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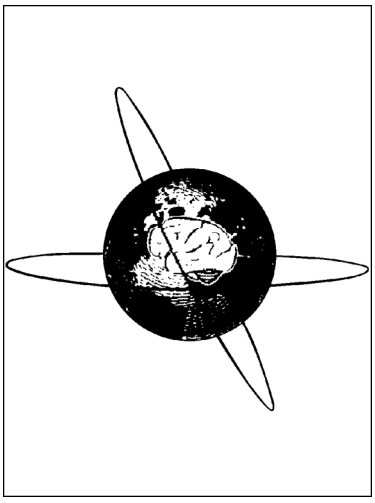

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Amyotrophic lateral sclerosis weakens spinal recurrent inhibition and post-activation depression

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Abstract

Objectives: Amyotrophic lateral sclerosis (ALS) disrupts motoneurons that control movement and some vital functions, however, exact details of the neuronal circuits involved in ALS have yet to be fully endorsed. To contribute to our understanding of the responsible neuronal circuits, we aimed to investigate the spinal recurrent inhibition (RI) and post-activation depression (P-AD) in ALS patients.

Methods: In two groups of ALS patients, i.e. lumbar-affected (clinical signs in leg muscles) and nonlumbar-affected (clinical signs in arms or bulbar region but not in the legs), RI and P-AD on the soleus muscle were investigated using single motor units and amplitude changes of H-reflex in surface electromyography, respectively. The data were compared with healthy subjects.

Results: Compared to controls, P-AD of H-reflex was reduced severely in lumbar-affected patients and reduced to a certain degree in nonlumbar-affected patients. Similarly, a significant reduction in the duration of RI on firing motoneurons was found in lumbar-affected patients (11.5 ± 2.6 ms) but not in nonlumbar-affected patients (29.7 ± 12.4 ms, $P<0.0001$) compared to controls (30.8 ± 7.2 ms, $P<0.0001$).

Conclusion: The current study revealed that spinal inhibitory circuits are impaired in ALS.

Significance: These findings may provide insight for proposing new therapeutic approaches and following disease progression in humans.

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Highlights

- Spinal recurrent inhibition of motoneurons is weakened in lumbar-affected ALS patients.
- Post-activation depression is ineffective to reduce spindle primary afferent input to motoneurons in ALS.
- Reduced inhibition in the spinal cord may provide valuable support for the excitotoxicity theory.

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Introduction

Motoneurons are the final common pathways to activate skeletal muscles. Malfunctions in motoneurons can lead to devastating consequences, including impairment of locomotion, speech, respiration, and death. One of the most studied motoneuron disorders is amyotrophic lateral sclerosis (ALS). Genetic studies investigating ALS improve our understanding of its pathophysiology. However, genetic mutations such as in superoxide dismutase-1 (SOD1), FUS/TLS and newly identified kinesin family member 5A (KIF5A) only account for about 10% of all ALS incidences (Rosen et al., 1993; Kwiatkowski et al., 2009; Vance et al., 2009; van Rheenen et al., 2016; Nicolas et al., 2018). The rest of the ALS prevalence has a sporadic background and displays varying clinical indications (Kiernan et al., 2011). Therefore, the exact pathophysiology of ALS has yet to be understood.

Some of the findings on its pathophysiology indicate that toxic gain of function of the SOD1 enzyme may eventually lead to damage in cellular structures and disrupts axonal transport (Bruijn et al., 1998; Vucic and Kiernan, 2009). Moreover, dysfunction of ionic homeostasis and mitochondrial abnormalities may also lead to cell death by disrupting intracellular ionic balance and cell structure (Wong et al., 1995; Sirabella et al., 2018; Tedeschi et al., 2019). Multicellular involvement of cellular damage, i.e. mutant damage in neighboring glial cells along with motoneurons, has also been proposed to lead to ALS (Taylor et al., 2016).

Glutamate-induced excitotoxicity may also kill neurons in the central nervous system where the glutamate is the main excitatory neurotransmitter. One of the mechanisms behind the excitotoxicity has been proposed to be an impairment in excitatory amino acid transporter of astrocytes. Malfunction in astrocytes may then lead to the accumulation of glutamate at synaptic cleft, resulting in persistent activation of the glutamate receptors (Schousboe and Waagepetersen, 2005; Selvaraj et al., 2018). Excessive activation of glutamate receptors leads to a rise in the intracellular calcium ions which then activate calcium-dependent enzymes that can eventually lead to apoptosis (Watkins and Evans, 1981; Heath and Shaw, 2002).

In addition to the impairment in glutamate uptake at synapses, malfunction in the inhibitory circuitries may also lead to a disruption of inhibition-excitation homeostasis on motoneurons (Brownstone and Lancelin, 2018). Fasciculation in muscles can be the earliest sign, preceding the

muscle weakness and atrophy in ALS. This activity may be due to the hyperexcitability of motoneurons due to impaired inhibition-excitation homeostasis (Kanai et al., 2012; de Carvalho et al., 2017; de Carvalho and Swash, 2017). Therefore, excitotoxicity due to disruption of the inhibitory neuronal circuits can cause an imbalance in inhibitory/excitatory inputs on motoneurons and may lead to ALS.

One of the spinal inhibitory interneurons, i.e., Renshaw cells, mediate spinal recurrent inhibition (RI) on motoneurons (Renshaw, 1941). It has been proposed in animal studies that the deficiencies in motor output to Renshaw cells and vice-versa are observed at the early stages of ALS (Chang and Martin, 2009; Wootz et al., 2013). Moreover, a reduction in the efficacy of RI in ALS patients has been shown in human subjects using paired H-reflex technique (Raynor and Shefner, 1994). Therefore, RI impairment could be considered to lead to excitotoxicity in motoneurons and eventually degeneration (Pasquali et al., 2009).

