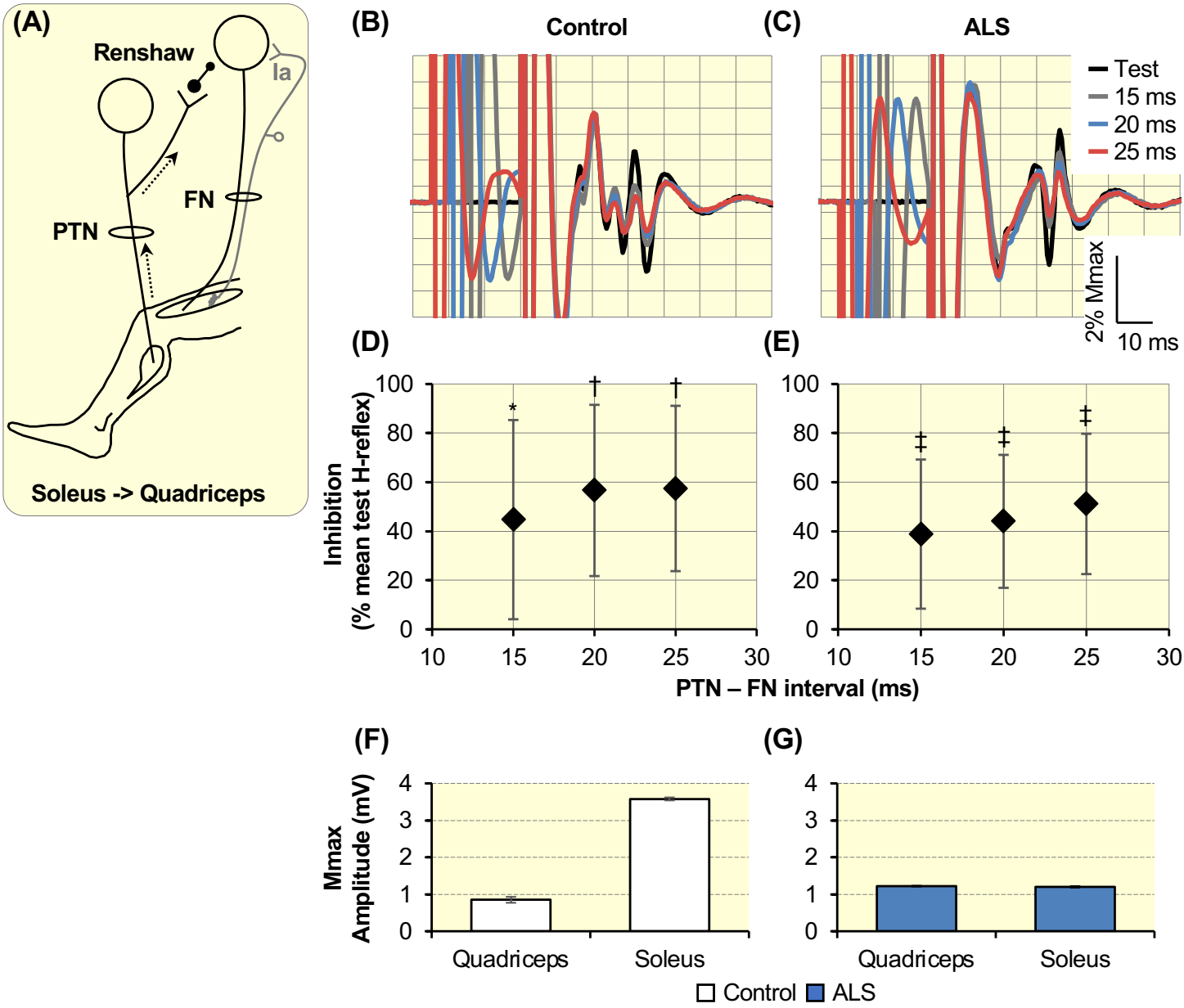
columns represent the repartition of the patients (% of full group; n = 38 participants for quadriceps and 17 for soleus) according to their modulation of maximal recurrent inhibition (RI) in quadriceps (left column) and soleus (right column) LMN pools, compared to the 95 % of confidence interval (CI95) of the mean inhibition in the control group. The number of patients (% of the full group of participants in the corresponding muscle) exhibiting a mean maximal recurrent inhibition below the CI95 in controls are in grey, those within the CI95, in blue, and those above the CI95, in white. **B,D**, Plots illustrating the projection of variable modalities in 2 dimensions corresponding to X and Y axis, according to their inertia (λ; deviation from independence: the greater the value the larger the dependency). Modalities include the modulation of recurrent inhibition (RI, black diamonds; ↔ for inhibition within the CI95 in controls, ↑ for inhibition above the upper limits of CI95 in controls, and ↓ for inhibition below the lower limits of CI95 in controls; **BD**), the target LMN pool (grey squares; soleus and quadriceps; **BD**), the size of test Mmax (blue triangles; ↔ and ↓ as for inhibition; **BD**), progression type according to total ALSFRS-r score (red X; slow and fast progressors; **B**), onset site in lower limbs (LL) or in other regions (Other = upper limbs and bulbar regions; blue circles; **D**), duration (blue squares; ≤ 1 year [y.] or < 1 year; **D**), progression in lower limbs (LL-progression; slow *vs.* fast; red diamonds; **D**) and clinical manifestation of UMN signs (Yes; right-orientated red cross; **D**). **C,E**, Bars illustrate the partial contribution of each modality of each parameter in dimension 1 (white bars) and in dimension 2 (blue bars). Bars illustrate the lines of the contingency table *i.e.*, target LMN pool (So. for soleus and Quad. or Q for quadriceps; **CE**), progression (**C**), and test Mmax (↔ for not depressed and ↓ for depressed; **CE**), duration (> 1 year and ≤ 1 year; **E**), LL-progression (Sl. for slow and F for fast; **E**), and UMN signs (N for no and Y for yes; **E**).

