Changes in corticospinal transmission following 8 weeks of ankle joint immobilization

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Objectives: Joint immobilization has previously been shown to modulate corticospinal excitability. The present study investigated changes in the excitability of distinct fractions of the corticospinal pathway by means of conditioning the H-reflex with transcranial magnetic stimulation (TMS) of the primary motor cortex (Hcond). This method allows assessment of transmission in fast (monosynaptic) and slow(er) (polysynaptic) corticospinal pathways.
Methods: 9 subjects underwent 8 weeks of unilateral ankle joint immobilization during daytime, 7 subjects served as controls. The measures obtained before and after immobilization included stretch- and H-reflexes assessing excitability of the spinal reflex circuitries, TMS recruitment curves estimating overall changes in corticospinal excitability, and Hcond.
Results: TMS recruitment curves showed an overall increase in corticospinal excitability following immobilization. Importantly, Hcond revealed significant facilitation of conditioned reflexes, but only for longer conditioning intervals, suggesting that immobilization increased excitability only of slower, indirect corticospinal pathways. No changes were observed in the control group. Immobilization had no significant effects on spinal reflex measures.
Conclusions: 8 weeks of ankle joint immobilization was accompanied by pathway-specific modulation of corticospinal transmission.
Significance: It is particularly interesting that fast corticospinal projections were unaffected as these are involved in controlling many, if not most, movements in humans.

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1. Introduction

Longer-term immobilization, whether it be bed rest or joint immobilization, causes degeneration of all systems of the human body including bones, muscles, the cardiovascular system and the central nervous system (CNS) (Convertino et al., 1997). Most previous studies concentrating on adaptations of the CNS following immobilization have focused on spinal reflex measures and the corticospinal pathway. Especially the latter is highly relevant for movement execution (Lemon et al., 1998, 1995, 2004). Therefore, electrophysiological techniques like transcranial magnetic stimulation (TMS) and peripheral nerve stimulation (PNS) have been applied to assess changes in spinal and corticospinal excitability, most often before and after limb or joint immobilization in humans for up to a few weeks.

The results derived from the TMS experiments are inconsistent:Liepert et al. (1995) found reduced motor cortical representational maps following 16 weeks of ankle immobilization, and both Facchini et al. (2002) and Ngomo et al. (2012) found reduced motor evoked potential amplitudes (MEPs) following 4 days of finger immobilization. Lundbye-Jensen and Nielsen (2008a) did not find changes in corticospinal excitability following 1 week of wrist immobilization, whereas increased corticospinal excitability was shown for arm and hand muscles following longer periods of immobilization ranging from 21 to 45 days (Clark et al., 2008; Zanette et al., 2004) and for leg muscles following 10 days of immobilization (Roberts et al., 2007).

In addition to corticospinal changes, spinal reflex circuitries have been investigated using PNS. Kaneko et al. (2003) observed no changes in H-reflexes following 3–6 weeks of arm immobilization, but the majority of studies have shown that H-reflex amplitudes increase following a period of immobilization. In the rat, Anderson et al. (1999) reported increases in the H-reflex gain following 3 weeks of hindlimb unloading, and in humans, Lundbye-Jensen and Nielsen (2008a) and Clark et al. (2008)similarly observed increased H-reflex amplitudes at rest following 1 week of wrist and hand immobilization and 4 weeks of lower limb suspension. More recently, it was demonstrated that 2 weeks of ankle joint immobilization causes changes in spinal interneuronal circuitries responsible for presynaptic control of sensory input to the spinal cord (Lundbye-Jensen and Nielsen, 2008b).

The reason for the differential findings, especially with respect to corticospinal plasticity, in previous immobilization studies is not clear. It may be speculated that the effects of immobilization relate to the specific limb or muscle, the duration, the immobilization procedure (e.g. without loading or partial loading of the limb), and possibly also depend on the tested population (e.g. age, the level of fitness, etc.). However, another confounding factor may be the method of how corticospinal excitability was assessed. One problem when interpreting compound MEPs elicited by single-pulse TMS is its non-specificity: the corticospinal pathway consists of a variety of different connections, i.e. monosynaptic, oligosynaptic and polysynaptic ones, from the motor cortex to the spinal motoneurons. The compound MEP reflects the net sum of all excitatory and inhibitory influences from the level of the primary motor cortex and downstream to the level of the spinal motoneurons. Thus, changes in the compound MEPs following immobilization may be caused by alterations either at a cortical or at the spinal level, or in direct or indirect corticospinal pathways to a varying extent. The precise description of the sites of action is important to be able to interpret neural plasticity following immobilization.

A possibility to test corticospinal transmission with sufficient temporal resolution is by conditioning H-reflexes with single pulse TMS over the motor cortex (Hcond) with different interstimulus intervals (ISIs) (Leukel et al., 2012; Nielsen and Petersen, 1995a; Nielsen et al., 1995, 1993; Petersen et al., 1998b; Taube et al., 2014, 2011). Using different ISIs by temporarily shifting the H-reflex relative to TMS reveals transmission in specific corticospinal pathways with different latencies and different synaptic connections (Nielsen et al., 1993). Thus, effects at "early" ISIs have been attributed to fast, most likely monosynaptic corticospinal projections whereas effects at "later" ISIs are thought to reflect excitation of slower and/or indirect pathways (Nielsen et al., 1995, 1993; Petersen et al., 1998a; Taube et al., 2006).

In order to investigate pathway-specific alterations in corticospinal transmission the Hcond technique was used in the present study. We immobilized the lower leg, i.e. ankle joint, of subjects during daytime for 8 weeks using a special designed cast (Hephaistos orthosis).