Another spinal circuit, post-activation depression (P-AD), is an effective presynaptic network that dampens the output of the primary afferents on motoneurons (Crone and Nielsen, 1989; Hultborn et al., 1996). It has been proposed that P-AD has been reduced in animals with ALS (Hedegaard et al., 2015). Like RI, reduction in P-AD may lead to excessive excitation of motoneurons, thus can disrupt the homeostasis.

Although some evidence has been proposed regarding the involvement of inhibitory circuits in ALS (Pasquali *et al.*, 2009; Wootz *et al.*, 2013; Hedegaard *et al.*, 2015), there is little information on spinal inhibitory networks which may disrupt the physiological equilibrium of motoneurons. In this study, we hypothesize that inhibitory RI and P-AD circuits are impaired in ALS patients. Using an optimized technique of stimulating the Renshaw cells to evoke RI on firing motoneurons (Özyurt et al., 2019) and utilizing an established method to elicit H-reflex, we aimed to investigate the involvement of RI and P-AD in ALS. Previously, the duration of the RI was shown to be directly related to the inhibitory conductance of a single Renshaw cell (Bhumbra et al., 2014). Therefore, changes in RI duration could provide substantial information about the inhibitory conductance, i.e. strength of inhibition, in ALS patients for the first time in the literature.

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Materials and methods

The Human Ethics Committee of Koç University approved the procedure following the Declaration of Helsinki. Before the experiments, subjects filled and signed informed consent forms. Control experiments were performed earlier and published before. For the RI experiments; 12 healthy control subjects (seven males and five females) (Özyurt *et al.*, 2019), and for P-AD experiments 11 control subjects (six males and five females) were recruited. On the other hand, there were two groups of ALS patients; i) symptoms only in the cervical and/or bulbar segments but no clinical signs in the lumbar segment-leg muscles (referred to as nonlumbar-affected patients) and ii) clinical signs observed in the leg muscles (referred to as lumbar-affected patients).

Participant selection

All patients were administered Riluzole at least one month preceding the experiments. Besides, Edaravone was started in one patient before the experiment. None of the patients were enrolled in interventional clinical trials.

UMN involvement was defined based on the findings of increased/hyperactive deep tendon reflexes (DTR) and/or pathological reflexes (i.e., in the upper extremity: palmomental and Hoffmann signs, and in the lower extremity: Babinski sign), in the neurological examination or abnormal findings in transcranial magnetic stimulation (TMS), i.e., absent or reduced motor evoked potential or prolonged central motor conduction time adjusted to the height of the subject (Groppa et al., 2012; Imajo et al., 2017). DTR was determined according to DTR scale (0: no response, 1+: sluggish, 2+: normal, 3+: slightly hyperactive/brisk, 4+: abnormally hyperactive with intermittent clonus) (Seidel et al., 2011).

Lower motoneuron involvement was defined based on the clinical (i.e., asymmetrical atrophy and weakness) and neurophysiological (i.e., reduced maximum M-response (Mmax) in nerve conduction studies, positive sharp waves, acute denervation or fasciculation indicating acute denervation accompanied by chronic neurogenic motor unit potentials in needle EMG studies) findings in the relevant extremity. One of the criteria, Mmax, was determined based on the reference values given by Leis and Schenk (2013). Symptom locations were determined based on these parameters and participants were grouped, i.e. lumbar-affected or nonlumbar affected. After the determination of the MRC scores for the soleus muscle (lower limb) and first dorsal

interosseous muscle (upper limb) (**Table 1**), we grouped the patients according to the scores. If there were no symptoms in leg muscles, then patients were included in the nonlumbar-affected group. If only one of the legs had symptoms, the patients were included in both groups (affected leg into the lumbar-affected group) as the experimental unit is the leg. However, if both legs were affected, the subjects were included in the lumbar-affected group. It is important to note that none of the nonlumbar-affected patients had acute denervation in the soleus muscle. It should be also noted that the reduction in Mmax was not used as the sole evidence of lower motoneuron involvement.

Table 1 shows patients sub-classified into possible, probable, or definite ALS based on Awaji criteria (de Carvalho et al., 2008) individually. Findings on UMN involvement accompanied by findings of denervation in lower motoneurons in one, two, or three segments out of four spinal segments (i.e., bulbar, cervical, thoracic, and lumbosacral) were defined as possible, probable, or definite ALS, respectively.

Some of the findings of control subjects for RI has been reported before (Özyurt *et al.*, 2019). Therefore, the control subjects were not age-matched with ALS patients. However, there are studies to claim that P-AD and RI values are similar in the young and the elderly (Chalmers and Knutzen, 2004; Trompetto et al., 2014). The exclusion criteria for control subjects were: (i) use of any medication, (ii) having any neuromuscular pathology including acute/chronic pain, and (iii) performed vigorous exercise in the last 24 hours.

Equipment

Software Spike2 version 7.20 was used to perform data acquisition and analyses. CED 1902 Quad System MKIII amplifier and CED 3601 Power 1401 MKII DAC (Cambridge Electronic Devices Ltd, Cambridge, U.K.) were used for recording and Digitimer DS7A (Digitimer Ltd, Hertfordshire, U.K.) was used for electrical stimulation. Custom made copper anode (10x12 cm) and the cathode (1×1 cm) were used to stimulate the tibial nerve through popliteal fossa. To record muscle activity, Ag/AgCl sticky electrodes (Redline, Istanbul, Turkey) for surface EMG (SEMG) and Teflon® insulated silver fine wires (75 μm core diameter and 3T, Leico, Medwire, New York, U.S.A.) for intramuscular EMG were used.

