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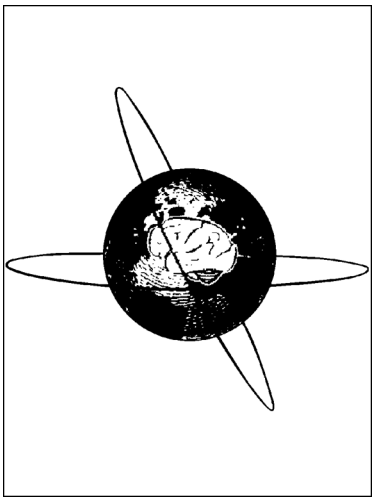

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Single motor unit estimation of the cutaneous silent period in ALS

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Highlights

- Cutaneous silent period (CSP) latency of single motor units is prolonged in severely affected ALS patients, whereas the duration of CSP remained similar to healthy controls.
- Discharge rate estimation revealed longer CSP duration compared to probabilistic methods.
- Severely and mildly affected ALS patients lose motor unit discharge rate vs. CSP duration inverse correlation.

Abstract

Objective: Recent evidence indicated that amyotrophic lateral sclerosis (ALS) also impairs spinal circuits, including those mediating cutaneous silent period (CSP). However, most studies utilised surface electromyography (sEMG), which needs more resolution to pinpoint changes at the single motoneuron level. We aimed to investigate CSP properties using single motor unit discharges in ALS.

Methods: In mild and severe ALS patients and controls, CSP was recorded in the first dorsal interosseus and analysed using the discharge rate method, which accurately shows the inhibitory postsynaptic potentials (IPSPs) profile.

Results: Our findings confirmed that the CSP latency was prolonged only in severe ALS patients. Moreover, the CSP duration was similar in each group, but late-stage ALS patients tend to have a longer CSP duration. The discharge rate method revealed a significantly longer duration (up to 150 ms) than the duration detected using sEMG. Strikingly, the motoneuron discharge rate - IPSP duration inverse relationship is lost in ALS patients, indicating a possible impairment in the motoneuron integrative properties.

Conclusions: Our data support previous findings of prolonged latency, presented input-output modifications of motoneurons, and revealed the entire course of the CSP, representing a much stronger inhibitory event than previously thought.

Significance: Motoneuron integrative property modification assessed by CSP could be a new biomarker for ALS.

Keywords: Amyotrophic Lateral Sclerosis, cutaneous silent period, single motor units, electromyography.

1. Introduction

Despite being a motoneuron disorder, amyotrophic lateral sclerosis (ALS) also affects other neurons and causes impairment in the functional balance between excitatory and inhibitory systems. Increased excitation or reduced inhibition could lead to overexcitation of the neuronal circuit, finally causing the death of the neuron (Remirez-Jarquin et al., 2013). For example, in one of the most studied spinal circuitries, recurrent inhibition by Renshaw interneurons affects the excitability of the motoneurons due to their functional positioning. Our previous study demonstrated that recurrent circuit is impaired in ALS (Özyurt et al., 2020). Frequency-based analysis showed that the Renshaw inhibition of soleus motoneurons was significantly weakened in ALS patients who show symptoms in lower limbs but not in patients without any symptoms in the legs. Similarly, post-activation depression, which reduces monosynaptic reflex transmission, decreased in symptomatic ALS patient groups (Özyurt et al., 2020), indicating increased net excitation acting on motoneurons.

Another valuable tool to investigate the excitability of the motoneurons is the cutaneous silent period (CSP). High-intensity electrical stimulation of the distal parts of the upper and lower limb generates a silence of the ongoing electromyography (EMG) activity in some muscles of the same or neighbouring dermatomes. (Kranz et al., 1973; Shahani and Young, 1973). CSPs occur with near-noxious stimulation and are thought to be mediated by A-delta fibres (Inghilleri et al., 1997; Uncini et al., 1991). Previous studies demonstrated that CSP latency was significantly prolonged in hand muscles in ALS and in some other motoneuron diseases compared to healthy control subjects; however, CSP duration did not differ between groups (Gilio et al.,2008; Cengiz et al., 2018; Castro et al., 2021) except for one study which showed a prolonged CSP duration and unchanged latency in ALS patients (Gutierrez et al., 2020).