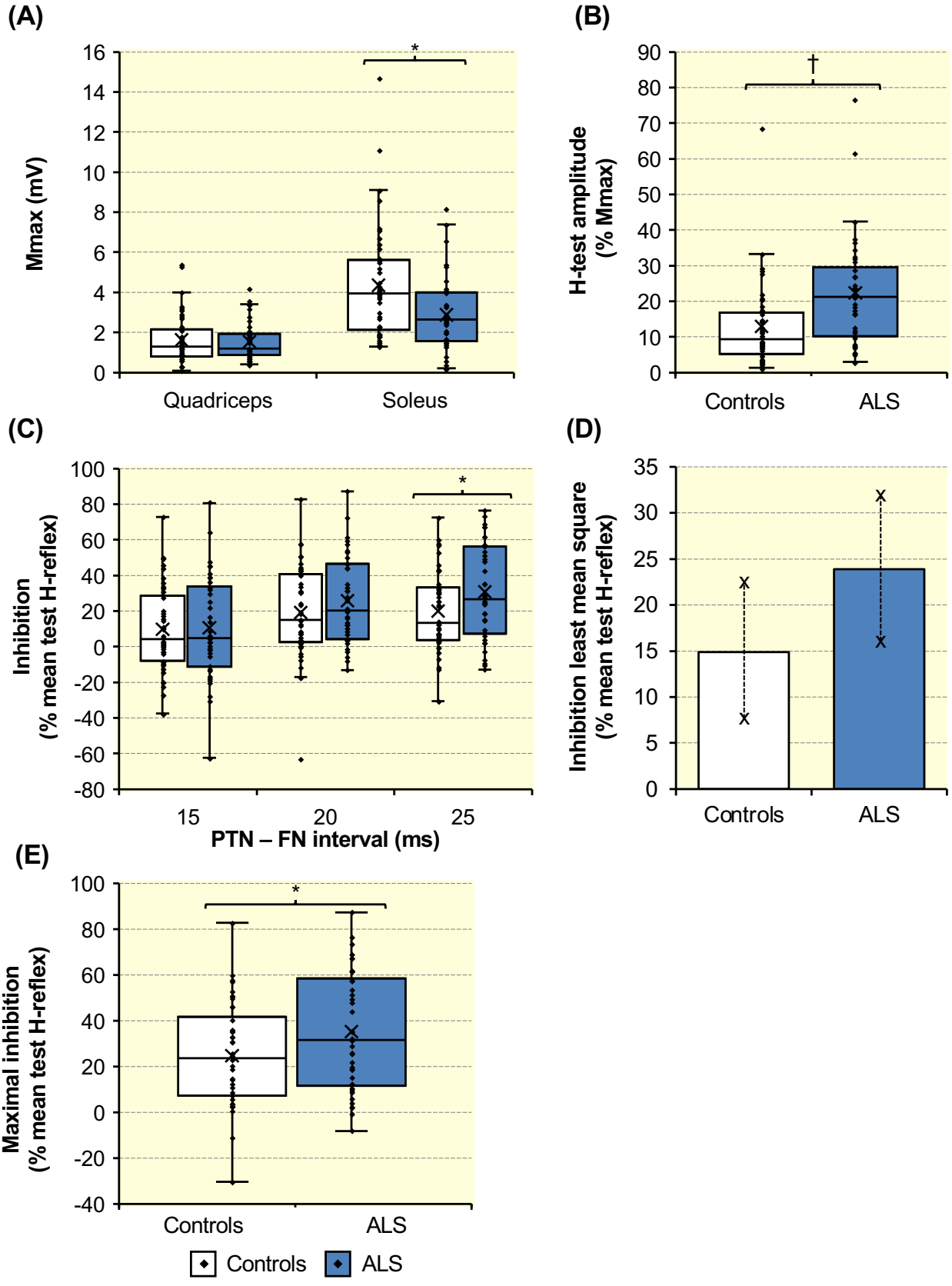
Figure 5: EMG recordings in one control. Mean EMG activities in vastus lateralis (VL) head of quadriceps (N = 20 stimuli; left panels) and in soleus (right panel) in one participant (control), when FN-test stimuli were delivered alone (top trace), and on combined stimuli (PTN + FN) at the 3 optimal