2. Methods

Nine healthy male subjects (31 ± 4 years) without neurological and orthopedic disorders were immobilized over a period of 8 weeks. The measurements conducted in the present study were part of a multidisciplinary research project at the German Aerospace Centre (DLR) in Cologne, investigating the physiological consequences of immobilization. This project only included immobilized subjects and no control subjects. Therefore, we recruited 7 additional subjects (27 ± 3 years) without immobilization as a control group after the measurements were conducted with the immobilized subjects to ensure that the electrophysiological results we obtained were not caused by other factors than immobilization (e.g. elapsed time between measurements).

Subjects in the immobilization group were psychologically screened including a standardized personality test (Freiburger personality inventory, FPI) and a 45-min interview with two psychologists. Exclusion criteria were smoking, participating in regular strength training, clotting disorders and consistent uptake of any prescribed medication. All subjects gave written informed consent prior to participation. The study was performed according to the Declaration of Helsinki (latest revision in Seoul, 2008) and approved by the local ethics committee (Ärztekammer Nordrhein). The Hephaistos study is registered at clinicaltrials.gov (NCT01576081).

2.1. General experimental protocol

Subjects wore a unilateral leg cast (see below) over a period of 8 weeks (see also (Weber et al., 2013)). The side of immobilization (left or right leg) was randomly chosen some weeks before the experiments started. The leg tested in the control group was also chosen in a randomized fashion.

During the 8 weeks of immobilization subjects followed their normal everyday activities while wearing the device in all activities that required loading of the leg. Subjects did not wear the orthosis during night-time sleep. This resulted in a "net wearing time" of 12–16 h per day, depending on their habitual activities.

Electrophysiological measurements were conducted 48 h before the start of the intervention (pre) and immediately after the end of the intervention (post, i.e. immediately after removing the cast). The order of electrophysiological measures in the pre- and post-measurement was as follows: (i) stretch reflexes, (ii) H-reflex recruitment curves, (iii) MEP recruitment curves, (iv) Hcond. Subjects in the control group were also tested two times (pre- and post-measurement) within a period of 8 weeks. We conducted the same measurements for the control subjects in the same order as for the immobilized subjects except the recording of stretch reflexes during sitting and stance and H-reflexes during stance (see below).

2.2. Orthosis

A novel unloading orthosis (Hephaistosor HEP cast, patent application No. 102011082700.5) was developed in the German Aerospace Center (DLR) in Cologne, Germany (Weber et al., 2013). Briefly, the Hephaistos cast significantly reduces the activation and force production of the calf muscles when performing leg movements where loading is required (e.g. locomotion) while it completely retains body mass impacts transferred through the bones. These specific effects of the orthosis were tested using biomechanical methods when designing the device (Weber et al., 2013 for more detailed description).

2.3. EMG

EMG recordings were obtained from the soleus muscle (SOL) and the tibialis anterior muscle (TA) of the respective leg. After preparation of the skin, bipolar surface electrodes (Blue Sensor P, Ambu®, Bad Nauheim, Germany) were attached longitudinally above the muscle belly (2 cm inter-electrode distance). The reference electrode was placed on the patella of the same leg. EMG signals were amplified (×1000), bandpass-filtered (10–1000 Hz) and sampled at 4 kHz. The EMG was stored and analyzed offline (IMAGO, pfitec®, Endingen, Germany).

2.4. Stretch reflex

Stretch reflexes in the SOL of the immobilized limb were elicited to elucidate changes in spinal reflex circuitry following immobilization. Measurements were conducted in a custom-built ankle ergometer (for more information about the technical specifications of the ergometer please contact the authors of the study). Subject's feet were placed on the left and right platform of the ergometer and were fixed with a snowboard binding system. The rotation axis of the upper ankle joint coincided with the rotation axis of the platform. Torque of the platform was assessed using a torque transducer (Burster®, Gernsbach, Germany) and displacement was measured with a goniometer (Megatron®, München, Germany). Both devices were mounted between the servomotor of the ankle ergometer and the platform.

Stretches were applied with two different velocities, 300° per second (fast stretch) and 150° per second (slow stretch). Displacement (toe up rotation) of the platform was always 10°. Both, the fast and the slow stretches were elicited while subjects were either sitting (knee angle at 90° and ankle angle at 90°) or standing (knee angle at 180° and ankle angle at 90°). During standing, subjects took hold on sidebars that were mounted at the level of the waist on the ergometer on left and right side of the standing subjects.

For each of the 4 conditions, a total of 10 consecutive stretches (pause between the stretches was set to 5 s) were applied in a randomized order meaning that the order of the 4 conditions was randomized.

Note that stretch reflexes were not recorded in subjects of the control group. As mentioned, the control group was tested after the immobilization group, and as we found no changes in spinal reflex circuitries (stretch reflexes and H-reflexes) in the immobilization group (see "Section 3") parameters were not tested in the control group.

2.5. H-reflex

The purpose of the H-reflex measurements was to detect changes in the strength of Ia afferent projections onto spinal motoneurons and therefore to reveal central adaptations in the spinal reflex circuitry following immobilization. Measurements were conducted while sitting (knee angle at 90° and ankle angle at 90°) in a custom built chair and also during stance (knee angle at 180° and ankle angle at 90°) in a randomized order (i.e. some subjects started with measurements during sitting and some with measurements while standing). H-reflexes in the right SOL were elicited with an electrical stimulator (constant current stimulator Digitimer® DS7a, Hertfordshire, UK) by stimulating the posterior tibial nerve in the popliteal fossa. Stimuli consisted of square-wave pulses of 0.5 ms duration. The anode, a graphite coated rubber pad of 5 × 5 cm, was fixed on the anterior aspect of the knee just underneath the patella. The site for the placement of the cathode was always searched during stance and thus, the optimal location for stimulation always refers to the stance condition. We moved the cathode (2 cm in diameter) stepwise until the optimal position for eliciting an H-reflex was found (the time interval between successive stimuli was 5 s). Care was taken that the stimulation did not activate the common peroneal nerve. We therefore recorded the TA EMG. A second criterion for the optimal position was that no or small M-waves in SOL were evoked with low stimulation intensities. After the optimal position was found, the cathode (Blue sensor N, Ambu®, Bad Nauheim, Germany) was fixed with tape.

