Determining the types of descending waves from transcranial magnetic stimulation measured with conditioned H-reflexes in humans

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Abstract
Non-invasive techniques are scarce with which human (motor) cortical mechanisms can be investigated. In a series of previous experiments, we have applied an advanced form of conditioning technique with transcranial magnetic stimulation (TMS) and peripheral nerve stimulation by which excitability changes at the laminar level in the primary motor cortex can be estimated. This method builds on the assumption that the first of subsequent corticospinal waves from TMS which is assessed with H-reflexes (called early facilitation) results from indirect excitation of corticospinal neurons in motor cortex (I-wave) and not direct excitation of corticospinal axons (D-wave). So far, we have not provided strong experimental evidence that this is actually the case. In the present study, we therefore compared temporal differences of the early facilitation between transcranial magnetic and electrical stimulation (TES). TES is known to excite the axons of corticospinal neurons. TES in our study caused a temporal shift of the early facilitation of H-reflexes in all subjects compared to TMS, which indicates that the early facilitation with TMS is indeed produced by an I-wave. Additionally, we investigated temporal shifts of the early facilitation with different TMS intensities and two TMS coils. It has long been known that TMS with higher intensities can induce a D-wave. Accordingly, we found that TMS with an intensity of 150% of resting motor threshold compared to 130%/110% results in a temporal shift of the early facilitation, indicating the presence of a D-wave. This effect was dependent on the coil type.

KEYWORDS
corticospinal, direct and indirect volleys, non-invasive, primary motor cortex, spinal motoneurons, TMS

1 | INTRODUCTION

Transcranial magnetic stimulation (TMS) artificially elicits a number of descending corticospinal waves (Kaneko et al., 1996; Nakamura et al., 1996). These waves have different but overlapping origins (Di Lazzaro et al., 2008, 2017; Di Lazzaro & Ziemann, 2013) as TMS excites different neural elements at different lamina in the brain. Studying these...
waves is interesting because the waves can reveal information about the connectivity and the behavioural significance of distinct neural circuits (Hannah, 2020). In a recent study (Kurz et al., 2019), we provided evidence that by investigating corticospinal waves with a non-invasive electrophysiological technique it is possible to infer activity changes at supra- and infragranular layers in the primary motor cortex. This non-invasive method consists of the combination of peripheral nerve stimulation (PNS) and TMS (Nielsen et al., 1993; van der Linden & Bruggeman, 1993) and was advanced for our purposes (Kurz et al., 2019; Wiegel et al., 2020; Wiegel & Leukel, 2020). A prerequisite for mechanistic interpretations about activity changes at different lamina in the motor cortex is that the earliest facilitation from TMS assessed with H-reflexes (termed early facilitation delay [EFD] 0 ms in our previous study, Kurz et al., 2019) is caused by the initial part of the first I-wave (I1-wave). This is important because the non-invasive method for humans builds on experiments in non-human primates (Kurz et al., 2019) in which the origin of the earliest part of the I1-wave (infragranular layer 5b) and the late part of the I1-wave (infragranular layers) was directly observed. We (Niemann et al., 2018) and another research group (Hannah et al., 2018) indeed did provide some evidence that EFD 0 ms is associated with the I1-wave. However, this evidence is based on modulations of TMS parameters such as coil orientation (Hannah et al., 2018; Niemann et al., 2018) where the origin of effects is still ambiguous. Consequently, strong experimental evidence concerning the EFD 0 ms origin is still missing but important for deriving solid mechanistic interpretations out of current and future investigations in which conditioned H-reflexes with TMS are applied. The comparison of TMS with transcranial electrical stimulation (TES) provides a basis for solid conclusions about the origin of EFD 0 ms. In several previous investigations, it has been consistently found that anodal TES with stimulation intensities around rMT produces D-waves whereas TMS with stimulation intensities around rMT produces I-waves (specifically early I-waves) but no D-wave (Di Lazzaro et al., 1998; Edgley et al., 1997; Kaneko et al., 1996; Nakamura et al., 1996; Patton & Amassian, 1954). In the mentioned studies on awake human subjects, the first descending wave with TES appeared around 1 ms earlier than the first wave with TMS. This means that if the assumption about the origin of the early facilitation of H-reflexes with TMS is true the onset of this facilitation would be temporally shifted with TES. Hence, the early facilitation would occur at EFD −1 ms with TES compared to TMS. This was the main hypothesis of the present study.