Preparation

Most of the subjects rested on a bed in the prone position. However, one ALS patient was assessed in the sitting position and another in the supine position due to tracheostomy. We excluded these 2 patients from P-AD experiments as posture may affect the H-reflex excitability (Chalmers and Knutzen, 2002; Cecen et al., 2018). Soleus muscles of both legs in ALS patients and only right leg of the control participants were prepared as follows; i) shaving the skin if necessary, ii) rubbing the skin with sandpaper to reduce impedance, iii) cleaning the skin with 70% alcohol and iv) application of conductance gel to further reduce impedance. Two surface EMG (SEMG) electrodes were placed on the lateral part of the soleus muscle 4 cm apart for better signal recognition (Tucker and Türker, 2005). Sterilized bipolar fine wires were inserted in between two sticky SEMG electrodes using 25 G surgical needle which was immediately withdrawn, leaving a pair of fish-hooked wires inside the soleus muscle (**Fig 1A**). Both SEMG and intramuscular electrodes were stabilized using surgical tape to minimize the movement artifact. After following the same preparation protocol, a sticky electrode was placed on the lateral malleolus to be used as a ground electrode and stabilized.

To stimulate the tibial nerve at the popliteal fossa, an anode was placed just above the patella while a cathode was located on the popliteal fossa crease per the recommendations of Özyurt et al. (2018). Specifically, the cathode was at the midpoint of the crease to evoke the H-reflex in the P-AD study and at a slightly lateral position from the midpoint to evoke M-only response in the RI study (**Fig 1A**). In both cases, square pulses with 1-ms width were delivered using the constant current stimulator.

Recording configuration

EMG was recorded with a sampling rate of 20,000 Hz for both surface and intramuscular EMG. The band-pass filter of 20-500 Hz and 200-5,000 Hz was used for SEMG and intramuscular EMG, respectively. The real-time stimulus-triggered averaging window of the SEMG during the experimental protocol was scrutinized to make sure that we induced H-reflex and M-response (whenever possible) that stayed similar during the experiment for P-AD and sole M-response without H-reflex for RI.

Experimental procedure

After the placement of recording and stimulating electrodes, we first determined the maximum isometric voluntary contraction (MVC) for soleus muscle by asking subjects to perform plantar flexion as strong as he/she could. MVC protocol was followed by finding Mmax and maximum H-reflex (Hmax) of the participants at rest. If we could reliably elicit M-only response, the subject was included for the RI experiments. However, if the H-reflex was observed, then the subject was included in the P-AD protocol. If by changing the position of the cathode we could elicit both M-only response and a clear H-reflex, the subject was included in both protocols.

We determined the stimulus intensity that induced half of the amplitude of the Hmax and used this level of stimulus intensity to evoke H-reflex (Hmax/2) in P-AD experiments. During these experiments, we also made sure that minimal M-response, which could be obtained in half of the experiments, stayed constant. It is useful to have a small but detectable M-response during H-reflex experiments as a point of reference to stimulus intensity. Although this is relatively easy to achieve for control subjects and even for some of the ALS patients, it was not always possible to obtain an M-response in some patients possibly due to a loss of larger motor units. We slightly increased the stimulus intensity until a small M-response was observed in half of the experiments without exceeding the Hmax/2 stimulus intensity level significantly. The average M-response obtained in these experiments was 4.4 ± 2.5 % of Mmax. For the rest of the experiments without M-response, we used the % coefficient of variation of the conditioning H-reflex (H_{FIRST}) within experiments to make sure that it stayed similar throughout the procedure.

Paired electrical stimuli with 1-sec interstimulus interval were delivered 5 times in the control subjects and 10-12 times in ALS patients. For both groups, inter-pair interval was 10 secs (**Fig. 1B**). This protocol was repeated for the other leg if it had a reliable H-reflex. Protocols for P-AD were always performed in the relaxed state of the muscle.

To study RI, we stimulated the largest diameter motor axons without evoking the H-reflex in the tibial nerve while recording from the smaller motor units. Stimulation of the motor axons antidromically activates motoneuron cell body and orthodromically activates Renshaw cells via axon collaterals which in turn inhibits firing motoneurons (Kudina and Pantseva, 1988; Özyurt *et al.*, 2019). There is also a chance that an F-wave may also activate Renshaw cells orthodromically. Online visual feedback of the action potentials of an easily recruited single motor unit (SMU) was

provided to subjects to ensure that they maintained regular firing of this low threshold SMU. As soon as SMU was maintained at a regular rate, 200 M-only stimuli with 1-2 secs of interstimulus interval were deliverd. This protocol was repeated for the other leg if we could elicit M-only response.

Analysis

The peak-to-peak amplitude of H-reflex in unrectified SEMG was recorded for P-AD experiments. Test H-reflex generated by the second stimulus of each stimulus pair (second H-reflex **Fig. 1B**) was normalized to H_{FIRST} which was not affected by any prior stimuli (second H-reflex divided by the H_{FIRST}), and the normalized values were averaged within each group, i.e. control, nonlumbar-affected or lumbar-affected. After determining the distribution of the data using the Shapiro-Wilk test, Kruskal-Wallis with Dunn's multiple comparison test was applied to determine the significance of the differences in H-reflex. To verify latency difference, we normalized the latency of H-reflex or RI to 180 cm height (actual latency*180/height of the subject). Ordinary one-way ANOVA with Tukey's multiple comparisons test was used to compare the normalized latency of H-reflex and RI between control, nonlumbar-affected, and lumbar-affected patients.

We have calculated variability between legs to test whether it is similar to the inter-subject variability. For that purpose, % coefficient of variation was calculated using 100*(standard deviation (SD)/mean) of normalized reduction in H-reflex for each subject (both legs pooled for each participant and presented as independent data points) and each leg (each leg was used as an independent data point) (6 subjects and 12 legs). We have then compared the variability across legs and inter-subjects using a parametric t-test. Similar variation analysis was also used to figure out the amount of change in M-response or H_{FIRST} during the P-AD procedure.

