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Estimating reflex responses in large populations of motor units by decomposition of the high-density surface electromyogram

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Key points

- Reflex responses of single motor units have been used for the study of spinal circuitries but the methods employed are invasive and limited to the assessment of a relatively small number of motor units.
- We propose a new approach to investigate reflexes on individual motor units based on high-density surface electromyography (HDsEMG) decomposition.
- The decomposition of HDsEMG has been previously validated in voluntary isometric contractions but never during reflex activities.
- The use of HDsEMG decomposition for reflex studies at the individual motor unit level, during constant force contractions, with excitatory and inhibitory stimuli, was validated here by the comparison of results with concurrently recorded intramuscular EMG signals.
- The validation results showed that HDsEMG decomposition allows an accurate quantification of reflex responses for a large number of individual motor units non-invasively, for both excitatory and inhibitory stimuli.

Abstract We propose and validate a non-invasive method that enables accurate detection of the discharge times of a relatively large number of motor units during excitatory and inhibitory reflex stimulations. High-density surface electromyography (HDsEMG) and intramuscular EMG (iEMG) were recorded from the tibialis anterior muscle during ankle dorsiflexions performed at 5%, 10% and 20% of the maximum voluntary contraction (MVC) force, in nine healthy subjects. The tibial nerve (inhibitory reflex) and the peroneal nerve (excitatory reflex) were stimulated with constant current stimuli. In total, 416 motor units were identified from the automatic decomposition of the HDsEMG. The iEMG was decomposed using a state-of-the-art decomposition tool and provided 84 motor units (average of two recording sites). The reflex responses of the detected motor units were analysed using the peri-stimulus time histogram (PSTH) and the peri-stimulus frequencygram (PSF). The reflex responses of the common motor units identified concurrently from the HDsEMG and the iEMG signals showed an average disagreement (the difference between number of observed spikes in each bin relative to the mean) of 8.2 ± 2.2% (5% MVC), 6.8 ± 1.0% (10% MVC) and 7.5 ± 2.2% (20% MVC), for reflex inhibition, and 6.5 ± 4.1%, 12.0 ± 1.8% and 13.9 ± 2.4%, for reflex excitation. There was no significant difference between the characteristics of the reflex responses, such as latency, amplitude and duration, for the motor units identified by both techniques. Finally, reflex responses could be identified at higher force (4 of the 9 subjects performed contraction up to 50% MVC) using HDsEMG but not iEMG, because of the difficulty in decomposing the iEMG at high forces. In

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conclusion, single motor unit reflex responses can be estimated accurately and non-invasively in relatively large populations of motor units using HDsEMG. This non-invasive approach may enable a more thorough investigation of the synaptic input distribution on active motor units at various force levels.

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Abbreviations CoV_{ISI}, coefficient of variation of the inter-spike interval; CPN, common peroneal nerve; CUSUM, cumulative sum; HDsEMG, high-density surface electromyography; iEMG, intramuscular EMG; MU, motor unit; MVC, maximum voluntary contraction; PNR, pulse-to-noise ratio; PSF, peri-stimulus frequencygram; PSTH, peri-stimulus time histogram; RA, rate of agreement; SIR, signal-to-interference ratio; TA muscle, tibialis anterior muscle; TN, tibial nerve.

Introduction

The analysis of reflex responses is one of the fundamental methods for the study of neuromuscular pathways. Although the interference surface EMG has been the most common technique for this analysis, it has been shown that single motor unit recordings are needed for an accurate interpretation of excitatory and inhibitory pathways (Miles & Türker, 1986; Semmler, 2002; Yavuz *et al.* 2014). The behaviour of individual motor units is usually studied with intramuscular EMG (iEMG) signals but this approach has limitations. Besides the invasiveness, one of the main problems of iEMG for the study of reflexes is the high selectivity that is needed for discriminating the action potentials of individual motor units. Selectivity is specifically important in reflex studies where the ability to distinguish between action potentials occurring at similar timings is crucial. The greater the selectivity, however, the smaller the number of motor units in the detection volume of the electrodes, so that reflex studies are usually based on 1–2 motor units per subject in each tested condition (Stephens *et al.* 1976; Buller *et al.* 1980; Miles & Türker, 1986; Awiszus *et al.* 1991; Devanne *et al.* 1997; Nicolas *et al.* 2001; Rogasch *et al.* 2012; Yavuz *et al.* 2014). These limited sample sizes do not allow investigations of non-uniform synaptic input to different motor neurons (Pierrot-Deseilligny & Mazevet, 2000) and, in general, they may not be representative of the pool of active units. In addition, the study of reflexes using single motor unit activity recorded from iEMG is further biased by the difficulties in decomposing the iEMG at high forces, so that most experimental studies are conducted at forces $<$10% of the maximum.

Surface EMG signals can also be decomposed into individual motor unit activities using multichannel electrodes (De Luca *et al.* 2006; Farina *et al.* 2010) and various surface EMG decomposition methods have been proposed in the last decade (Rau & Disselhorst-Klug, 1997; Gazzoni *et al.* 2004; De Luca *et al.* 2006; Holobar & Zazula, 2007; Kleine *et al.* 2007). Moreover, there has been an extensive validation effort for surface EMG decomposition (Holobar *et al.* 2010, 2014; Nawab *et al.* 2010; Hu *et al.* 2013), so that the approach has recently begun to be accepted for drawing conclusions of physiological interest in healthy subjects (Beck *et al.* 2005; De Luca & Hostage, 2010; Laine *et al.* 2014) as well as patients (Suresh *et al.* 2011; Holobar *et al.* 2012). However, there has not been a discussion and validation on the use of surface EMG decomposition for reflex studies at the individual motor unit level. Translating the results obtained in voluntary contractions to reflex activations is not simple because reflex responses, especially excitatory ones, pose challenges for the decomposition, due to the high degree of superimposition of action potentials.

Here we propose and investigate, for the first time, reflex responses in several motor units whose behaviour has been concurrently identified by decomposition of high-density surface EMG (HDsEMG) signals at various forces. The reflexes were elicited by the electrical stimulation of the mixed nerve bundle. To validate the use of the HDsEMG decomposition method in reflex studies, the results were compared with those obtained by iEMG decomposition. Due to limitations in the force levels for which iEMG decomposition is reliable, the present study primarily focused on the validation of the HDsEMG decomposition of the reflex activity at low contraction forces (5, 10 and 20% of MVC). The main advantages of the proposed method over the iEMG approach to single motor unit reflex analysis are the larger number of motor units that can be investigated concurrently and the greater forces for which the EMG signals can be decomposed.

Methods

Nine healthy subjects (males, age: 29.6 $\pm$ 5.6 years) took part in the main experiments and two additional healthy subjects (male, ages 33 and 27 years) participated

in separate tests at greater contraction forces (see 'Procedures' for details). The protocols were approved by the Human Ethics Committee of the University Medical Centre, Georg-August-University of Göttingen, and were in accordance with the *Declaration of Helsinki*. Each subject provided informed written consent prior to the experiments.