H/*M* recruitment curves were obtained by applying approximately 20–50 electrical stimuli over a range of stimulation intensities ranging from 1 mA to the maximum of 100 mA. The pause between successive stimuli was 5 s. Note that, based on unchanged H-reflexes in the immobilization group, H-reflexes were only applied during sitting in the control group for normalization of electrophysiological data (MEP recruitment curve and Hcond, respectively).

2.6. TMS

A Magstim® Rapid Rate Stimulator (Magstim® Company Ltd., Whitland, UK) with a figure of eight coil (Magstim SP16097) was used for TMS. Measurements were conducted while subjects were sitting (knee angle at 90° and ankle angle at 90°). The optimal individual coil position (hotspot) for evoking MEPs in the SOL muscle was determined together with the resting motor threshold (1.0 MT) in each subject.

The SOL hotspot was determined by setting the initial stimulation point approximately 0.5 cm anterior to the vertex and over the interhemispheric midline and then going through a mapping procedure while monitoring the amplitude of the evoked MEP in SOL. For the mapping procedure, the coil was moved lateral from the vertex and anterior–posterior. The coil orientation was tangential to the scalp with the handle pointing backwards and centered at 0° angle with respect to the midline, inducing a posterior–anterior directed current in contralateral hemisphere M1 to activate preferentially intracortical neurons and, at higher stimulation intensities, corticospinal neurons trans-synaptically (Di Lazzaro et al., 2004; Terao et al., 2000). The handle of the coil was fixed to a stand (Manfrotto®, Italy). The coil was fixed with velcro® strips to the subject's head. The head of the subjects was fixed with velcro® strips to the headrest of the chair. This ensured a constant position of the coil relative to the head. Additionally, the position of the coil was marked on the scalp with a marker and monitored repeatedly throughout the experiment by the experimenter. With the hotspot set, 1.0 MT was determined as the minimum intensity of magnetic stimulation required to evoke MEPs of 50 μV peak-to-peak amplitude in at least 3 of 5 consecutive trials.

Two different conditions were tested with TMS, which are explained in the following.

2.7. MEP recruitment curve

MEP recruitment curves were recorded to test for overall changes in corticospinal excitability following immobilization according to previous studies (e.g. (Clark et al., 2008; Ngomo

et al., 2012)). Single-pulse magnetic stimuli were applied over M1 with different stimulation intensities ranging from 0.8 to 1.5 MT in steps of 0.1 with a 5 s interval between subsequent stimuli. We recorded a total of 5 stimuli at each stimulation intensity in a pseudo-randomized sequence meaning that we changed the stimulation intensity after each stimulus in a pre-defined order and did not apply 5 stimuli with the same stimulation intensity.

2.8. Conditioned H-reflexes (Hcond)

The conditioning technique aimed to evaluate pathway-specific plasticity of corticospinal transmission following immobilization and was applied in accordance with previous studies (Nielsen and Petersen, 1995a,b; Nielsen et al., 1993; Petersen et al., 1998a; Schubert et al., 2008). Electrical stimuli (pulse width of 0.5 ms) with an intensity to evoke SOL H-reflexes of 15–25% of the respective maximum M-wave in the pre- and post-measurement (Crone et al., 1990) and TMS with an intensity of 1.0 MT were combined at different ISIs (−5, −4, −3, −2, −1, 0, 4, 8, 12, 16, and 20 ms). Negative ISIs (in ms) indicate that the electrical stimulus was elicited before TMS. The reason for starting with the ISI of −7 ms was to ensure recording of the early facilitation of the conditioned H-reflexes as this most likely indicates activation of the spinal motoneurons by the fastest (monosynaptic) corticospinal fibers (Nielsen et al., 1993). In most subjects, the early facilitation can be observed when PNS precedes TMS by 2–4 ms.

Each ISI was measured 10 times in a randomized order including the same number of unconditioned H-reflexes and MEPs induced by single pulse TMS. The time interval between successive stimuli was 5 s.

2.9. Data analysis and statistics

2.9.1. Stretch reflexes

The peak-to-peak amplitude was calculated for each stretch reflex based on the unrectified SOL EMG. The amplitudes of 10 stretch reflexes were averaged in each condition and this mean was expressed as a percentage of the Mmax obtained in the pre- and post-measurement, respectively. Importantly, the stretch reflex values recorded during sitting were expressed relative to Mmax obtained during sitting and the stretch reflex values recorded during stance were expressed relative to Mmax obtained during stance. This normalization procedure aimed to account for altered recording conditions (e.g. position of the surface electrodes) in the pre-measurement compared to the post-measurement. These normalized values were entered into a repeated-measures analysis of variance (ANOVA) to test differences in the stretch reflex size between pre- and post-measurement with the within-subject factors TIME (pre- versus post-measurement) and VELOCITY (stretch with 300° per second and 150° per second).

