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DIFFERENT EEG EFFECTS OF LONG-INTERVAL PAIRED PULSE TMS IN AWAKE AND NREM STATE

Master thesis

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Running head: Effects of ppTMS in different brain states

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Abstract

Different EEG effects of long-interval paired pulse TMS in AWAKE and NREM state.

Occipital transcranial magnetic stimulation (TMS) applied in a task-free experimental setup during wakefulness leads to relative more positive slow-wave brain potentials in frontal and central areas the of brain (Stamm, Aru, & Bachmann, 2011). Current study tested whether paired pulse TMS during wakefulness is associated with increase of slow negative processes compared to the deep sleep (NREM state). Interestingly, qualitatively and quantitatively different processes take place when second TMS impulse is added. Here it is shown that long-interval paired pulse TMS (with inter-pulse-interval 100 ms) targeted at V1 (calcarine area) will trigger slow wave potential during sleep while single pulse TMS fails to do so. It is proposed that globally expressed differences in the TMS-induced potentials may be due to inhibitory and excitatory effects of the thalamocortical system. The ability to evoke slow waves noninvasively at much lower intensities of the TMS than it was previously thought is important for several reasons (as discussed in this work) and could be used to develop clinical tools.

Kokkuvõte

Pika ajaintervalliga paaris-impulssidega TMS kutsub ärkveloleku- ja uneseisundis esile erinevad EEG-vastused.

Oktsipitaalne transkraniaalne magnetstimulatsioon (TMS) rakendatuna ülesandevabas katseseadistuses suurendas ärkvelolekus aeglase potentsiaali suhtelist positiivsust frontaalses ja tsentraalses ajupiirkonnas (Stamm, Aru, & Bachmann, 2011). Käesolev töö uuris, kas ärkveloleku ajal esitatud paaris-impulssidega TMS on seotud aeglaste negatiivsete protsesside suurenemisega võrrelduna rahuliku unega (NREM faasis). Selgus, et teise impulsi lisamisel toimuvad kvalitatiivselt ja kvantitatiivselt teistsugused protsessid. Kasutades pika impulsside vahelise intervalliga paaris TMSi (impulsside vaheline intervall 100 ms) sihituna V1-te (kalkariinvao piirkond) kutsub une ajal esile aeglase laine, mida aga üksikimpulsiga TMS-i puhul ei teki. Töös leitud tulemustest järeldub, et globaalselt väljendunud erinevused TMSi poolt esile kutsutud potentsiaalides võivad olla seotud talamo-kortikaalse süsteemi pidurdus- ja erutusefektidega. Võimalus esile kutsuda aeglaseid laineid mitteinvasiivselt ja palju madalamal intensiivsusel kui seda varasemalt on sobivaks peetud, on oluline mitmel töös käsitletaval põhjusel ja seda nähtust annab rakendada kliiniliste meetodite väljatöötamiseks.

Introduction

Our knowledge of how one state of mind (sleep, wakefulness, vegetative state, etc.) differs from another is still quite inadequate. We can presume that if a person drifts from wakefulness to sleep (Massimini et al., 2005), from wakefulness to pharmacologically induced loss of consciousness (Ferrarelli et al., 2010) or to vegetative state (Laureys, Owen, & Schiff, 2004) this person is unconscious. However, this does not mean that brain simply shuts down – the brain remains active and different processes take place (Steriade, Timofeev, & Grenier, 2001; Hobson & Pace-Schott, 2002; Steriade, McCormick, & Sejnowski, 1993; Muzur, Pace-Schott, & Hobson, 2002; Gennaro et al., 2007; Baars, Ramsøy, & Laureys, 2003). The problem is that we fully do not understand what happens and how it happens.

According to the "Integrated information theory" consciousness depends on information integration in the brain (Tononi, 2004) and not so much on sensory activity, or neural processes at specific frequencies. If that information integration established within different cortical regions, consciousness should fade (Massimini et al., 2005). Therefore changes in consciousness may be associated with changes in cortical effective connectivity (Massimini et al., 2010; Massimini et al., 2005; Nir & Tononi, 2010). By effective connectivity it is meant that a subset of neural systems has the ability to causally influence the activity of other neural populations within a system (Lee, Harrison, & Mechelli, 2003). In order to measure this, one could perturb a specific brain area and measure how evoked activity spreads to other regions (Esser, Hill, & Tononi, 2009).