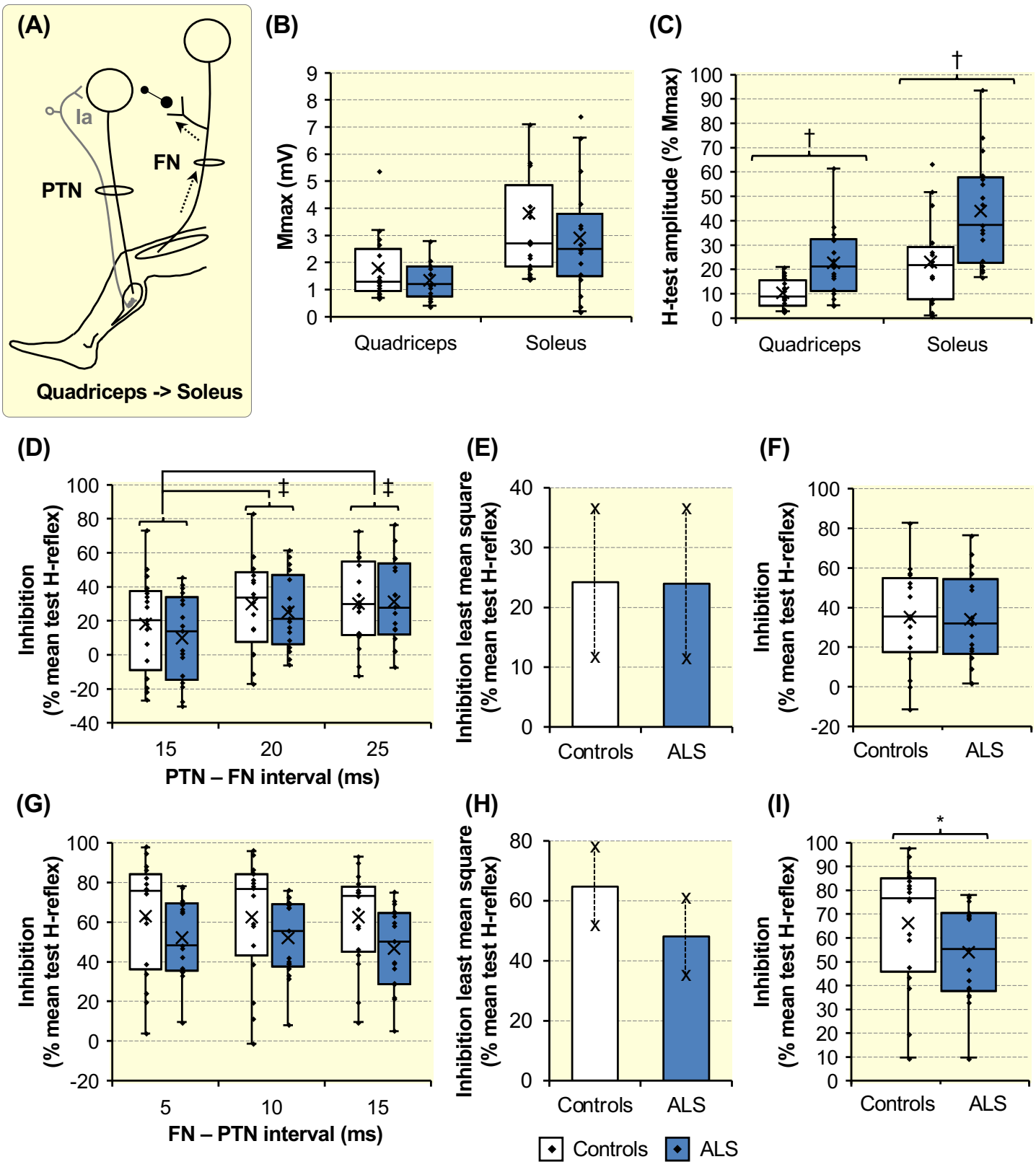
ISIs for producing recurrent inhibition in quadriceps LMNs: 15 ms (upper trace), 20 ms (middle trace) and 25 ms (lower trace).