Experimental setup

Subjects were seated in the chair of a Biodex System 3 (Biodex Medical Systems Inc., Shirley, NY, USA), which allowed stable fixation of the right leg and foot. Ankle dorsiflexion force was measured using an ATI Omega160 force transducer attached to the foot plate. To record motor unit activity, two bipolar Teflon insulated silver wire electrodes (75 μm in core diameter) were inserted into the tibialis anterior (TA) muscle with two 25 gauge needles. After fixation of the wires, the HDsEMG electrodes (13 × 5 electrode grid, 10 mm interelectrode distance, ELSCH064NM2; OT Bioelettronica, Torino, Italy) were mounted on top of the wire electrodes, parallel to the tibia, and covering the belly of the tibialis anterior muscle. An ankle strap electrode was used as a reference electrode (WS2; OT Bioelettronica). For stimulating the tibial nerve (TN), the anode of the stimulation probe was a dampened 12 cm × 8 cm pad (Spes Medica s.r.l., Battipaglia, Italy) placed over the patella. The metal cathode of the stimulation probe was placed on the popliteal fossa (Fig. 1*A*). For stimulating the common peroneal nerve (CPN), metal pin anode and cathode were placed posterior and anterior, respectively, to the head of the fibula. All signals were filtered, amplified and recorded at 10,240 samples s^{-1} using an EMG-USB2 data acquisition system (OT Bioelettronica). The iEMG signals and the HDsEMG signals were band-pass filtered with 100–4400 Hz and 10–500 Hz cut-off frequencies, and amplified by a factor of 300 and 1000, respectively.

Procedures

The nine subjects of the main experiment were asked to perform ankle dorsiflexion contractions at 5%, 10% and 20% of the maximum voluntary contraction (MVC)

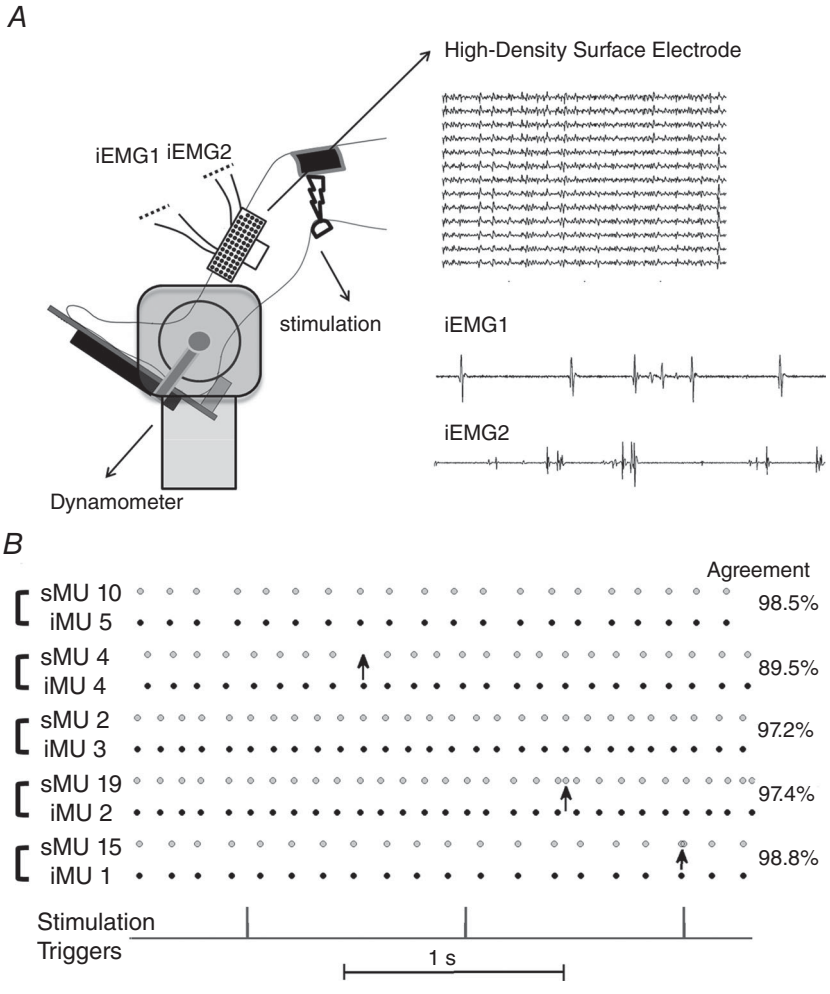

Figure 1. Signal acquisition and identification of the matched motor units for the two modalities
A, experimental setup. On the right-hand side the segments of raw signals are shown from the HDsEMG (from only the middle column of matrix electrode) and iEMG electrodes. *B*, example of motor unit spike trains (2.5 s window) common for the two methods (iEMG and HDsEMG) during 10% MVC contraction. Black and grey circles indicate motor unit spike trains decomposed from iEMG and HDsEMG, respectively. Vertical arrows show disagreements. The numbers on right-hand side show the rate of agreement between matched motor unit couples.

force. The MVC was measured as the maximum peak of three consecutive maximal contractions with 2 min of rest after each trial. The subjects were asked to maintain the contraction force at fixed MVC level (5%, 10% and 20%, ordered randomly) while an average of 150 electrical stimuli were delivered to the mixed nerve (TN for inhibition, CPN for excitation), with a 100 μs pulse duration and a 2–3 s random inter-pulse interval. Thus, the average duration of each contraction was approximately 225 s. The subjects had 5 min of resting period between each contraction. To identify the stimulation intensity, the Hoffman reflex (H-reflex) and direct motor response (M-wave) were recorded on the homonymous muscle before each trial. The intensity of the stimulation was set corresponding to the intensity that elicited an H-reflex with size equal to 10% of the maximum M-wave. For the stimulation of the CPN (excitation on TA), the H-reflex elicited on TA was the reference while during stimulation of the TN, the H-reflex of the soleus muscle was the reference. TN was stimulated to elicit inhibition (reciprocal inhibition) on the TA in 6/9 subjects and CPN was stimulated to elicit excitation (H-reflex) on TA in 5/9 subjects. In addition to these measures, the protocol was repeated on four additional subjects for a contraction at 50% MVC during stimulation of inhibitory and excitatory pathways. Two of these four subjects repeated the lower level contractions in addition to the 50% MVC tasks. The inter-pulse interval was reduced to 1–2 s for the 50% MVC contraction in order to reduce the contraction duration to 120 s on average.

Analysis

The HDsEMG was decomposed by the convolution kernel compensation (CKC) technique (Holobar & Zazula, 2007) providing fully automatic decomposition. However, if an abnormal firing frequency was observed after the stimulation instant, it was considered as an obvious false identification. In these special cases, the result of the surface EMG decomposition near the stimulation instant was examined carefully and the motor unit discharge was manually removed if verified as a clear false identification. For decomposition, the EMG signals were divided into intervals of 60 s with 10 s overlap and decomposed individually. In order to track the same motor unit spike trains between segments, the correlation coefficient between the discharge timings estimated in the time interval of overlapping has been computed. Spike trains with a correlation greater than 90% were merged. iEMGs were decomposed using the EMGLAB tool, which has been extensively validated in previous work (McGill *et al.* 2005). Afterwards, the iEMG decomposition results were extensively examined and each motor unit discharge pattern was manually edited in order to increase the decomposition accuracy.