Changes in RI between groups were tested using SMU analysis. After SMU extraction, we performed peristimulus time histogram (PSTH) to determine the latency of inhibition and peristimulus frequencygram (PSF) (Turker and Cheng, 1994; Turker and Powers, 1999) to calculate the duration of the RI as proposed by Özyurt *et al.* (2019). PSTH calculates the stimulus-correlated changes in the firing "probability" of the SMUs while the PSF takes the instantaneous discharge rate of SMUs into account and determines the variation in discharge rate following stimulation (Turker and Powers, 2005) (**Fig. 1C**). We used a window of 600 ms (300 ms

prestimulus + 300 ms poststimulus) to construct PSTH and PSF in which binwidth was selected to be 0.1 ms.

(FIGURE 1)

To find out subtle but persistent changes in spike frequency and probability, we used the cumulative sum (CUSUM) of the analyzed data (Ellaway, 1978). To calculate CUSUM, prestimulus average value was subtracted from each bin value during the entire analysis period and the residual values were summed to obtain CUSUM. Any poststimulus deflection that was larger than the maximal prestimulus deflection (error box; Türker *et al*., 1997) was considered as a significant event (Turker and Powers, 2003). The latency of inhibition was the turning point at the beginning of the downward deflection whereas the duration was the horizontal distance between the latency and the next turning point. First using the Shapiro-Wilk test we found the distribution of the data. Then, we tested the differences in the background discharge rates, latencies and durations of RI, stimulus intensities, and Mmax between experimental groups using one-way ANOVA. The relationship between the duration of the RI and Mmax was tested using linear regression for all participants. For all statistical analyses, the level of significance was set to $P<0.05$, and GraphPad Prism v8 (San Diego, CA, USA) was used.

To determine the reflection of SMU action potentials on SEMG, we used the spike-triggered averaging method where SMU potentials were used as triggers and SEMG as the source. Then, we calculated the peak-to-peak amplitude of SMU reflection on the SEMG and divided it by the background EMG activity. This value is presented as a signal-to-noise ratio of spike-triggered average and compared using Kruskal-Wallis with Dunn's multiple comparison test between groups.

Results

Participant demographic information

In two of the ALS patients, UMN involvement was confirmed by TMS. Clinical deterioration in follow-up examinations (range 10–48 months) of the patients confirmed the progression of the disease. We repeated the experimental protocol in both legs of some of the patients while this was

only done in the right leg of the control subjects. We could not test both legs in all patients either because some patients could not activate single motor units due to severe motoneuron loss, had difficulty in cooperation, had no M-only response, or had no clear H-reflex. Four nonlumbar-affected male patients (number of legs: six) and seven lumbar-affected patients (seven males, number of legs: 10) participated in P-AD experiments. For RI, a total of 11 SMUs from seven males and 10 SMUs from six subjects (five males and one female) were recorded from nonlumbar and lumbar-affected ALS patients, respectively. Detailed demographic and clinical information about the patients and controls are presented in **Table 1**.

Table 1. Demographic and clinical information of the participants for both recurrent inhibition (RI) and post-activation depression (P-AD).

ID	Age (years)	Gender	Disease Duration (months)	Onset Region	Awaji Subgroup	Symptom Locations	FASC	ACT DNV	MRC Score			
									RA	LA	RL	LL
1¥	65	M	23	Bu	Definite	Bu	Bu, RA, RL	LA	5	5	5	5
2¥	55	M	10	Bu	Possible	Bu	Bu, LA, LL	RA, LL	5	5	5	5
3¥	59	M	16	LA	Possible	LA	LA	LA	5	4	5	5
*4**¥	47	M	10	LA	Definite	LA, RA, RL	LA, LL	Bu, LA, LL	4	4-	5-	5
5¥	55	M	19	Bu	Definite	Bu	Bu, LA, LL	LA, LL, RL	5	5	5	5
*6**¥	37	M	38	Bu	Probable	Bu, LA, RA, RL	Bu, LA, RA, LL, RL	-	3+	3	4	5
*7**¥	73	M	12	RL	Probable	RL	-	LA, RA, LL, RL	5	5	5-	5
*8**	43	M	72	RA	Probable	Bu, RA, LL, RL	LA, RA, LL, RL	RA, LL, RL	4	5	5-	5-
*9**	62	F	48	RA	Possible	LA, RA, LL, RL,	-	LA	3	4	4	4
*10**	53	M	13	RA	Probable	LA, RA, LL, RL	RA, LL	LA, LL, RL	3	4	4	4
*11**	63	M	32	RL	Probable	LL, RL	LL	LA, RA, LL, RL	5	5	4-	4
*12**	54	M	13	LL	Definite	LA, LL, RL	Bu, LA, RA, RL	LA, RA, LL, RL	4	4	4	4-
*13**	40	M	23	RA	Probable	LA, RA, LL, RL	LA, LL	LA, LL, RL	3	4	3	3
*14**	34	M	11	LL	Definite	LA, RA, LL, RL	Bu, LA, RA, LL	LA, RA, LL, RL	5-	5-	4	3
CTRL	26.0 ± 3.9	13 M 10 F										

Notes: *All subjects use Riluzole, participant number 11 also uses Edaravone. Only participant number 11 had SOD1 mutation, the rest of the subjects had no mutations. Average value: Mean ± Standard Deviation. CTRL: Control subjects, M: Male, F: Female, Bu: Bulbar, LA: Left arm, RA, Right arm, LL: Left leg, RL: Right leg, FASC: Fasciculation, ACT DNV: Acute denervation. Experimental groups were determined by the symptom locations which were determined by neurophysiological evaluations and MRC scores. Only the legs with an MRC score of 5 are included in the nonlumbar-affected group. The legs with a score of 5- (five minus) and below are included in the lumbar-affected group.*
Lumbar-affected
¥Nonlumbar-affected
**¥Both lumbar-affected and nonlumbar-affected*