Transcranial magnetic stimulation was also reported to be capable of inducing D-waves in case higher stimulation intensities of around 130% of active motor threshold are applied (Di Lazzaro et al., 1998; Di Lazzaro & Rothwell, 2014; Di Lazzaro & Ziemann, 2013). Hence, in the second part of the study we systematically assessed how a stepwise increase in stimulation intensity affects the onset of the early facilitation of the H-reflex in temporal terms. The hypothesis concerning this part of the study was that EFD 0 ms would be shifted by around 1 ms with higher TMS intensity, which indicates that TMS is producing a D-wave. This hypothesis was tested using two different TMS coils, as coil geometry might influence the intensity at which D-waves can be evoked (Deng et al., 2013).

2 | MATERIALS AND METHODS

2.1 | Ethical approval

The present study conformed to the standards set by the Declaration of Helsinki (latest revision in Fortaleza, 2013), except for registration of the study in a database. The study was approved by the local ethics committee of the Albert-Ludwigs-University in Freiburg (approval number 327/18). All subjects provided written informed consent for the procedures performed in the study. None of the participants had contraindications to TMS and TES (Rossi et al., 2009).

2.2 | Subjects

A total of 23 healthy subjects (14 males and 9 females, aged between 23 and 31 years) participated. The study consisted of two parts. In part 1_TES/TMS, TMS was compared to TES (8 subjects, 6 males and 2 females). In part 2_intensity/coil type, different TMS intensities with two different coil types were tested (15 subjects, 8 males and 7 females). We included only subjects in whom H-reflexes in the M. flexor carpi radialis (FCR) could be elicited and H-reflex onsets could be determined.

2.3 | Surface electromyography and kinematics

Surface electromyography (EMG; EISA, Pfitz Biomedical Systems) was recorded from the FCR and the M. extensor carpi radialis (ECR) of the left arm. Bipolar surface EMG electrodes (Blue sensor P, Ambu®) were placed over the muscle bellies (electrode distance: 2 cm) and one common ground electrode was placed at the caput ulnae. Impedances were kept below 5 kΩ. Signals were pre-amplified (100x), further amplified (2x), bandpass filtered (10–1,300 Hz) and stored on a computer for offline analyses. All data were sampled at 10 kHz.
2.4  Peripheral nerve stimulation

FCR H-reflexes were elicited by stimulating the nervus medianus of the left arm just above the elbow joint. For this purpose, we used a constant current stimulator (DS7a, Digitimer®) that produced square wave-pulses of 0.2 ms in duration. A bipolar electrode configuration was applied. A graphite-coated rubber pad of 2 × 5 cm was used as anode and placed proximal to the olecranon. The best stimulation position for the cathode was determined during a search procedure, which involved moving a custom-made round pad (1 cm diameter) on the skin surface at the medial area of the os humeri just above the elbow joint. The optimal position was defined as the site where low stimulation intensities (5–30 mA, monophasic pulse) elicited no or minimal M-wave, while H-reflex sizes remained constant. Furthermore, H-reflexes had to be clearly distinguishable from the M-wave, so that H-reflex onsets could be determined. It was also ensured that no H-reflexes were elicited in the antagonistic ECR muscle. We attached a self-adhesive cathode (Blue sensor P, Ambu®) at the optimal position, and this electrode was used for the remainder of the experiment. At the beginning of each experiment, maximal M-waves and H-reflexes were recorded, and the required stimulation intensity for TMS-conditioned H-reflex measurements was calculated.