To our knowledge, all the studies to date investigated CSP in patients using the surface EMG (sEMG) method, which is effective at investigating the global motoneuron populations but lacks the resolution to detect modifications at different motoneurons. Therefore, we aimed to investigate CSP characteristics in ALS patients using single motor unit (SMU) recordings. SMU analysis highlights significant events with minimal cross-talk from surrounding muscles and allows us to observe possible differential effects of the stimulus on individual motoneurons. To analyse the responses of SMUs to the stimulus, we used a probability-based method, peristimulus time histogram (PSTH), and a discharge rate-based method, peristimulus frequencygram (PSF), for obtaining CSP latency and duration, respectively, from the first dorsal interosseous (FDI) muscle. The rationale for using two different methods to analyse the SMU responses to the stimulus is as follows: The PSTH is similar to the sEMG in reflecting the stimulus-induced firing probability of underlying motor units. These methods have been claimed to contain significant errors in estimating underlying synaptic potentials (Türker and Cheng, 1994; Türker and Powers, 1999 and 2003). Using regularly discharging motoneurons in rat brain slices, these authors have shown that these errors can be minimised if the instantaneous discharge rate of single motor units (PSF) is used to estimate underlying synaptic potentials. Therefore, as well as the classical methods, we used the PSF method, which has been shown to mirror the stimulus-correlated discharge alterations at the single motoneuron level, reflecting the synaptic potential developed on the motoneuron (Türker and Powers, 2005).

2. Methods

Experiments were performed at Marmara University Pendik Training and Research Hospital and Koç University Neurophysiology Laboratory. Koç University Human Ethics Committee approved the experimental procedure (2017.124.IRB2.038), which conformed with the

Declaration of Helsinki. Subjects read the information form and gave their written consent before experiments.

2.1 Participants

Thirty subjects participated in the study. Twenty-one were patients (thirteen males, eight females aged 38-69 years), and nine were healthy controls (five males, four females, aged 28-53 years). ALS patients had no known diseases that can affect peripheral nerves (diabetes, kidney failure, thyroid gland disorders, alcohol abuse, glucose intolerance, ulnar entrapment, C8-T1 level radiculopathy, or vitamin deficiencies). Except for five new patients, all patients were admitted to riluzole treatment (16 patients). ALS patients were classified as possible, probable, and definite with Awaji criteria (de Carvalho et al., 2008). The onset region of the disease, progression regions (upper and lower motoneuron involvement), disease duration, fasciculations, acute/chronic denervation, ALS Functional Rating Scale (ALFRS), and ALS Functional Rating Scale-Revised (ALFRS-R) (Cedarbaum et al., 1999) were recorded. The control group had no diseases affecting the peripheral nerves or neurodegenerative disorders. The patients were selected based on their hand conditions: mildly and severely affected by the disease; details of the clinical profiles of the patients are provided in Table 1. The severely affected patient group had an onset of disease and showed apparent symptoms at the cervical level and other possible levels. In contrast, the mildly affected patient group had no visible symptoms at the cervical level but showed denervation at the lumbosacral cord (Figure 1A).

2.2 Surface electromyography recording – direct motor response, motor unit number index and cutaneous silent period

Compound muscle action potential (CMAP – direct motor response, M-response) amplitude and area, motor unit number (MUNIX), motor unit size index (MUSIX) and CSP were calculated using sEMG (Nicolet, Natus Neurology, US) through a pair of self-adhesive (Ag/AgCl) electrodes which were placed on the belly and tendon (Figure 1B). A ground electrode was also placed between the recording and stimulation electrodes. Subjects were seated upright on a comfortable chair with their elbows fixed during the experiments.