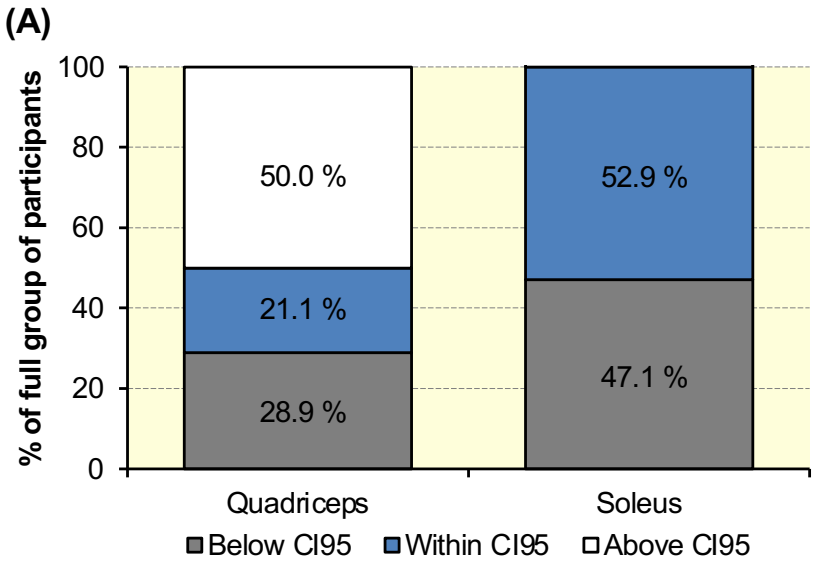
PHYSIOLOGICAL RELEVANCE

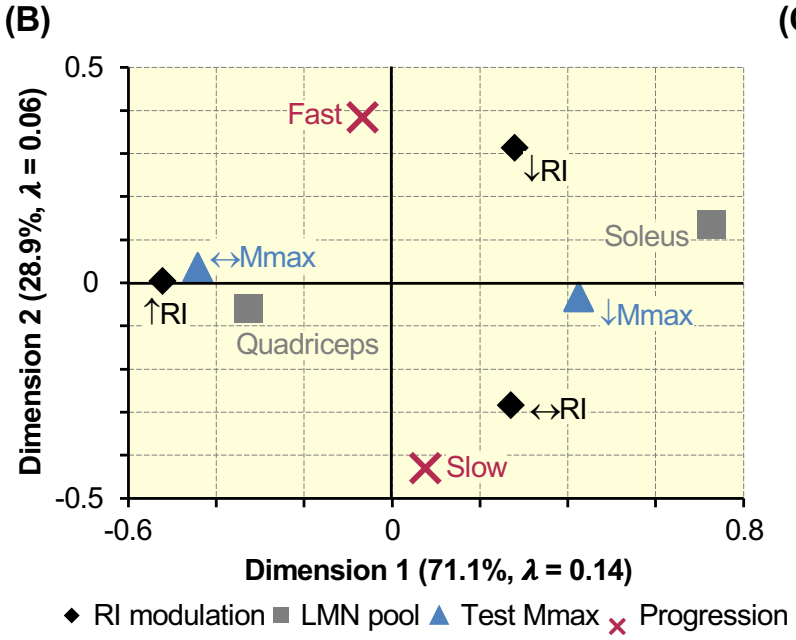
The present study allows to reconsider the role of Renshaw cells in the pathophysiology of ALS and shows that adaptative mechanisms transiently enhance recurrent inhibition of LMNs at early disease stage, which can counteract PICs amplification, and likely contribute to maintain homeostasis and motor output before degeneration.

