Assessing TMS-induced D- and I-waves with spinal H-reflexes

Running head: Spinal H-reflexes to dissect D- and I-waves

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Abstract

Transcranial magnetic stimulation (TMS) of motor cortex produces a series of descending volleys known as D- (direct) and I- (indirect) waves. In the present study, we questioned whether spinal H-reflexes can be used to dissect D-waves, early and late I-waves from TMS. We therefore probed H-reflex facilitation at arrival times of D- and I-waves at the spinal level and thereby changed TMS parameters that have previously been shown to have selective effects on recruitment of D- and different I-waves. We changed TMS intensity and current direction, and applied a double-pulse paradigm known as short-interval intracortical inhibition (SICI). Experiments were conducted in flexor carpi radialis (FCR) in the arm and soleus (SOL) in the leg.

There were two major findings: I) In FCR, H-reflex facilitation showed characteristic modulations with altered TMS-parameters that correspond to the changes of D- and I-wave recruitment. II) H-reflexes in SOL did not, possibly because of increased interference from other spinal circuits. Therefore, the most significant outcome of this study is that in FCR, H-reflexes combined with TMS seem to be a useful technique to dissect TMS-induced D- and I-waves.

New and noteworthy:

Questions that relate to corticospinal function in pathophysiology and movement control demand sophisticated techniques informing about corticospinal mechanisms. We introduce a non-invasive electrophysiological technique that may be useful in describing such mechanisms in more detail, by dissecting D- and I-waves from transcranial magnetic stimulation (TMS). Based on the combination of spinal H-reflexes and TMS in the flexor carpi radialis muscle, the technique showed to measure selective effects on D- and I-waves from changing TMS parameters.

Keywords

transcranial magnetic stimulation (TMS); spinal H-reflex; motor cortex

Introduction

A single pulse of transcranial magnetic stimulation (TMS) over the primary motor cortex (M1) produces several descending volleys, termed D- (direct) and I- (indirect) waves, that can be measured by invasive recordings at spinal cord. TMS around threshold intensity preferentially evokes I-waves (Di Lazzaro et al. 2008). D-waves, early and later I-waves are argued to be produced by at least partially independent mechanisms (Di Lazzaro et al. 2012). D-waves are thought to originate from direct stimulation of corticospinal axons in the subcortical white matter or axon initial segment (Di Lazzaro et al. 1998a). Early and later I-waves are thought to result from the stimulation of less (early I-waves) and more (late I-waves) complex neural circuits of motor cortex and their descending connections to spinal motoneurones (Di Lazzaro et al. 2012). Investigating D- and I-waves has provided useful insight into the physiological mechanisms of TMS (Di Lazzaro and Rothwell 2014). However, a significant limitation is that these experiments are invasive and require patients who have implants in the spinal cord.

In healthy individuals, recruitment of spinal motoneurones from D- and different I-waves can be studied using single motor unit recordings (Day et al. 1989), but measurements are time-consuming and results biased towards the contribution of early arriving inputs. A potentially valuable and more easily applicable approach to dissect D- and I-waves in healthy individuals may be by assessing the time course of facilitation of spinal H-reflexes from TMS (Nielsen et al. 1993). A single TMS pulse facilitates H-reflexes for several milliseconds in the upper limb muscle flexor carpi radialis (FCR) and the lower leg muscle soleus (SOL) (Nielsen et al. 1995; Nielsen et al. 1993). In the present study, we questioned whether probing of H-reflexes in FCR and SOL at the arrival times of D- and I-waves at the spinal level would allow us to dissect these different waves. This cannot be taken for granted, as many spinal mechanisms like reciprocal (Cowan et al. 1986), presynaptic (Meunier and Pierrot-Deseilligny 1998) and Ib inhibition (Iles and Pisini 1992), as well as the contribution from propriospinal connections (Pauvert et al. 1998) can interfere with the synaptic input from D- and I-waves to spinal motoneurones and thus obscures contributions from the different

waves. To test our idea about the dissection of D- and I-waves with H-reflexes, we used TMS parameters that have previously been shown to have selective effects on recruitment of different D- and I-waves, and assessed whether we could see the same characteristic changes in H-reflex facilitation.

D- and early I-waves have been shown to be modulated by altering TMS current direction and stimulation intensity. A posterior-anterior (PA) directed TMS pulse tends to recruit I1 waves at threshold intensity, whereas an anterior-posterior (AP) directed pulse tends to recruit only later I-waves (Di Lazzaro et al. 2001a; Di Lazzaro et al. 2001c). Furthermore, AP pulses especially with higher TMS intensity were more likely to recruit D-waves than PA pulses (Di Lazzaro et al. 2001c). According to these findings, we would expect a smaller H-reflex facilitation at the arrival time of the I1 wave at the spinal level with AP than PA stimulation. Further, we would expect the first H-reflex facilitation to occur earlier with higher intensity AP pulses than with PA pulses.

To investigate the contribution of later I-waves to recruitment of spinal motoneurones with spinal H-reflexes, we applied a known paired-pulse protocol termed short interval intracortical inhibition (SICI), consisting of a subthreshold conditioning TMS pulse followed 2 to 5 ms later by a suprathreshold test TMS pulse (Kujirai et al. 1993). SICI was shown to suppress later I-waves but leaves earlier I-waves unchanged (Di Lazzaro et al. 2000; Di Lazzaro et al. 2001b; Di Lazzaro et al. 1998b). According to these findings, we would expect a smaller H-reflex facilitation at arrival times of later I-waves but not at arrival times of D-waves and earlier I-waves.

A second minor aim of the present study was to assess facilitatory effects of the H-reflex that occur immediately after the arrival of the last I-waves. H-reflex facilitation lasts much longer (> 20 ms) than the duration of D-and I-waves (around 6-8 ms). We wondered whether changes in TMS parameters would influence early and late facilitatory effects in a different manner with regards to their direction and magnitude. If the effects differ, we argue that it is

likely that the mechanism of late H-reflex facilitation differs from that of early H-reflex facilitation.

Materials and methods

Experiments and subjects

We performed two sets of experiments. In the first, we investigated the effect of TMS coil orientation (AP/PA) and TMS intensity, while in the second we applied SICI. In both sets, we collected separate measurements for the upper limb muscle FCR and for the lower limb muscle SOL. Thus, there were four types of experimental sessions, APPA_FCR (N = 15), APPA_SOL (N = 15), SICI_FCR (N = 17), and SICI_SOL (N = 16). In APPA experiments, all subjects (N = 15) participated in both FCR and SOL measurements. In SICI experiments, many of the subjects (N = 9) participated in both the FCR and SOL measurements. The FCR and SOL measurements in those subjects were conducted on different days with a minimum of 48 hours in between measurements. The order of measurements was randomized across subjects.

All participants were young (aged between 23 and 27 years), healthy, and had no contraindications to TMS (Rossi et al. 2009). All participants gave written informed consent to the procedures, which were approved by the local ethics committee of the Albert-Ludwigs-University in Freiburg (423/15).

Electromyography (EMG)

Surface EMG (EISA, Pfitec Biomedical Systems, Endingen, Germany) was recorded from the left flexor carpi radialis muscle (in experiments on FCR) and the left soleus (SOL) and tibialis anterior (TA) muscles (in experiments on SOL) using bipolar surface electrodes (Blue sensor P, Ambu®, Bad Nauheim, Germany). The preference for the left side was due to the arrangement of the setup. The skin was prepared (abrasion, cleaning) and electrodes were attached over the muscle belly with 2 cm interelectrode distance. A ground electrode was placed at the caput ulnae (in experiments on FCR) and at the tibial plateau (in experiments

on SOL). Impedance was below 10 kΩ. EMG signals were pre-amplified (FCR and SOL x 100; TA x 500), further amplified (2 x), bandpass filtered (10 – 1300 Hz) and sampled at 2 kHz. TA data were not further analysed since monitoring of TA activity was solely required for peripheral nerve stimulation (see below).

Electrophysiological stimulation techniques

Measurements were performed with subjects at rest. Subjects were seated comfortably in a custom-built laboratory seat with headrest. The subjects' legs were placed on a custom-built footboard in a stretched but relaxed position. The left arm was slightly flexed and pronated and placed on the subjects' lap. Subjects wore a forearm bandage which was stabilized with tape mounted to the chair (only in experiments on FCR).

Peripheral nerve stimulation (PNS)

H-reflexes were elicited with a constant current stimulator (DS7a, Digitimer®, Hertfordshire, UK) by stimulating the median nerve approximately 1-3 cm proximal to the elbow joint (in experiments on FCR) and the posterior tibial nerve at the popliteal fossa (in experiments on SOL). Stimuli consisted of square wave-pulses of 0.2 ms duration (median nerve) and 0.5 ms (tibial nerve) (Leukel et al. 2015). A graphite coated rubber pad of 5 x 5 cm was used as anode and was fixed proximal to the olecranon (in experiments on FCR) and at the anterior aspect of the knee just underneath the patella (in experiments on SOL). A custom-made round pad (1 cm diameter) was used as cathode and moved stepwise to detect the optimum position for eliciting H-reflexes in the respective muscle. The optimum was defined as the site where low stimulation intensity (in between 5 and 30 mA) elicited a consistent H-reflex with minimal M-wave. Further, in experiments on SOL, stimulation at this optimum site did not or only little activate the common peroneal nerve, which was tested with parallel recordings from TA (TA H-reflex and TA M-wave). Note that the latter was not tested for FCR, as we unfortunately did not record from the antagonist muscle extensor carpi radialis. After the

optimum site was found, a self-adhesive cathode (Blue sensor P, Ambu®, Bad Nauheim, Germany) was fixed at this site.

We determined the maximum H-reflex (Hmax) and the maximum M-wave (Mmax) after recording an H/M recruitment curve at the beginning and at the end of an experiment. Hmax and Mmax values obtained at the beginning of the experiment were required for setting the PNS intensity when recording conditioned H-reflexes (see "*Conditioned H-reflexes by TMS*").

Transcranial magnetic stimulation (TMS)

Single-pulse and paired-pulse TMS were applied over the contralateral M1 hand/arm area (experiments on FCR) and leg area (experiments on SOL) using a Magstim® 200^2 stimulator with a BiStim unit (Magstim® Company Ltd., Whitland, UK) and a 70-mm figure-of-eight batwing coil for experiments APPA_FCR, APPA_SOL, SICI_SOL, and a 50-mm figure-of-eight coil for experiment SICI_FCR. The reason for using a smaller coil was that we performed SICI experiments after completing APPA experiments, and only after the APPA experiments realized that a 50-mm coil, producing a more focal stimulation, is sufficient for our purpose. The handle of the coil was mounted to a stand that was positioned on top of the chair (Manfrotto® Magic Arm, Lino Manfrotto & Co, Cassola, Italy). Brainsight TMS navigation (Brainsight 2®, Rogue Research, Montreal, Canada) was used to monitor the position of the coil relative to the skull to ensure that the set coil position remained the same throughout all stimulations.

The optimum site for evoking motor evoked potentials (MEPs) was determined by a mapping procedure. The optimum was defined as the site where clear MEPs could be evoked with the lowest possible stimulation intensity. For FCR, the coil was held tangentially on the scalp at an angle approximately 45° to the mid-sagittal plane with the handle pointing laterally and posteriorly (inducing a PA directed current). For SOL, the coil was placed tangentially on the scalp, the handle pointed posteriorly at an angle of 0° with respect to the midline (inducing a PA directed current).

Resting motor threshold (RMT) was determined as the minimum stimulator output (in % of

maximum stimulator output, MSO) required to evoke MEPs of ~50 µV in at least three out of five consecutive trials applied at the same intensity (Rossini et al. 1994). In experiments APPA_FCR and APPA_SOL, resting motor thresholds (RMT) were determined separately for PA and AP stimulation. For the AP condition, the position of the coil was identical but rotated by 180°.

Conditioned H-reflexes by TMS

Conditioning of H-reflexes with TMS was applied in accordance with previous studies (e.g. Nielsen et al., 1993; Leukel et al., 2012). Two stimuli were applied together: PNS and TMS. The objective of this technique is to promote coincidence of TMS-induced activity and afferent activity by PNS at the spinal level (see Figure 1 A). Therefore, PNS was applied relative to TMS with different temporal delays, termed interstimulus intervals (ISIs). Negative ISIs indicate that PNS precedes TMS and positive ISIs indicate the opposite.

The combination of TMS and PNS produces a conditioned H-reflex. The TMS-induced activity triggers a changed recruitment of spinal motoneurones compared to recruitment of spinal motoneurones from PNS alone (see Figure 1B).