The motor units that were discriminated from HDsEMG and iEMG concurrently were matched by calculating the number of matched discharges (CD_j) with the discharge timing tolerance set to $\pm$5 ms. The rate of agreement RA_j was defined for each motor unit j as the number of matched discharges, normalized by the sum of the matched discharges, the number of discharges of the j-th motor unit identified from HDsEMG (S_j) only, and the number of discharges (I_j) identified from iEMG only (Holobar *et al.* 2010):

$$RA_j = \frac{CD_j}{CD_j + I_j + S_j} \times 100 \quad (1)$$

Two motor units detected from HDsEMG and iEMG were considered the same if their agreement rate (RA_j) was >30% (Fig. 1*B*). Additionally, by following the methodology in Holobar *et al.* (2014), the pulse-to-noise ratio (PNR) of the motor units identified from HDsEMG was calculated as an alternative metric for global HDsEMG to evaluate the decomposition accuracy. This signal-based metric enables the accuracy of the identification of each detected motor unit to be tested without the need for concurrent intramuscular recording. As validated on extensive synthetic and experimental signals from the TA muscle, a PNR >30 dB corresponds to a sensitivity in identification of motor unit discharges >90% (Holobar *et al.* 2014). However, the PNR metric can only be applied to the results of the CKC-based decomposition and cannot be calculated for motor units identified by EMGLAB (Holobar *et al.* 2014). In this study, the PNR metric was used to test whether the global decomposition accuracy of the HDsEMG signal influenced the disagreement between the two modalities. The linear regression between PNR and the average disagreement was calculated to quantify this influence.

The reflex responses of the matched motor units were studied with the peri-stimulus time histogram (PSTH) and the peri-stimulus frequencygram (PSF). The PSTH represents the number of motor unit spike occurrences in a particular bin width around the stimulus (Davey *et al.* 1986), whereas the PSF method represents the instantaneous discharge rates of single motor units against the time instant of the stimulus (Awiszus *et al.* 1991; Türker & Cheng, 1994). These two methods are usually presented together with their cumulative sums (CUSUM) that make any subtle but persistent changes detectable (Türker *et al.* 1996):

$$\mathrm{CUSUM}(t_{\mathrm{pre}}) = 0,$$

$$\mathrm{CUSUM}(t) = \sum_{t_{\mathrm{pre}}}^{t_{\mathrm{post}}} (x(t) - M) \quad (2)$$

where $x(t)$ is the instantaneous discharge rate at time t and M is the mean pre-stimulus discharge rate. t_{pre} and

t_{post} are the 'pre' and 'post' time intervals around the stimulation, respectively, and the result is given relative to the pre-stimulus mean (M). Thus, consecutive data points denote the sums of the differences from the pre-stimulus mean. Peaks and troughs after the stimulus in the PSF-CUSUM were considered significant excitations and inhibitions only if the vertical distance between two consecutive PSF-CUSUM turning points was greater than 100% of the maximum pre-stimulus variation. In this way, the reflex parameters (latency, duration and amplitude) were retrieved from the PSF-CUSUM. The first deflection point for a peak or trough was considered the onset of the reflex. The distance between the peak and the onset value was measured as the reflex amplitude. The reflex duration was defined as the time interval between the onset and the next turning point. The reflex responses estimated for the common motor units, as identified by the two recording techniques, were compared on the basis of the extracted reflex parameters as well as on the similarity between the post-stimulus periods of estimated PSTHs. For the latter comparison, the difference between values of post-stimulus bins (number of observed spikes) was calculated relative to the mean bin values and will be referred to as disagreement (disA):

$$\mathrm{disA}(\tau) = \frac{|X_{\mathrm{HDsEMG}}(\tau) - X_{\mathrm{iEMG}}(\tau)|}{\frac{X_{\mathrm{HDsEMG}}(\tau) + X_{\mathrm{iEMG}}(\tau)}{2}} \times 100 \qquad (3)$$

where X denotes the number of discharges in the τ-th PSTH bin in the 200 ms time interval after stimulation. Five motor units that presented a clear M-wave were excluded from the calculation of average disagreement rates since the presence of the M-wave is not desired in single motor unit reflex studies and therefore single motor unit reflexes are commonly analysed only when the M-wave is not present.

For the tests at 50% MVC force, the recorded iEMG signals were highly interferential, despite the selective wire electrodes, hindering their accurate decomposition into contributions of individual motor units. Consequently, mutual comparison of HDsEMG and iEMG decomposition results was not possible. For this reason, the coefficient of variation of the inter-spike interval ($\mathrm{CoV_{ISI}}$) for motor units identified from the two decomposition modalities was used as an additional indirect performance measure (Holobar *et al.* 2010; Marateb *et al.* 2011*a*). Indeed, high coefficients of variation are probably associated with frequent decomposition errors. For the two subjects who performed the contractions at all force levels, the reflex latencies measured at 50% MVC were compared with those measured at lower contraction forces. The latency (onset of the reflex) did not depend on the contraction force whereas the amplitude and duration of the reflex were influenced by force. In order to study the decomposition accuracy specifically at the reflex time, the signal-to-interference ratio (SIR) was calculated for the 200 ms interval following the stimulus. The SIR score is defined as follows (Holobar *et al.* 2010):

$$\mathrm{SIR}(i) = \left(1 - \frac{\sum_{T}^{T+200\mathrm{ms}} \left| (x_i(n) - \sum_{j} Y_{ij}(n))^2 \right|}{\sum_{T}^{T+200\mathrm{ms}} \left| (x_i(n)^2 \right|}\right) \times 100 \qquad (4)$$

where $x_i(n)$ represents the EMG signal at i-th channel, $Y_{ij}(n)$ denotes the motor unit action potential train and T the stimulation time.

Statistical analysis

One trial at 20% MVC was excluded from the analysis because the iEMG could not be decomposed due to poor signal quality. The latency (response onset time minus stimulation time), the reflex amplitude normalized to the number of stimulations (amplitude/number of stimulations) and the duration (response peak time minus response onset time) of the reflex responses as estimated from PSF-CUSUM were compared to test the similarity between the motor units that were identified in common by both decomposition methods. The comparisons were performed using the paired t test for each of the three contraction forces. A one-way ANOVA was used to test the differences between RA values, disagreement between PSTHs and PNR indices across contraction forces (5%, 10% and 20%) and stimulation type (excitation and inhibition). Moreover, the motor units (MUs) identified at different force levels (10% and 20% MVC) were determined by comparing their action potentials with cross correlation. The total disagreement in the PSTHs of MUs for the two force levels was tested with one-way ANOVA. Since it was hypothesized that the decomposition of EMG signal in intervals containing the stimulation time would be the most critical for accuracy, one-way ANOVA and least significant difference (LSD) *post hoc* analysis were used to examine the differences among the bins of the average disagreement around the stimulation timing. In order to investigate the correlation between the PNR and the disagreement, the significance of their linear regression was tested for inhibitory and excitatory reflex responses using a regression test. Finally, the difference between the $\mathrm{CoV_{ISI}}$ for the two decomposition modalities was tested using one-way ANOVA. In all tests, the significance level of $P < 0.05$ was chosen.