2.9.2. H-reflexes and M-waves

The peak-to-peak amplitude was calculated for each H-reflex and each M-wave based on the unrectified surface EMG. The largest H-reflex of the H-reflex recruitment curve in the pre- and post-measurement was selected and named Hmax. The corresponding procedure was performed for the largest M-wave (Mmax). Finally, Hmax in each subject was divided by the Mmax to obtain the Hmax/Mmax ratio. Individual responses obtained in the sitting position (Hmax/Mmax and also Mmax) were entered into a repeated-measures ANOVA with the between-subject factor GROUP (immobilization versus control) and the within-subject factor TIME (pre- versus post-measurement). All post hoc pairwise comparisons were performed using Bonferroni corrected Student's *T*-tests. The H-reflexes data recorded during standing were tested using Bonferroni corrected paired Student's *T*-tests (pre-measurement against post-measurement) as the data during standing were only obtained for the immobilization group.

2.9.3. MEP recruitment curves

The peak-to-peak amplitude was calculated for each recorded MEP based on the raw SOL EMG. Five MEPs obtained at each stimulation intensity were averaged. The averaged value was individually normalized to Mmax obtained during sitting. For statistical analysis, we were interested in changes caused by the immobilization. Therefore, we subtracted the individual mean value of the post-measurement from the corresponding individual mean value (i.e. same stimulation intensity) of the pre-measurement. These data were entered into a repeated-measures ANOVA with the between-subject factor GROUP (immobilization versus control), and the within-subject factor STIMULATION INTENSITY (0.8–1.5 MT-pre-measurement). Additionally, we performed a repeated-measures ANOVA for each group with with-subject factors TIME (pre-measurement vs post-measurement) and STIMULATION INTENSITY. All post hoc pairwise comparisons were performed with Bonferroni corrected Student's *T*-tests.

2.9.4. Hcond

The peak-to-peak-amplitude of the conditioned H-reflexes and unconditioned control H-reflex was calculated based on the unrectified SOLEMG. Ten conditioned H-reflexes at each ISI and 10 unconditioned control H-reflexes were averaged in each subject. The individual mean of the conditioned H-reflexes (at each ISI) was divided by the individual mean of the unconditioned control H-reflex (Leukel et al., 2012; Taube et al., 2014, 2011). The unconditioned control H-reflex served as a reference for the conditioned H-reflexes. For statistical analysis, we subtracted the individual mean value of the post-measurement from the corresponding mean value of the pre-measurement. The first analysis was conducted to evaluate differences in the early facilitation between the immobilization group and the control group. We pointed out previously (Leukel et al., 2012; Taube et al., 2011) that there is a small individual variability in the onset of the early facilitation of about 1–2 ms caused by variations of the anatomical (e.g. trunk length, leg length) and physiological parameters of the subjects, which results in differences in conduction times of the corticospinal and (Ia) afferent volley to reach the spinal a-motoneurons. Therefore, we selected the early facilitation intra-individually as the first increase of conditioned H-reflexes starting from ISI −7 ms by visual inspection of the conditioning curves. An unambiguous detection was possible in all subjects. The early facilitation was determined in the pre- and post-measurement and ranged from −4 to −2 ms in all tested subjects. The corresponding ISI did not change in the post-measurement in the immobilization group and the control group, respectively. Therefore, data at the ISI corresponding to the early facilitation was compared between the two groups using a Bonferroni corrected unpaired Student's *T*-test. In a second analysis we compared the ISIs after the early facilitation, reflecting the excitability of slower (polysynaptic) corticospinal pathways. These data were not normalized to the early facilitation. With normalization group-wise comparisons would be impossible since the time lag between different ISIs was not constant. We used a repeated-measures ANOVA with the factors ISI (−2, −1, 0, 4, 8, 12, 16, 20 ms) and GROUP (immobilization versus control group).All post hoc pairwise comparisons were performed as Bonferroni *T*-tests.

Statistics were performed using SPSS 21 (IBM®, Armonk, NY, USA). Values are reported as mean ± standard deviation (SD).

3. Results

3.1. Spinal adaptations

3.1.1. Stretch reflexes

Stretch reflexes aimed to elucidate changes in spinal reflex circuitry following immobilization. With the fast and slow stretches applied in the present study we detected a short latency response (SLR) in the SOL EMG but no medium latency response (MLR). The SLR has been mainly attributed to Ia afferent fibers (Berardelli et al., 1982; Gottlieb and Agarwal, 1979). Changes in the reflex size could therefore be caused by modulations in the sensitivity of muscle spindles in response to immobilization and/or neural modulations at the spinal level.

For stretches applied during sitting, the ANOVA revealed no effect for TIME ($F_{1,8} = 1.11$, $P = 0.32$) and no effect for VELOCITY ($F_{1,8} = 1.38$, $P = 0.28$). This means that stretch reflexes during sitting were not different between pre-and post-measurement and between the two velocities. For stretches applied during stance, the ANOVA revealed no effect of TIME ($F_{1,8} = 0.12$, $P = 0.73$), but an effect of VELOCITY ($F_{1,8} = 32.17$, $P < 0.001$). This means that stretch reflexes during stance for both velocities were not different between pre- and post-measurement. However, as indicated by the significant effect for VELOCITY, stretch reflex size for fast stretches was larger than for slow stretches.

Mean ± SD stretch reflex amplitudes were as follows: High velocity sitting pre: 7.9 ± 3.9% Mmax versus post: 11.3 ± 8.5% Mmax; high velocity stance pre: 15.9 ± 4.7% Mmax versus post: 15.9 ± 7.5% Mmax; low velocity sitting pre: 8.4 ± 6% Mmax versus post: 8.2 ± 5% Mmax; low velocity stance pre: 11.5 ± 6.4% Mmax versus post: 9.9 ± 5.2% Mmax (Fig. 1).

In conclusion, stretch reflexes were not affected by immobilization, and this was independent of the velocity with which the stretches were applied. The only difference we found was a larger reflex size with high stretch velocity than with low stretch velocity during stance. This finding is in accordance with previous studies (Leukel et al., 2009).