A bar stimulator was used to stimulate the ulnar nerve (0.5 ms pulse duration) to evoke CMAP and calculate MUNIX. Amplitudes higher than one mV were used to calculate the CMAP area and MUNIX. The negative phase of the potential of CMAP amplitude and area was used for calculations (Güneş et al., 2021). The subjects performed voluntary isometric contractions at 10%, 25%, 50%, 75%, and maximum, guided by audio feedback. Isometric voluntary contractions were performed by pinching movement (index finger against the thumb). sEMG signals were rectified and smoothed (0.2 s). Subjects were asked to contract until around the required level (±5% of the given levels), and when this level was reached, audio feedback was provided to keep contraction as long as this indicator sound was present. Ten sEMG interference patterns (SIP) were recorded, i.e., two repeats per contraction level, giving 30 s of rest between each bout to obtain SIP. SIP area of >20 mV/ms, ideal case MU count (ICMUC) of <100, and SIP area/CMAP area of >1 were accepted (Güneş et al., 2021).

2.3 Single motor unit recording – cutaneous silent period

Spike 2 software and CED hardware were used for data acquisition and analysis of CSP (CED 1902 Quad System MKIII amplifier and CED 3601 Power 1401 MKII DAC; Cambridge Electronic Design). A constant current stimulator (model DS7A; Digitimer Ltd, UK) was used for electrical stimulation.

SMU activity was recorded via two silver fine-wire electrodes coated with Teflon (75 μm in core diameter; Ag3T, Leico Industries, USA). These two wires were placed into a 25 G surgical needle and sterilised. The needle was inserted into the FDI muscle (between two sEMG electrodes), followed by an immediate withdrawal, leaving "fish-hooked" electrodes inside the muscle. A bandpass filter of 200-5,000 Hz and a 20,000 Hz sampling rate was used in intramuscular recordings.

ID	Age (Years)	Gender	Disease Duration (months)	ALFRS inc	Awaji	Onset Region	Spread in Follow up	Acute Den.	Chronic Den.	Drug Treatment
1	46	F	4	42	Pb	LS	C, LS	C,T,LS	B,C,LS	Y
2	44	M	5	42	D	B	B, C, LS	B,C,LS	B,C,LS	Y
3	51	F	72	36	D	LS	B,C,LS	C,LS	B,C,LS	Y
4	64	F	25	35	Pb	C	C,LS	C,LS	C,LS	Y
5	64	F	48	30	D	C	B,C,LS	B,C,LS	B,C,LS	Y
6	38	M	68	25	D	B	B,C,LS	B,C,LS	B,C, LS	Y
7	69	M	8	43	Pb	C	B,C,LS	C,LS	B,C,LS	Y
8	46	M	2	42	Po	C	B,C	C	C	Y
9	45	M	18	21	Pb	C	B,C,LS	C, LS	C, LS	N
10	60	F	24	33	Po	LS	LS	LS	LS	Y
11	54	M	12	45	Po	B	B,C,LS	B,C,LS	B	N
12	58	F	6	46	Pb	LS	LS	LS	LS	N
13	54	M	8	42	D	B	B,C,LS	B,C,LS	B,C,LS	Y
14	60	F	9	40	D	B	B,C,LS	B,C,LS	B,C,LS	Y
15	43	M	6	43	Po	LS	B,LS	C,LS	LS	N
16	56	M	18	34	D	B	B,C,LS	B, C, LS	B,C,LS	N

17	74	F	15	43	Po	C	B,C	C	C	Y
18	56	M	23	43	Po	C	B,C	B,C	B,C	Y
19	56	M	24	28	Pb	C	C,LS	C,LS	B, C, LS	Y
20	68	M	12	43	D	B	B,C,LS	B,C,LS	B,C,LS	Y
21	63	M	48	44	D	LS	C,LS	C,LS	LS	Y

Table 1*. Clinical and Demographic Information of Patients: Notes: M: Male, F: Female, Pb: Probable, D: Definite, Po: Possible, Bu: Bulbar, C: Cervical, LS: Lumbosacral, T: Thoracal. Sixteen subjects use Riluzole as a drug treatment.*

Subjects were asked to contract their hands slightly by pinching to recruit their motor units during the experiment. Participants were given visual feedback from the screen to activate a detectable SMU steadily. More than one motor unit activity was accepted if it was separable via SMU analysis (Figure 1B – right side). During this brief FDI contraction, electrical stimuli were delivered from the participants' fifth digit via two ring-shaped adjustable electrodes placed approximately 2 cm apart. The fifth digit was chosen for stimulation since it is located at the C8 dermatome, including cutaneous innervations that supply the FDI muscle (Figure 1B – left side). We have recorded FDI muscle bilaterally in patients and only one hand in control subjects. The intensity of the electrical stimulus was gradually increased until the subjects first perceived the stimulus, "Sensory Threshold (ST)," and 10 x ST stimulus intensity was used as a working intensity to induce CSP. Approximately 250 stimuli per SMU were delivered randomly at 1-2 seconds intervals, and a pulse width of 0.2 ms (Corsi et al., 2002; Kofler et al., 2019a; Shefner and Logigian, 1993), and the sEMG signals were obtained in parallel with SMU recordings. The stimuli were sufficient for all subjects to analyse CSP by PSTH and PSF methods.