Results

The mean stimulation intensities were 14.6 ± 7.1 mA for inhibition and 13.1 ± 3.4 mA for excitation. In total, 416

Table 1. Comparison of two decomposition modalities for inhibitory (Inh) and excitatory (Exc) reflexes at three contraction levels (5, 10 and 20% MVC)

Reflex	% of of MVC	No. of HDsEMG MUs	No. of iEMG1 MUs	No. of iEMG2 MUs	No. of common MUs	RA (%)	TdisA (%) PSTH (200 ms)	TdisA (%) PSTH (70 ms)	PNR (dB)
Inh	5	15	4	3	5	84.7 ± 2.7	8.2 ± 2.2	—	47.8 ± 6.8
	10	92	20	23	21	94.5 ± 4.9	6.8 ± 1.0	—	49.6 ± 6.1
	20	133	30	21	18	90.0 ± 6.4	7.5 ± 2.2	—	48.1 ± 3.8
Exc	5	15	2	5	3	92.5 ± 1.2	6.5 ± 4.1	11.4 ± 8.9	47.7 ± 5.6
	10	83	21	15	13	91.4 ± 3.6	12.0 ± 1.8	18.0 ± 4.4*	48.5 ± 5.8
	20	78	14	10	9	85.4 ± 10.9	13.9 ± 2.4	17.0 ± 6.6	44.6 ± 8.7

The total number of motor units identified from high-density surface EMG (HDsEMG) and intramuscular EMG (iEMG) and the numbers of common motor units are presented. Mean ± 95% confidence interval of the global rate of agreement (RA), total-disagreement (TdisA) estimated as sum of disagreement values for 200 ms and 70 ms post-stimulus time histogram (PSTH) bins were provided in the table (columns 6,7 and 8). The pulse-to-noise ratio (PNR) as calculated for motor units identified from HDsEMG is also shown in the table (last column). *Significantly different value.

motor units were identified from the HDsEMG, whereas on average 84 motor units were identified from each iEMG recording site (91 from the first and 77 from the second recording site) from the nine subjects. Each subject performed three contractions during inhibitory and excitatory reflex stimulation. Sixty-nine of the identified motor units were detected by both techniques (on average, 4 motor units per subject, per contraction force) (Table 1). For these motor units in common, the RA in discharge patterns identified from global HDsEMG and iEMG was not significantly different across contraction forces for inhibitory (range = 71.2–99.0%) and excitatory (range = 62.7–96.2) reflexes (Table 1).

To validate the accuracy of decomposition for post-stimulation time, the PSTH and PSF-CUSUM of the common motor units were compared for inhibition

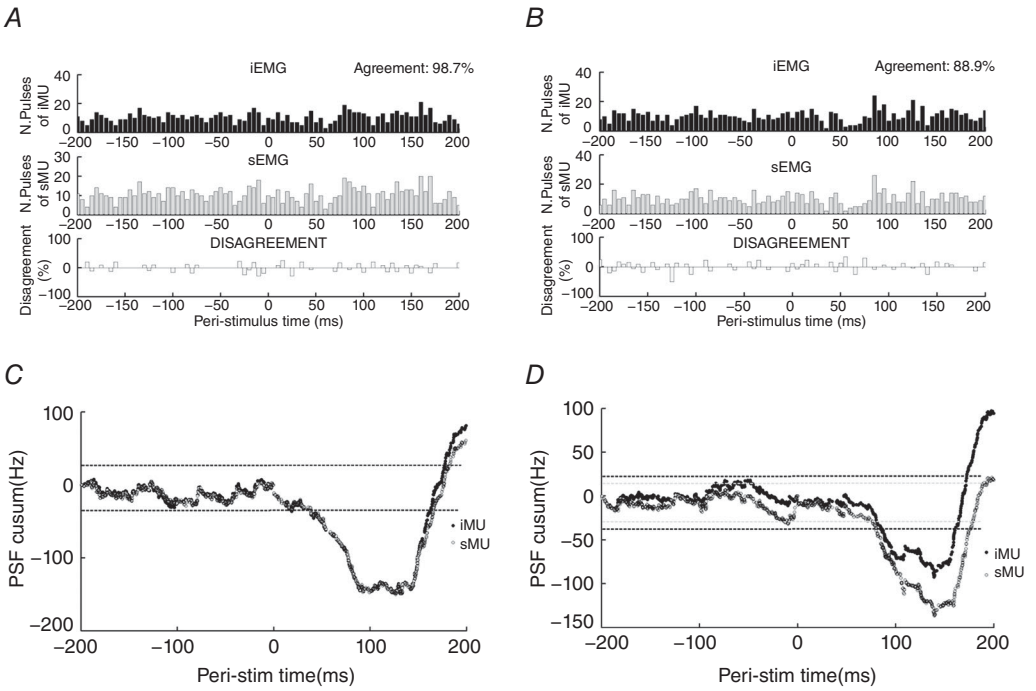

Figure 2. Inhibitory reflex example
PSTH (black and grey) and disagreement (white) graph of common motor units with higher (*A*) and lower (*B*) agreement score. N. Pulses refers to the number of motor unit discharge pulses (spikes) observed in each bin of the PSTH. The lower panels depict the PSF-CUSUM of common motor units with higher (*C*) and lower (*D*) agreement rate. The horizontal dashed lines in *C* and *D* refer to the range of the maximum pre-stimulus variation that was used to determine significance of reflex response. The intramuscular EMG and high-density surface EMG are labelled iEMG and sEMG, respectively.

(Fig. 2*A–D*) and H-reflex (Fig. 3*A–D*) responses. The ranges of total disagreement as calculated by summing up the disagreement values over all the bins were 1.40–13.30% and 2.5–24.1% for inhibition and excitation, respectively (Table 1, column 7 and 8). The greatest disagreement level (24.1%) in excitation reflex corresponded to the MUs with the lowest RA (62.7%). The total disagreement calculated for the 200 ms window after the stimulation instant did not change with the muscle contraction force (inhibition: $P = 0.40$; excitation: $P = 0.18$) for inhibition and excitation individually. On the other hand, the total disagreement was greater for excitation than inhibition at 10% and 20% MVC ($P < 0.05$; Fig. 4*A*). The total disagreement in excitation reflex was not different between the same MUs

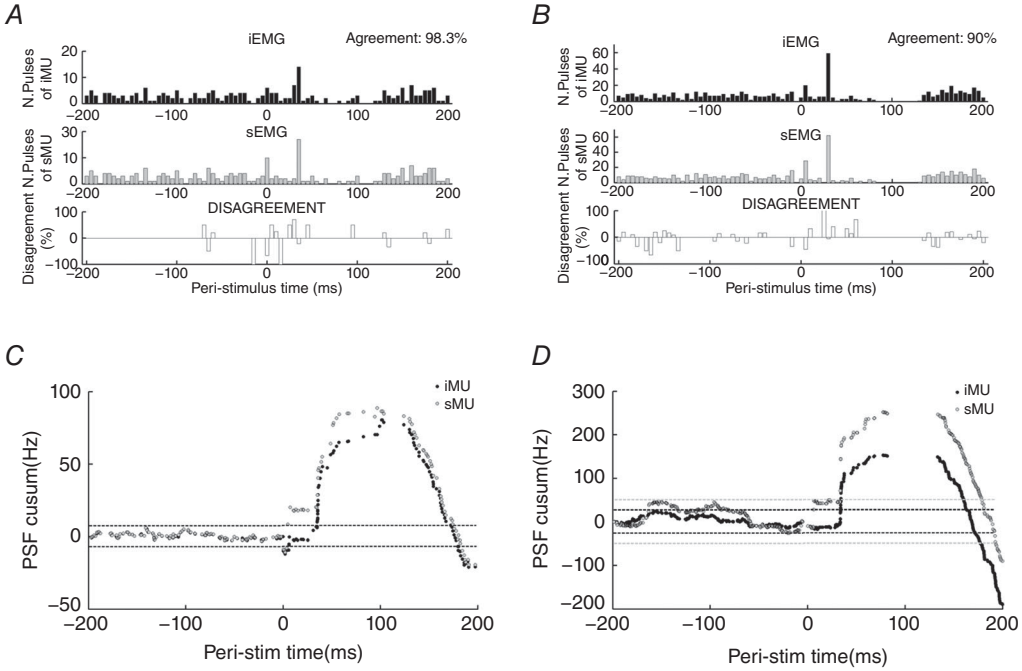

Figure 3. Excitatory reflex example
PSTH (black and grey) and disagreement (white) graph of common motor units with higher (*A*) and lower (*B*) agreement score. N. Pulses refers to the number of motor unit discharge pulses (spikes) observed in each bin of the PSTH. The lower panels depict the PSF-CUSUM of common motor units with higher (*C*) and lower (*D*) agreement rates. The horizontal dashed lines in *C* and *D* refer to the range of the maximum pre-stimulus variation that was used to determine the significance level of the reflex response. The intramuscular and high density surface EMGs are labelled iEMG and sEMG, respectively. The sharp increase of PSTH and PSF-CUSUM at the stimulation moment is the stimulation artefact.