3.1.2. H-reflexes and M-waves

The H-reflex aimed to test for changes in the excitability of the central part of the spinal stretch reflex circuitry, i.e. the projection of Ia afferents onto spinal motoneurons. For *H*/*M* ratios obtained during sitting the ANOVA revealed no significant effect of GROUP ($F_{1,6} = 1$, $P = 0.35$)and TIME ($F_{1,6} = 0.28$, $P = 0.62$). In the immobilization group, the *H*/*M* ratio was 0.63 ± 0.17 in the pre-measurement and 0.63 ± 0.18 in the post-measurement. For the control group, the *H*/*M* ratio was 0.72 ± 0.13 in the pre-measurement and 0.71 ± 0.12 in the post-measurement. This means that the strength of Ia afferent excitation of spinal motoneurons did not change by immobilization (Fig. 1).

H/*M* ratios obtained during stance, only tested in the immobilization group, were 0.55 ± 0.12 in the pre-measurement and 0.63 ± 0.19 post immobilization ($P = 0.20$).Therefore, like with measurements obtained during sitting, immobilization had no effect on the H-reflex recorded during stance.

Regarding Mmax, the ANOVA revealed no effect of the factor GROUP ($F_{1,6} = 0.18$, $P = 0.69$) but a significant effect of the factor TIME ($F_{1,6} = 16.8$, $P < 0.01$) and a significant GROUP × TIME interaction ($F_{1,6} = 10.9$, $P < 0.05$). Post-hoc comparisons revealed a significant reduction of Mmax only for the immobilization group (pre: 13 ± 3.4 mV, post: 11.7 ± 3.2 mV, $P < 0.01$) but no change of Mmax in the control group (pre: 10.2 ± 2.3 mV, post: 10.6 ± 3 mV, $P = 0.99$). The reduced Mmax indicates immobilization-induced morphological alterations at the level of the stimulated motor

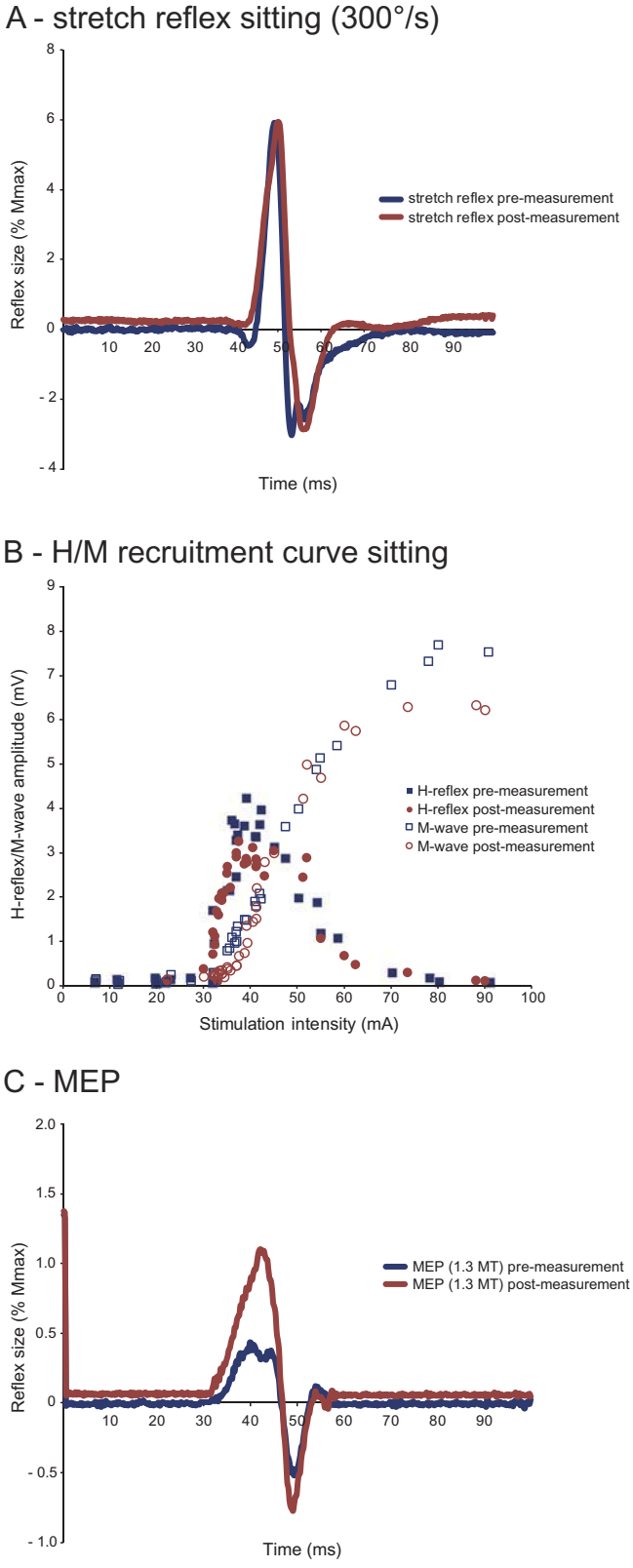

Fig. 1. Data from a single subject. (A) Shows the mean of 10 stretch reflexes applied during sitting in the pre-measurement (blue line) and the post-measurement (red line). Stretches to the calf muscles were applied with 300° per second and an amplitude of 10°. (B) Depicts the *H*/*M* recruitment curve during sitting in the pre- and post-measurement (squares: pre-measurement; circles: post-measurement). (C) Shows the mean of 5 MEPs elicited with 1.3 × motor threshold (MT). Stretch reflexes and MEPs are normalized with reference to Mmax elicited in the pre- and post-measurement, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