In humans, direct perturbation of a certain brain area has been done by using transcranial magnetic stimulation (TMS). By combining TMS with functional brain imaging techniques such as electroencephalography (EEG) it is possible to map TMS-evoked neural processes in different brain states (Komssi & Kähkönen, 2006). Previous works, where TMS and EEG have been combined, have shown different state dependent effects. Massimini et al. (2005) found that when rostral part of the right pre-motor cortex was stimulated by TMS during slow wave sleep, the response to TMS was local, with shorter duration and remained confined to the site of stimulation, indicating a reduction in effective connectivity in this state, whereas during wakefulness TMS-evoked activity propagated beyond the site of stimulation. Similar kind of breakdown of cortical effective connectivity can be also induced by pharmacologic agents (Ferrarelli et al., 2010) or can be seen when person is in the vegetative state (Rosanova, Gosseries, Casarotto, Boly, & Casali, 2012). More widespread patterns of

activations were also seen after TMS stimulation during REM sleep, which makes this state more similar to wakefulness (Massimini et al., 2010).

Changes in connectivity also take place when ethanol is induced for lowering arousal (Kähkönen et al., 2001) or when caffeine was used for inducing a higher arousal state (Murd, Aru, Hiio, Luiga, & Bachmann, 2010). Murd et al. (2010) found that occipitally delivered TMS evoked brain potentials with increased global negativity, but no speed-up of the latency of the potentials occurred. On the other hand, occipitally delivered TMS during NREM sleep led to relative more positive slow-wave brain potentials in frontal and central areas of the brain, compared to wakefulness (Stamm, Aru, & Bachmann, 2011). These two findings suggest that absence of negativization will indicate the loss of consciousness and negativization related signatures could be a marker of the breakdown of cortical connectivity.

Other specific state dependent EEG patterns can be also found. Slow waves (SW) are well-known EEG features of NREM sleep. Slow waves emerge spontaneously from the synchronization of large populations of slowly oscillating neurons during deep sleep (Amzica & Steriade, 1995) and travel across the brain (Massimini, Huber, Ferrarelli, Hill, & Tononi, 2004). EEG studies have shown that this traveling is not random but involves specific parts of higher order cortical structures (Massimini, Huber, Ferrarelli, Hill, & Tononi, 2004). Interestingly, visual and sensory areas are less involved with SW (Murphy et al., 2009). It has been proposed that this is due to the fact that during wakefulness these areas may have less plasticity than the higher order cortical areas and therefore may be less involved in SW (Murphy et al., 2009) This indicates that SW plays an important role in the cellular plasticity and can be used to investigate changes in neuronal excitability and connectivity – especially when taking into account that SW are not only spontaneous events but can be also induced with TMS (Massimini et al., 2007). Interestingly and importantly for the present work, Massimini and colleagues (Massimini et al., 2007) were unable to induce SW from the visual cortex.

To summarize, the TMS-EEG studies discussed so far have provided significant hints about the mechanisms that may be critical for sleep and consciousness and how different conscious states are expressed in the EEG brain activity. Studies of decreased (Ferrarelli et al., 2010; Massimini et al., 2010; Massimini et al., 2005; Rosanova, Gosseries, Casarotto, Boly, & Casali, 2012; Stamm, Aru, & Bachmann, 2011) or increased (Murd, Aru, Hiio, Luiga, & Bachmann, 2010) states of arousal can give us a better insight how one state of mind differs from another. More precisely – these TMS-EEG studies help us to understand why unconsciousness (in this case NREM sleep) rises and how it differs from consciousness (in

this case wakefulness). This is particularly important because with traditional perception experiments (modal stimulation through senses) behavioral tasks are seriously confounded with brain state dependent effects and therefore underlying state dependent effects are difficult to investigate.