Post-activation depression is reduced in both ALS groups

To make sure that the stimulus intensity remained similar during the experiment, the best way is to keep M-response similar in size. Since we could evoke measurable M-response only in half of the experiments without exceeding the Hmax/2 stimulus intensity, we investigated the variability of the conditioning stimulus intensity (H_{FIRST}). Coefficient of variation for H_{FIRST} was 10.8 ± 2.8 % (with and without simultaneous M-response), which was similar to the variability of the M-response (11.3 ± 6.7 %). Therefore, we decided to use the size of H_{FIRST} as an indicator of the stimulus intensity in those subjects where we could not elicit M-response. Paired primary afferent stimulation of all groups revealed significant reduction in the second H-reflex within a pair (**Fig. 2A**). Firstly, we calculated variance across legs and between subjects. Since the legs may be affected asymmetrically by ALS, we investigated whether the experimental unit can be legs rather than subjects. The percent coefficient of variation was similar when legs or subjects were used as independent data points (P=0.6974), allowing us to use each leg as an experimental unit (**Fig. 2B**). We have also calculated the latency of H-reflex between groups (**Fig. 2C**). Although there was no difference between patient groups (P=0.9696), both lumbar-affected (35.9 ± 2.4 ms, mean ± SD, P=0.0009) and nonlumbar-affected patients (35.7 ± 1.9 ms, P=0.0069) had longer latency compared to controls (32.3 ± 1.6 ms). This finding may indicate a sign of degeneration in large diameter axons involved in H-reflex, i.e. motoneurons and/or primary afferents.

The most inefficient P-AD was detected in the lumbar-affected group in which the second H-reflex was 79.6 ± 30.2% of the H_{FIRST}. That value was significantly larger than the values of both the control group (31.9 ± 18.7% of H_{FIRST}, P<0.0001) and nonlumbar-affected group (54.3 ± 35.1%

of H_{FIRST}, P<0.0001). Also, P-AD was significantly weaker in nonlumbar-affected patients compared to controls (P=0.0014) (**Fig. 2D**).

(FIGURE 2)

Recurrent inhibition is reduced in lumbar-affected but not in nonlumbar-affected ALS patients

The latency and duration of RI were calculated from PSTH-CUSUM and PSF-CUSUM, respectively (**Fig. 3**). After making sure that M-only response was elicited for each of the SMUs (**Fig. 3A-C, top left traces**), we delivered electrical stimuli to study the properties of the synaptic potentials of Renshaw cells on firing motoneurons. We first detected the latency of RI in PSTH-CUSUM (**Fig 3A-C, the first blue arrow at traces on the left**), and then we calculated the RI duration using PSF-CUSUM (**Fig. 3A-C, boxes at traces on the right**).

(FIGURE 3)

We compared the background discharge rates between experimental groups and found no difference (**Fig. 4A**), discharge rates were around 6.2 Hz (all: P>0.05). In addition, latency of RI (normalized to 180 cm of height) was similar in all groups (**Fig. 4B**), i.e., 39.2 ± 2.7 ms (control), 38.9 ± 5.3 ms (nonlumbar-affected), and 38.5 ± 4.7 ms (lumbar-affected) (all: P>0.05). Also, the average stimulus intensity of M-only stimulation was similar between each group (P>0.05 in all groups) (**Fig. 4C**).

A significant effect was observed in the duration. The duration of RI was dramatically reduced in lumbar-affected patients (11.5 ± 2.6 ms; **Fig. 4D**). This duration was significantly shorter compared to the duration of RI in nonlumbar-affected patients (29.7 ± 12.4 ms, P<0.0001) and control subjects (30.8 ± 7.2 ms, P<0.0001). Yet, the duration in control subjects was similar to the duration in nonlumbar-affected patients (P=0.9034). Moreover, the duration of the RI is correlated with the size of the Mmax (P=0.0208) (**Fig. 4E).** Lumbar-affected patients had significantly lower Mmax amplitude (4.9 ± 2.8 mV) than controls (13.1 ± 8.7 mV, P=0.0430) and nonlumbar affected patients (13.4 ± 4.6 mV, P=0.0113) whereas control and nonlumbar-affected patients had similar Mmax (P>0.9999) (**Fig. 4F**). The Mmax value can be affected by the skin resistance and the thickness of the fat tissue under the electrodes. However, these factors would be similar in all subjects as we use the same preparation procedure for all the subjects and most of the subjects had

a similar body-mass index. When the Mmax is large (more motor units surviving) the duration of inhibition is longer, i.e. less affected by the ALS. However, when the motor units die and hence Mmax becomes smaller, the duration of inhibition is shortened.

(FIGURE 4)

Single motor unit potentials of lumbar-affected ALS patients are well-reflected to the surface recording

In most of the lumbar-affected ALS patients, we detected a clear representation of SMU potentials on SEMG (**Fig. 5**) which could be due to the reduced number and/or reinnervated SMUs (**Fig. 5C**). However, in control subjects (**Fig. 5A**) and nonlumbar-affected patients (**Fig. 5B**), we could not detect clear SMUs on SEMG. Signal-to-noise value in lumbar-affected patients was significantly higher than both the controls ($P=0.0111$) and nonlumbar-affected patients ($P=0.0149$), whereas controls and nonlumbar-affected participants had similar signal-to-noise ratio ($P>0.9999$) (**Fig. 5D)**.

(FIGURE 5)

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The major findings of this study for each subject are presented in **Table 2**.

Table 2. The major findings for both recurrent inhibition (RI) and post-activation depression (P-AD) experiments.