2.4 Analysis

Spike 2 software has been used to extract the single motor units (SMUs) based on their shapes using a template-matching algorithm. In total, 40 SMUs from 21 ALS patients (21 SMUs from mild, 19 SMUs from severe patients) and 18 SMUs from nine control subjects were recorded. Rectified averaged sEMG, PSTH, and PSF were constructed to calculate CSP latency and duration. PSTH is a probability-based method that shows the timing of motoneuron discharges before and after the electrical stimulus. Thus, it gives us the stimulus-correlated firing probability of the units around the timing of the stimulus. In PSTH analysis, peaks and troughs are interpreted as excitation or inhibition.

On the other hand, PSF is a discharge-based method that gives the units' discharge rate superimposed around stimuli. In PSF analysis, increases and decreases in SMU discharge rate are evaluated as excitatory and inhibitory events, respectively. Six hundred msec (300 msec prestimulus and 300 msec poststimulus) window was used in PSTH and PSF using a bin width of 0.1 ms. sEMG signals were rectified and averaged to calculate the CSP's latency and duration.

The cumulative sum (CUSUM) method was used in EMG records (for all three analysis methods) to reveal subtle but persistent changes (Ellaway, 1978) and used to indicate significant diversions from the prestimulus baseline (error box approach of Türker et al., 1997; Brinkworth and Türker, 2003). The maximum level of prestimulus deflection was determined (either upwards or downwards) and used to build the error box in both directions. Deflections larger than this box are considered significant events. In our case, decreases larger than the error box is considered significant inhibition.

2.5 Statistical Analysis

GraphPad Prism v8 (San Diego, CA, USA), OriginPro 2021 (OriginLab Corporation, Northampton, MA, USA), and MATLAB R2021a (Mathworks, Natick, MA) were used for statistical analysis. The Shapiro Wilk and D'Agostiono & Pearson normality tests have been used to evaluate the distribution. Two ALS groups and controls were compared via Kruskal-Wallis. Each group was compared using Dunn's multiple comparisons tests or ordinary one-way ANOVA and means comparisons with the Bonferroni test. Duration to compare the effect of medication was tested using unpaired t-test, and analysis methods were compared using Wilcoxon matched-pairs signed rank test or paired t-test. PSF duration vs. background discharge rate was analysed using linear regression. Analysis methods (sEMG vs PSTH and PSF) within groups were compared using Repeated-Measures one-way ANOVA with Tukey's multiple comparisons test. The significance level was $P < 0.05$ for all standard descriptive statistical analyses.

Descriptive statistics were strengthened using estimation statistics to pinpoint how much a group differs. The samples were first bootstrapped 10,000 times in MATLAB, and the distribution, 95% confidence intervals, and mean difference were calculated. In the estimation graphs, the lower panel illustrates the effect size by how much the values differ (Ho et al., 2019). For the estimation plots, the effect sizes that do not include zero (95% confidence interval not crossing 0 mean difference) would be considered biologically significant (Ho et al., 2019).

3. Results

The mean age of ALS patients is 54.74 ± 9.49, and for the control group, 40.66 ± 8.54 years. The mean disease duration is 21.67 ± 20.43 months (a range between 4 to 72 months), and the mean ALFSR score is 38.31 ± 7.02.

A sample recording and analysis from each group are shown in Figure 2. The top traces are from a control subject, the middle from a mildly affected patient, and the bottom from a severely affected patient).