Using task-free, non-sensory stimulation (TMS) of cortical tissue together with EEG to investigate neural correlates of consciousness offers several further specific advantages. First, using controlled perturbations allows studying reactivity of any cortical region in the intact brain (Komssi & Kähkönen, 2006). Second, neuronal effects of TMS in the brain can be measured in a millisecond time scale and from multiple scalp locations (Komssi & Kähkönen, 2006). Third, it represents an effective way to understand brain rhythm interactions at the whole-brain level without unwanted peripheral effects (Ferrarelli et al., 2010; Komssi & Kähkönen, 2006) by bypassing sensory pathways, primary cortical areas or/and thalamic gate (Ferrarelli et al., 2010; Esser, Hill, & Tononi, 2009). Fourth and most importantly, directly probing cortical circuits with TMS/EEG can help us to arrive at a better understanding of what the underlying processes of consciousness are and accomplish this without any task- and environmental stimulus related confounds. Therefore combining TMS with experimentally controlled arousal states (wakefulness vs. sleep) effective connectivity (causal interactions) can be distinguished from functional connectivity (Alkire, Hudetz, & Tononi, 2008; Murd, Aru, Hiio, Luiga, & Bachmann, 2010; Stamm, Aru, & Bachmann, 2011).

TMS could be applied in single or paired pulses, but also repetitively (rTMS) by regularly repeating stimuli to a single scalp site. Paired-pulse techniques have mainly been used for studying of the function of human motor cortex (Sparing et al., 2005). These investigations have shown that the effect of paired-pulse TMS may be inhibitory (intracortical inhibition, ICI) or excitatory (intracortical facilitation, ICF), depending on the interstimulus interval (ISI) (Ziemann, 2002). Similar patterns of inhibition and facilitation can be induced in motor (Maeda, Gangitano, Thall, & Pascual-Leone, 2002), prefrontal (Oliveri et al., 2000), parietal (Oliveri et al., 2000) and visual cortex (Sparing et al., 2005).

However, little or no research has done to investigate paired-pulse effects of TMS (ppTMS) by means of sleep studies. Our aim is to study state dependent effects of single and paired pulse TMS on primary visual cortex (V1 and V2). We chose visual cortex because although ppTMS related studies have mainly focused on stimulation of motor cortex (Overgaard, Nielsen, & Fuglsang-Frederiksen, 2004), the visual system is probably the most studied system in cognitive neuroscience. Furthermore, with this research we extend our earlier studies with occipital TMS/EEG where single TMS pulses were used (Murd, Aru,

Hiio, Luiga, & Bachmann, 2010; Stamm, Aru, & Bachmann, 2011). More importantly, there is a long tradition of studying the effects of using two stimuli in visual system in the form of visual masking where the interactions of two visual stimuli depend on the timing between them (Bachmann, 2000). It would be interesting to see how two amodal "visual" TMS-evoked impulses interact and how this compares to the interaction of two modal visual stimuli.

Moreover, when analyzing the pertinent literature on paired-pulse TMS effects on EEG responses it can be noticed that (i) mostly stimulation protocols with very short inter-pulse intervals ranging from a couple to about a dozen milliseconds (ms) have been used and (ii) post-TMS epochs subjected to TMS evoked potential (TEP) analysis have been short in duration (up to about 200-400 ms) (Ferreri et al., 2011; Ilmoniemi & Kičić, 2010; Kimura, Ogata, Nakazono, & Tobimatsu, 2013; Kojima et al., 2013). This seems to be a certain limitation because as shown with clarity and sufficient power of replication in modal ERP experiments, conspicuous state dependent signatures of brain activity sensitive to the consciousness level unfold slowly (He & Raichle, 2009; Hudetz, Vizuete, & Imas, 2009; Riedner, Hulse, Murphy, Ferrarelli, & Tononi, 2011). Therefore, it should be natural to try observe the effects of first pulse on the second pulse evoked TMS potentials also over the range of longer inter-pulse-intervals (IPI).

It would be desirable to test various IPI-s between the two TMS impulses just as different stimulus onset asynchronies are used in visual masking. However, for reliable EEG analysis we need a high number of trials and therefore we settled for one particular IPI. We chose the IPI of 100 ms. This is because on the one hand 100 ms covers an interesting late portion of the traditional masking functions (Bachmann, 2000) but on the other hand 100 ms is the cycle of the alpha oscillation, whose effects on visual perception have been studied well over the recent years (Busch, Dubois, & VanRullen, 2009). Taken together, our main interest is whether and how the ppTMS with the IPI of 100 ms differs between the awake and the NREM sleep states.