		MRC Score					
ID	**Symptom Locations**	**RL**	**LL**	**P-AD (% of H_{FIRST})**	**RI Duration (ms)**	**Hmax (mV)**	**Mmax (mV)**
1 [¥]	Bu	5	5	L: 46.7	L: 49.2	L: 1.8	L: 7.7, R: 11.0
2 [¥]	Bu	5	5	L: 83.8	L: 23.2, R: 24.2	L: 6.0, R: 2.7	L: 10.8, R: 6.7
3 [¥]	LA	5	5	-	R: 30.0	-	L: 8.7, R: 11.8
4* [¥]	LA, RA, RL	5-	5	L: 56.2, R: 90.8	L: 12.7	L: 1.6, R: 2.0	L: 17.2, R: 13.8
5 [¥]	Bu	5	5	L: 23.5, R: 19.2	L: 28.0, R: 42.3	L: 2.6, R: 4.1	L:15.3, R: 21.0
6* [¥]	Bu, LA, RA, RL	4	5	-	L: 28.4, R: 9.2	-	L:16.6, R: 8.9
7* [¥]	RL	5-	5	-	L: 11	L: 0.7, R: 1.6	L: 7.3, R. 4.1
8*	Bu, RA, LL, RL	5-	5-	L: 104.9, R: 27.7	R: 6.4	L: 1.9, R: 2.3	L:9.9, R: 8.5
9*	LA, RA, LL, RL,	4	4	-	L: 8.4	L: 1.1, R: 3.1	R:5.9, L:5
10*	LA, RA, LL, RL	4	4	R: 50.9	R: 15.0	R: 3.5	R: 9.5
11*	LL, RL	4-	4	-	L: 12.3, R: 13.6	L: 2.0	L: 4.0, R: 2.7
12*	LA, LL, RL	4	4-	L: 85.2, R: 100.5	L: 12.5, R: 12.4	L: 1.6, R: 1.9	L: 3.6, R: 0.9
13*	LA, RA, LL, RL	3	3	L: 92.9, R: 62.5	-	L: 1.0, R: 1.1	L: 1.6, R: 5.8
14*	LA, RA, LL, RL	4	3	L: 81.5, R: 96.2	-	L: 8.4, R: 1.0	L: 12.1, R: 2.0
CTRL	-	-	-	31.9 ± 18.7	30.8 ± 7.2	4.5 ± 1.4[a]	13.11 ± 8.7[b]

***Notes**: Average values: Mean ± Standard Deviation, CTRL: Control subjects, Bu: Bulbar, LA: Left arm, RA: Right arm, LL: Left leg, RL: Right leg, L: Left, R: Right, P-AD: Post-activation depression, RI: Recurrent inhibition, Hmax: Maximum H-reflex, Mmax: Maximum M-response. Experimental groups were determined by the symptom locations which were determined by neurophysiological evaluations and MRC scores. All the values were obtained from the soleus muscle of the participants. The average value for RI has been given from the legs where more than one SMUs were recorded. Only the legs with an MRC score of 5 are included in the nonlumbar-affected group. The legs with a score of 5- (five minus) and below are included in the lumbar-affected group.*

***Lumbar-affected group**

[¥]*Nonlumbar-affected group*

[*¥]*Both lumbar-affected and nonlumbar-affected group*

[a]*Only P-AD subjects*

[b]*Only RI subjects*

Discussion

In this study, we have investigated P-AD using paired H-reflex method and RI using the M-only stimulation method in nonlumbar and lumbar-affected ALS patients and compared their results with the healthy individuals. Firstly, we found a significantly longer latency of the H-reflex and a substantial reduction in the efficiency of P-AD in both groups of ALS patients. Secondly, P-AD was even lower in lumbar-affected ALS patients compared to nonlumbar-affected. Thirdly, we showed a large reduction in RI duration only in the lumbar-affected ALS patients and this duration was correlated with the Mmax size. Lastly, we reported a clear reflection of the SMUs of lumbar-affected ALS patients in SEMG recordings. These results support our original hypothesis that clear disinhibition takes place in the spinal cord during motoneuron degeneration in ALS.

Post-activation depression and amyotrophic lateral sclerosis

H-reflex is often used as a probe to assess many spinal circuits. In this study, we evoked two consecutive H-reflexes (with 1 second of interval in between) where the first stimulus activated the P-AD mechanism, and the second stimulus induced an H-reflex whose amplitude was reduced by P-AD. The extent of this reduction can be claimed to be proportional to the strength of the P-AD.

Although there is no consensus about the mechanisms underlying P-AD, several hypotheses have been put forward to explain its background. It has been proposed that P-AD is a presynaptic phenomenon and can be due to neurotransmitter depletion at the Ia-motoneuron synapse (Hultborn *et al.*, 1996) or increased presynaptic inhibition (Crone and Nielsen, 1989). Also, Ishikawa et al. (1966) proposed that the underlying mechanism behind P-AD could be RI. However, the duration of RI was too short to inhibit H-reflex for up to 8 secs (Hultborn *et al.*, 1996; Özyurt *et al.*, 2019). Despite the lack of consensus about its mechanism, P-AD has been shown to be affected by various neurological disorders along with this study.

It has been claimed that during the development of spasticity, new primary afferent synapses develop on motoneurons and these synapses lack presynaptic connections responsible for presynaptic inhibition (Calancie et al., 1993). Also, reduction in P-AD is highly correlated with the severity of the spasticity and the interactions between P-AD and presynaptic inhibition has been proposed (Davies et al., 1985; Lamy et al., 2009; Achache et al., 2010; Yang et al., 2015).

Therefore, reduction of P-AD in ALS patients could be due to a similar change in presynaptic inhibition as in the development of spasticity (lack of P-AD on newly developed Ia terminals) and/or reduced inhibition of Ia terminals in the existing pool (Fig 6).