When both TMS and PNS are applied at the same time, the fastest corticospinal volley typically recruits FCR and SOL spinal motoneurones earlier than recruitment from afferent fibres. The time interval when the earliest arriving synaptic input from the descending corticospinal volley coincides with the earliest arriving synaptic input from afferent volleys at the spinal level has been termed “early facilitation” in previous studies (e.g. Leukel et al. 2015; Nielsen et al. 1993; Taube et al. 2015b) (see also “*Data analysis*”).

ISIs of -7/-6 ms to +8 ms (in experiments on FCR) and -5 ms to +8 ms (in experiments on SOL), in 1 ms steps, were tested in the present study. The range of ISIs for SOL was selected based on our experience (Taube et al., 2011; Leukel et al., 2012; Leukel et al., 2015; Taube et al., 2015) that the early facilitation occurs at around ISI -3 ms (± 2 ms) in most of the subjects. Thus, this range of ISIs with the most negative ISI at -5 ms allows to detect the early facilitation. For FCR, based on a lack of prior experience with this muscle,

we decided to include more negative ISIs for testing, and additionally used ISIs -7 ms and -6 ms (in experiments APPA), and ISI -6 ms (in experiments SICI), respectively. For all measurements, electrical stimulation was adjusted at an intensity to evoke H-reflexes of 15 to 25% of the respective Mmax (Crone et al., 1990), on the upsloping part of the H/M recruitment curve. For experiments APPA and SICI, TMS was applied at suprathreshold and subthreshold intensity (see "*conditioned H-reflex protocols*").

Short interval intracortical inhibition (SICI)

In experiments SICI_FCR and SICI_SOL, SICI was combined with H-reflexes. This means a second, subthreshold TMS pulse (S1) was included which preceded the suprathreshold TMS pulse (S2) used for H-reflex conditioning (both with PA current direction). S1 preceded S2 by 2.5 ms (see Figure 1C).

The intensity of the conditioning S1 pulse was determined by a testing procedure that was performed before recording conditioned H-reflexes. This test procedure consisted of several blocks of trials. In each block, S2 alone and the combination of S1 and S2 with a delay of 2.5 ms ($SICI_{2.5}$) were applied in a randomized order. Twenty MEPs (10 for S2 alone, 10 for $SICI_{2.5}$) were recorded in each block. The pause between successive trials was 4 s. The stimulation intensity for S1 was varied in-between blocks, ranging from 55% of RMT to 80% of RMT. The objective of this testing procedure was to find the highest decreasing effect of S1 on the MEP size produced by S2. The stimulation intensity of S1 producing the maximum reduction of the S2 MEP was used for H-reflex conditioning (see Table 1).

Conditioned H-reflex protocols

For experiments APPA_FCR and APPA_SOL: Conditioned H-reflexes at each ISI were recorded 15 times with 110% and also 90% RMT (both with PA and AP coil orientation). Unconditioned H-reflexes (for PA and AP conditions, respectively) and unconditioned MEPs (PA and AP, both with 110% and 90% RMT) were also recorded 15 times. All parameters were tested at once, in a pseudo-randomized design, to avoid biased results by changes in

basic parameters like the H-reflex size and/or possible interference effects induced by the different conditions. We applied 15 recording blocks for each coil orientation. One recording block consisted of randomized testing of conditioned H-reflexes at all ISIs (1 x each ISI) with both stimulation intensities plus control parameters (1 x unconditioned H-reflex and 1 x unconditioned MEPs) with a given coil orientation (PA and AP). Five continuous recording blocks with PA and AP stimulation were performed alternatingly. We started either with PA or AP stimulation in a pseudorandomized order. The delay between subsequent stimuli was always 4 s to avoid changes in post activation depression of the H-reflex (Crone and Nielsen 1989).

For experiments SICI_FCR and SICI_SOL: Conditioned H-reflexes at each ISI were recorded 15 times for each of the three different conditions: S2 stimulation (baseline condition), S1 stimulation, and S1/S2 combined stimulation (SICI delay of 2.5 ms). Unconditioned H-reflexes and MEPs (S2 stimulation, S1 stimulation, SICI) were also recorded 15 times. All parameters were tested at once, in a pseudo-randomized design, to avoid biased results by changes in basic parameters like the H-reflex size and/or possible interference effects induced by the different conditions. We applied 15 recording blocks. One recording block consisted of randomized testing of conditioned H-reflexes at all ISIs (1 x each ISI with S2 stimulation, S1 stimulation, SICI) plus control parameters (1 x unconditioned H-reflex and 1 x MEPs (from S2 stimulation, S1 stimulation, SICI). The delay between subsequent stimuli was always 4 s to avoid changes in post activation depression of the H-reflex (Crone and Nielsen 1989).

Data analysis

Peak-to-peak amplitudes of all electrophysiological responses were calculated from the unrectified FCR and SOL EMG.

We identified the early facilitation in each experiment for the baseline conditioned H-reflex curve (APPA experiments: PA 110% RMT; SICI experiments: S2 stimulation). We therefore computed uncorrected paired Student’s t-tests for conditioned H-reflexes between all

consecutive negative ISIs (e.g. for SOL: -5 ms vs. -4 ms, -4 ms vs. -3 ms, …), and between conditioned H-reflexes at all negative ISIs and the unconditioned H-reflexes (e.g. for SOL: -5 ms vs. unconditioned H-reflexes, -4 ms vs. unconditioned H-reflexes, ...). The first significant increase in the size of the conditioned H-reflexes from more negative to less negative ISIs (i.e. for SOL: -5 ms, -4 ms, -3 ms) was denoted early facilitation ($p < 0.05$ in one or both of the aforementioned t-tests). Usually, the statistical result matches with the visual impression of a sharp facilitation of mean conditioned H-reflexes at this ISI (early facilitation) and non-facilitated values at more negative ISIs. However, in 8 measurements the statistical tests yielded no significant result. In these measurements, we denoted the early facilitation solely based on visual inspection of the conditioned H-reflex plot (Taube et al. 2015a).

The ISI denoted as early facilitation in the baseline condition (APPA experiments: 110% RMT; SICI experiments: S2 stimulation) of each experiment was also taken as “early facilitation” for the other conditions tested in the same experiment. For statistical comparison, there is no benefit to denote the early facilitation also for the other conditions. It could even be a disadvantage, as the denotation may contain an error, in case no statistical significance can be reached.

Mean conditioned H-reflexes at each ISI were expressed as the percentage of the intra-individual reference H-reflex. The reference H-reflex was computed as the mean of the unconditioned H-reflexes.

Finally, the referenced conditioned H-reflex curves of the subjects were aligned to the ISI of the individual early facilitation. The ISIs in the *“Results”* section refer to this alignment, and are consequently named EFD (delay with respect to the early facilitation in ms) rather than ISI.

In summary, this normalization procedure described in the previous paragraphs contains three steps: first, we determined the early facilitation for the baseline conditioning curve and used this ISI as “early facilitation” also for the other conditions tested in the same measurement. Second, we referenced the mean conditioned H-reflex at each ISI to the mean unconditioned H-reflex. Third, we aligned the H-reflex conditioning curves to the individual

early facilitation and named the ISI according to this alignment EFD (early facilitation delay) to allow for statistical comparisons across subjects.

Statistics

All data sets showed normality and homogeneity, tested by the Kolmogorov-Smirnov test and the Levene's test, respectively.

For referenced conditioned H-reflexes in the APPA_FCR and APPA_SOL experiments, we performed a three-way repeated measures ANOVA for FCR and SOL separately with factors COIL ORIENTATION (PA, AP), INTENSITY (110% RMT, 90% RMT) and EFD (EXP_SOL: 2 x 2 x 12; EXP_FCR: 2 x 2 x 12). For FCR, the factor EFD contained all intervals from EFD -2 ms to EFD +9 ms whereas for SOL the factor EFD encompassed all intervals from EFD -1 ms to EFD +10 ms. These were time intervals with no missing values from subjects. Missing values in experiments APPA_FCR resulted in case the early facilitation occurred at a more positive ISI than -2 ms. This was the case in one subject, displaying the early facilitation at ISI -1 ms. Missing values in experiments APPA_SOL resulted in case the early facilitation occurred at a more negative or positive ISI than -3 ms. This was the case in six subjects, three subjects where the early facilitation occurred at ISI -4 ms and three subjects where the early facilitation occurred at ISI -2 ms.

For referenced conditioned H-reflexes in the SICI_FCR and SICI_SOL experiments, we performed two-way repeated measures ANOVAs for FCR and SOL separately with factors TMS PULSE (S2 stimulation, S1 stimulation, SICI) and EFD (SICI_SOL: 2 x 10; SICI_FCR: 2 x 13). The factor EFD for FCR contained all intervals from EFD -2 ms to EFD +10 ms. For SOL, the factor EFD encompassed all intervals from EFD 0 ms to EFD +9 ms. These were time intervals with no missing values from subjects. Missing values in experiments SICI_SOL resulted in case the early facilitation occurred at a more negative ISI than -4 ms or a more positive ISI than -2 ms. This was the case in three subjects, one subject where the early facilitation occurred at ISI -5 ms and two subjects where the early facilitation occurred at ISI -1 ms.

Paired Student's t-tests were performed for all other a-priori and post-hoc analyses. Results obtained from multiple comparisons were corrected by the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995).

The level of significance was set to $p < 0.05$ for all tests. Mean values and standard error of the mean (SEM) are reported. Greenhouse-Geisser corrected values for ANOVAs are reported in case sphericity of the tested samples was violated (Mauchly's test). Data were statistically analysed with SPSS software 24.0 (SPSS®, Chicago, IL, USA).

Results

APPA_FCR and APPA_SOL

TMS conditioned H-reflexes

Results from ANOVAs (Table 2) and post-hoc t-tests (Figure 2 and Figure 3) can be summarized as follows:

- In FCR, TMS at 110% RMT facilitated H-reflexes more than at 90% RMT at all time intervals from EFD 0 ms to EFD +11 ms. Importantly, AP stimulation at 110% RMT also facilitated H-reflexes at EFD -1 ms.
- In SOL, stimulation at 110% RMT facilitated H-reflexes more than at 90% RMT for EFD 0 ms to EFD +5 ms (PA stimulation) and +6 ms (AP stimulation). In contrast, at EFDs +7 ms to +11 ms the amount of H-reflex facilitation did not differ between 110% RMT and 90% RMT.
- Changes in coil orientation yielded no significant test outcome from Benjamini-Hochberg corrected t-tests. Indeed, for SOL none of the p-values dropped below 0.05 (the uncorrected level of significance). However, for FCR this was very different. In fact, comparison at EFD 0 ms revealed that there was significantly weaker H-reflex facilitation with AP stimulation compared to PA stimulation at both stimulation intensities (Figure 2B). Conversely there was more facilitation at EFD -1 ms using AP stimulation at 110% RMT.

MEP amplitude

In FCR and SOL, the amplitude of MEPs evoked at 110% RMT did not differ between PA and AP stimulation (t-tests FCR: $p = 0.56$; SOL: $p = 0.53$). The EMG level was significantly smaller at 90% RMT compared to 110% RMT in FCR (t-tests PA: $p < 0.001$; AP: $p < 0.001$) and SOL (t-tests PA: $p < 0.01$; AP: $p < 0.001$). In fact, subthreshold TMS at 90% RMT produced no MEP (Figure 4).

H-reflex/M-wave

In FCR, Hmax and Mmax were significantly lower at the end compared to the beginning of the measurement (Student's t-test Hmax: $p < 0.05$; Mmax: $p < 0.01$). In SOL, Hmax and Mmax were not different between pre- and post-measurement (Student's t-test: Hmax: $p = 0.7$; Mmax: $p = 0.25$). Importantly, during H-reflex conditioning measurements, the size of FCR and SOL unconditioned H-reflexes did not differ between PA and AP stimulation (Student's t-test FCR: $p = 0.53$; SOL: $p = 0.81$). H-reflex/M-wave amplitudes are presented in Figure 4.

<u>SICI FCR and SICI SOL</u>

TMS conditioned H-reflexes

Results from ANOVAs (Table 2) and post-hoc t-tests (Figure 5) can be summarized as follows:

- In FCR, SICI reduced facilitation of H-reflexes only at later time intervals. This depression started at EFD +3 ms.
- The effects of SICI were different in SOL. At EFD +1 ms, H-reflexes tended to be facilitated. Thereafter, SICI reduced H-reflex facilitation at EFD +2 ms, EFD +3 ms and EFD +4 ms. Interestingly, facilitation of H-reflexes at late time intervals (from EFD +8 ms) was again strengthened by SICI.