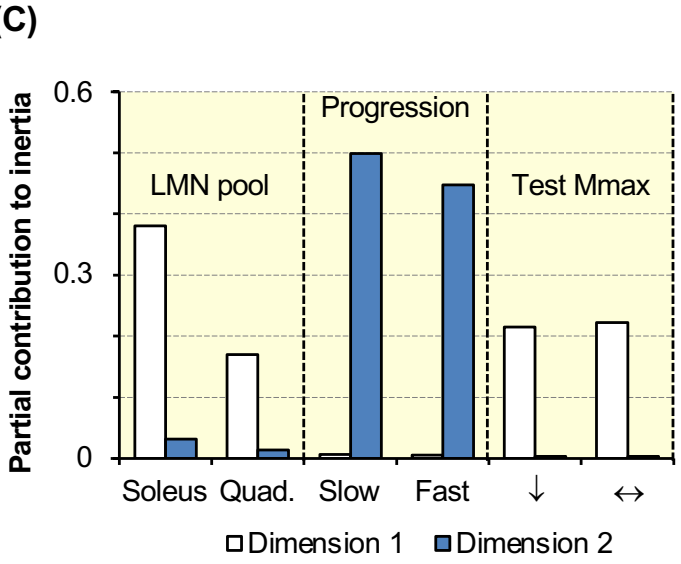

(A)
Renshaw
Ia
FN
PTN
Soleus -> Quadriceps
(B)
Control
(C)
ALS
Test
15 ms
20 ms
25 ms
2% Mmax
10 ms
(D)
(E)
Inhibition
(% mean test H-reflex)
100
80
60
40
20
0
10
15
20
25
30
PTN – FN interval (ms)
(F)
(G)
Mmax
Amplitude (mV)
4
3
2
1
0
Quadriceps
Soleus
Control
ALS

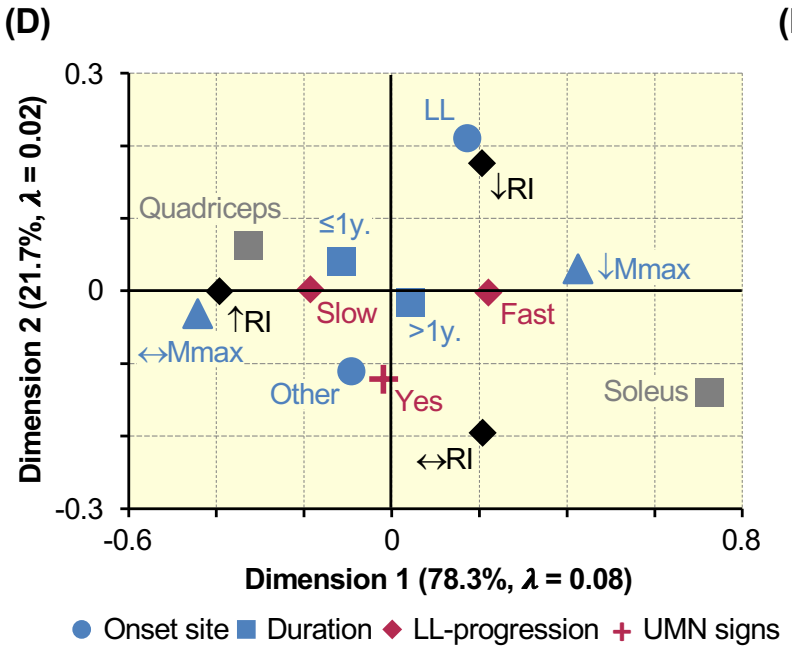

(A)
Mmax (mV)
16
14
12
10
8
6
4
2
0
*
Quadriceps
Soleus
(B)
H-test amplitude (% Mmax)
90
80
70
60
50
40
30
20
10
0
†
Controls
ALS
(C)
Inhibition (% mean test H-reflex)
100
80
60
40
20
0
-20
-40
-60
-80
*
15
20
25
PTN – FN interval (ms)
(D)
Inhibition least mean square (% mean test H-reflex)
35
30
25
20
15
10
5
0
Controls
ALS
(E)
Maximal inhibition (% mean test H-reflex)
100
80
60
40
20
0
-20
-40
*
Controls
ALS
Controls
ALS