Taking into account our previous discussion we put forward two simple general hypotheses: 1) when assessed in sleep, globally spreading positivity (measured by slow potentials and N1) should increase after TMS; 2) compared to the NREM state ppTMS in awake state is associated with an increase of slow negative process, such that this increase is more than simply the sum of the differences between single TMS pulse effects of NREM and awake state.

Materials and methods

Participants

Fifteen healthy adults (8 male and 7 female, age range 20–29) participated in a one-day experimental study. Before the experiment all subjects gave written, informed consent and they were paid for participation. The protocol was approved by the Ethics Review Committee on Human Research of the University of Tartu and the experiments were undertaken in compliance with national legislation and the Declaration of Helsinki.

Prior to the experiment a neurological screening was performed to exclude potential adverse effects of TMS and according to the following criteria: 1) not using a cardiac pace-maker, a hearing aid or other surgical implants or installed surgical staples, 2) no neurological or psychiatric disorders, 3) no history of head trauma or surgery, severe chronic seizures (including anxiety disorders), seizures, paralysis, high or low blood pressure, migraine headaches, hearing problems; 4) no pregnancy, 5) no self-reported sleep complaints, 6) non-smoking.

All participants were right handed, reported normal or corrected to normal vision and did not report any sleeping disorders. All subjects were instructed to maintain regular sleep-wake schedule and they filled sleep diary during the 4 days before the experiments. Participants refrained from caffeine and alcohol on the day during the experiment and were not allowed to engage in excessive physical activity 72 hours prior to the study.

In one case experiment was repeated after some weeks because the subject could not fall asleep. In other cases where subjects could not fall asleep or only attained brief periods of light sleep subjects were excluded from later study; 4 male and 4 female subjects were excluded from the analysis due to insufficient sleep trials. Data presented here are from 7 subjects (4 male and 3 female) who were able to maintain NREM sleep for a sufficient duration under the inconvenient recording conditions and from whom sufficient data could be recorded for robust statistical evaluation.

General experimental procedures

Before coming to the study session participants were instructed not to drink anything containing caffeine, not to smoke and participate in intense physical activity. On the

experimental night, participants arrived at the laboratory between 18:00 and 19:00 and were outfitted with 60-channed EEG cap with a set of necessary electrodes for sleep-TMS-EEG recordings (Siebner et al., 2009). After preparations, where stimulation parameters (target location, stimulation intensity and masking noise volume) were adjusted, we digitized electrode positions and started the main experiment between 21:00 and 22:00. Participants were instructed to fall asleep as soon as they could. Stimulation was started while the subject was still awake and while the subject progressed from wakefulness to NREM sleep TMS stimulation blocks were continuously delivered and the spontaneous EEG was monitored. Throughout the experiment EEG data were digitized and were used later for the analysis.

During the experiment TMS pulses were delivered by the same persons. Subjects were laying eyes closed in semi-horizontal position on the chair of the Eximia EEG/TMS set (Nexstim Ltd, Finland), with a special head-rest that allowed a comfortable and stable head position and fixed TMS coil position. All participants were blindfolded and masking noise was played through in-ear headphones. In addition, subjects were covered with a blanket so that they would be more comfortable to fall asleep and stay asleep. Lights were turned off and room was dimly lit.

All TMS pulses were delivered on the same target area (middle of calcarine fissure) (see Fig. 1). MRI image of the brain of each subject was scanned previously and targets for TMS were marked individually for each subject in the right location.