2.5  Transcranial magnetic stimulation

Single-pulse TMS was applied over the right motor area of the wrist muscles using a Magstim 2002 stimulator with a BiStim unit (Magstim) and a 50 mm figure-of-eight coil (in part 1_TES/TMS and part 2_intensity/coil type) and a 70 mm figure-of-eight coil (in part 2_intensity/coil type). The stimulation position was determined during a mapping procedure at the beginning of each experiment. We recorded MEPs in the EMG of the FCR at several positions and stimulation intensities. The optimal position was defined as the site where TMS elicited clear MEPs at the lowest possible stimulation intensity. This optimal position was targeted during the entire experiment with the help of a Brainsight TMS navigation system (Brainsight 2®, Rogue Research). A stand (Manfrotto® Magic Arm, Lino Manfrotto & Co.) stabilised the coil on the subjects’ head. The coil was held tangentially on the scalp at an angle of 45° to the mid-sagittal plane. The induced current direction was posterior-anterior. Resting motor threshold (rMT) was defined as the minimum required percentage stimulator output to evoke MEPs of at least 50 μV in at least three out of five subsequent stimulations at a certain intensity (Rossini et al., 1994). Note that the searching procedure in part 2_intensity/coil type was only performed with the small coil (same hotspot for both coils) but that rMT was defined for the smaller and larger coil separately.

2.6  Transcranial electrical stimulation

Anodal stimulation of the motor cortex was performed with a Digitimer D180A stimulator (DS7a, Digitimer®) and a 50 ms time constant. The cathode was placed at the vertex and the anode was placed 7 cm laterally. Stimulation intensity was continuously increased until clear MEPs of 50 μV amplitude in the FCR could be evoked in at least three out of five consecutive sweeps with the subjects at rest (Di Lazzaro et al., 1998).

2.7  TMS and TES H-reflex conditioning

Transcranial magnetic stimulation and TES H-reflex conditioning was performed in accordance with previous studies (Kurz & Leukel, 2019; Kurz et al., 2019; Wiegel et al., 2020). The method aims for segregation of corticospinal waves that are elicited through TMS and TES (Di Lazzaro & Ziemann, 2013) with H-reflexes (Figure 1a). PNS from which H-reflexes are evoked and TMS are timed, so that distinct corticospinal waves coincide with the induced afferent volley at the spinal motoneurons. Detailed explanations and illustrations of the H-reflex conditioning method can be found in previous publications (Kurz & Leukel, 2019; Kurz et al., 2019; Wiegel et al., 2020). All measurements were performed with the subjects at rest. Delays between TMS/TES and PNS were tested in the range between −5 to 0 ms in steps of 0.5 ms (negative delays indicate that PNS was applied prior to TMS/TES). Twenty sweeps at each delay and also 20 unconditioned H-reflexes were recorded in a block design. One block consisted of a single sweep of all delays and an unconditioned H-reflex. The different delays were randomly applied in each block.

The interval between stimulations was 4 s to avoid changes in the post-activation depression of the H-reflex (Crone & Nielsen, 1989). The intensity for PNS was set, so that H-reflex sizes were between 15% and 25% of Mmax. At this intensity range, H-reflexes typically correspond to the upsloping part of the H/M-recruitment curve and are linearly modulated by additional (TMS) input to spinal motoneurons (Crone et al., 1990).

The different conditions tested in part 1_TES/TMS and also in part 2_intensity/coil type were randomly executed. There were two conditions in part 1_TES/TMS, namely H-reflex conditioning with TMS and TES. TES and TMS intensity were set to 110% rMT. In part 2_intensity/coil type, eight conditions were tested, namely H-reflex conditioning
with TMS intensities of 90%, 110%, 130% and 150% of rMT, all with the 50 mm and the 70 mm figure-of-eight coils.

2.8 Data analysis and statistics

The onset of the H-reflex was visually determined from superimposed unconditioned reflexes and the averaged unconditioned H-reflex. The mean H-reflex onset was 16.7 ms (standard deviation: 1.4 ms) in subjects participating in part 1_TES/TMS, and 16.2 ms (standard deviation: 1.2 ms) in subjects participating in part 2_intensity/coil type. EMG values were corrected for EMG offsets (y-position) by shifting the EMG signal at H-reflex onset to zero. This is important because EMG offset at the H-reflex onset may change between trials because of uncontrollable electrical noise. H-reflex magnitudes (conditioned and unconditioned) were analysed by calculating the root mean square values (RMS) from the initial 0.5 ms of the H-reflex from the unrectified EMG. This analysis ensures that H-reflex magnitudes only include the earliest monosynaptic components of the reflex (Nielsen et al., 1993, 1995).