- In FCR and SOL, S1 stimulation produced smaller H-reflex facilitation than S2 stimulation. It is noteworthy that the conditioning S1 pulse given alone facilitated H-reflexes in some subjects.

MEP amplitude

In FCR and SOL, MEPs were different between tested conditions. The SICI MEP was smaller than the MEP with S2 stimulation (Student's t-test FCR: $p < 0.001$; SOL: $p < 0.001$). S1 stimulation did not produce a MEP (Figure 6).

H-reflex and M-wave

In FCR and SOL, Hmax and Mmax were not different between the pre- and post-test (Student's t-test Hmax FCR: $p = 0.71$; SOL: $p = 0.23$; Mmax FCR: $p = 0.74$; SOL: $p = 0.65$). H-reflex/M-wave amplitudes are presented in Figure 6.

Discussion

The main objective of the present experiments was to test whether H-reflexes can be useful to dissect D- and I-waves from TMS. We therefore compared the facilitation of H-reflexes with two different current directions of TMS and two levels of TMS intensity in the first set of experiments, and then explored the effects of SICI in a second set of experiments. In both sets, we evaluated effects on H-reflex facilitation in FCR and SOL. This resulted in a number of interesting findings:

Experiments APPA:

- In FCR but not SOL, stimulation with AP current facilitated the H-reflex less than PA stimulation at EFD 0 ms.
- In FCR but not SOL, AP stimulation with higher TMS intensity facilitated H-reflexes at EFD -1 ms, which is a time interval immediately preceding the presumed arrival of the first I-wave.

- Increasing stimulation intensity from 90% RMT to 110% RMT strengthened facilitation of H-reflexes at all time intervals, except in SOL where H-reflex facilitation at later time intervals (EFDs +7 ms to +11 ms) remained unchanged.

Experiments SICI:

- In FCR, the reduction of H-reflex facilitation by SICI started at EFD +3 ms.
- In SOL, the reduction in H-reflex facilitation started earlier than in FCR, at EFD +2 ms. Interestingly, we also observed facilitation of H-reflexes by SICI, at the late time point EFD +8 ms, and a trend towards a facilitation at EFD +1 ms.
- The subthreshold conditioning S1 pulse given alone facilitated H-reflexes, suggesting that it can induce descending activity even at a mean intensity of around 70% RMT.

Altogether, these results indicate that contribution of D- and different I-waves to recruitment of spinal motoneurones can be assessed with spinal H-reflexes in the arm muscle FCR, but not in the lower leg muscle SOL. Further, according to the second aim of the study, in SOL later H-reflex facilitation that occurs after the arrival of D- and I-waves seems to be caused by different mechanisms than early H-reflex facilitation.

<u>Changing TMS current direction and intensity</u>

It is known that AP TMS at stimulation intensity around threshold tends to recruit only later I-waves, whereas PA TMS preferentially recruits early I-waves (Di Lazzaro et al. 2001c; Di Lazzaro et al. 2012). Furthermore, AP stimulation to the arm/hand area at higher TMS intensities can recruit D-waves (Di Lazzaro et al. 2001a; Di Lazzaro et al. 2001c). According to these findings, at low TMS intensity we would expect the earliest facilitation of H-reflexes, which has been considered to be generated by transsynaptic activation of fast conducting corticospinal output neurons (Nielsen et al. 1995; Nielsen et al. 1993), to be smaller with AP compared to PA stimulation. We would expect this effect because early descending corticospinal volleys would dominate after PA TMS compared to AP TMS. Furthermore, we

444 would expect higher intensity AP stimulation to facilitate H-reflexes even earlier than the
445 facilitation from the I1-wave, compatible with H-reflex facilitation from a D-wave. Indeed, our
446 results confirm these hypotheses. AP stimulation produced less H-reflex facilitation than PA
447 TMS at EFD 0 ms. Further, AP stimulation at 110% RMT facilitated H-reflexes at EFD -1 ms
448 compared to AP stimulation with 90% RMT and PA stimulation. Regarding the latter result,
449 future studies may additionally apply TMS with latero-medial (LM) current flow to investigate
450 the contribution of D-waves in more detail (Di Lazzaro et al. 2001c).
451 Interestingly, we saw these effects only in FCR but not in SOL. This difference between
452 muscles may be caused by the anatomy of the arm and leg regions of the motor cortex. In
453 the arm area, neural elements may exist that are more sensitive to the AP/PA direction of
454 stimulus current. If the same elements exist in the leg area, then their orientation may be
455 different, perhaps because they are positioned within the bank of the longitudinal fissure
456 rather than exposed on the lateral surface of the brain.
457
458 Another difference between the two muscles we observed was that only in SOL higher TMS
459 intensity did not increase H-reflex facilitation at later time intervals albeit facilitation was
460 increased at early intervals. This finding suggests that H-reflex facilitation at early and later
461 time intervals is produced by different mechanisms. We will refer to this issue again in the
462 following paragraph.
463
464 Applying SICI
465 SICI in FCR reduced facilitation of H-reflexes only at later time intervals (EFD +3 ms and
466 more positive EFDs). By definition, the time interval EFD +3 ms tests synaptic input to spinal
467 motoneurones that occurs 3 ms after the fastest corticospinal volley reached the spinal level.
468 The reduction in H-reflex facilitation at EFD +3 ms is therefore consistent with the timing
469 shown with direct recordings of descending volleys. SICI in most cases depressed I3-waves
470 and subsequent I-waves (Di Lazzaro et al. 2000; Di Lazzaro et al. 2012; Di Lazzaro et al.
471 1998b). Keeping in mind that distinct I-waves are typically 1.5 to 1.6 ms apart, the I3-wave

represents neural activity that descends with a delay of approximately 3 ms after the fastest conducted corticospinal volley.

In contrast to clear timing effects in FCR that were consistent with the literature, SICI in SOL produced inconsistent results. The depression of H-reflexes by SICI started at EFD +2 ms, and this is earlier than the onset of suppression of I-waves reported in the literature (Di Lazzaro et al. 2001b). Further, we observed an increased facilitation of the H-reflex with SICI at EFD +1 ms (only trend) and at later time intervals (significant difference at EFDs +8). The unexpected facilitation of H-reflexes at EFD +1 ms with SICI may result from a spinal effect. Effects at EFD +1 ms can be prone to disynaptic reciprocal inhibition from TA interneurons, acting depressive at SOL spinal motoneurones (Cowan et al. 1986). In the SICI condition, the S1 pulse is applied 2.5 ms before S2. Thus, at EFD +1 ms in the SICI condition, to estimate the contribution from the S1 pulse we have to look at EFD +3.5 ms. As we can see in Figure 5, the S1 pulse given alone facilitates H-reflexes at EFDs +3 and +4 ms. Thus, the S1 effect in the SICI condition at EFD +1 ms is presumably facilitatory. The S1 pulse in the SICI condition may counteract the depression from reciprocal inhibition at EFD +1 ms, and this would appear like a higher facilitation of conditioned H-reflexes as shown in Figure 5. In contrast to EFD +1 ms, we have no mechanistic explanation for the strengthened facilitation at EFDs +8. However, this finding together with our findings about the differential effect on H-reflex facilitation by changes in TMS intensity (APPA experiments) support different underlying mechanisms of early and later H-reflex facilitation in SOL. Clearly, future studies should investigate the origin of H-reflex facilitation at early and later time intervals in SOL in more detail.

<u>Subthreshold TMS can trigger descending activity</u>

We observed that stimulation with 90% RMT in the APPA experiments and S1 stimulation in SICI experiments induced descending activity. Thus, the subthreshold pulse was not truly subthreshold for evoking subcortical activity. This finding is not surprising, as several studies before emphasized that TMS not producing a compound potential is nevertheless capable of

inducing significant downstream activity (Day et al. 1989; Nielsen et al. 1993; van der Linden and Bruggeman 1993). Concerning the results of the present study, the finding of descending activity induced by the S1 pulse in the SICI experiments does of course not indicate that SICI effects are spinal, but they do mean that the effects are not necessarily purely cortical. Thus, the possibility of a spinal origin should be considered when interpreting e.g. treatment/training-induced changes of SICI. Certainly, effects at some EFDs in our study are more likely to have a strong cortical component. For instance, the reduction of H-reflex facilitation at EFD +3 ms in FCR is likely to be of cortical origin, simply because S1 alone triggers a facilitation at the spinal level which is opposite to the reduced facilitation seen when combining S1 and S2.

One may think that the higher the S1 intensity relative to RMT the more likely it is that S1 induces downstream activity. However, this was not the case, there was no correlation between the two measures (data not shown in this manuscript). The practical result is that the estimate of whether subcortical activity is induced by S1 cannot be based on the stimulation intensity alone. Potential effects have to be measured.

Limitations

When corticospinal contributions to recruitment of spinal motoneurones are assessed with H-reflexes, a significant limitation is the potential influence of other spinal circuits. We discussed this for SOL in the previous paragraphs, but spinal mechanisms could of course also contribute to changes in H-reflex facilitation in FCR. For instance, presynaptic inhibition of Ia afferents was shown to be modulated in FCR by descending activity from TMS (Meunier 1999). TMS was reported to increase presynaptic inhibition in FCR, and to decrease presynaptic inhibition in SOL (Meunier and Pierrot-Deseilligny 1998). Further, the strength of depression of spinal motoneurone activity from Ib afferents can be changed by descending input and thus modulate the H-reflex size. The H-reflex is not truly a monosynaptic response produced by Ia afferent input but may involve contribution from Ib afferents, depending on the balance of Ia afferent and Ib afferent excitation (Marchand-Pauvert et al. 2002; Pierrot-

Deseilligny and Burke 2005). Strong descending activity can interact with strong group I inhibitory activity and reduce spinal inhibition, thus increase the H-reflex size (Iles and Pisini 1992; Lundberg and Voorhoeve 1962). Such spinal effects (changes in presynaptic inhibition, Ib inhibition) could contribute to the time course of H-reflex facilitation in response to the TMS test pulse. In fact, out of the main results of the present study in FCR, the reduced facilitation of H-reflexes with SICI at EFD +3 ms could be explained by a spinal effect, caused by increased presynaptic inhibition from the conditioning (S1) pulse (Meunier and Pierrot-Deseilligny 1998). It takes several milliseconds from the arrival of the descending volley at the spinal level to change presynaptic inhibition (Meunier and Pierrot-Deseilligny 1998), and thus the S1 pulse is suitable as it arrives some milliseconds earlier at the spinal level than the S2 pulse. However, this would require that S1 causes a depression of the H-reflex prior to and/or at the time when the depression with SICI occurs, i.e. at and/or before interval EFD +5.5 ms in the S1 condition in the present experiments. As can be seen from Figure 5, there is no such a depression from the S1 pulse. Thus, in the present experiments, spinal mechanisms could potentially bias but are unlikely to explain main results obtained in FCR. The timing of effects in H-reflexes fits very accurately to the timing of effects found with direct recordings at the spinal cord. D- and I-waves measured at the spinal level are not influenced by spinal mechanisms that we discussed, and thus our results are assumed to be significantly caused by cortical origin.

Another issue that needs also to be considered when mechanistically interpreting effects is the potential contribution from propriospinal neurons to recruitment of spinal motoneurones. TMS may excite the propriospinal system (Mazevet et al. 1996; Pauvert et al. 1998), and this can interfere with the contribution from cortically-generated D- and I-waves to facilitation of H-reflexes.

Conclusions

Altogether, our results indicate that in FCR, conditioning of H-reflexes with TMS can be a useful technique to dissect out individual effects of D-waves, early and late I-waves. In SOL,

this method is not so useful, as H-reflex facilitation appears to be more strongly influenced by spinal circuits. Furthermore, our results indicate that in SOL, mechanisms underlying H-reflex facilitation are different at later time intervals compared to earlier time intervals. Finally, our results confirm that a TMS pulse subthreshold for triggering a FCR and SOL compound potential may still be able to induce significant subcortical activity.

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Disclosures

The authors declare no conflict of interest.