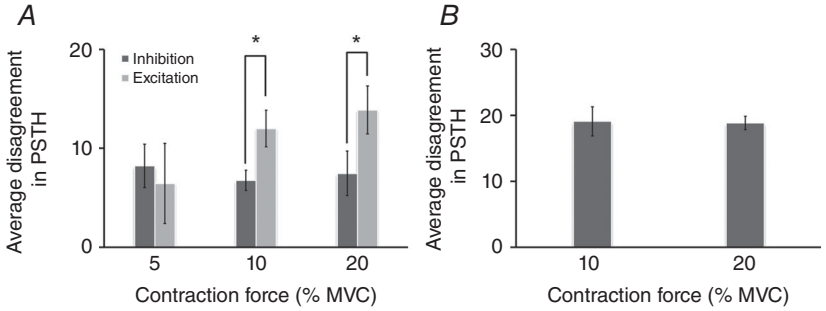

Figure 4. Comparison of average disagreement at the post-stimulus time interval of the PSTH across contraction levels
The error bars refer to the 95% confidence interval (CI) of data. *A*, average disagreement on inhibition and excitation reflex at 5%, 10% and 20% MVC contraction force. There was no significant difference across contraction levels, either for inhibition or for excitation. The disagreement was greater in excitation at 10% and 20% MVC ($P < 0.01$). *B*, average disagreement on excitation reflex for MUs common at 10% and 20% MVC (no significant difference; $P > 0.05$). *Significantly different at $P < 0.05$.

at two contraction forces (10% and 20% MVC) ($P > 0.05$; Fig. 4*B*). The regression analysis between PNR and the disagreement index showed that there was no relation between the accuracy of HDsEMG decomposition and the error at reflex time, either for inhibition or for excitation reflexes ($P > 0.05$, Fig. 5). The stimulation artefact and the M-wave were the main source of disagreement in the 0–10 ms interval for some of the MUs (sharp increase at 0–10 ms in Fig. 3*A*–*D*). Despite the fact that the stimulation intensity had been adjusted to elicit only the H-reflex in the present study, the M-wave was observed on five common MUs (two MUs at 10% MVC and three at 20% MVC for one subject). The total disagreement was greater for the MUs for which the M-wave was observed (mean disagreement in MUs without M-wave: $10.8 \pm 4.1\%$ and with M-wave: $20.1 \pm 4.0\%$; $P < 0.01$). The distribution of average disagreements and the total number of spikes for each individual bin is shown in Figs 6 (inhibition reflex) and 7 (excitation reflex). For the excitation reflex (Fig. 7), although the total disagreement was greater on average for all contraction forces in the first 70 ms of post-stimulus time compared with the total disagreement estimated for 200 ms of post-stimulus time, the difference was significant only at 10% MVC force ($P < 0.05$, Table 1). For inhibition, there was a uniform distribution of average disagreement at the post-stimulation time (Fig. 6). Thus, none of the bins exhibited a significantly different disagreement value from the others. However, those factors did not influence the estimation of the reflex amplitude (reflex amplitude ratio of common MUs

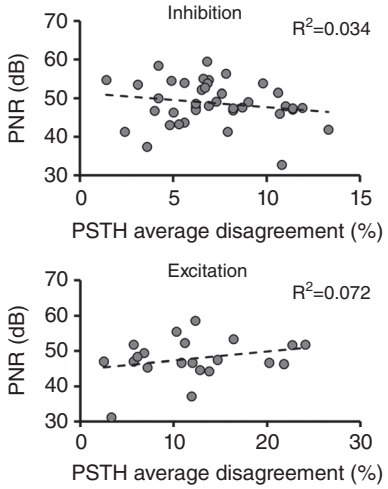

Figure 5. Correlation between the pulse-to-noise ratio (PNR) and PSTH percentage disagreement
The regression line (black dashed line) is shown. There was no significant linear correlation between PNR and average disagreement on PSTH for either reflex type ($P > 0.05$).

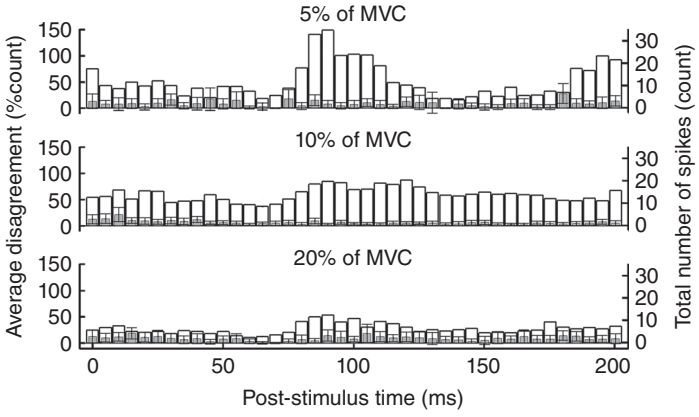

Figure 6. Average percentage disagreement histogram after stimulation instant for the inhibition reflex
Histograms show percentage disagreement values (grey filled bars) on 5%, 10% and 20% of MVC (from top to bottom) averaged in each bin across all subjects. Open bars show the total number of spikes (right *y*-axis) identified in the iEMG and HDsEMG averaged across all subjects. Bins represent 5 ms time intervals. Error bars refer to standard deviations. There is no statistical difference between bins ($P > 0.05$).

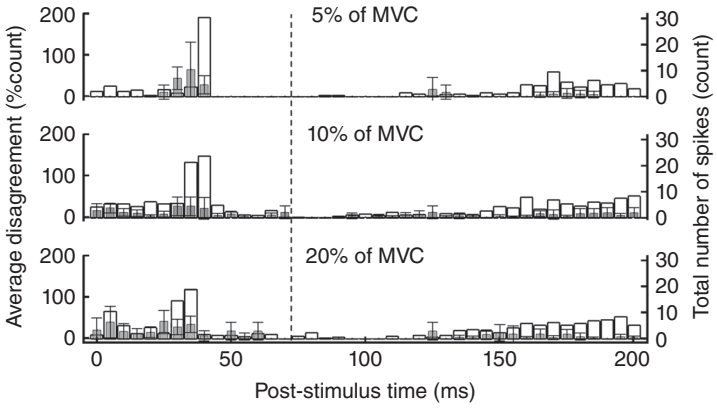

Figure 7. Average percentage disagreement histogram after stimulation instant for the excitation reflex
Histograms show percentage disagreement values (grey filled bars) on 5%, 10% and 20% of MVC (from top to bottom) averaged in each bin across subjects. Open bars show the total number of spikes (right *y*-axis) identified in iEMG and HDsEMG averaged across all subjects. Bins indicate 5 ms time intervals. Error bars refer to standard deviation. The total disagreement in the first 70 ms (until vertical dashed line) of post-stimulus time histogram was greater for 10% MVC ($P < 0.05$).