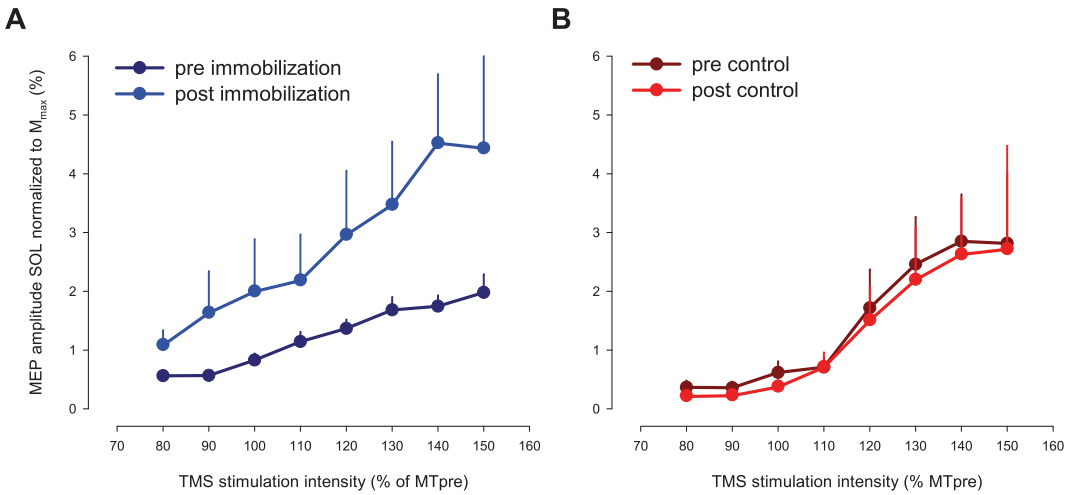

Fig. 2. TMS recruitment curves were generated based on motor-evoked potential (MEP) amplitudes obtained in SOL at rest in the pre and post-measurement for both the immobilization group and the control group. (A) Shows MEP amplitudes obtained in the immobilization group before (dark blue) and after immobilization (blue). (B) Shows corresponding data for the control group in the pre (dark red) and post-measurement (red). All MEP amplitudes are normalized to the corresponding individual Mmax and presented as group mean and standard deviation of the mean (error bars). In both panels, the abscissa represents the TMS stimulation intensity normalized to the individual MT in the pre-measurement. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

axons, the neuromuscular junction and/or associated muscle fibers i.e. muscle atrophy in response to immobilization.

3.2. Corticospinal excitability

3.2.1. MEP recruitment curves

The MEP recruitment curves served to test overall changes in corticospinal excitability. Group mean MEP recruitment curves are illustrated in Fig. 2 and single subject data is shown in Fig. 1. Within the immobilization group there was a significant main effect of STIMULATION INTENSITY ($F_{1,7} = 12.86$, $P < 0.001$), not TIME ($F_{1,7} = 4.56$, $P = 0.065$) but a significant STIMULATION INTENSITY × TIME interaction ($F_{1,7} = 3.13$, $P = 0.007$). Posthoc Bonferroni *T*-tests revealed that MEP amplitudes were significantly facilitated following immobilization for stimulation intensities 130% MT ($P < 0.05$), 140% MT ($P < 0.01$) and 150% MT ($P < 0.05$). Within the control group, there were no significant effect of TIME ($F_{1,7} = 0.23$, $P = 0.68$) or STIMULATION INTENSITY ($F_{1,7} = 2.27$, $P = 0.091$) and no interaction ($F_{1,7} = 0.27$, $P = 0.95$).

When comparing differences between pre and post-measurements a main effect of GROUP ($F_{1,6} = 17.64$, $P < 0.001$), but no effect of STIMULATION INTENSITY ($F_{1,6} = 0.4$, $P = 0.90$) was detected meaning that the observed increase in MEP-amplitudes from the pre- to post-measurement in the immobilization group was significantly different from the control group. These results demonstrate that immobilization resulted in an increased overall corticospinal excitability, which was not observed in the control group.

3.2.2. Hcond

We applied Hcond to test pathway-specific changes in corticospinal transmission. We first analyzed Hcond corresponding to the early facilitation. The mean value of the early facilitation was at −3 ms (see Fig. 3), with an individual range between −4 and −2 ms (see Section 2.9). Data corresponding to that ISI were not different between the two groups ($P = 0.65$), indicating that the early facilitation did not change after immobilization (see also Fig. 3).

The second analysis compared Hcond for the ISIs after the early facilitation, reflecting the excitability of slower (polysynaptic) corticospinal pathways. The ANOVA revealed no effect for GROUP ($F_{1,4} = 3.27$, $P = 0.15$) but an effect for ISI ($F_{7,28} = 2.49$, $P < 0.05$) and also a significant ISI × GROUP interaction ($F_{7,28} = 2.83$, $P < 0.05$). Besides the trivial finding that conditioned H-reflexes at the different ISIs changed in size (indicated by a significant effect for ISI), the striking result was the significant ISI × GROUP interaction. This finding indicates that there was a significant difference of Hcond between the two groups only at specific ISIs. We unmasked these ISIs by comparing Hcond at each ISI between the two groups. There were 3 ISIs showing significant differences, namely at ISI −1 ms ($P < 0.01$), ISI 0 ms ($P < 0.05$) and ISI 4 ms ($P < 0.01$). These results indicate that immobilization caused a significant increase of Hcond for ISIs −1, 0, and 4 ms, respectively (see Fig. 3).

The finding is strengthened when comparing Hcond at each ISI of the post-measurement with the corresponding Hcond of the pre-measurement for both groups separately. For the immobilization group, Hcond was different only at ISI −1 ms ($P = 0.04$), ISI 0 ms ($P = 0.03$), and ISI 4 ms ($P = 0.01$) (Fig. 3). There were no differences of Hcond at each of the tested ISIs for the control group.