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662 **Figure legends**

663 Figure 1 A illustrates the electrophysiological method of combining TMS with H-reflexes
664 (TMS H-reflex conditioning). TMS and PNS were applied together with different delays
665 between the two stimuli (in 1 ms steps), so that TMS-triggered activity and the afferent volley
666 from PNS coincided at the spinal motoneurones (here illustrated for SOL). Part B of the
667 graph shows the electrophysiological responses recorded with surface EMG. TMS triggered
668 an MEP when applied above threshold intensity, PNS generated a H-reflex. TMS (with
669 stimulation intensities above (110% RMT) and below (90% RMT) threshold intensity)
670 combined with PNS produced a conditioned H-reflex. Note the higher peak-to-peak
671 amplitudes of conditioned H-reflexes as compared to the unconditioned H-reflex. Part C of
672 the figure displays the three stimulation conditions applied in the SICI experiments. Note that
673 the vertical bars indicate the relative instants when the stimuli were triggered. The charts
674 illustrate testing at ISI -3 ms. For SICI, the delay between the S1 pulse and the S2 pulse was
675 kept constant (2.5 ms) throughout the stimulations.
676
677 Figure 2 A shows referenced conditioned H-reflexes (grand mean values and SEM) of APPA
678 experiments. The graphs display comparisons between coil orientations PA and AP, for FCR
679 (left side) and SOL (right side). Results from post-hoc Student's t-tests (p-values) and the
680 corresponding corrected significance levels (correct.) are illustrated in the tables at the
681 bottom. Part B of the figure displays single subject differences of referenced conditioned H-
682 reflexes at the early facilitation (EFD 0 ms) between conditions AP stimulation and PA
683 stimulation. Negative values indicate higher H-reflex facilitation by PA stimulation.
684
685 Figure 3 shows referenced conditioned H-reflexes (grand mean values and SEM) of APPA
686 experiments. The graphs display comparisons between stimulation intensities 110% RMT
687 and 90% RMT, for FCR (left side) and SOL (right side). Results from post-hoc Student's t-
688 tests (p-values) and the corresponding corrected significance levels (correct.) are illustrated
689 in the tables at the bottom. Significant differences between conditions are marked in green.

690

691 Figure 4 displays grand mean values and SEM of control parameters of APPA experiments:
692 MEP amplitude (upper part) and Mmax, Hmax and reference H-reflex (Href) (lower part).

693

694 Figure 5 The upper part shows referenced conditioned H-reflexes (grand mean values and
695 SEM) of the three conditions tested in the SICI experiments, for FCR (left side) and SOL
696 (right side). Results from post-hoc Student's t-tests (p-values) and the corresponding
697 corrected significance levels (correct.) are illustrated in the tables at the bottom. Significant
698 differences between conditions are marked in green. The lower part of the figure displays
699 differences in mean referenced conditioned H-reflexes between the SICI and the S2
700 stimulation condition. Negative values indicate lower H-reflex facilitation by SICI.

701

702 Figure 6 displays grand mean values and SEM of control parameters of SICI experiments:
703 MEP amplitude (upper part) and Mmax, Hmax and reference H-reflex (Href) (lower part).

704

705 Table 1 shows TMS intensities (in % of the maximum stimulator output) and how these relate
706 to resting motor threshold (RMT). Data display grand mean values and SEM.

707

708 Table 2 shows results of the ANOVAs performed for the APPA experiments and the SICI
709 experiments. Significant results are marked in green.

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719 Table 1

720

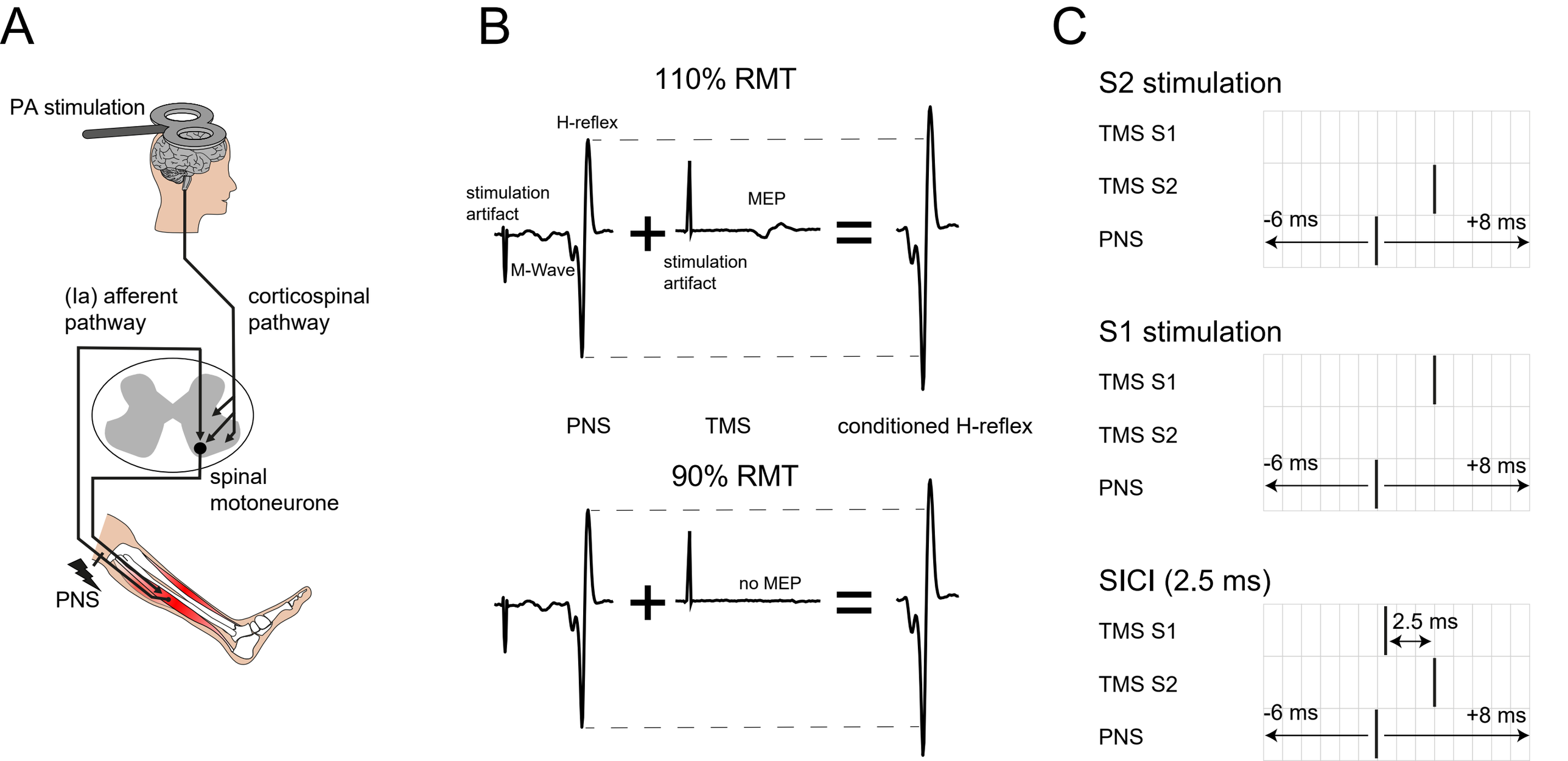
	FCR	***SOL***
APPA experiments		
RMT (PA)	38 ± 2	60 ± 2
RMT (AP)	47 ± 2	60 ± 3
High stimulation intensities (110%)		
PA (% of RMT)	43 ± 2 (111.9 ± 0.7)	67 ± 2 (112.2 ± 1.1)
AP (% of RMT)	52 ± 2 (110.9 ± 0.3)	67 ± 3 (111.7 ± 1.0)
Low stimulation intensities (90%)		
PA (% of RMT)	34 ± 1 (88.4 ± 0.8)	54 ± 2 (89.5 ± 0.3)
AP (% of RMT)	41 ± 2 (87.5 ± 0.8)	54 ± 2 (89.6 ± 0.3)
SICI experiments		
RMT	55 ± 1	60 ± 2
S2 Intensity (% of RMT)	65 ± 2 (116.7 ± 1.0)	68 ± 2 (113.1 ± 1.0)
S1 Intensity (% of RMT)	38 ± 1 (69.4 ± 1.3)	40 ± 1 (67.5 ± 1.0)