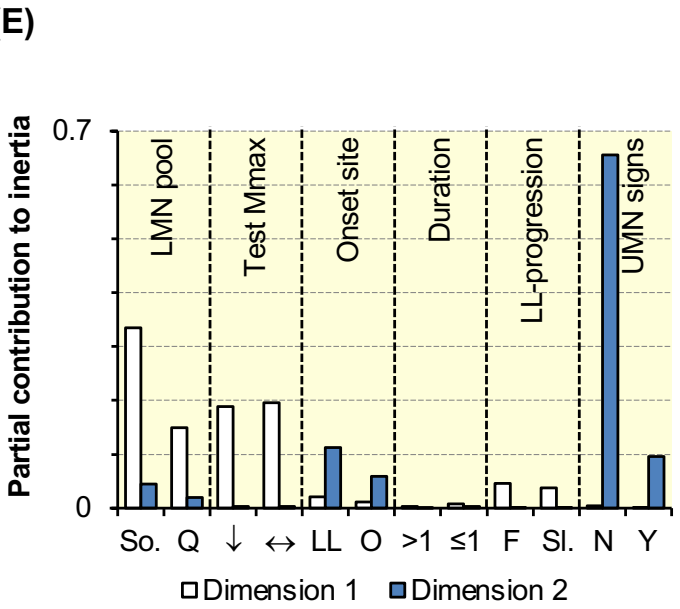

(A)
Ia
FN
PTN
Quadriceps -> Soleus
(B)
Mmax (mV)
Quadriceps
Soleus
(C)
H-test amplitude (% Mmax)
†
Quadriceps
Soleus
(D)
Inhibition (% mean test H-reflex)
‡
PTN – FN interval (ms)
(E)
Inhibition least mean square (% mean test H-reflex)
Controls
ALS
(F)
Inhibition (% mean test H-reflex)
Controls
ALS
(G)
Inhibition (% mean test H-reflex)
FN – PTN interval (ms)
(H)
Inhibition least mean square (% mean test H-reflex)
Controls
ALS
Controls
ALS
(I)
*
Inhibition (% mean test H-reflex)
Controls
ALS

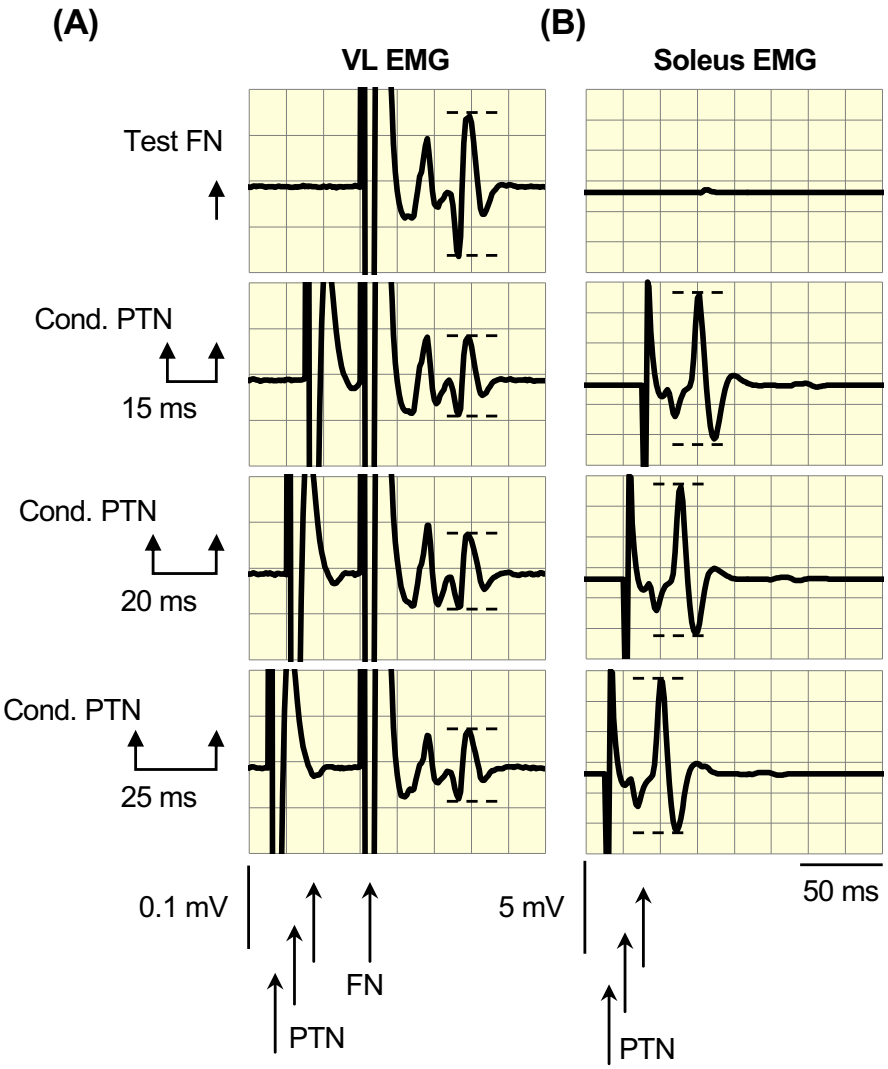

(A)
% of full group of participants
100
80
60
40
20
0
50.0 %
21.1 %
28.9 %
52.9 %
47.1 %
Quadriceps
Soleus
Below CI95
Within CI95
Above CI95