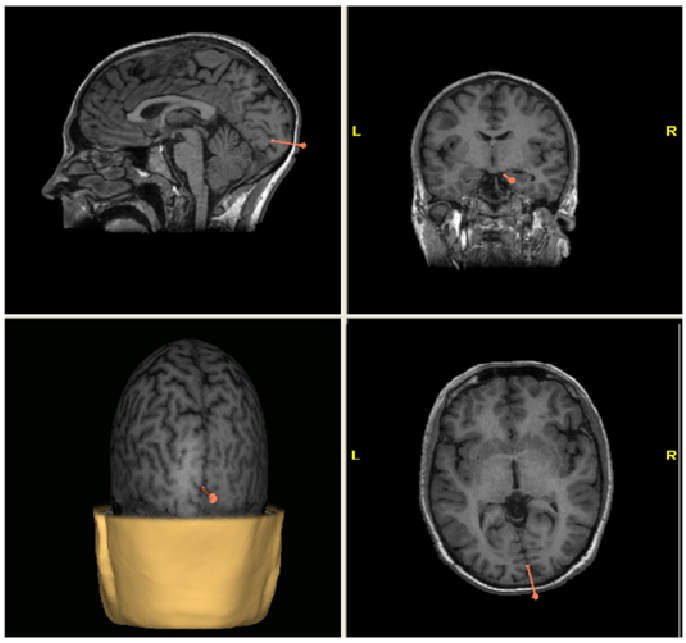

Figure 1. Example view of the brain image that assisted neuronavigation of the TMS pulses to V1 in the calcarine area.

Two alternations of TMS blocks were used – with single-pulse TMS (spTMS) and with paired-pulse TMS (100 ms between stimulations) (ppTMS). Each block consisted of 60 trials and lasted approximately 4 minutes. There was a brief pause between each block, during which the TMS coil was repositioned precisely to the correct position (if correction was needed). The pauses also helped us to avoid overheating of the coil. Stimulation was restarted according to the EEG pattern of interest. In addition, if a movement occurred during a recording session, the session was interrupted and the coil was repositioned to the precise specified stimulation coordinate. At the end of the experiment, the session parameters (stimulation coordinate, stimulation intensity and electrode position) were recorded, digitized and were made available for later analysis.

All studies started with single stimulation block and blocks were given in ABAB design. At least 3 blocks (180 trials) of each condition (spTMS wakefulness, spTMS NREM sleep, ppTMS wakefulness and ppTMS NREM sleep) were recorded. Therefore at least 720 trials were recorded for each subject.

TMS

In order to ensure the accuracy and reproducibility of stimulation target in the brain and reduce effects of uncontrolled variability (such as inaccurate positioning of the stimulation coil) (Casarotto et al., 2010), MRI-based Navigated Brain Stimulation (NBS) system (Nexstim Ltd., Helsinki, Finland) was used to locate the TMS target on an individual subject. This system has several advantages: it enables 1) precise (maximal inaccuracy is typically 1–2 mm, plus a possible inaccuracy of few millimeters on registration) on-line navigation of relative positions and orientation of the subject's head and of the TMS coil in 3D space (Julkunen et al., 2009); 2) estimation of the NBS calculated intracranial electric field intensity (V/m) and distribution induced on the surface of the brain by the TMS individually for each subject's brain; 3) that the exact location of the maximum electric field on the cortical surface (hot-spot) could be monitored in on-line; 4) real-time control over the reproducibility of stimulation parameters across sessions (Casarotto et al., 2010).

Individual TMS target locations were marked down using brain MRI visualization software. TMS target location in V1 was placed in middle of the calcarine fissure (see Fig. 1) for each of the subjects and were slightly adjusted across subjects to adapt to inter-individual differences (brain size and anatomy, or cortex morphology). The MR-images and the

coordinates of stimulation (coil position, tilting and orientation) for each individual subject were stored to a software aiming tool. This allowed reliably repeating TMS pulses to a certain anatomical structure and made markers visible for the experimenter on computer monitor throughout the session. In addition, by using NBS software, spatial EEG electrode positions for each individual subjects was registered and stored.

During the experiment all subjects wore spectacles with special multiple small spherical reflectors mounted on it. Similar reflectors were mounted on biphasic figure-of-eight coil (wing diameter of 70 mm). Using special spectacles and coil with the dedicated system of an infrared stereo camera, supported by special software and TMS-system (Eximia TMS Stimulator; Nexstim Ltd., Helsinki, Finland) enabled to maintain the spatially stable and reliable TMS coil position throughout the experiment.

Stimulation parameters

Stimulation parameters were chosen in accordance with the internationally accepted guidance document (Anand & Hotson, 2002; Awiszus, 2003; Hallet, Wassermann, Pascal-Leone, & Valls-Sole, 1999; Keel, Smith, & Wassermann, 2000; Wassermann, 1998, 2002). Two kinds of stimulation-parameters were applied: single pulses with duration less than 1 ms and double pulses with delay of 100 ms between pulses. Stimulation intensity was 50% of the maximum output of the stimulator (maximal output 0.7 T in cortex) and corresponded to a maximum estimated electric field on the target between 17 and 67 V/m (average 43 V/m) on the target location (average peeling depth 22 mm), as estimated by the NBS system. This kind of pulse intensity guaranteed that subjects did not experience scotomas, phosphenes or any kind of other inconveniences (like neck muscle contractions). In order to avoid unwanted reorganization or plasticity processes that might possibly interfere with the longitudinal measurements or affect EEG responses to TMS (Casarotto et al., 2010) single and paired TMS pulses were delivered at an inter-stimulation frequency randomly jittered between 3500 – 5000 ms (equivalent to ca. 0.3–0.2 Hz).