We then investigated the number of motor units in FDI muscles in three groups. First, we compared the FDI's maximum peak-to-peak M-responses. Results revealed that the controls had a larger M-response than the severely affected group ($p=0.003$). Also, mildly affected patients had a larger M-response than the severely affected patients ($p=0.035$). MUNIX revealed that mild and severe ALS groups have fewer functional motor units than controls based on the estimation plots. Descriptive statistics do not show significant differences between the controls and mild ALS group ($p=0.190$), but severely affected patients had smaller MUNIX compared to controls ($p<0.0001$) and mildly affected patients ($p=0.026$). Similarly, MUSIX, a hallmark of reinnervation observed in ALS patients, reveals that severely affected ALS groups have larger MUSIX than the control (Figure 3, Table 2).

Next, we performed SMU analyses using PSTH and PSF-CUSUM to calculate the CSP latency and duration. We observed a prolonged CSP latency in patients with severely affected hands compared to controls and patients with mildly affected hands ($p=0.007$ and $p=0.038$, respectively, Figure 4A). However, mild patients had similar latency with controls ($p=0.7275$). Then, we investigated the duration characteristics of the CSP. Given that background discharge rate may affect the duration of the synaptic potentials calculated using PSF (Ozyurt et al., 2019a), we first checked whether the average background firing rate of SMUs differed between groups and confirmed no difference (Figure 4B). CSP duration (Figure 4C) was acquired from PSF-CUSUM, and with classical (all $p>0.5$) and estimation statistics, no changes were observed between groups. Then, we aimed to show whether the use of medication (Riluzole) affected the duration and observed no difference in duration between patients who used Riluzole or not (Figure 4D). Interestingly, linear correlation revealed that despite the similar CSP duration across groups, CSP duration was longer for smaller Mmax, which indicates motoneuron degeneration (Figure 4E). Thus, this suggests a possible increase in CSP duration as the disease progresses. Despite the similar CSP duration in all the groups, we compared the duration obtained from probability (PSTH) and frequency (PSF) based methods in all groups (Figure 5A). The average durations obtained using PSTH versus PSF in controls were 90 vs. 151 ms, mildly-affected patients were 101 vs. 158 ms, and severely-affected patients were 103 vs. 158 ms. PSTH and sEMG indicated a significantly shorter duration in all the groups than PSF (all $p<0.01$). In contrast, sEMG and PSF had similar duration ($p>0.05$) except for mildly-affected patients where sEMG duration was slightly shorter.

Lastly, we investigated the dependence of CSP duration on background discharge rate, which is known to affect the duration of the IPSP (Ozyurt et al., 2019a). Although CSP duration in controls was inversely correlated to the background discharge rate, ALS patients (especially the severely affected) lost this dependence (Figure 5B). The overall findings of this study and the corresponding statistics are summarised in Table 2.

	Control	**Mildly Affected**	**Severely Affected**	**P**
Background Discharge Rate (Hz)	8.76 ± 1.78	9.65 ± 2.13	9.80 ± 3.04	p (mild vs severe) =1
				p (control vs severe) = 0.810
				p (control vs mild) = 0.427
Mmax (mV)	16.83 ± 5.33	7.98 ± 3.72	4.22 ± 3.51	p (mild vs severe) = 0.035
				p (control vs severe) = 0.0003
				p (control vs mild) = 0.080
MUNIX	218.80 ± 75.91	110.29 ± 60.65	44.92 ± 42.39	p (mild vs severe) = 0.026
				p (control vs severe) <0.0001
				p (control vs mild) = 0.190
MUSIX	50.66 ± 31.54	91.00 ± 26.36	124.92 ± 76.86	p (mild vs severe) = 0.932
				p (control vs severe) = 0.020
				p (control vs mild) = 0.148
PSTH				
CSP Latency (ms)	55.52 ± 9.87	59.04 ± 15.55	70.53 ± 16.47	p (mild vs severe) = 0.038
				p (control vs severe) = 0.007
				p (control vs mild) = 0.7275
CSP Duration (ms)	90.38 ± 22.32	99.57 ± 28.07	102.91 ± 38.30	p (mild vs severe) = 1

				p (control vs severe) = 0.2094
				p (control vs mild) = 0.5763
PSF				
CSP Duration (ms)	151.44 ± 25.69	163.59 ± 35.51	158.73 ± 55.08	p (mild vs severe) = 0.925
				p (control vs severe) = 0.850
				p (control vs mild) = 0.624

Table 2. Patient motor unit properties and CSP characteristics in all groups. CSP: Cutaneous silent period; Mmax: Maximum peak-to-peak direct motor responses (M-responses); MUNIX: Motor unit number index; MUSIX: Motor unit size index. Values are mean ± SD.