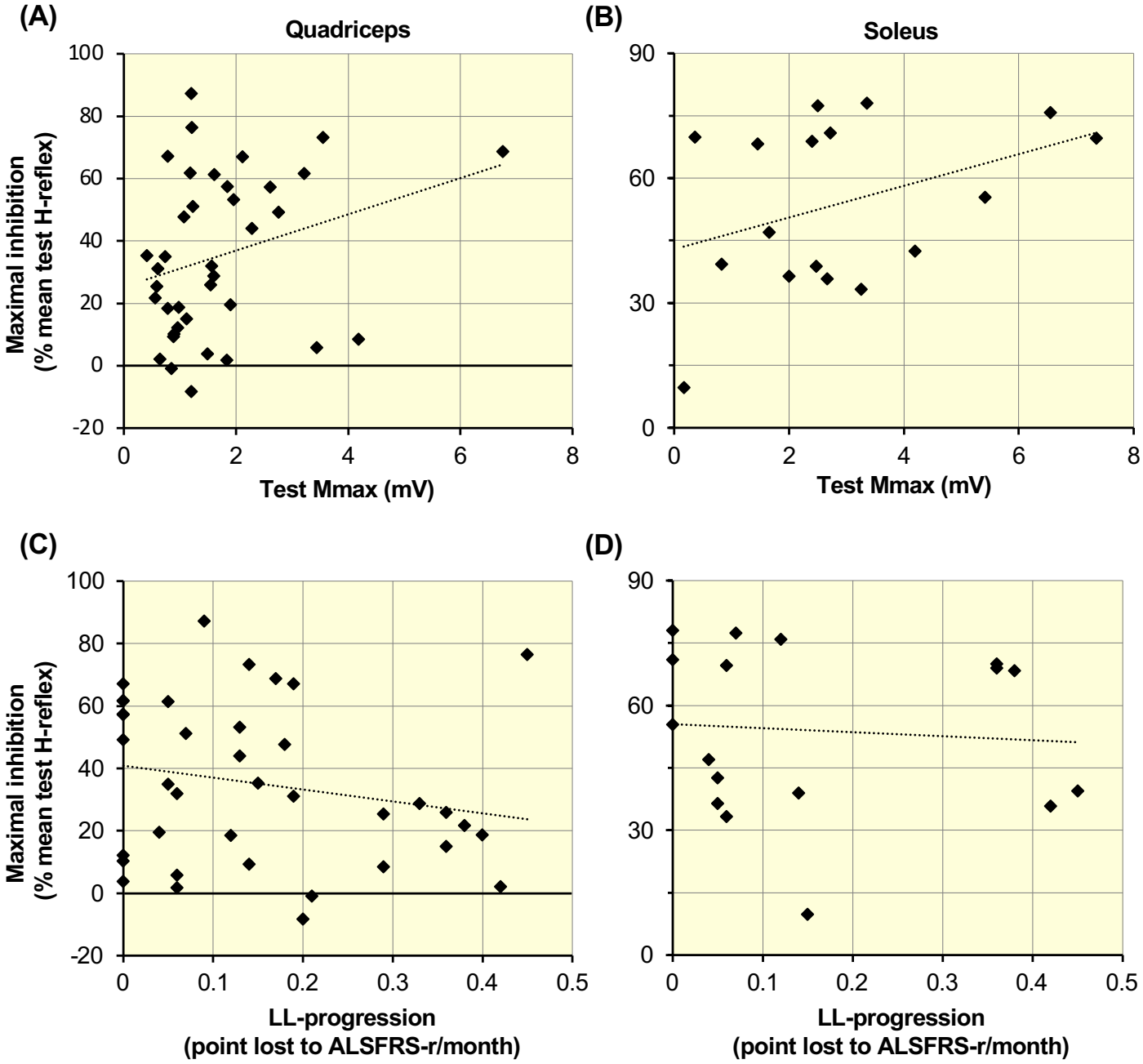

(B)
Dimension 2 (28.9%, λ = 0.06)
0.5
0
-0.5
-0.6
0
0.8
Fast
↓RI
Soleus
↔Mmax
↑RI
Quadriceps
↓Mmax
↔RI
Slow
Dimension 1 (71.1%, λ = 0.14)
RI modulation
LMN pool
Test Mmax
Progression


(C)
Partial contribution to inertia
0.6
0.3
0
Progression
LMN pool
Test Mmax
Soleus
Quad.
Slow
Fast
↓
↔
Dimension 1
Dimension 2


(D)
Dimension 2 (21.7%, λ = 0.02)
0.3
0
-0.3
-0.6
0
0.8
LL
↓RI
Quadriceps
≤1y.
↓Mmax
↑RI
Slow
>1y.
Fast
↔Mmax
Other
Yes
Soleus
↔RI
Dimension 1 (78.3%, λ = 0.08)
Onset site
Duration
LL-progression
UMN signs