Table 2. Comparison of reflex response parameters for inhibitory (Inh) and excitatory (Exc) reflexes at three contraction levels (5, 10 and 20% of MVC)

Reflex	Contract. level, % of MVC	Lat (ms)	Dur (ms)	Amp (Hz/N.Stim)	Lat ratio	Dur ratio	Amp ratio
Inh	5	61.0 ± 10.4	29.7 ± 10.9	0.25 ± 0.09	100.4 ± 0.4	109.4 ± 13.2	123.8 ± 59.5
	10	65.2 ± 9.4	65.4 ± 34.5	0.54 ± 0.23	100.1 ± 2.8	101.5 ± 11.2	99.3 ± 18.2
	20	67.2 ± 13.9	51.1 ± 32.9	0.86 ± 0.68	99.7 ± 3.4	95.8 ± 15.0	97.8 ± 20.03
Exc	5	33.2 ± 1.4	4.2 ± 1.1	0.49 ± 0.11	103.2 ± 2.6	89.2 ± 6.8	114.7 ± 12.7
	10	37.6 ± 2.3	9.51 ± 11.5	0.74 ± 0.26	99.2 ± 2.8	93.0 ± 24.0	100.2 ± 17.7
	20	35.5 ± 2.7	23.9 ± 10.3	1.67 ± 0.70	98.7 ± 3.0	91.4 ± 10.5	104.3 ± 20.5

The mean ± SD is shown for the latency (Lat), duration (Dur) and amplitude (Amp) of the reflex response. N.Stim, as a denominator in the calculation of the amplitude, refers to the number of stimulation. The last three columns show the ratio between latency, duration and amplitude of the reflexes measured by the two methods (for example, Lat ratio = Lat HDsEMG/Lat iEMG).

without interference: 97.1 ± 19.4%, and with interference: 110.3 ± 17.7%; $P > 0.05$) that was calculated as the distance between the reflex peak and onset value. The latency, amplitude and duration of the reflex responses were compared for the common MUs identified by the two decomposition methods. The ratio of the reflex parameters (i.e. 100 × Amplitude in HDsEMG/Amplitude in iEMG) is reported in Table 2. For both inhibitory and excitatory reflex responses, there were no significant differences between these reflex characteristics at all contraction forces.

Finally, we analysed the possibility of extracting reliable information on reflex activations at greater forces. For this purpose, four subjects performed ankle dorsi-flexions at 50% MVC force. The CoV_{ISI} of the motor units identified by the two decomposition modalities were compared (Fig. 8). The results showed that 98 out of 167 motor units identified from HDsEMG had a CoV_{ISI} lower than 30%, while all the 157 motor units identified from the iEMG had a CoV_{ISI} greater than 30%. Moreover, the one-way ANOVA test showed that CoV_{ISI} was greater for motor units decomposed from the iEMG compared to HDsEMG ($P < 0.01$). This high variability of inter-spike interval was due to errors in the identification of discharges, especially at the excitation reflex where most MUs discharged synchronously. Accordingly, the post-stimulus SIR score of the iEMG for the excitation reflex was lowest for the 50% MVC contraction ($P < 0.05$), whereas it did not depend on the contraction force for the HDsEMG (Fig. 9) Figure 10*A–D* shows the concomitant discharges of four MUs around stimulation moment that were identified by the two decomposition methods at 50% MVC. With HDsEMG, the average latency and normalized amplitude of the PSF-CUSUM in the inhibitory reflex were 54.9 ± 6.7 ms and 0.60 ± 0.41 Hz, respectively. For the excitatory reflex, the average latency and normalized amplitude of the PSF-CUSUM was 36.9 ± 2.6 ms and 0.89 ± 0.42 Hz, respectively. Conversely, no significant reflex response was observed when using the intramuscular decomposition method, which was presumably due to poor decomposition accuracy. Therefore mutual comparison for testing the accuracy of reflex response

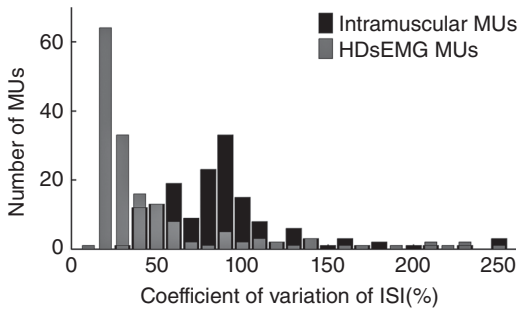

Figure 8. Coefficient of variation of inter-spike interval (CoV_{ISI})
Histogram shows the number of motor units in specified CoV_{ISI} intervals. Black and grey bars represent motor units identified from iEMG and HDsEMG during 50% MVC contraction, respectively.

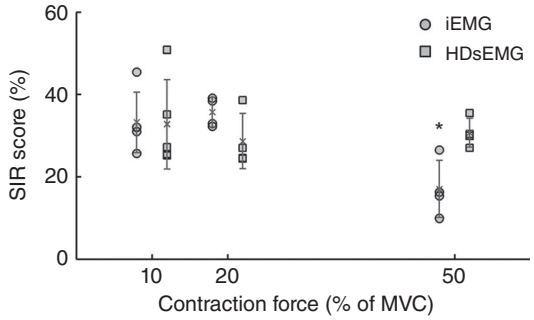

Figure 9. Post-stimulus signal-to-interference ratio (SIR) at three contraction forces
The circles (iEMG) and squares (HDsEMG) denote the average SIR over EMG channels for each subject. The crosses mark the mean SIR. The error bars represent the 95% confidence interval. The SIR values at 50% MVC are significantly lower than at the other contraction forces for iEMG. *Significant difference at $P < 0.05$.

estimation was not possible at this contraction force. However, the latency of the reflexes measured from HDsEMG were compared with the latencies measured at lower contraction forces (5%, 10%, 20% MVC) for the two subjects who performed all trials. There was no significant difference between latencies, either for the inhibition or for the excitation reflex ($P > 0.05$).

We also investigated whether the excitation and inhibition reflex inputs to motor neuron pools were uniform or showed a distribution of values across motor units. For this purpose, the probability density histogram of the reflex amplitudes (measured from the PSF-CUSUM, as explained in Methods) and the fitting distributions were estimated for both reflexes at the two contraction forces (Fig. 11*A*–*D*). In total, 56 MUs at 10% MVC and 74 at 20% MVC force level in excitation, and 68 MUs at 10% MVC and 65 at 20% MVC in inhibition, were decomposed from three representative subjects and pooled for analysis. The bin sizes for the histograms were determined using the Freedman–Diaconis rule (Birgé & Rozenholc, 2006). The gamma distribution function, that is defined by the 'k' (skewness) and 'θ' scale parameters, was fitted to the experimental histograms (log-likelihood > -67 dB). The distribution curve was skewed on the left for excitation (for 10% MVC, $k = 2.3 \pm 0.4$ and for 20% MVC, $k = 2.1 \pm 0.3$) while it approached a normal distribution for inhibition (for 10% MVC, $k = 6.2 \pm 1.0$ and for 20% MVC, $k = 3.5 \pm 0.6$).

Discussion

Reflex responses have been extensively used to investigate physiological (Kahya *et al.* 2010; Gervasio *et al.* 2013; Laine *et al.* 2014; Yavuz *et al.* 2014) and pathological (O'Dwyer *et al.* 1996; Veltink *et al.* 2000; Stubbs *et al.* 2012) activities of spinal circuitries in humans. Recordings of single motor unit reflex activations provide detailed information on interneuronal circuitries at the spinal level and allow the investigation of the distribution of sensory input to the motor neurons in relation to their intrinsic properties (Miles & Türker, 1986; Binboğa & Türker, 2012).