The delay between TMS/TES and PNS at which the fastest induced corticospinal wave causes a change in the recruitment of spinal motoneurons induced by PNS is called early facilitation (Kurz et al., 2019; Nielsen et al., 1993). The exact time lag between PNS and TMS at which this earliest facilitation occurs is different between individuals because of anatomical variations like the trunk length and therefore the conduction time of the fastest conducting TMS-induced volley. We assessed the time lag between TMS and PNS that relates to the early facilitation in each individual and labelled the corresponding value early facilitation delay (EFD) 0 ms according to previous studies (Kurz et al., 2019; Niemann et al., 2018; Wiegel et al., 2018, 2020). To assess the individual early facilitation, uncorrected paired Student’s t tests were calculated between all trials of conditioned H-reflexes corresponding to a particular delay and all trials of unconditioned H-reflexes. The first delay (starting from −5 ms to less negative delays) at which the size of the conditioned H-reflexes was larger than the unconditioned H-reflexes (p < 0.05), and which was the first out of a minimum of three consecutive delays with conditioned H-reflexes being significantly larger than unconditioned H-reflexes, was denoted EFD 0 ms (see Figure 1b [blue box]). Note that when displaying the results from the different parts of the study, EFD 0 ms refers to the interval with TMS at 110% of rMT. Intervals assessed with other conditions (TES and different TMS intensities) are expressed relative to this interval and the corresponding coil (Figure 1b [red box]).

Normal distribution and homogeneity were confirmed in all datasets with the Kolmogorov–Smirnov and Levene tests, respectively. In part 1_TES/TMS, a repeated measures (rm) ANOVA was performed with a 2 × 5 within-subject design with the dependent variable H-reflex facilitation and the independent factors STIMULATION (TES and TMS) and EFD (EFD −2 ms, EFD −1.5 ms, EFD −1 ms, EFD 0.5 ms and EFD 0 ms). According to our a priori hypothesis, we further applied post hoc t tests to identify the intervals at which the TMS and TES conditioned

FIGURE 1 Conditioning of the H-Reflex with transcranial magnetic stimulation (TMS) and transcranial electrical stimulation (TES). (a) Illustration of experimental setup. (b) Illustration of the methodological concept. The early facilitation delay (EFD) 0 ms was determined by testing different delays between TMS and PNS (negative delays indicate that TMS/TES was trigger after PNS; top panel). EFD 0 ms in this example was at the delay of −3.5 ms with TMS at 110% of rMT (middle panel, blue arrow). Intervals assessed with TES and other stimulation intensities are expressed relative to this interval assessed with TMS at 110% of rMT. We assumed that the initial H-reflex facilitation with TES would be elicited by a D-Wave (bottom panel, red arrow) and therefore temporally shifted by −1 ms compared to TMS. Hence, the early facilitation would occur at a delay of −4.5 ms, which refers to EFD −1 ms with TMS
H-reflexes were increased compared to the unconditioned H-reflexes. In part 2_intensity/coil, we performed a rmANOVA with a $2 \times 4 \times 5$ within-subject design with the dependent variable H-reflex facilitation and the independent factors COIL (50 mm and 70 mm), INTENSITY (90% rMT, 110% rMT, 130% rMT and 150% rMT) and EFD (EFD −2 ms, EFD −1.5 ms, EFD −1 ms, EFD 0.5 ms and EFD 0 ms). According to our a priori hypothesis, we additionally applied post hoc $t$ tests to identify the intervals at which conditioned H-reflexes were significantly increased compared to the unconditioned H-reflexes for each coil and intensity, respectively. Partial eta-squared values ($\eta^2$) were calculated to estimate the effect sizes of the results of the ANOVAs. The level of significance was set to $p < 0.05$ and adjusted for multiple comparisons according to Benjamini and Hochberg (Benjamini & Hochberg, 1995). Note that raw $P$-values are presented, and statements about the significance are made in the text.