4. Discussion

This study investigated CSP characteristics in ALS patients with SMU recordings and evaluated the latency and duration using PSTH-CUSUM and PSF-CUSUM. A probability-based PSTH method has been shown previously to obtain latency of short-latency inhibition or excitation quite accurately, and discharge rate-based PSF has been known to be more precise in detecting the profile of the stimulus-induced postsynaptic potential in motoneurons (Türker and Powers, 2005). Therefore, we used PSTH-CUSUM to detect the onset of the synaptic potentials, whereas PSF-CUSUM was used to analyse the duration. Our SMU recordings from FDI muscles of control and ALS patients discovered that 1) the latency of the CSP is prolonged in severely affected ALS patients, 2) the duration was similar across groups, but the duration tends to be longer for advanced disease stages, 3) the use of Riluzole was not a factor affecting the duration of the CSP, 4) probability-based methods (sEMG and PSTH) underestimate the complete profile of the inhibitory postsynaptic potential (IPSP) generated by noxious stimuli, and PSF can be used to detect the rising phase of the IPSP, and 5) ALS patients lose dependence of IPSP duration calculated by PSF to background discharge rate of motor unit.

The primary method to test CSP is sEMG, which is easy to apply in clinics (Cengiz et al., 2018; Castro et al.,2021). Although the sEMG is more advantageous in detecting the motoneuron population response noninvasively, SMU recordings are helpful to pinpoint the alterations i) with a better resolution, ii) with minimal cross-talk from other muscles, and iii) in various size motor unit populations despite the limitation of mainly detecting the low threshold motor units since they are recruited earlier (Henneman, 1957). Also, SMU firing properties present a valuable tool for understanding the time course of inhibitory potentials. Therefore, we used the SMU approach to investigate the temporal properties of the CSP and employed the frequency-based analysis method, which was shown to detect the time course of the IPSPs more accurately (Türker and Cheng, 1994; Türker and Powers, 1999).

One of the parameters we investigated in ALS patents was the onset of the CSP, i.e., the latency. We have found prolonged CSP latency in ALS patients but only in those with apparent symptoms and denervation at their hands (severely affected patient group). There was also a hint that these severe patients also had longer CSP latency than other ALS patients who showed little to no symptoms at the muscle CSP was recorded. Our latency findings align with other studies utilising different methods (Cengiz et al., 2018; Castro et al., 2021; Castro et al., 2023).

CSP latency seems mainly related to the conduction velocity of A-delta fibres and alpha motoneurons and the synaptic delay between sensory axons to inhibitory interneurons and motoneurons. Given that cutaneous afferents (Mulder et al., 1983) and spinal sensory pathway (Cohen et al., 2013) are also affected in ALS, prolonged CSP latency in ALS patients may be due to sensory, motoneuronal, or intraneuronal deterioration or a combination of these.

CSP was used to test the spinal excitability of the motoneurons in addition to testing other clinical, pathophysiological conditions (Kofler et al. 2019b), and the duration of CSP was used as a neurophysiological signature of this excitability in clinical studies (Cengiz et al., 2018; Gutierrez et al., 2020). Excitotoxicity could occur due to the overstimulation of glutamate receptors and is thought to play an important role in ALS (Van der Bosch et al., 2006). Although there is evidence about the increased excitation or reduced inhibition of the motoneurons in ALS at different disease stages (Geevasinga et al., 2016; King et al., 2016; Shibuya, 2017; Özyurt et al., 2020), this and previous studies show no differences in CSP duration in ALS. Interestingly, there is an inverse relationship between the duration of CSP (in PSF) and Mmax, suggesting that towards the advanced stages of ALS, the CSP tends to last longer. This could be due to the multiphasic excitability changes in motoneurons during ALS, observed in mice (Huh et al., 2021) and humans (Geevasinga et al., 2016). That is, neuronal hyperexcitability in ALS could be seen in the early stages of the disease, and hypoexcitability occurs by disease progression (this could explain the prolonged CSP at later stages), possibly due to an adaptation to prevent cell death (Araki T., 2021). Another explanation for CSP duration findings could be the Riluzole treatment (16 of 19 patients used Riluzole). Riluzole antagonises Na^+ channels and inhibits glutamate transmission (Geevasinga et al., 2016). However, our comparisons show that patients with Riluzole treatment have similar CSP duration compared to those who do not use Riluzole, indicating that, at least in our experimental groups, Riluzole does not significantly affect CSP duration.