It has also been reported previously that UMN degeneration could lead to spasticity similar to primary lateral sclerosis (Pringle et al., 1992). In the present study, although all ALS patients had UMN degeneration signs, a reduction in P-AD was observed mostly in lumbar-affected patients. We propose that the difference in P-AD between nonlumbar and lumbar-affected ALS patients could be due to damage either in the lumbar spinal networks responsible for P-AD and/or loss of soleus motoneurons and/or their primary afferents. Moreover, for those with only UMN signs (i.e. nonlumbar-affected patients), both local networks and spinal motoneurons may be unaffected, so that descending influences may also play a role. Along these lines, it has been suggested that P-AD may be affected by a reduction in the cortical input to motoneurons (Andrews et al., 2015).

In an animal study, the reduction of P-AD in the pre-symptomatic stage of ALS was previously reported (Hedegaard *et al.*, 2015). At this stage, some motoneurons would also be degenerated, specifically larger motoneurons, as they are prone to excitotoxicity due to relatively lower calcium buffering capacity than the smaller motoneurons (Palecek et al., 1999).

Loss of motoneurons and primary afferents are expected to affect the amplitude and latency of the H-reflex. Therefore, to determine the axonal degeneration, we checked the latency between the two groups of ALS patients and compared the values with the findings of the control group. We found longer reflex latency in both patient groups suggesting a malfunction in the conduction of primary afferents and/or motor axons. However, since the latency of RI is similar in all groups and the only difference between these two circuits is the primary afferents along with Renshaw cells, the most likely explanation for increased H-reflex latency could be degeneration of the large diameter primary afferents.

Duration of the recurrent inhibition in amyotrophic lateral sclerosis

Previous animal studies revealed that Renshaw cell functions are impaired in the early stages of ALS (Wootz *et al.*, 2013). Besides, a reduction in Renshaw cell functions was reported in ALS patients using paired H-reflex methodology (Mazzocchio and Rossi, 2010). In this study, we investigated the temporal properties of RI in ALS patients to identify the level of impairment using

antidromic stimulation of the Renshaw cells, in turn, recording inhibition that they induced on firing motoneurons. Duration of RI, a key parameter of the current study, has been proposed to be directly related to the inhibitory conductance of the Renshaw cells (Bhumbra *et al.*, 2014). Our results revealed a significant reduction in RI duration only in lumbar-affected ALS patients. It has been suggested that the loss of larger motoneurons during the development of ALS may lead to damage in Renshaw cell function as these cells are tightly coupled (Friedman et al., 1981). If this is the case, we would expect normal RI duration in nonlumbar-affected patients, as we found in the current study. This finding may provide an insight about the role of Renshaw cells in ALS.

Another possibility would be the impairment in Renshaw cells or their outputs to motoneurons before the degeneration of motoneurons (**Fig. 6**). In that case, we would expect a decrease in the duration of RI even before the degeneration of motoneurons. Some of the nonlumbar-affected patients exhibited a shorter duration, around 11 ms, which was similar to the results of the lumbar-affected patients. This could be an early sign of Renshaw cell malfunction without (or minimal) motoneuron loss as we have never recorded such short durations in the control subjects. This hypothesis is in line with the fasciculations observed in ALS patients which could be due to reduced inhibition on motoneurons even before the loss of motor function. However, not all nonlumbar-affected patients had lower durations of RI, therefore, the current study cannot provide evidence whether the impairment in ALS occurs first in motoneurons or Renshaw cells.

Abnormal chloride homeostasis may also lead to reduced RI in ALS. KCC2 co-transporter plays an important role to maintain low intracellular chloride concentration (Payne et al., 1996; Uvarov et al., 2005). Therefore, any malfunction in KCC2 co-transporter on motoneurons may result in higher intracellular chloride concentration. Higher intracellular chloride concentration, in turn, decreases the driving force of chloride into the cell, hence can reduce the effect of RI.

(FIGURE 6)

Single unit reflection to surface recordings

SEMG recordings did not show any clear surface reflection of SMU potentials in control and nonlumbar-affected patients. Yet, in lumbar-affected patients, there was a clear reflection of the SMU potentials on SEMG. This could be explained by either increased synchronization between motor units or increased size of SMU potentials due to reinnervation. During muscle fatigue, large

reflections of SMU potentials can be seen on SEMG due possibly to the synchronous discharge of motor units (Yao et al., 2000). However, this reflection was observed even at the beginning of the experiment, therefore, fatigue might not be the main reason behind the SMU reflection in lumbar-affected patients.

Reinnervation of motor units can compensate the pool for as much as 50% of motor loss (Emeryk-Szajewska et al., 1997; Henderson and McCombe, 2017). This happens due to reinnervation of neighboring skeletal muscle fibers by surviving motoneurons which in turn results in increased motor unit potential size (Krarup, 2011; Henderson and McCombe, 2017) that reflect directly on SEMG. Related to this issue, another possibility is the reduced number of motor units that affect background EMG activity. Due to a lower number of motor units in the motor pool of lumbar-affected patients, background SEMG activity would be relatively lower and therefore SMU potentials could easily be exposed on SEMG amongst only a few active motor units.

Direct motor axon stimulation in patients with motoneuron loss: a technical remark

Although we could evoke M-only response easily in most of the control participants, evoking M-only response in patients, especially in lumbar-affected ALS patients, was comparatively challenging. Due to UMN degeneration in ALS patients which may result in spasticity, low-intensity stimulation of the tibial nerve generates H-reflex, especially when there is voluntary contraction. This may be one of the reasons why we had difficulties evoking M-only response in some of the ALS patients.

Another difficulty in recording M-only response in patients could be the selective degeneration of the motoneurons. It has been reported that larger motoneurons are degenerated first in the pre-symptomatic stage of ALS while the smaller motoneurons are more resistant to degeneration until the terminal stage (Frey et al., 2000; Pun et al., 2006; Nijssen et al., 2017; Brownstone and Lancelin, 2018). Many of the lumbar-affected subjects in this study had severe motoneuron loss determined by clinical observations and electrodiagnostic evaluations. To evoke a low amplitude M-only response, we had to deliver relatively strong electrical stimuli, which in turn, resulted in more primary afferents to be activated resulting in no M-only response in some ALS patients. Therefore, caution should be taken while studying RI in ALS patients using the M-only technique.