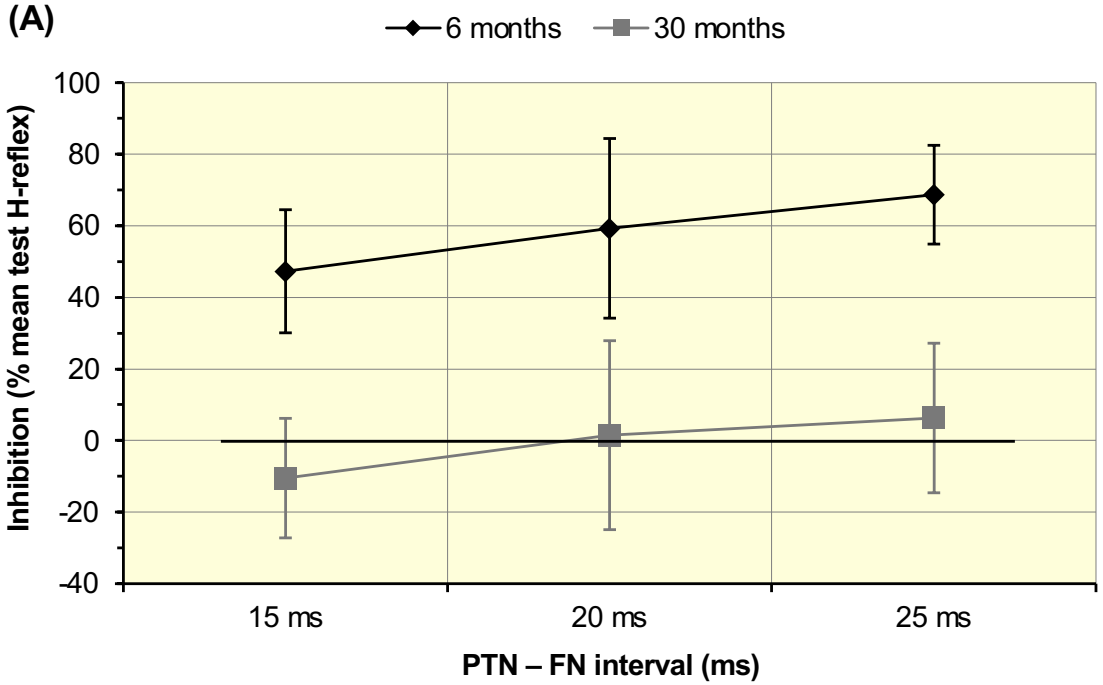

(E)
Partial contribution to inertia
0.7
0
LMN pool
Test Mmax
Onset site
Duration
LL-progression
UMN signs
So.
Q
↓
↔
LL
O
>1
≤1
F
Sl.
N
Y
Dimension 1
Dimension 2


(A)
(B)
VL EMG
Soleus EMG
Test FN
Cond. PTN
15 ms
Cond. PTN
20 ms
Cond. PTN
25 ms
0.1 mV
5 mV
50 ms
PTN
FN
PTN



Supplemental material 1. Maximum inhibition (% mean test H-reflex) is plotted against the test Mmax (mV; AB) or the progression of motor dysfunctions in lower limbs (LL; mean loss of points to ALSFRS-r/month; CD). AC show the results obtained in quadriceps (38 patients, test Mmax in VL EMG). BD show the results obtained in soleus (17 patients; test Mmax in soleus EMG). Each dot represents one patient. Interrupted lines represent the linear regression curves.

(A)



(B)

		6 months	30 months
	Mmax VL (mV)	0.68 ± 0.02	0.50 ± 0.03
	VL H-reflex (% Mmax)	11.49 ± 5.72	22.43 ± 5.35
	Mmax soleus (mV)	2.84 ± 0.14	0.08 ± 0.003
	ALSFRS-r (total)	47	40
	Sub-score ALSFRS-r	7	3
MRC	Quadriceps	5	5
	Soleus	5	5
	TA	5	1
	EHL	5	3

Supplemental material 2. A, Mean inhibition (% mean test H-reflex) plotted against the ISI between conditioned PTN stimuli and test FN stimuli (ms) in patient #13 investigated at 24-month interval: 6 months (black diamonds and line) and 30 months after the first symptoms (grey squares and line). Vertical bars are ± 1 SD. **B**, Table summarizing the following measures in patient #13, 6 (left column) and 30 (right column) months after the first symptoms (from 1st to 9th row): the mean amplitude (± 1 SD) of test Mmax in VL EMG (mV), of test H-reflex in VL EMG (% Mmax; ± 1 SD), of conditioning Mmax in soleus EMG (mV; ± 1 SD), and scores to ALSFRS-r (total), to the items for lower limb functions in ALSFRS-r (sub-score for lower limbs), to muscle testing (according to MRC scale) in quadriceps, soleus, tibialis anterior (TA) and extensor hallucis longus (EHL) muscles.