3 | RESULTS

3.1 | Part 1_TES/TMS

The mean rMT with TMS was 48.4% (standard deviation: 8.1%) of the maximum stimulator output. The rmANOVA yielded significant effects for STIMULATION ($F_{1,7} = 29.34$, $p < 0.01$, $\eta^2 = 0.81$) and EFD ($F_{4,4} = 58.41$, $p < 0.01$, $\eta^2 = 0.98$) and a significant STIMULATION × EFD interaction ($F_{4,4} = 13.29$, $p = 0.01$, $\eta^2 = 0.93$). Post hoc analyses revealed that conditioned H-reflexes at EFD 0 ms were significantly increased both with TMS ($p < 0.01$) and TES ($p = 0.02$). However, only TES conditioned H-reflexes yielded a significant facilitation at EFD −1 ms ($p = 0.01$) and EFD −0.5 ms ($p < 0.01$, see Figure 2a). Note that the most negative interval at which the H-reflex facilitation was observed with TES but not TMS was at −1 ms, but that there were quite substantial individual differences concerning the

![FIGURE 2](image-url) Results of conditioned H-reflexes recorded with transcranial magnetic stimulation (TMS) and transcranial electrical stimulation (TES). (a) Graphs display FCR H-reflex facilitation for TMS and TES conditioning. Open circles represent grand mean values ± SEM of all subjects (*$p < 0.05$). Filled circles represent single subject mean data. The table displays results from post hoc $t$ tests ($p$-values) and the corresponding corrected significance levels (corr.). Grey boxes indicate significant differences between conditioned H-reflexes and unconditioned H-reflexes. Note that EFD 0 ms refers to the condition with TMS at 110% of rMT. (b, c) Graphs display FCR H-reflex facilitation for TMS at intensities 90%, 110%, 130% and 150% of rMT. Open circles represent grand mean values ± SEM of all subjects (*$p < 0.05$). Filled circles illustrate single subject data. The table displays results from post hoc $t$ tests ($p$-values) and the corresponding corrected significance levels (corr.). Grey boxes indicate significant differences between conditioned H-reflexes and unconditioned H-reflexes.
most negative deflection (Table 1). This interval ranged from EFD −1.5 ms to EFD −0.5 ms between subjects.

3.2 | Part 2_intensity/coil type

The mean rMT with the 50 mm TMS coil was 49.5% (standard deviation: 6.6%) of the maximum stimulator output and the mean rMT with the 70 mm TMS coil was 31.7% (standard deviation: 3.8%) of the maximum stimulator output. The rmANOVA yielded no significant effect for COIL ($F_{1,14} = 3.60, p = 0.08, \eta^2 = 0.21$), a significant effect for INTENSITY ($F_{3,12} = 6.05, p < 0.01, \eta^2 = 0.60$) and a significant effect for EFD ($F_{4,11} = 4.18, p < 0.05, \eta^2 = 0.60$). There were no significant interaction effects: COIL × INTENSITY ($F_{3,12} = 2.01, p = 0.17, \eta^2 = 0.34$), COIL × EFD ($F_{4,11} = 1.34, p = 0.39, \eta^2 = 0.29$), INTENSITY × EFD ($F_{4,11} = 1.27, p = 0.48, \eta^2 = 0.84$), COIL × INTENSITY × EFD ($F_{12,3} = 0.83, p = 0.65, \eta^2 = 0.77$).

3.2.1 | 50 mm coil

Post hoc analyses for TMS at 90% rMT yielded no significant facilitation of H-reflexes. TMS above rMT caused a significant facilitation of conditioned H-reflexes only at EFD 0 ms (110%: $p < 0.05$; 130%: $p < 0.01$; 150%: $p < 0.01$, see Figure 2b).