721

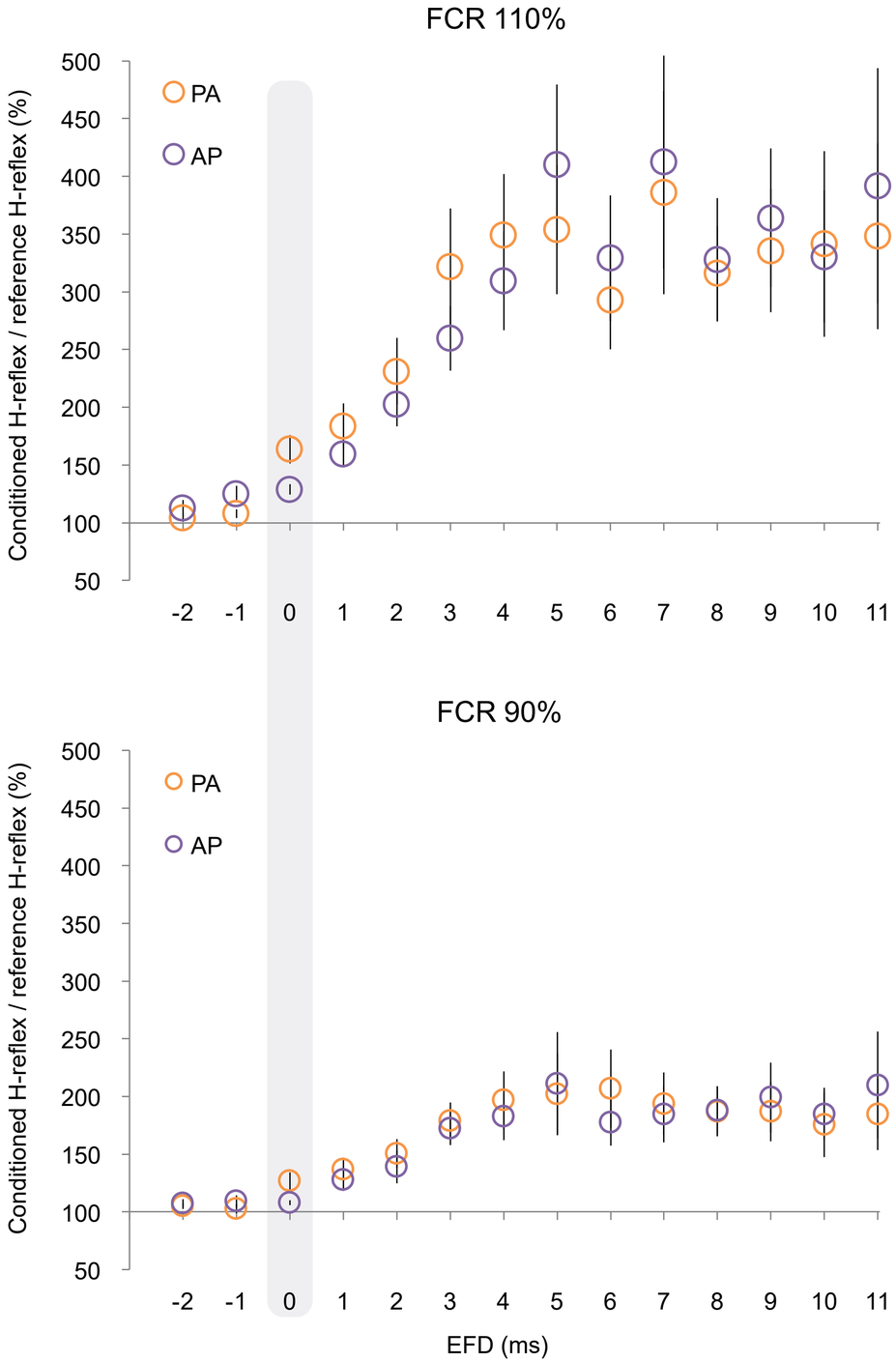
722

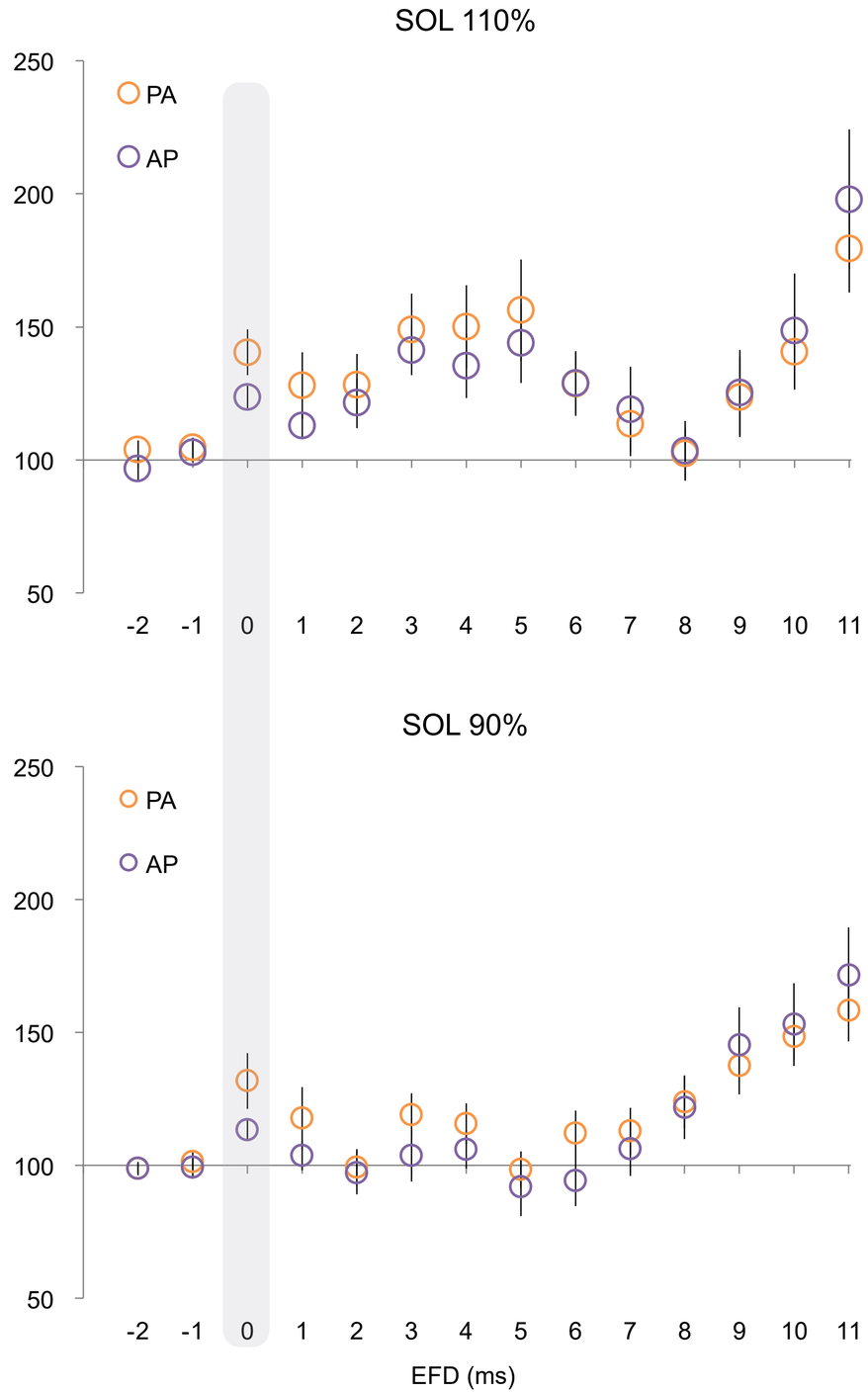
Table 2

	FCR	***SOL***
APPA experiments		
Main effects:		
COIL ORIENTATION	$F_{1,14} = 0.38$, $p = 0.55$	$F_{1,14} = 0.06$, $p = 0.81$
INTENSITY	$F_{1,14} = 18.2$, $p < 0.01$	$F_{1,14} = 13.7$, $p < 0.01$
EFD	$F_{1.5,20.6} = 12.1$, $p < 0.001$	$F_{2.6,35.8} = 4$, $p < 0.05$
Interactions:		
COIL ORIENTATION x INTENSITY	$F_{1,14} = 0.6$, $p = 0.45$	$F_{1,14} = 1.1$, $p = 0.31$
COIL ORIENTATION x EFD	$F_{3.3,45.5} = 2.16$, $p = 0.10$	$F_{3.3,45.8} = 1.62$, $p = 0.19$
INTENSITY x EFD	$F_{2.4,33.7} = 6.8$, $p < 0.01$	$F_{3.7,51.6} = 8.1$, $p < 0.001$
COIL ORIENTATION x INTENSITY x EFD	$F_{3.1,42.9} = 2.22$, $p = 0.10$	$F_{3.6,49.9} = 0.87$, $p = 0.48$
SICI experiments		
Main effects:		
TMS PULSE	$F_{1,15.8} = 30.3$, $p < 0.001$	$F_{1.5,22} = 2$, $p = 0.16$
EFD	$F_{2.1,30.4} = 13$, $p < 0.001$	$F_{2.4,36.6} = 2.2$, $p = 0.11$
Interactions:		
TMS PULSE x EFD	$F_{24,360} = 9.1$, $p < 0.001$	$F_{4.6,69} = 8.6$, $p < 0.001$

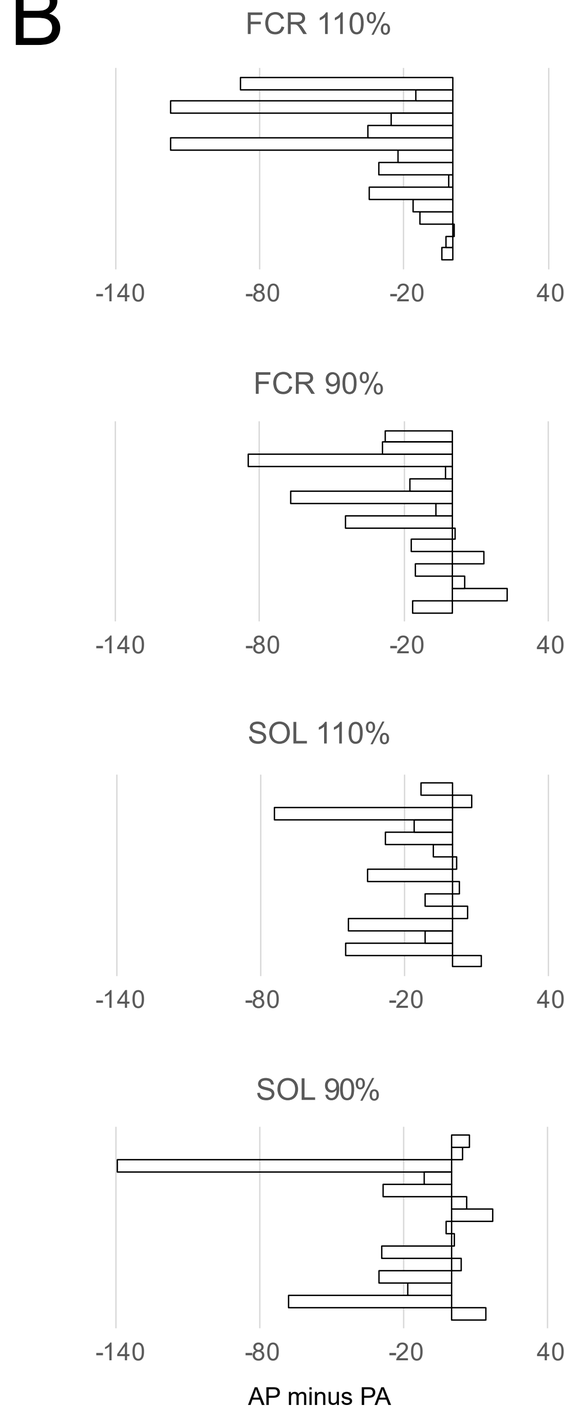

A
PA stimulation
(Ia) afferent pathway
corticospinal pathway
spinal motoneurone
PNS
B
110% RMT
H-reflex
stimulation artifact
M-Wave
MEP
stimulation artifact
PNS
TMS
conditioned H-reflex
90% RMT
no MEP
C
S2 stimulation
TMS S1
TMS S2
PNS
-6 ms
+8 ms
S1 stimulation
TMS S1
TMS S2
PNS
-6 ms
+8 ms
SICI (2.5 ms)
TMS S1
TMS S2
PNS
2.5 ms
-6 ms
+8 ms