TMS stimulation area was middle of right and left hemisphere calcarine fissure. We chose this location for several reasons: 1) it stimulates both hemispheres of the brain at a time (because TMS focus is approximately one square centimeter); 2) it allowed comfortable stimulation and is far from any major head or facial muscle whose unwanted activation may affect EEG recordings; 3) the reproducibility of the stimulation coordinates across subjects was easily obtained.

Management of confounding factors

With each TMS pulse unwanted side effects, such as sensory, somatosensory and auditory artifacts, could arise (Bergmann et al., 2012; Esser, Hill, & Tononi, 2009; Ferrarelli et al., 2010; Tononi et al., 2005). Confounding factors could lead to misinterpreting the EEG data and/or disturb the sleep pattern and need to be avoided. Therefore we took special care to reduce this. The following precautions were used in order to avoid different artifacts.

The main unwanted side effects in addition to the magnetic artifacts are eye blinks and skeletal muscle tension (Komssi & Kähkönen, 2006). Firstly, all subjects were blindfolded in order be sure that the TEP was not due to the interaction with visual sensory stimulation.

Movement of head could cause movement of the electrodes. This can also happen due to coil vibration causing direct-current shifts in the signals of the electrodes near the coil. Direct TMS manipulation can also induce unwanted contraction of head muscles that may contaminate the EEG data. In order to reduce somatosensory stimulation there was left a little space between the coil and skull (Komssi & Kähkönen, 2006). Moreover, this artifact can be also minimized if stimulation is done at lower intensity. Therefore stimulation intensity, which did not cause any head muscle contractions, was chosen.

EEG recordings could be also disturbed by auditory sound of TMS coil click. More precisely the click associated with the coil's discharge propagates through air and bone and can elicit an auditory N1-P2 complex at latencies of 100-200 ms (Nikouline, Ruohonen, & Ilmoniemi, 1999). Using in-ear earplugs, continuous masking sound was played in order to remove the subject's perception of the coil's click and reduce its disturbance of spontaneous sleep EEG patterns. Two types of masking sound were played simultaneously: sequence of previously recorded TMS clicks and neutral monotonic music. Sound volume was adjusted in accordance with each subject so that they reported not perceiving the TMS click (always kept below 90dB) but not higher where it could distract person to fall asleep (Tononi et al., 2005). All subjects reported not to have heard the TMS-clicks.

EEG recording

Spontaneous and TMS-evoked EEG data were recorded using a 60-channel Nexstim eXimia EEG-system with carbon electrodes cap and specifically designed TMS-compatible EEG amplifier (Nexstim Ltd, Helsinki, Finland). To further optimize TMS compatibility, the impedance at all electrodes was kept below 10KΩ. All EEG signals (resting-state and sleep),

were referenced to an additional reference electrode placed on the forehead. Signals were amplified with a gain of 2000 and with hardware based band-pass filter of 0.1-350 Hz and sampled at 1450 Hz. To control for eye-blinks two extra sensors were used to record the vertical electrooculogram (VEOG).

EEG preprocessing

All EEG data were analyzed with FieldTrip open source toolbox (Oostenveld, Fries, Maris, & Schoffelen, 2011) running under Matlab (Mathworks, Inc.). EEG data were filtered with 30 Hz high cutoff filter. Continuous EEG recordings gathered during TMS stimulation were split into epochs (between -1200 to +1500 ms) around TMS pulses. The time interval used for baseline correction was set at -200 – -100 ms as measured with regard to TMS stimulation onset. All data were additionally visually inspected for artifacts. Trials containing artifacts, such as muscle activity, eye blinks (movements), were visually detected and manually rejected.