3.2.2 | 70 mm coil

Post hoc analyses for TMS at 90% rMT yielded no significant facilitation of H-reflexes. TMS above rMT caused facilitation of the H-reflex at EFD 0 ms (110%: $p < 0.01$; 130%: $p < 0.05$, 150%: $p < 0.01$). Additionally, TMS at 150% rMT caused facilitation of H-reflexes also at EFD −1 ms ($p < 0.05$) and EFD −0.5 ms ($p < 0.05$, see Figure 2c).

4 | DISCUSSION

The main aim of the present study was to provide evidence concerning the EFD 0 ms origin with TMS applied at intensities around motor threshold, namely that H-reflex facilitation at EFD 0 ms is caused by the earliest part of the I1-wave and not by a D-wave. This claim was put forward in previous publications in which H-reflex conditioning with TMS was advanced for laminar-specific investigations in the human motor cortex (Kurz & Leukel, 2019; Kurz et al., 2019; Niemann et al., 2018; Wiegel et al., 2018, 2020; Wiegel & Leukel, 2020). It is important that the H-reflex facilitation at EFD 0 ms results from the I1-wave in order to make valid conclusions about the underlying mechanisms. We provide this missing evidence in this paper. Future studies interested in laminar circuitry in the human motor cortex concerning behaviour, diseases or aspects about brain connectivity, can therefore be more confident when making mechanistic conclusions from results obtained with the H-reflex conditioning technique with TMS. In the following, we summarise the main findings of the current study, then discuss potential inconsistencies of the results, and limitations of the experiments.

The main findings of the present study were as follows: the EFD of the initial H-reflex facilitation was on average shifted by −1 ms with TES compared to TMS. Further, the EFD of the initial H-reflex facilitation with TMS at the intensity of 150% rMT was on average shifted by −1 ms compared to TMS with 110% and 130% of rMT. This second finding was observed with the larger 70 mm but not the smaller 50 mm TMS coil. These findings indicate that TMS with intensities around rMT produces I-waves and not a D-wave, and hence that H-reflex facilitation at EFD 0 ms can be ascribed to the initial part of the I1-wave. TES shifted the onset of the H-reflex facilitation by approximately 1 ms, which fits to previous findings and the different origins of TMS and TES, namely indirect excitation of corticospinal neurons (TMS) versus direct excitation of corticospinal axons (Di Lazzaro et al., 1998). Further, TMS with high intensity in our study also caused this temporal shift, which again fits to previous findings about the capability of high-intensity TMS to induce a D-wave (Di Lazzaro et al., 2012, 2017).

Although the mean values are in line with previous results about the temporal shift of the earliest H-reflex facilitation between TES and TMS, we found a considerable inter-individual variability of this delay ranging between −0.5 and −1.5 ms. The most plausible explanation for this variability relates to the testing steps of 0.5 ms between TES/TMS and H-reflexes and further the time window of 0.5 ms after H-reflex onset during which the H-reflex was analysed. These rough timings naturally lead to less precise categorisation of the earliest facilitation, in contrast to the temporally precise recordings of TMS-evoked volleys from the spinal cord in patients with chronic implants at the spinal level (Di Lazzaro

| Subject | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---------|---|---|---|---|---|---|---|---|
| EFD TES (ms) | −1 | −1 | −1.5 | −0.5 | −0.5 | −1.5 | −0.5 | −1 |

Note: Shows the temporal EFD shift with TES (mean EFD 0 ms with TES relative to mean EFD 0 ms with TMS) for all participants.
et al., 1998). This issue can be illustrated with two imaginary subjects in which the true earliest facilitation with TES starts at −1.2 and −0.9 ms, respectively, in relation to TMS. The true temporal difference between the two subjects is only 0.3 ms. However, according to the time windows used in the present study, one subject would be assorted to EFD −1.5 ms (the conditioned H-reflexes start to rise in this time window) and the other subject would be assorted to EFD −1.0 ms. The EFDs in the present study may thus not reflect the true timings. However, despite this temporal imprecision, the interpretation of the findings and its significance are not curtailed. We observed a temporal shift of the earliest H-reflex facilitation between TES and TMS in every measured subject, and this supports the notion of the different origins of the corticospinal waves and the I1-wave origin of EFD 0 ms with TMS at rMT.