A



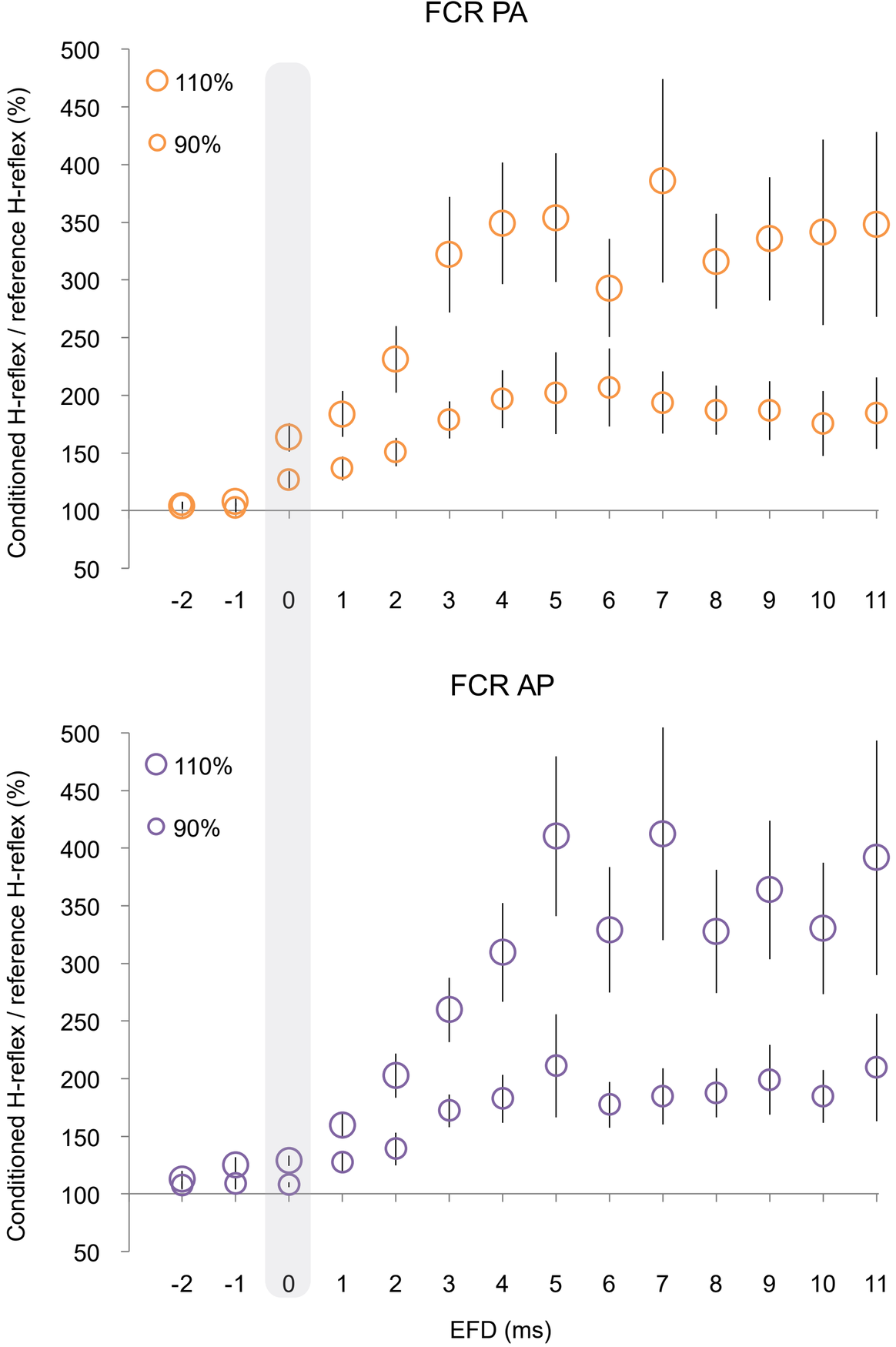


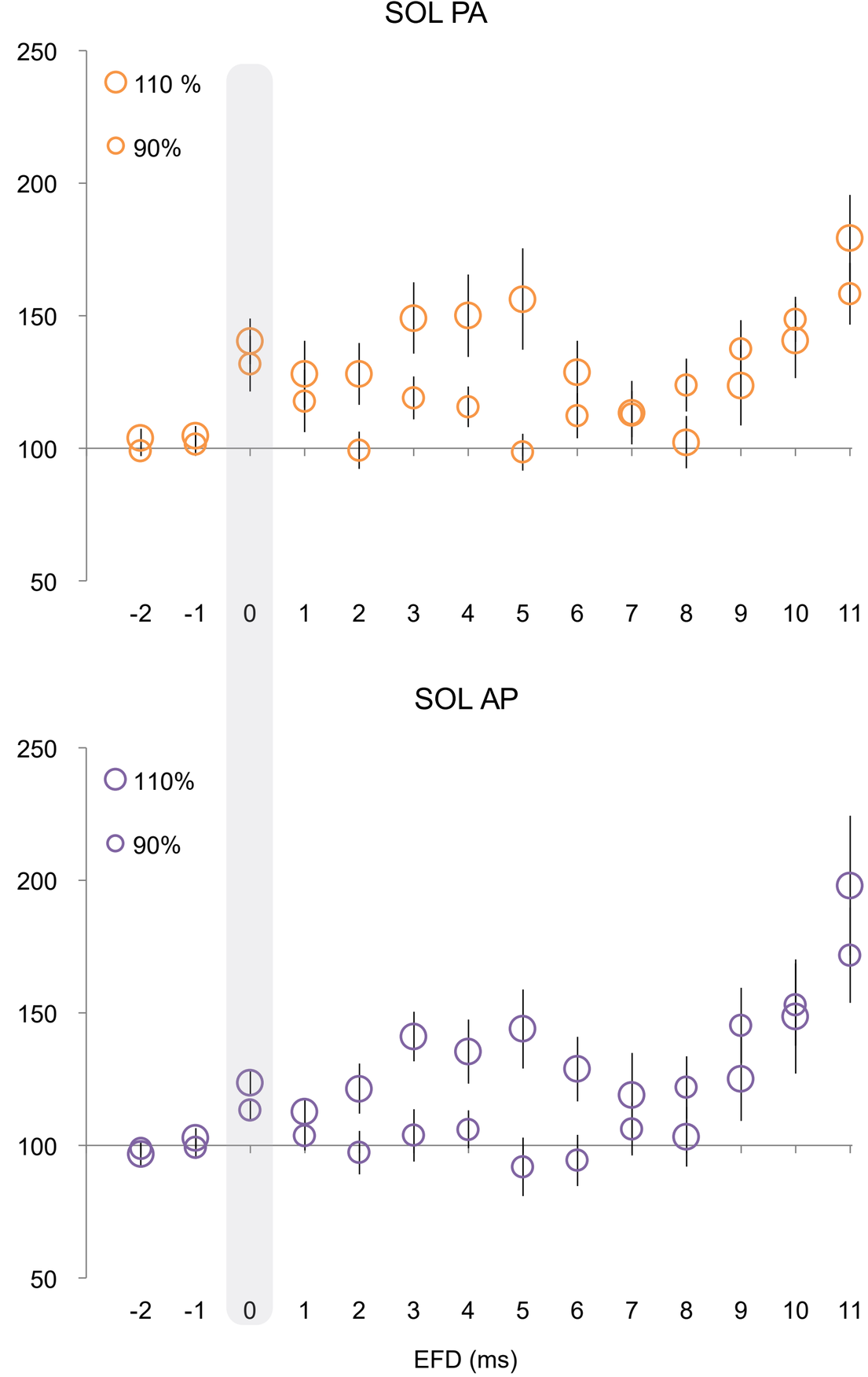
B



EFD	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	correct.
110% (PA and AP)	.42	.02	<.01	.10	.08	.06	.11	.27	.34	.66	.80	.24	.48	.47	-
90% (PA and AP)	.96	.58	.01	.34	.14	.70	.37	.99	.20	.39	.62	.73	.95	.71	-

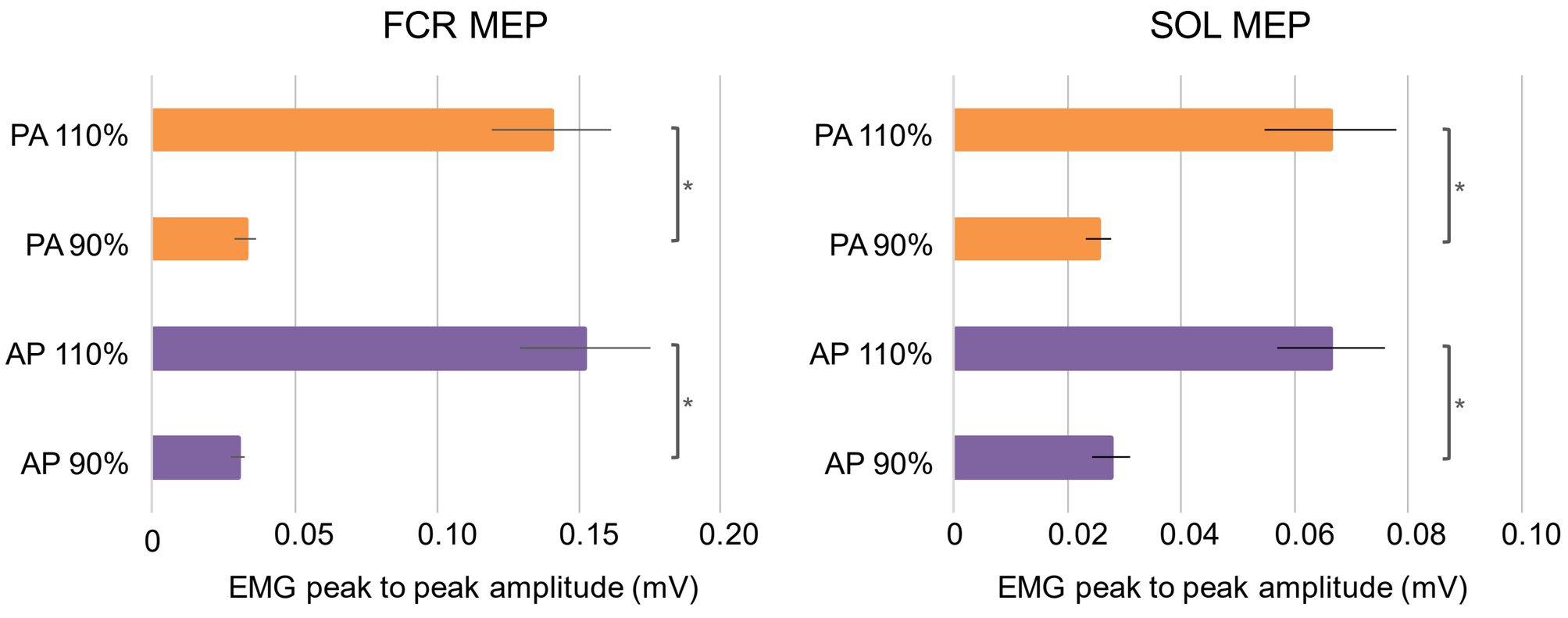
-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	correct.
.78	.50	.39	.38	.93	.96	.78	.91	.28	.05	.78	.37	.16	.10	-
.31	.53	.39	.43	.71	.51	.54	.77	.07	.92	.77	.12	.32	.10	-