Finally, we tested whether the size of the control H-reflex was different between the pre- and post-measurement. Note that the control H-reflex was adjusted in each experiment to 15–25% of the respective Mmax (Crone et al., 1990). As the control H-reflex served as a reference for all conditioned reflexes it should be comparable between the two tests. This was indeed the case for the immobilization group (pre control H-reflex: 26% Mmax; post control H-reflex: 26.6% Mmax; $P = 0.86$) and also for the control group (pre control H-reflex: 25% Mmax; post control H-reflex: 25.3% Mmax; $P = 0.89$).

4. Discussion

The main finding of the present study was a pathway-specific change in corticospinal excitability following immobilization evidenced by an increase in Hcond at specific ISIs. This pathway-specific modulation at ISIs −1, 0, and 4 ms, respectively, is most likely causally related to the overall increase in corticospinal excitability evidenced by the selective change in the MEP recruitment curves following immobilization. The ISI corresponding to the early facilitation was not affected by immobilization. Besides corticospinal adaptations, there were no changes in the excitability of the spinal reflex circuitry that was estimated based on stretch- and H-reflexes. All variables were referenced to Mmax in the pre- and post-measurement, respectively. Otherwise our interpretations would not be reasonable as Mmax changed following

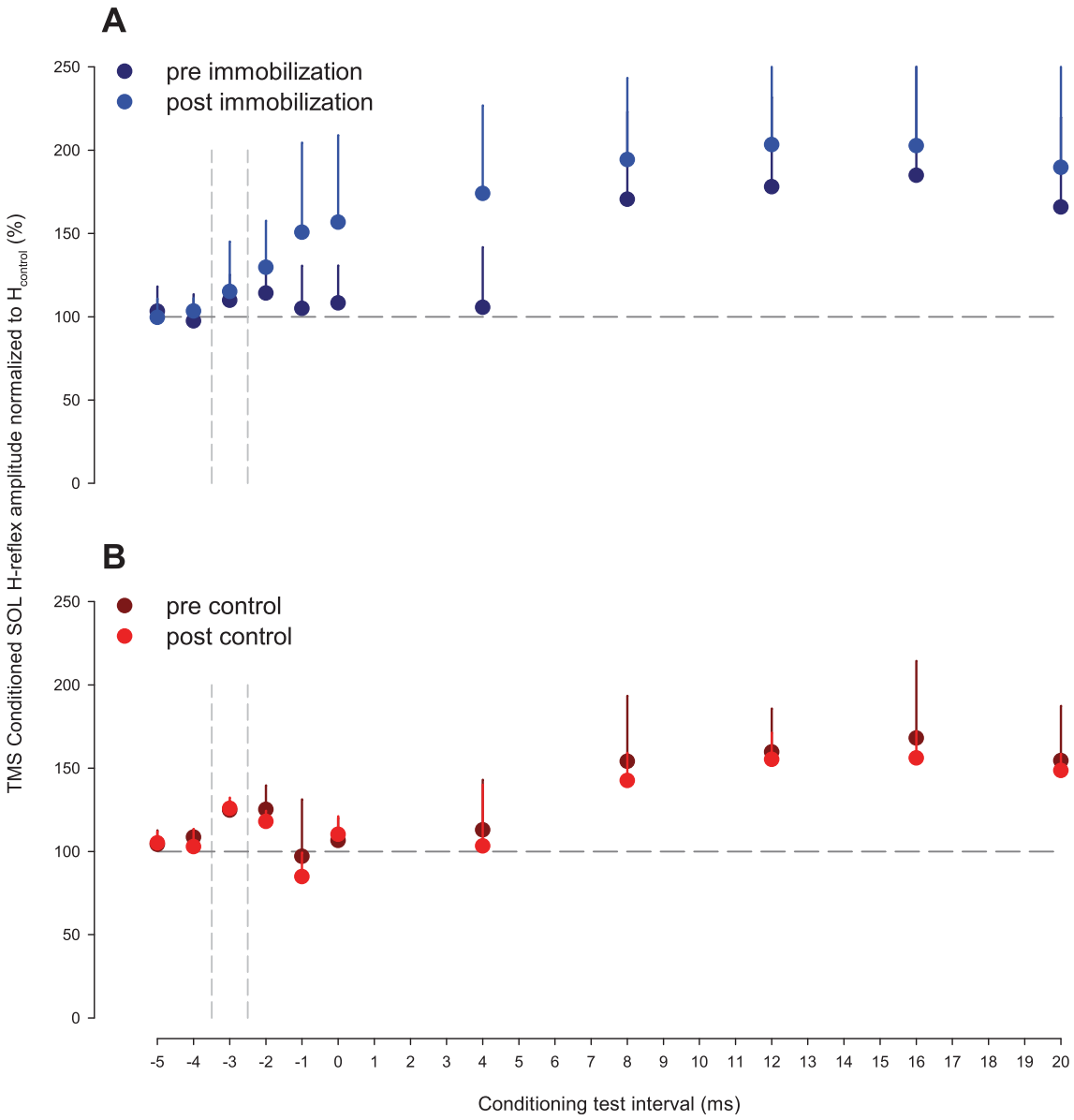

Fig. 3. Mean conditioned H-reflexes (Hcond) are illustrated for the immobilization group (A) and the control group (B). The mean value of the early facilitation, corresponding to the first increase in Hcond from baseline, can be visually detected at an ISI of −3 ms. The early facilitation most likely reflects excitation of spinal motoneurons by direct (monosynaptic) corticospinal projections. Note that at 3 ISIs there was a significant increase in Hcond following immobilization: −1, 0, and 4 ms. No changes were observed in the control group. Importantly, there was no change at the ISI corresponding to the early facilitation. Bars indicate standard deviation of the mean.

immobilization, reflecting modulations of intrinsic properties of the muscle distal to the stimulation site, most likely caused by atrophy.