Despite the dependence of the CSP duration on several stimulation parameters, such as stimulus intensity and pulse duration (Kim et al., 2009), most of the studies in the literature use probabilistic methods (generally sEMG) and report a wide range of duration from 50 to ~80 ms in upper or lower limb muscles, in healthy people and patients with motoneuron diseases (Cengiz et al., 2018; Gilio et al., 2018; Castro et al., 2021 and 2023). It has been previously reported that probability-based methods in SMUs (using PSTH) and sEMG cannot estimate the tail of the IPSP (Ozyurt et al., 2019b, Türker and Powers, 2002 and 2005). Although these methods are easier to apply (especially sEMG) in clinics, they lack pinpointing the IPSP duration with its complete profile. Our results indicate that motoneurons can evoke action potentials towards the tail of the IPSP but with a lower frequency than the background, resulting in a total duration of around 150 ms in controls and ALS patients (Figure 6). Our previous reports in control subjects also showed longer CSP duration in response to electrical or laser stimulations using frequency-based analysis methods similar to the current study, 100-125 ms (Kahya et al. 2010 and 2016). This is about 60% longer CSP duration compared to probabilistic methods, indicating a more robust nature of this inhibitory event than previously thought; therefore, frequency-based methods provide a more detailed estimation of the central synaptic transmission using SMU firing properties.

In contrast to the classical probabilistic methods, IPSP duration calculated using PSF-CUSUM depends on the background discharge rate. We have previously shown that recurrent IPSP durations were longer for motor units firing with lower frequency (Ozyurt et al., 2019b). Interestingly, although the control subjects have CSP duration dependence on background discharge rate, the duration of CSP in ALS patients does not have this correlation. Like the latency modifications, this loss of correlation could be explained at three levels: sensory fibres, interneurons or motoneurons. However, given that the total duration is similar for controls and ALS patients, the modifications causing the loss of correlation are unlikely to be at the sensory fibres or interneurons generating CSP.

On the other hand, this phenomenon could be explained at the motoneuron level. In ALS, motoneurons tend to have variable excitability states depending on the stage of the disease (Huh et al., 2021; Geevasinga et al., 2016). Some spinal circuits and intrinsic properties are altered, leading to increased or decreased excitability of motoneurons (Ozyurt et al., 2020; Sangari et al., 2022; Martínez-Silva et al., 2018). Especially in the hyperexcitable state, a reduction in the net IPSP could be expected due to extra excitatory PSPs or reduced IPSPs contributing to prolonging the ongoing IPSP. In severely affected ALS patients, extremely low CSP durations were never seen in controls or even mildly affected patients (<100 ms, see Figure 5). This could be one of the explanations behind the loss of an inverse relationship between CSP duration and background discharge rate. This dependence could be further investigated for other spinal circuits in ALS. In the future (with other spinal circuitries also tested), discharge rate vs. postsynaptic potential duration tests could be used to detect motoneuron health/functionality, especially for early detection, given that even Mmax is not always sensitive enough to detect early motoneuron loss/dysfunction, likely due to reinnervation which compensates for the reduction of Mmax. Thus, measuring this relationship and detecting the loss of this correlation could be used as a new biomarker in clinical neurophysiology.

5. Conclusion

In summary, our study confirms previous findings of CSP latency modifications and similar duration in ALS at a single motoneuron level. We provided a detailed analysis of the SMU discharges. Previous work used probability-based methods to estimate CSP duration and hence cannot represent the actual duration of this inhibitory event. Our results are unique in that they estimate the duration without count- and synchronisation-based errors embedded in the classical methods; thus, we depicted the complete synaptic profile of the IPSP mediated by noxious stimuli in ALS patients and control variables dependent on CSP duration on background discharge rate.