Limitations of the study

Some of the limitations of the study include the number of stimuli delivered in the experiments on ALS patients and the age of controls. Due possibly to the degeneration of the larger diameter motor axons, our technique for stimulating those motor axons and recording from the lowest threshold motor units was challenging in the ALS patients.

Another limitation was that the controls and patients were not age-matched. Studies investigating the effect of age on P-AD in humans were limited. However, when the stimulus rate was 1 Hz, similar to our protocol, a recent study showed similar P-AD values in the upper extremities of young (around 30 years of age) and the elderly (around 70 years of age) (Trompetto *et al.*, 2014). For the aspect of RI, even though our control subjects were remarkably younger than ALS patients (both groups), the nonlumbar-affected patients had similar RI durations to controls. Moreover, Chalmers and Knutzen (2004) investigated RI in soleus muscle using paired H-reflex technique in the elderly and young adults and found no significant difference. Both findings may rule out the possible effect of age on the reduced effectiveness of the circuitries in the patients.

Another limitation was the evaluation of UMN degeneration. In this study, we used clinical examination to determine UMN involvement in most of the patients, but a few supported with the TMS. Yet, to understand the impact of UMN involvement on spinal circuits, it would be better to quantify UMN degeneration using TMS in all the subjects rather than by only clinical examination.

Conclusion

This study revealed the significant involvement of spinal networks in ALS. We discovered a large reduction of P-AD as well as inefficient RI in lumbar-affected ALS patients. These results support the insight about the excitotoxicity hypothesis due to the disruption of neuronal inhibition/excitation homeostasis. Current findings suggest that both techniques may be used to assess the disease state, prognosis, and even to follow treatment progress. Although RI testing requires a relatively challenging protocol, P-AD testing is easy to use. Furthermore, other novel approaches may be developed as a result of the current findings to test the efficiency of the spinal inhibitory networks to fully understand the underlying reasons for motoneuron degeneration.

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Competing interests

Authors declare that they have no conflict of interest to disclosure.

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Figure Legends

Figure 1. **The experimental protocol for post-activation depression (P-AD) and recurrent inhibition (RI)**. A) Placement of stimulating and recording electrodes. B) Sample H-reflex-evoking stimuli to test the P-AD. C) Superimposed instantaneous firing frequency of single motor units (SMUs) to develop prestimulus frequencygram (PSF) (upper trace), their waveforms in intramuscular electromyography (EMG) channel (middle trace) to produce peristimulus time histogram (PSTH) and surface EMG (SEMG) (lower trace). M-only response in SEMG is indicated and the duration of RI is shown.

Figure 2. Post-activation depression (P-AD) in all experimental groups. A) Sample recordings of paired stimuli with 1-sec of the interstimulus interval from the control subjects and nonlumbar-affected and lumbar-affected patients. B) The percent coefficient of variation to investigate the experimental unit for patients we could evoke reliable H-reflex from both legs (N=6 for subjects, N=12 for legs). C) Comparison of the normalized latency of H-reflex between amyotrophic lateral sclerosis (ALS) patient groups and control participants. D) Change in H-reflex due to P-AD in all experimental groups. Number of trials: control = 55 (N = 11), nonlumbar-affected = 58 (N = 6) and lumbar-affected = 92 (N = 10). In all graphs, error bars are standard deviation. $^{ns}P>0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

Figure 3. The latency and duration characteristics of recurrent inhibition (RI). Figures on the left are surface electromyography (SEMG) to show direct motor response only (M-only) (top-left) and peristimulus time histogram (PSTH) with its cumulative sum (CUSUM-black line) where blue arrows indicate the onset and end of RI. Figures on the right show the peristimulus frequencygram (PSF) with its CUSUM (black line) in which boxes are the duration of RI. The yellow horizontal dashed line is the average background discharge rate. Each trace indicates a sample from an experimental group, specifically A) control, B) nonlumbar-affected and C) lumbar-affected.

Figure 4. Motor unit and recurrent inhibition (RI) characteristics of experimental groups. A) The background discharge rate, B) the normalized latency of RI to 180 cm height calculated using the cumulative sum of the peristimulus time histogram (PSTH-CUSUM), C) the stimulus intensity as % of maximum M-response (Mmax) and D) duration of RI determined using the cumulative sum of the peristimulus frequencygram (PSF-CUSUM). The number of single motor units (SMUs) for control subjects: 54 SMUs, nonlumbar-affected: 11 SMUs; and lumbar-affected: 10 SMUs. E) Linear regression between RI and Mmax. For the legs we recorded more than one SMU, we averaged their RI duration to obtain one value for that particular Mmax. F) Comparison of the Mmax values between groups. $^{ns}P>0.05$, *P<0.05, ****P<0.0001.

Figure 5. Reflections of single motor unit (SMU) potentials on surface electromyography (SEMG). Sample 1-sec of the recording of surface and intramuscular EMG shows the slight reflection of SMUs in SEMG recordings in A) control subjects and B) nonlumbar-affected patients but highly represented in C) lumbar-affected patients. Figures on the right are the surface reflection of SMU via spike-triggered averaging and signal-to-noise ratios. D) Signal-to-noise values for single units from each experimental group. $^{ns}P>0.05$, *P<0.05.

Figure 6. **Spinal networks and their involvement in amyotrophic lateral sclerosis (ALS)**. Upper drawing shows the recurrent inhibition (RI) and presynaptic mechanisms of post-activation depression (P-AD) and/or presynaptic inhibition in control subjects while lower figure indicates the proposed changes in the ALS patients (especially lumbar-affected patients) due to reduced inhibition/increased excitation, in light of the current findings.

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