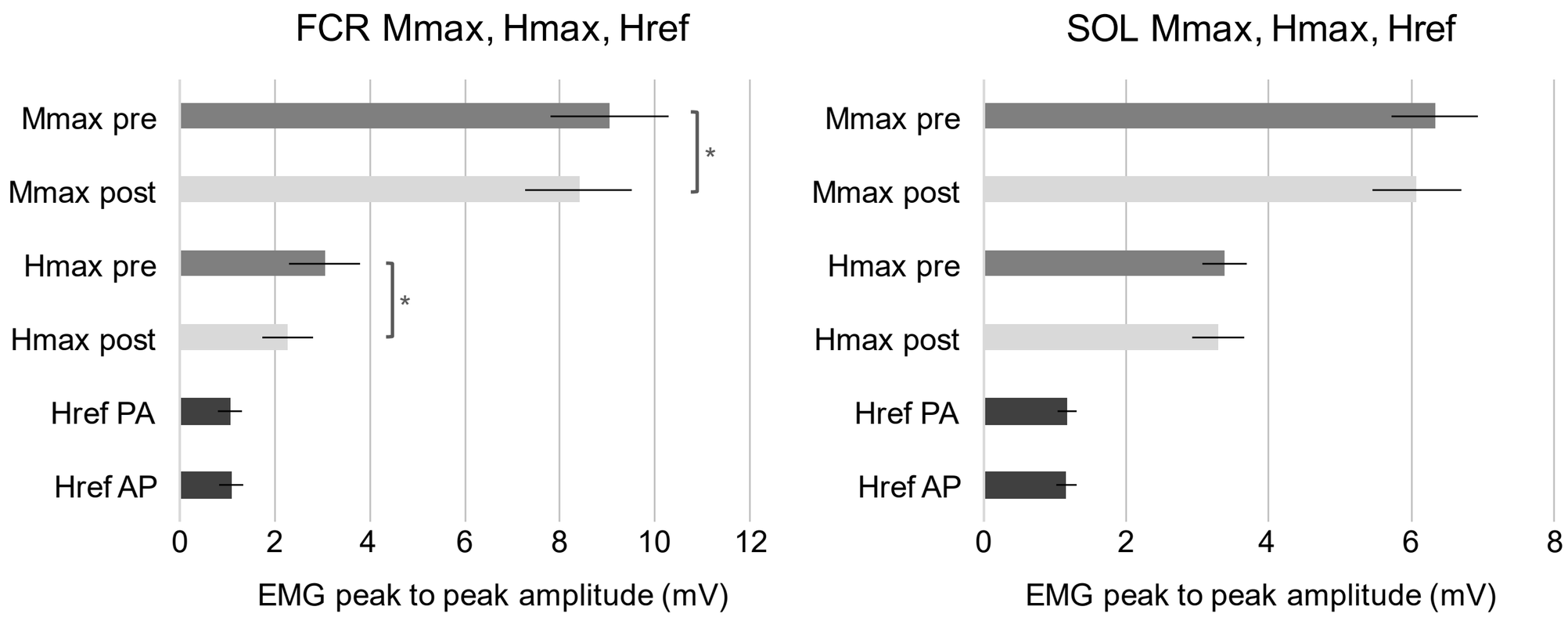


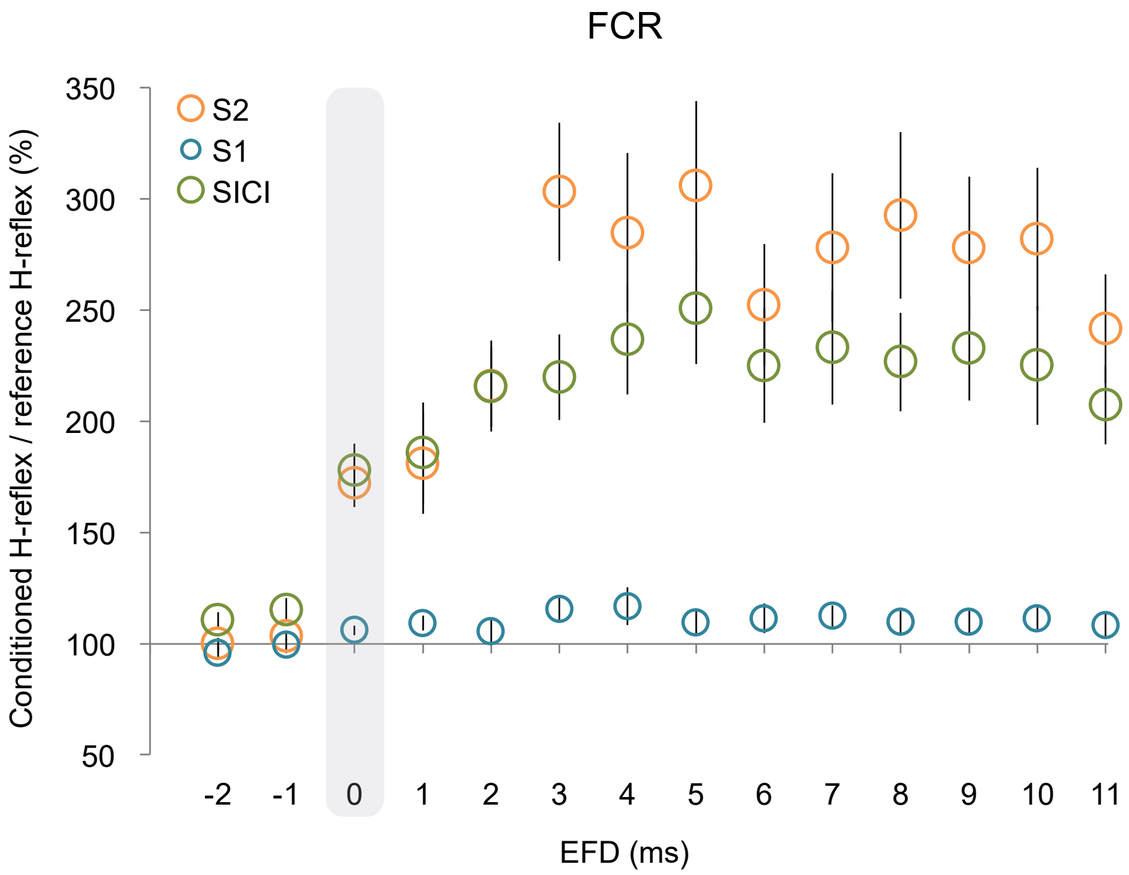


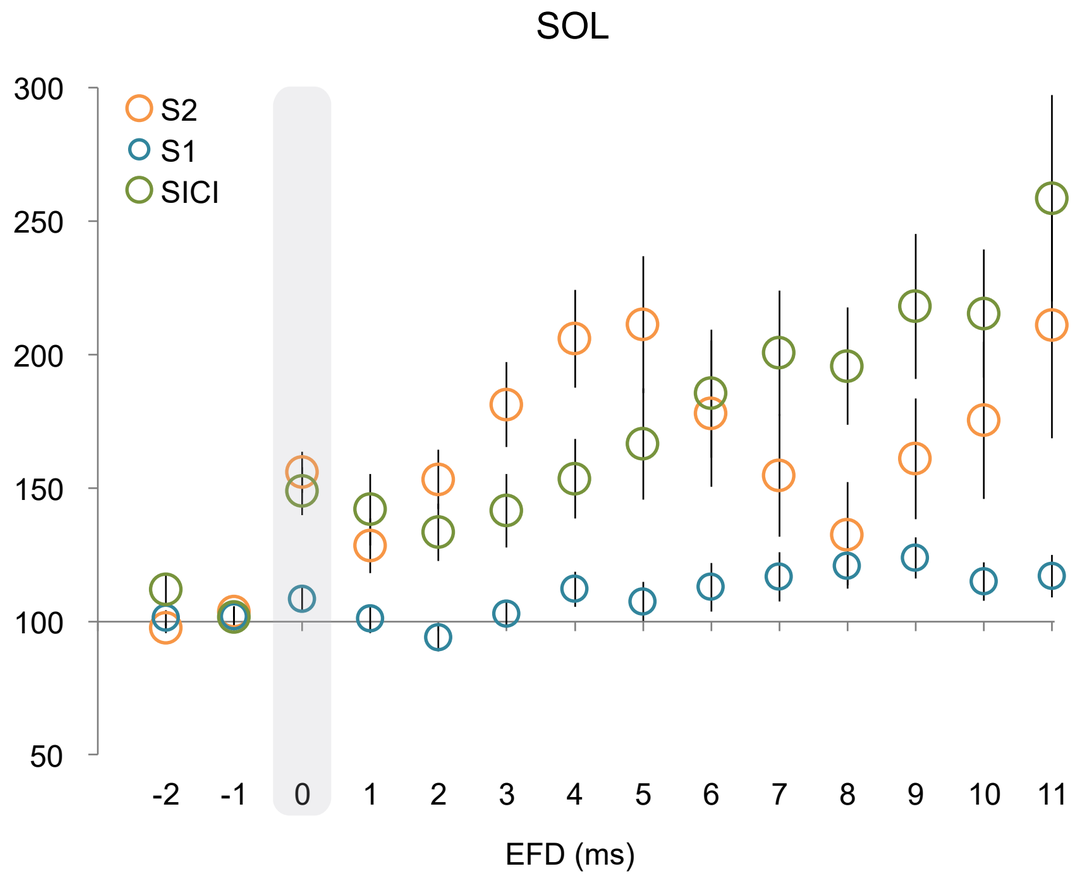
EFD	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	correct.
PA (110% and 90%)	.75	.19	<.01	.01	<.01	<.01	.01	<.01	.05	.01	<.01	<.01	.01	.01	<.039
AP (110% and 90%)	.36	.02	<.01	<.01	<.01	<.01	<.01	<.01	.01	.01	.02	<.01	<.01	.01	<.046

-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	correct.
.21	.33	.01	.01	<.01	<.01	.01	.01	.09	.80	.03	.25	.51	.10	<.021
.38	.12	.01	.07	.01	<.01	<.01	<.01	<.01	.19	.03	.16	.62	.20	<.021


FCR MEP
PA 110%
PA 90%
AP 110%
AP 90%
*
*
0
0.05
0.10
0.15
0.20
EMG peak to peak amplitude (mV)
SOL MEP
PA 110%
PA 90%
AP 110%
AP 90%
*
*
0
0.02
0.04
0.06
0.08
0.10
EMG peak to peak amplitude (mV)

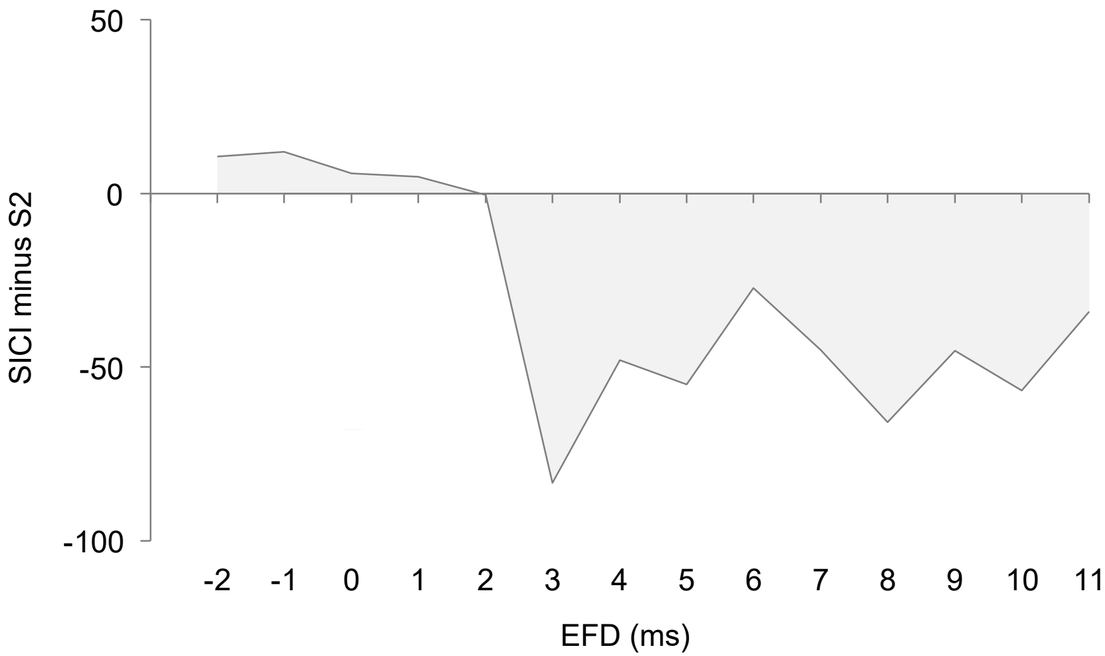

FCR Mmax, Hmax, Href
Mmax pre
Mmax post
Hmax pre
Hmax post
Href PA
Href AP
*
*
0
2
4
6
8
10
12
EMG peak to peak amplitude (mV)
SOL Mmax, Hmax, Href
Mmax pre
Mmax post
Hmax pre
Hmax post
Href PA
Href AP
0
2
4
6
8
EMG peak to peak amplitude (mV)