In order to apply data analysis to the same number of channels across sessions and subjects, we have interpolated bad channels by replacing them with the average of its neighbors weighted by distance with nearby electrodes. In no case the number of bad channels exceeded 5% of all channels. (Picton et al., 2000).

Sleep stages were scored according to adult sleep stage scoring rules (Fraiwan, Khaswaneh, & Lweesy, 2009; Hobson & Pace-Schott, 2002; Hori et al., 2001; Šušmáková, 2004). All artifact-free segments were pooled into four conditions and were averaged separately. Thus, no separations between NREM sleep stages 2-4 were done. NREM sleep stage 1 trials were visually detected and manually rejected during artifact rejection phase. Trials where spontaneous slow wave appeared right before TMS impulses were rejected because it is known that TMS cannot evoke a slow wave right after them (Massimini et al., 2007). Altogether, at least 153±34 trials (mean±standard error across all conditions and subjects) contributed to the averages per condition that were later used for analyses (range 101-219 trials).

On the average 30% of trials were excluded from wake trials and 34% of trials were excluded from sleep trials due to artifacts. In sum we had 2342 trials for wakefulness condition (of which 1288 were single and 1054 were double pulse TMS) and 1931 trials for NREM sleep condition (of which 974 were single- and 957 were double-pulse TMS).

EEG analysis

The experimental conditions were manipulation/session (wake vs. NREM) and inter-stimulus interval (single vs. double-pulse). After the preprocessing average TEP were computed for each subject in each condition and for each electrode. For plotting average of all electrodes was also calculated, which resulted in one single TEP potential per condition. Time range -200 ms to +1500 ms was used for plotting TEP; baseline correction was set against a -100 ms interval at -200 ms – 100 ms before TMS onset.

Statistical analysis

For statistical analysis of TEP amplitudes EEG signals spanning the time range +200 ms to 1000 ms post stimulation were analysed without using filtering or re-referencing. A nonparametric cluster based two-way analysis of variance (ANOVA) with repeated measures was used with condition manipulation/session (wake vs. NREM) and pulse delivery regime (single vs. double pulse) as within factors. Statistical significance was determined with the cluster randomization method computed over all electrodes and time-points. Given the impracticality of computing all possible combinations, 5000 unique combinations were ran for each comparison in order to approximate the actual cluster distribution. Single samples were considered significant and included into their respective cluster if they exceeded a p-value threshold of 0.01. Clusters revealing a p-value below 0.05 were considered significant.

Results

Expected as well as unexpected results were found. First we report results on main effects. Main effect of arousal state (sleep vs. wakefulness) in time range 206 ms – 383 ms ($p<0.05$ corrected for multiple comparisons) and inter-stimulus interval (spTMS vs. ppTMS) in time range 310 ms – 563 ms ($p<0.05$ corrected for multiple comparisons) were found. Also an interaction between arousal state and inter-stimulus interval was found in time range 213 ms – 512 ms ($p<0.05$ corrected for multiple comparisons), indicating that in different arousal states the brain reacts differently when spTMS or ppTMS is used. These effects are expressed over all electrodes.

EEG traces evoked by single-pulse or paired-pulse TMS from both sleep and awake conditions are illustrated on Figure 2 A. Voltage maps with 100 ms steps for the time frame 0

ms – 1000 ms post stimulation were also plotted to show the difference of wakefulness and NREM sleep conditions for all the electrodes (Fig. 2B). These plots show that ppTMS during sleep evokes a negative peak at 290 ms ($p<0.05$ corrected for multiple comparisons) followed by a positive slow wave ($p<0.05$ corrected for multiple comparisons), whereas spTMS evokes even larger negative peak ($p<0.05$ corrected for multiple comparisons) but no positive slow wave follows.