It has previously been demonstrated that corticospinal excitability may increase following joint immobilization (Clark et al., 2008; Roberts et al., 2007; Zanette et al., 2004). Previous studies analyzing compound MEP should not specify the site (i.e. the exact pathway(s)) underlying the adaptation. The really novel and exciting finding of the present study is that based on the results obtained from Hcond, we demonstrate that only a specific fraction of corticospinal pathways was affected following immobilization.

The early facilitation was similar in pre- and post-measurement and occurred on average at the ISI of −3 ms in both groups. An ISI around −3 ms for the early facilitation was similarly reported in previous studies (Leukel et al., 2012; Taube et al., 2011). The early facilitation has been argued to reflect excitation of spinal motoneurons by direct (monosynaptic) corticospinal projections (Nielsen and Petersen, 1995a; Nielsen et al., 1995, 1993). It is interesting that immobilization did not influence excitability and/or transmission in this part of the corticospinal pathway. Direct corticospinal pathways have been argued to be involved in many – if not most – forms of complex human movements such as walking (Petersen et al., 1998b) hopping (Taube et al., 2012), ballistic movements (Taube et al., 2011), and fine coordinated finger tasks (Porter and Lemon, 1993). Thus, when investigating effects of disuse (joint immobilization) we would have hypothesized beforehand that immobilization is accompanied by changes in the excitability and/or transmission in these direct pathways.

Instead of changes of the early facilitation we observed an increase in slower/longer latency corticospinal pathways at ISIs 0, 1, and 4 ms.

Although the conditioning technique used in the present study allows differentiation of different fractions of the corticospinal pathway it does not allow to deduce the exact site of these adaptation(s). Considering H-reflex conditioning curves it is well accepted that the first (early) facilitation is in all likelihood

propagated via direct (monosynaptic) corticospinal pathways (Nielsen and Petersen, 1995a; Nielsen et al., 1995, 1993; Petersen et al., 1998b). The subsequent inhibition occurring around 1–2 ms after the early facilitation was argued to be caused by disynaptic reciprocal inhibition (Nielsen and Petersen, 1995a; Nielsen et al., 1995). From this ISI onwards, slower and more indirect pathways were thought to be involved (Petersen et al., 1998b). Interestingly, the contribution of these indirect pathways seems to greatly depend on the task leading to a facilitation of the H-reflexes during tonic contractions and at rest but suppression during dynamic movements (Taube et al., 2011). Recently we demonstrated that this inhibition is most likely taking place at the cortical level as H-reflex conditioning with magnetic stimulation over the cervicomedullary junction (CMS-conditioning, where effects of the tested ISIs are not dependent on excitability of cortical cells) did not result in comparable inhibition but rather in a facilitation (Taube et al., 2011). Referring to the present experiment, the HEP orthosis prevented dynamic movements but allowed tonic contractions of plantar flexor muscles and therefore it may be argued that the kind of inhibition of slower corticospinal pathways just described was not needed. The significant facilitation of Hcond at later ISIs after immobilization might then be seen as a cortical dis-inhibition. However, the experimental setup, in particular the fact that we did not apply CMS-conditioning, does not allow testing this assumption, as we cannot exclude spinal adaptations. Recently, we provided evidence by means of CMS-conditioning that both direct monosynaptic (Taube et al., 2014) as well as indirect polysynaptic corticospinal projections (Leukel et al., 2012) undergo plasticity at the spinal level in response to repetitive activation. These observations make it likely that immobilization also results in changes of corticospinal transmission that are related to spinal mechanisms. Spinal changes may relate to adaptations of propriospinal neurons (Alstermark et al., 1999, 2007) or other segmental interneurons. In line with this, we previously found a decrease in presynaptic inhibition of Ia afferents in response to immobilization (Lundbye-Jensen and Nielsen, 2008b). Presynaptic inhibition was not monitored in the present study, but we did not observe changes in the H-reflex parameters.

The unchanged H-reflexes in the present study are in contrast to other studies that found increased excitability in the central part of the spinal stretch reflex circuitry and increased H-reflex amplitudes following joint immobilization (Clark et al., 2008; Lundbye-Jensen and Nielsen, 2008a,b). It may be that this discrepancy relates to the duration of the joint immobilization period or to the immobilization paradigm. In previous studies the ankle joint was immobilized for the full intervention period and also the lower limb was suspended whereas subjects were allowed weight-bearing activities in the present study. Additionally, subjects did not wear the orthosis overnight in the present study. This is also in contrast to previous studies in which subjects wore a cast for 24 h per day. Consequently, the immobilization paradigm in itself may have implications for the sensorimotor activation patterns and subsequently also for the plastic changes accompanying immobilization.

The fact that the electrophysiological measurements were conducted at rest and not during movement constitutes a methodological limitation of the current study. The present study was designed to cover a wide range of possible neural sites of adaptations. Based on our findings of pathway-specific modulation of corticospinal transmission after immobilization future work should look into "functional" corticospinal plasticity following immobilization.

In relation to the "functional" corticospinal plasticity, a further limitation is that we are unable to link the neural adaptations reported in the present study to behavior. For instance, the increase in Hcond at specific ISIs could mean a decrease in the performance of fine motor skills, walking, and/or balance control.

In summary, the current study is to our knowledge the first to demonstrate that the overall increase in corticospinal excitability observed following immobilization that was reported in previous experiments is in fact a pathway-specific corticospinal adaptation. Interestingly, we show that transmission in indirect but not direct (monosynaptic) corticospinal connections were affected.

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