The classic method of single motor recordings by iEMG is invasive and highly selective. For example, the present study demonstrated that the total number of motor units identified from one iEMG recording was approximately one-fifth of the units identified from HDsEMG (Table 1, columns 2, 3 and 4). Despite this limitation, many iEMG decomposition tools enable the user to edit and verify the results in each segment manually, so that the decomposition accuracy and number of accurately extracted units may increase (McGill *et al.* 2005). However, the accuracy of iEMG decomposition degrades rapidly with increasing contraction force, mainly due to the temporal superimposition of several motor unit discharges (Merletti & Farina, 2009; Marateb *et al.* 2011*b*). Conversely, the CKC-based decomposition of HDsEMG is not sensitive to the proportion of superimpositions (Holobar *et al.* 2012) and allows the identification of a greater number of motor units. For example, at 50% MVC contraction force, the CoV_{ISI} was outside the physiological range for the discharge timings of the motor units identified from the iEMG, whereas the motor units identified from the HDsEMG exhibited regular discharges (Fig. 8). Accordingly, the reflex responses identified from the HDsEMG at 50% MVC could not be detected using the motor units decomposed from the iEMG, presumably because of the high percentage of iEMG decomposition errors (Fig. 10).

Obtaining reflex responses from a relatively large sample of active motor units at a particular contraction force may help in describing the behaviour of spinal circuitries, which is of the utmost importance in understanding the functional synaptic organization of the spinal cord in humans. Intracellular recordings from many motor units in animal preparations have extensively revealed the relation between the synaptic input strength from stretch reflex afferents to motor neurons and their type (Burke,

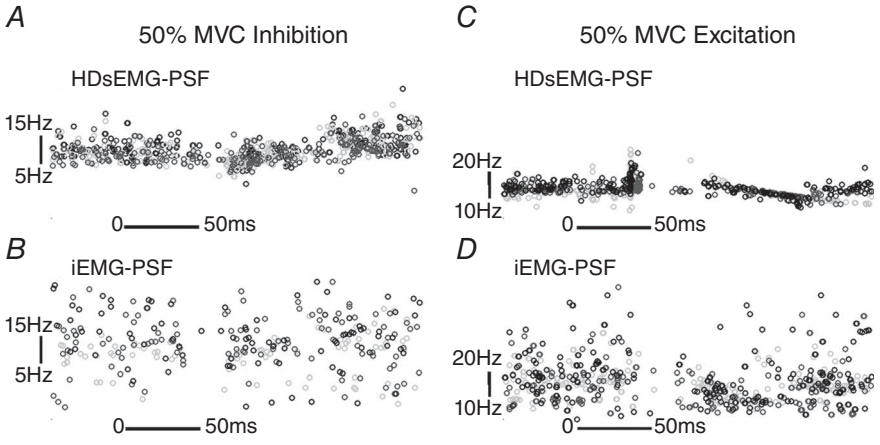

Figure 10. PSFs for the motor units identified by the two decomposition techniques at 50% MVC contraction
Each PSF contains the instantaneous discharge frequencies (*y*-axis) of three MUs (each MU has the marker in a different tone of grey) around stimulation instant (0 ms). MUs were concurrently identified in HDsEMG (*A* and *C*) and iEMG (*B* and *D*) at 50% of contraction force during inhibition and excitation. While the motor units identified in HDsEMG exhibited reflexes accurately, no reflex was observed in iEMG motor units that had very high discharge rate variability.

1967, 1970). In humans, the commonly used method is to compare the reflex amplitudes estimated by pairs of motor units with respect to their discharge rate (Miles & Türker, 1986) or recruitment threshold (Buller *et al.* 1980; Binboğa & Türker, 2012). However, the non-linear input–output relation in the pathways due to the contribution from different afferent input (Pierrot-Deseilligny & Mazevet, 2000) or recurrent effects (Hultborn & Pierrot-Deseilligny, 1979) makes it difficult to estimate the excitability of motor units by analysing only a few motor units. Larger populations, as can be identified using the proposed technique, probably reveal a better representation of the motor neuron pool behaviour. This may also explain contradictory results between animal (Burke, 1967, 1970) and human (Buller *et al.* 1980; Binboğa & Türker, 2012) studies when correlating motor unit size and reflex amplitude. The present study presents a tool that allows these issues to be addressed. For example, the distribution of monosynaptic input from muscle spindles (H-reflex) and reciprocal inhibition have been estimated in the present study for several motor units concurrently. The results showed that the synaptic distribution of both monosynaptic excitation and reciprocal inhibition fitted to the gamma distribution with different skewness (Fig. 11*A*–*D*). This difference between distributions was probably due to the differentiated synaptic projection of inhibitory and excitatory afferents over different types of motor units (Miles & Türker, 1986). However, further evaluation is necessary. Since the validation of the method was the main aim of this study, future work will be dedicated to its application for the analysis of the distribution of synaptic input to motor neurons from afferent activity.

The validation of the accuracy of the proposed method was mainly performed by comparing the single motor unit reflex results obtained from HDsEMG with those from iEMG. This comparison was limited to the contraction forces at which iEMG decomposition was reliable (up to 20% MVC). For these forces, the RA used as accuracy