The small sample size of eight participants in part 1_TES/TMS is problematic because of a potential type 2 error due to low statistical power. The reason for the small sample size is that TES is perceived as unpleasant by most people, and this makes it difficult to recruit a larger number of subjects. However, we observed an unambiguous temporal difference between TES- and TMS-induced H-reflexes in every subject. This finding makes us confident that our interpretations are solid.

The temporal shift of the earliest H-reflex facilitation between high intensity (150% rMT) TMS and TMS with lower intensities was only found for the larger 70 mm TMS coil. The simplest explanation for this finding relates to the penetration depth of electrical currents between the two coils. Most likely, the electrical currents produced with the smaller coil at 150% rMT at the required depth were insufficient/lacking to activate corticospinal axons, produce a D-wave and recruit a measurable amount of spinal motoneurons. This assumption is supported by modelling data analysing the penetration depths and electrical field strength with smaller TMS coils (Deng et al., 2013).

There was no significant facilitation of the H-reflex at 90% rMT with both coil types. This finding is contradictory to previous investigations showing a facilitation at similar delays in lower extremity muscles (Day et al., 1989; Nielsen et al., 1993; van der Linden & Bruggeman, 1993). The most likely explanation for the discrepancy is the different type of analysis of the H-reflex between the current and previous studies. Only the earliest portion of the H-reflex response (0.5 ms) was analysed in the current study, in contrast to the full response analysed in previous studies (Day et al., 1989; Nielsen et al., 1993; van der Linden & Bruggeman, 1993). We analysed only the initial portion to exclude non-mono-synaptic components of the H-reflex (Pierrot-Deseilligny & Burke, 2005). When analysing the full response for a time interval between PNS and TMS where the fastest corticospinal connections did not yet arrive simultaneously with the fastest arriving Ia volley from PNS, the corticospinal inputs can still be covered in the readout. These facilitated H-reflexes are not caused by the coincidence of the earliest possible corticospinal input and the earliest possible input from PNS at the spinal motoneurons, but instead because the earliest possible corticospinal input coincides with the excitation of motoneurons by PNS (either directly, and/or indirectly via spinal networks) at instants later than the earliest Ia input. Consequently, determining the early facilitation defined as the coincidence of the earliest possible corticospinal input with the earliest possible input from PNS at the spinal motoneurons requires the analysis of short time windows after H-reflex onset.

In conclusion, this study revealed temporal differences of the earliest facilitation of H-reflexes between TES and TMS and between lower and higher intensity TMS. These results indicate that H-reflex facilitation at EFD 0 ms with TMS intensities around rMT is caused by the initial part of the I1-wave. Because mechanistic interpretations from results of the H-reflex conditioning technique build on direct measurements in the monkey (Kurz et al., 2019) in which the association between I-waves and lamina in motor cortex was directly observed, future studies in humans using the non-invasive technique can now be more confident when interpreting their results with regards to laminar-specific activity modulations (Kurz & Leukel, 2019; Kurz et al., 2019; Wiegel et al., 2020; Wiegel & Leukel, 2020). Consequently, questions concerning laminar circuitry in the human motor cortex in relation to connectivity, behaviour and/or diseases can indeed be addressed by the advanced H-reflex conditioning technique which was applied in previous publications of our group (Kurz & Leukel, 2019; Kurz et al., 2019; Niemann et al., 2018; Wiegel & Leukel, 2020; Wiegel et al., 2018, 2020).

**COMPETING INTERESTS**

There are no conflicts of interest.

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**AUTHOR CONTRIBUTIONS**

CL and AK conceived and designed research; CL and AK performed experiments; CL and AK analysed data; CL and AK interpreted results of experiments; AK prepared figures; CL drafted manuscript; CL and AK edited and revised the manuscript; CL and AK approved the final version of manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy
or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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DATA AVAILABILITY STATEMENT
Raw data can be accessed upon request by contacting the corresponding authors.

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