5.1 Limitations of Study

The main limitation of this study was that the control group was younger than ALS patients, but age was not reported as a factor that influenced the CSP (Han et al., 2007; Yaman et al., 2007; Isoardo et al., 2012; Tekatas et al., 2014; Baek et al., 2016, Kofler et al., 2019a). Another limitation of this study includes the low sample size to perform MUNIX analysis (lack of data from some patients) and Riluzole effect comparison.

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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. *Experimental groups and cutaneous silent period (CSP) recording setup.* ***A)*** *Experiments included three distinct groups: the control group (left), amyotrophic lateral sclerosis (ALS) patients with their hands mildly affected by the disease (middle), and ALS patients with their hands severely affected (right).* ***B)*** *CSP was evoked by delivering high-intensity electrical stimuli to the fifth digit via ring electrodes and recording the response of the stimulus from the first dorsal interosseus (FDI) muscle via intramuscular electromyography (EMG) (single unit electrodes). The fifth digit was located on the C8 dermatome, including motor innervations that supply the FDI muscle (left drawing). The instantaneous firing rate during CSP is decreased following the stimuli (black horizontal double-headed arrows). An example of raw data (trace on the right) from a patient severely affected by the disease shows suppression of single motor units (SMUs) due to a strong electrical stimulus (purple arrow represents the timing of the stimulus). SMUs were converted into accepted pulses (top trace) described in Methods.*

Figure 2*. Example analysis from each experimental group. Peristimulus time histogram (PSTH- left) and Peristimulus frequencygram (PSF-right) graphs indicate the stimulus onset (orange vertical dashed line), the cutaneous silent period (CSP) latency and its duration (purple vertical dashed lines). Horizontal red lines in PSF show the cumulative sum (CUSUM) error box.*

Figure 3. *Motoneuron denervation properties. Plots along with the estimation stats (below) show maximum direct motor response (left plot), motor unit number index (MUNIX - middle plot) and motor unit size index (MUSIX - right plot), n indicates number of motor units.*

Figure 4*. A comparison of A) The cutaneous silent period (CSP) latency from peristimulus time histogram-cumulative sum (PSTH-CUSUM), B) Single motor unit (SMU) discharge rate, C) CSP duration from peristimulus frequency-cumulative sum (PSF-CUSUM) and D) duration of CSP from PSF-CUSUM between amyotrophic lateral sclerosis (ALS) patients based on Riluzole usage is presented. N indicates number of motor units. E) Correlation between maximum direct response (Mmax) and CSP duration obtained from PSF.*

Figure 5*. Duration characteristics of the cutaneous silent period (CSP).* ***A)*** *Duration of CSP obtained using surface electromyography (SEMG), peristimulus time histogram (PSTH), and peristimulus frequencygram (PSF).* ***B)*** *Dependence of PSF duration on background discharge rate.*

Figure 6*. The mechanism of action of the cutaneous silent period (CSP) and the reflection of CSP inhibitory neurotransmission to single motor unit (SMU) firings. The drawings on the left indicate the primary mechanism of the CSP and the estimation of its inhibitory postsynaptic potential (IPSP) using probability-based methods, such as surface electromyography (sEMG)*

suppression, and frequency-based methods, such as reduced discharge rates. Spike with a dashed line shape would show the expected spike based on background interspike interval (ISI) if there were no IPSP. Stimulus-induced IPSP moves the spike to occur later, where the IPSP effect is reduced. The plots on the right show a sample CSP evoked by noxious stimuli in probability-based methods in upper traces: rectified averaged surface EMG (sEMG), SMU average and peristimulus time histogram -cumulative sum (PSTH-CUSUM). The lower trace indicates the discharge rate analysis using peristimulus frequencygram-cumulative sum (PSF-CUSUM). The horizontal dashed line is the average background activity in sEMG. The horizontal lines in PSTH-CUSUM are the error box, and the red horizontal line in PSF shows the background discharge rate. The blue arrow represents the CSP duration calculated by the probability-based methods, whereas the black arrow shows the CSP duration calculated by PSF.