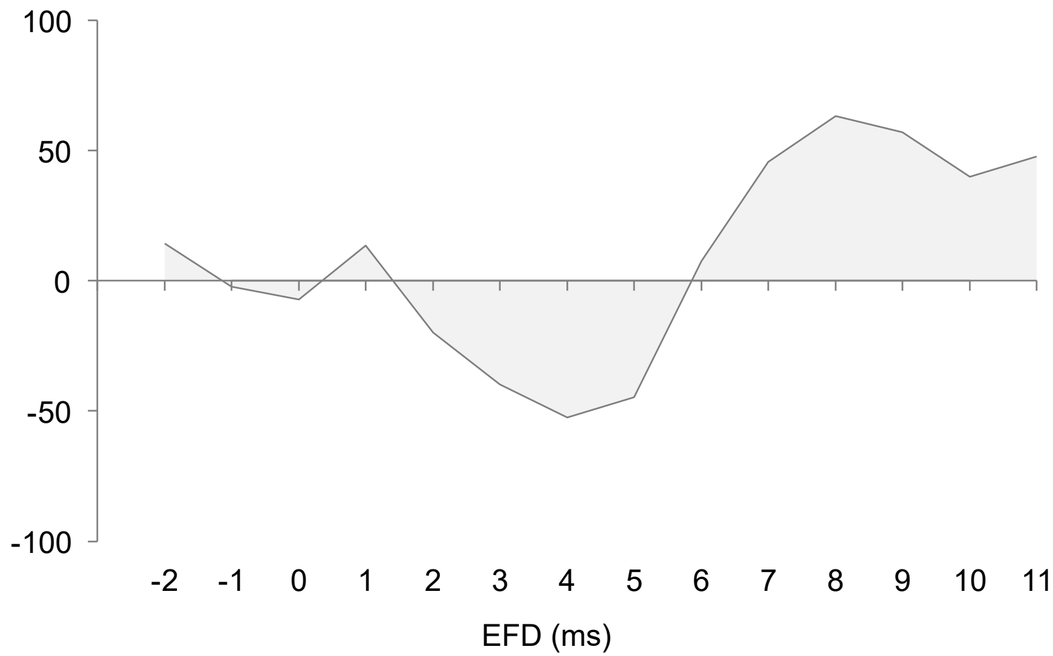


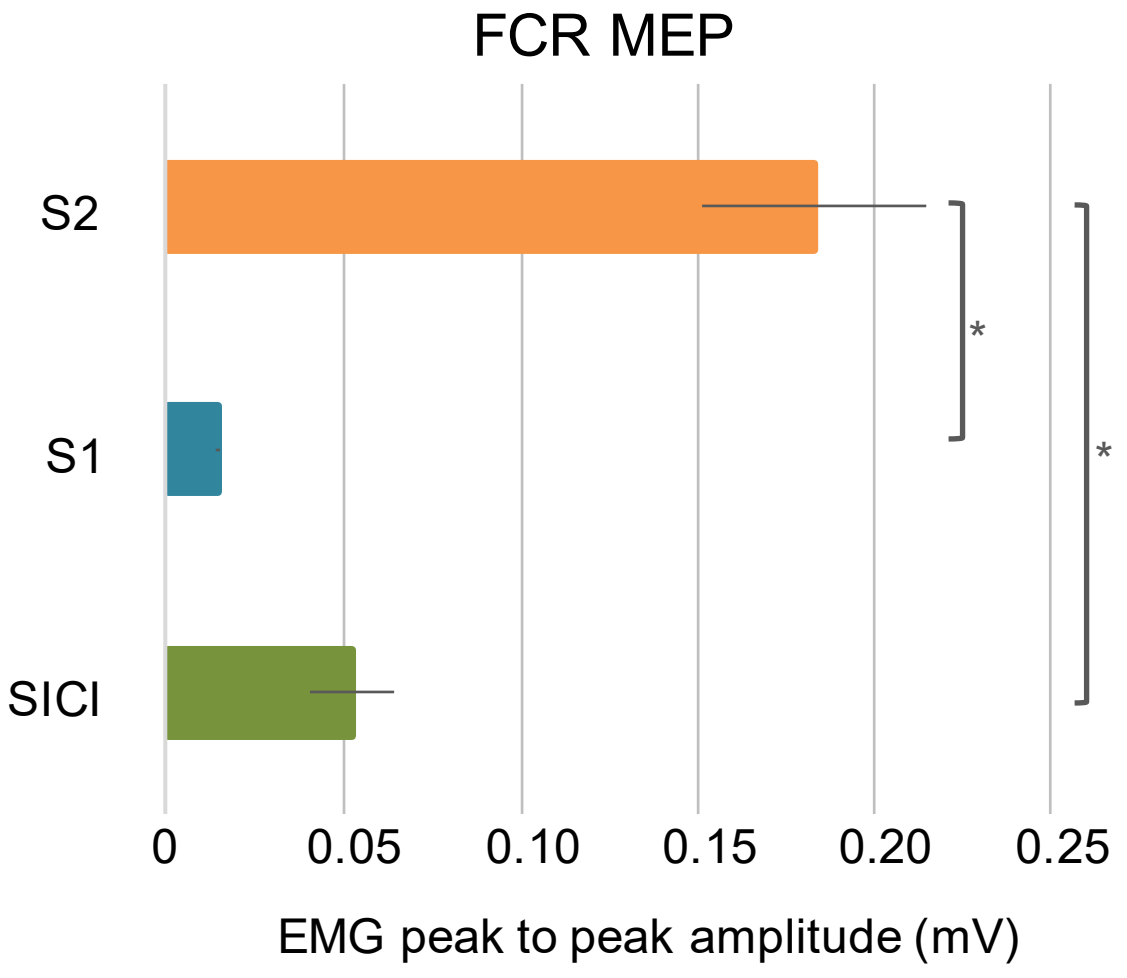


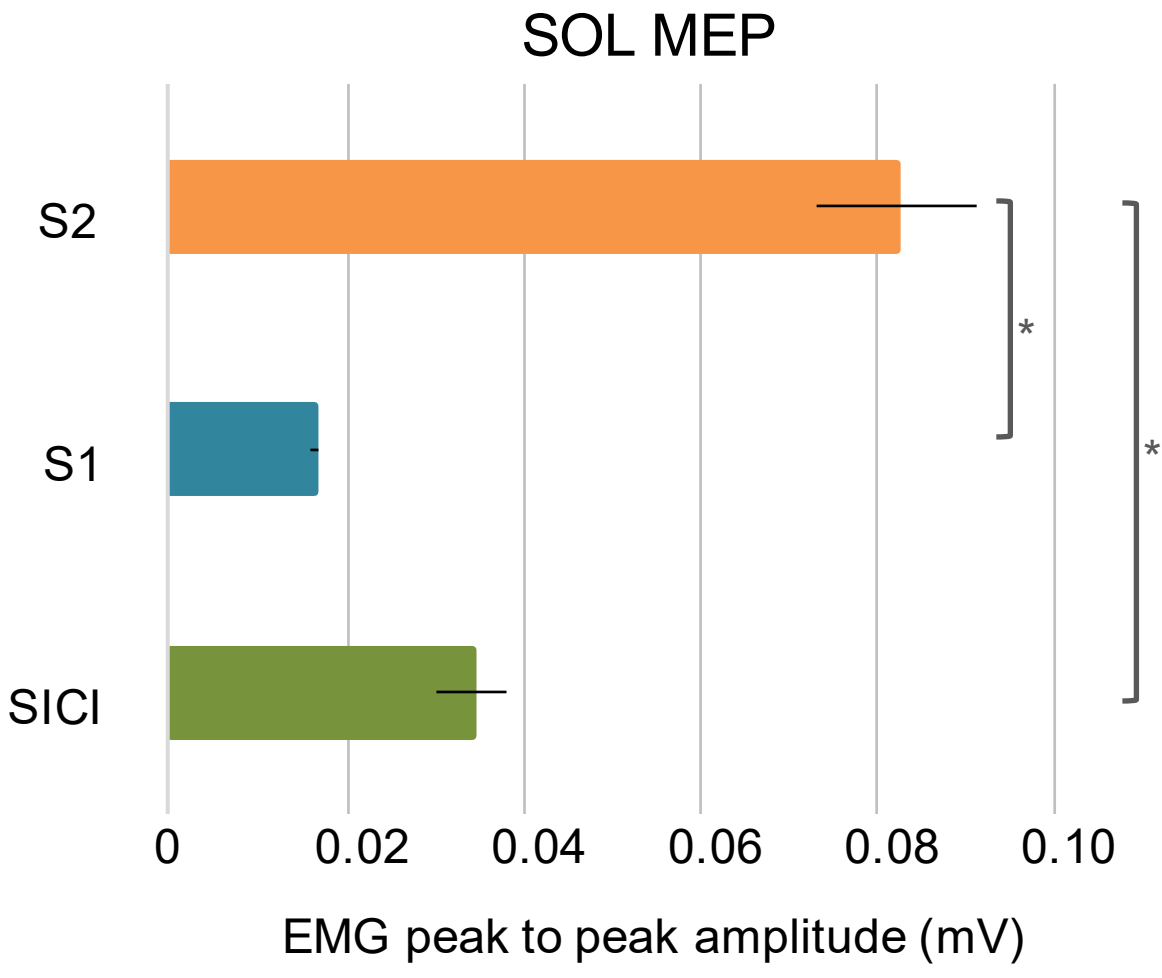
EFD	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	correct.
S2 and SICI	.04	.05	.23	.42	.95	<.01	.02	.02	<.01	<.01	<.01	<.01	<.001	.01	<.03
S2 and S1	.26	.37	<.001	<.01	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.04

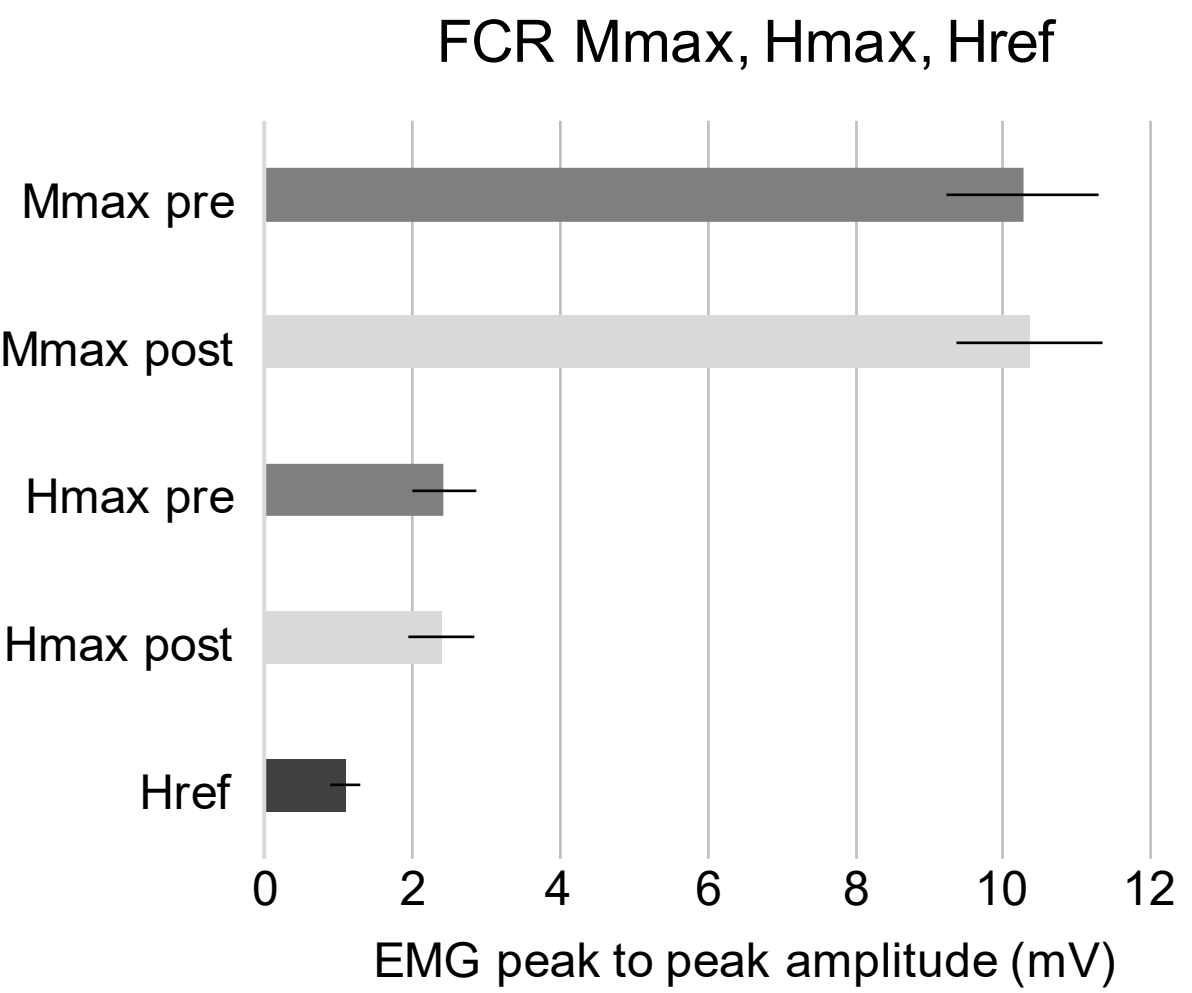
-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	correct.
.03	.62	.13	.03	<.01	<.01	<.01	.04	.71	.04	<.01	.02	.12	.25	<.014
.33	.66	<.001	<.01	<.001	<.001	<.001	<.01	.03	.16	.53	.12	.06	.07	<.021

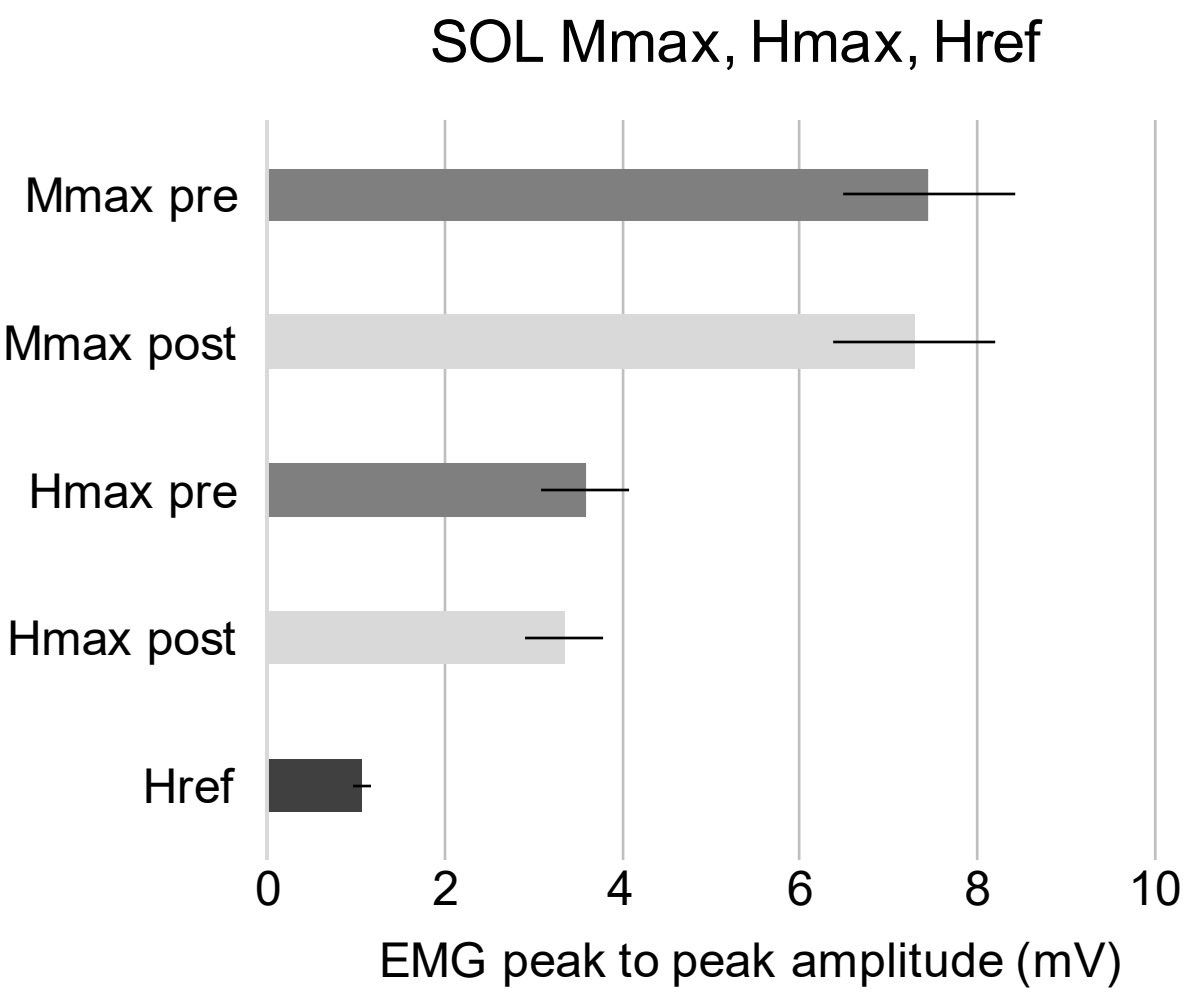





FCR MEP
S2
S1
SICI
*
*
0
0.05
0.10
0.15
0.20
0.25
EMG peak to peak amplitude (mV)


SOL MEP
S2
S1
SICI
*
*
0
0.02
0.04
0.06
0.08
0.10
EMG peak to peak amplitude (mV)


FCR Mmax, Hmax, Href
Mmax pre
Mmax post
Hmax pre
Hmax post
Href
0
2
4
6
8
10
12
EMG peak to peak amplitude (mV)


SOL Mmax, Hmax, Href
Mmax pre
Mmax post
Hmax pre
Hmax post
Href
0
2
4
6
8
10
EMG peak to peak amplitude (mV)