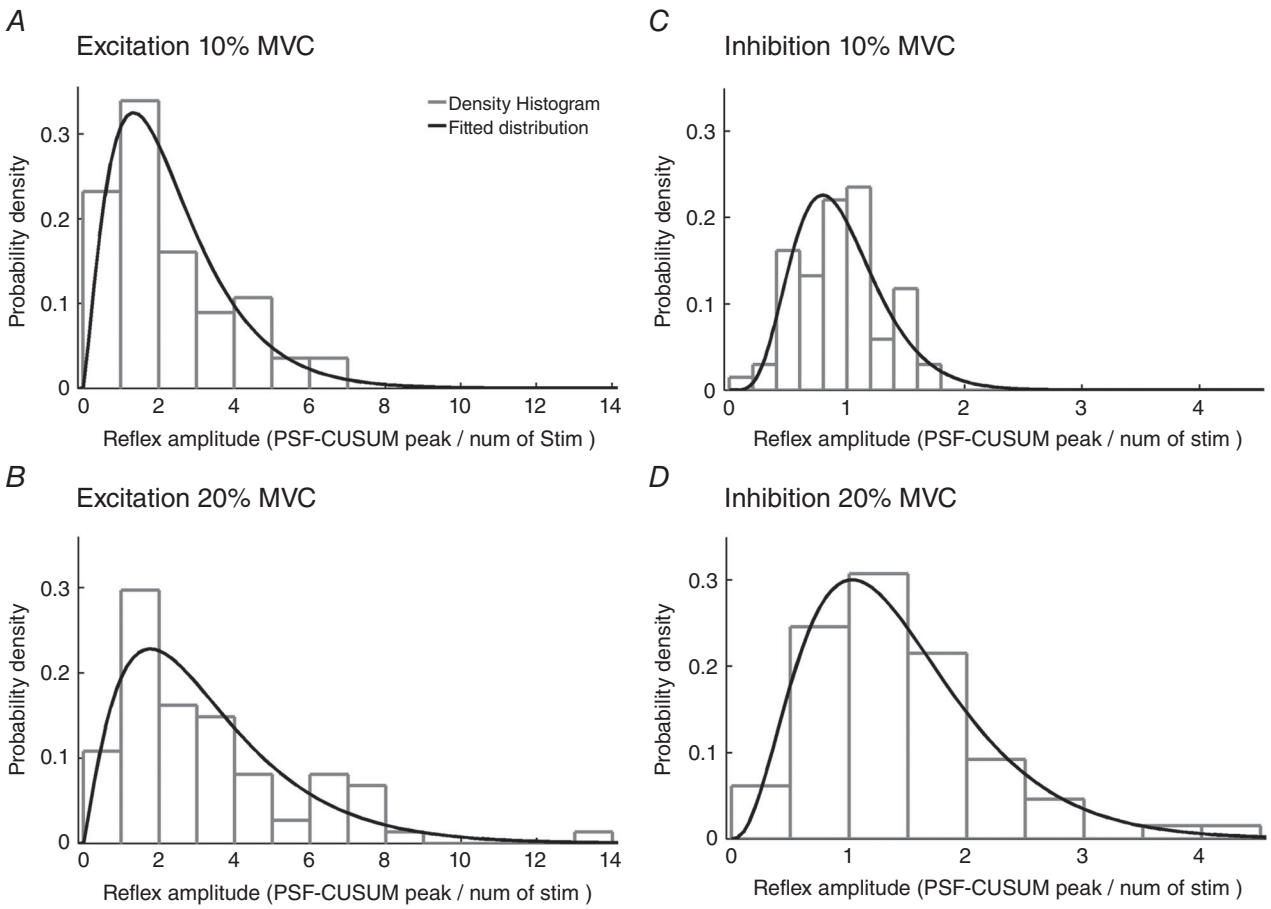

Figure 11. Distribution of excitation and inhibition reflex inputs to motor units (MUs)
The histograms represent the probability density of the reflex amplitude calculated as peak measured from PSF-CUSUM divided by the number of stimuli at 10% MVC and 20% MVC for excitation (*A* and *B*) and for inhibition (*C* and *D*). The black curves indicate the distribution functions were used to fit the histograms. Bin-width of each histogram was determined using the Freedman–Diaconis rule which is based on interquartile range of the data.

index should be considered as a conservative estimate of decomposition accuracy since it probably overestimates the decomposition errors. Namely, any disagreement between the two methods is considered an error while there are presumably cases in which one of the two methods is correct and the other incorrect. The agreement between the two decomposition methodologies in terms of concurrently detected discharges was above 82% in all cases, which is similar to the results obtained previously for decomposition of voluntary isometric contractions (Holobar *et al.* 2010). These results are in agreement with those by Holobar *et al.* (2012) on pathological tremor and prove that the high synchronization of motor unit discharges due to the electrical stimulation did not substantially influence the HDsEMG decomposition. The SIR results that have been calculated for 100 ms of the post-stimulus time verified this assertion (Fig. 9). For example, the post-stimulus SIR was lowest at 50% MVC for iEMG, whereas this score did not change with force for HDsEMG. This result is also in agreement with previous results on HDsEMG decomposition in tremor patients (Holobar *et al.* 2012). However, the SIR values of the two modalities cannot be compared because the signal-to-noise ratio of the surface EMG is much greater compared to that of the iEMG (Holobar *et al.* 2007; Nawab *et al.* 2010).

Two essential peri-stimulus time analyses, PSTH and PSF, were used to compare the characteristics of the reflex responses estimated with the two decompositions. While PSTH demonstrates the occurrence probability of motor unit discharges in peri-stimulus time, the PSF indicates their instantaneous discharge rates (Türker & Cheng, 1994). The disagreement values for PSTH were lower for inhibition than excitation, most likely due to a greater number of synchronous discharges after stimulation as well as to the stimulation artefact. Accordingly, the results showed that the disagreement factor between the two modalities was greater for excitation reflexes where an M-wave and/or stimulation artefact was observed. The stimulation artefact was indeed a disadvantage for the accuracy of the HDsEMG method (see Fig. 3*C* and *D*, the PSF-CUSUM of the HDsEMG shows rises at the stimulation moment) with respect to iEMG, since the surface electrodes have greater detection volume compared to fine wire EMG (Merletti *et al.* 1992). On the other hand, the M-wave can be avoided by tuning the stimulation intensity to elicit only the H-reflex. Indeed this is a common experimental condition for single motor unit reflex investigations since the occurrence of M-wave suppresses the H-reflex by the antidromic propagation of the action potentials. In this study, we have monitored the post-stimulus bipolar surface EMG and arranged the stimulation intensity to elicit only the H-reflex. However, despite the setting of the stimulation intensity below the M-wave elicitation, it could not be avoided in some cases that a small amplitude M-wave occurred, most likely due to small shifts in the location of the stimulation electrode during the experiment. The average disagreement was estimated in each bin to rule out MUs with a clear M-wave (Figs 6 and 7). For the excitation reflex the total disagreement was greater (70 ms of the PSTH) in the bins where the sharp synchronous responses were present, compared to the rest of the PSTH. However, this difference was significant only at 10% MVC force. The averaged disagreements were distributed uniformly with respect to the 200 ms post-stimulus time where the reflex responses and their derivatives were observed for both excitation and inhibition reflexes. These disagreements may not necessarily originate from HDsEMG decomposition errors only. It is indeed likely that the decomposition errors increased more with the force level for the iEMG than for the HDsEMG, as we have indirectly shown with the results obtained for 50% MVC force.

Latency, amplitude and duration of the reflexes, as measured from the PSF-CUSUM, were also compared between the two decomposition methods (Table 2). The results suggested that, although the shapes of the reflex responses did not match perfectly, the characteristics of the reflex that define synaptic delay (latency), strength of afferent input (amplitude), and the response time of the reflex system (duration) were the same for the two methods. The correlation between the accuracy of HDsEMG decomposition (as measured by PNR metric) and the PSTH disagreement was not significant (Fig. 5), suggesting that the loss in accuracy was not specifically associated with the decomposition errors during the post-stimulus time.

For the decomposition of the iEMG, several tools enable the user to edit and verify the results in each segment manually to improve the decomposition. However, this procedure is reliable only at low ($<$20% MVC) contraction forces. At higher contraction levels, the number of identified motor units and the level of superimposition are very high and it is usually not easy to rely on visual editing of the decomposition results. In these challenging conditions it is, in theory, possible to rely on the statistical properties of the residual EMG (after subtraction of the identified motor unit action potentials). The approach could be generalized to the multi-channel case for the surface EMG signals. This may provide an index of accuracy that could be calculated for selected segments of the decomposition.

In conclusion, the decomposition of the HDsEMG was validated in MU reflex responses in comparison with the decomposition of the iEMG. This comparison was mainly done at low forces. The proposed approach for the non-invasive investigation of single motor unit reflex responses is an accurate method when used to study reflex responses during low to moderate contraction forces. This

non-invasive method allows an accurate quantification of reflex responses for a larger number of individual motor units and thus provides a more representative alternative to iEMG decomposition for reflex studies.

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Additional information

Competing interests

The authors have no competing interest to declare.

Author contributions

U.Ş.Y., F.N. and O.S. contributed to the conception and design of the experiments, collection and assembly of data, data analysis and interpretation, and the writing of the manuscript. A.H., C.F., K.S.T. and D.F. contributed to the conception and design of the experiments, data interpretation and the writing of the manuscript. All authors approved the final version of the manuscript. The experiments were carried out in Department of Neurorehabilitation Engineering Laboratories, University Medical Centre Göttingen, Georg-August University, Göttingen, Germany.

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