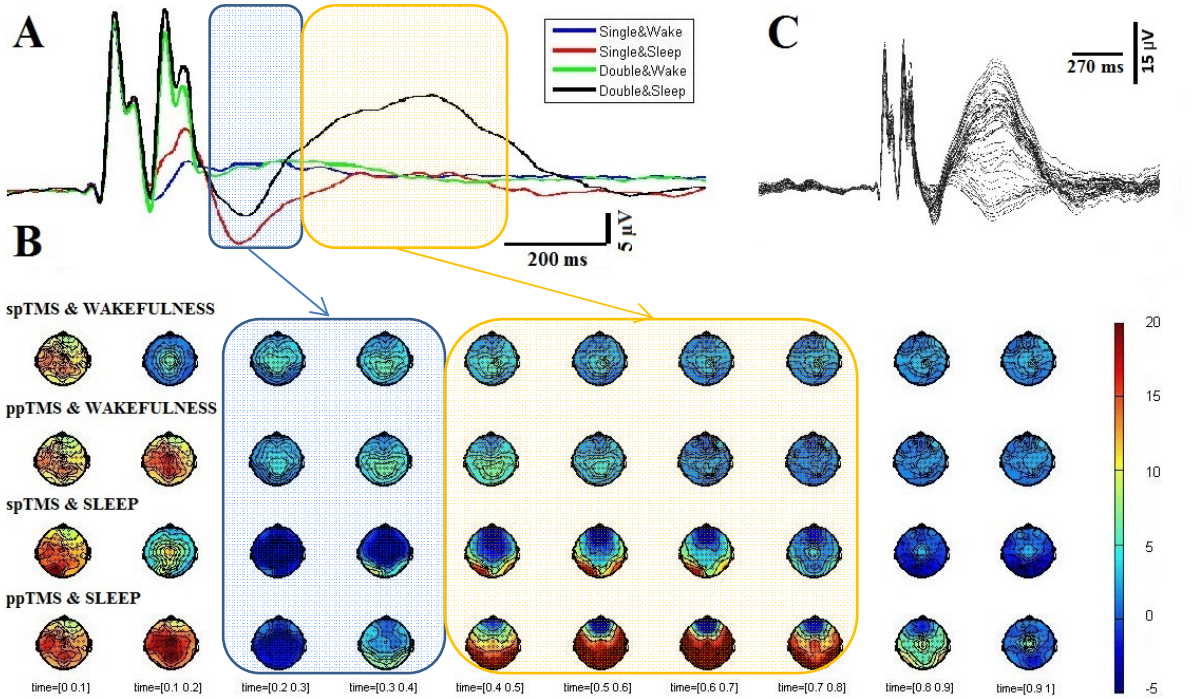

Figure 2. Examples of grand average TEPs recorded from all channels for four conditions (A). Blue EPs showing spTMS in wakefulness condition, red spTMS in NREM sleep condition, green ppTMS in wakefulness condition and black ppTMS in NREM sleep condition. (B) Plots of TEP cerebral distribution in wakefulness and NREM sleep conditions, data pooled over all subjects at different progressive time epochs after V1 stimulation, shown with 100 ms steps; plots between 0 to 1000 ms. (C) Examples of grand average ppTMS-evoked potentials recorded from all channels for sleep condition. TMS-evoked slow waves were detected when ppTMS was used during NREM sleep.

However, slow potential data did not confirm the first hypothesis because spTMS stimulation during NREM sleep did not induce globally spreading positivity as it was expected. On the contrary, in sleep TMS evoked a negative peak at 290 ms ($p<0.05$ corrected for multiple comparisons), which had a significantly larger overall peak amplitude, started later and had a longer latency when spTMS was used ($p<0.05$ corrected for multiple comparisons). Reason why relative negativity was not observed for the awake condition might be because different stimulation parameters (higher stimulation intensity and longer latency

between stimulations) were used compared to our previous study (Stamm, Aru, & Bachmann, 2011).

Our second hypothesis claimed that compared to the NREM state ppTMS in awake state is associated with an increase of slow negative process, such that this increase is more than simply the sum of the differences between single TMS pulse effects of NREM and awake state. By simply comparing spTMS with ppTMS TEP, however, one cannot test whether the second hypothesis is true or not, because effects that go beyond the simple sum of the differences of two single pulse effects remain hidden. To put it differently, we have not yet shown that the second TMS pulse causes a different phenomenon from the first pulse. In order to unravel which neural processes are uniquely elicited by the second TMS impulse the following procedure was adopted. Within every single subject spTMS TEP was subtracted from ppTMS TEP separately for wake and sleep conditions. After that single pulse condition was shifted by +100 ms so that single pulse TMS onset was aligned with second TMS pulse onset of the ppTMS condition (see Fig. 3). From this figure one can see the effect of the second TMS impulse as compared to the first TMS pulse and how this difference is modulated by the state of the brain.

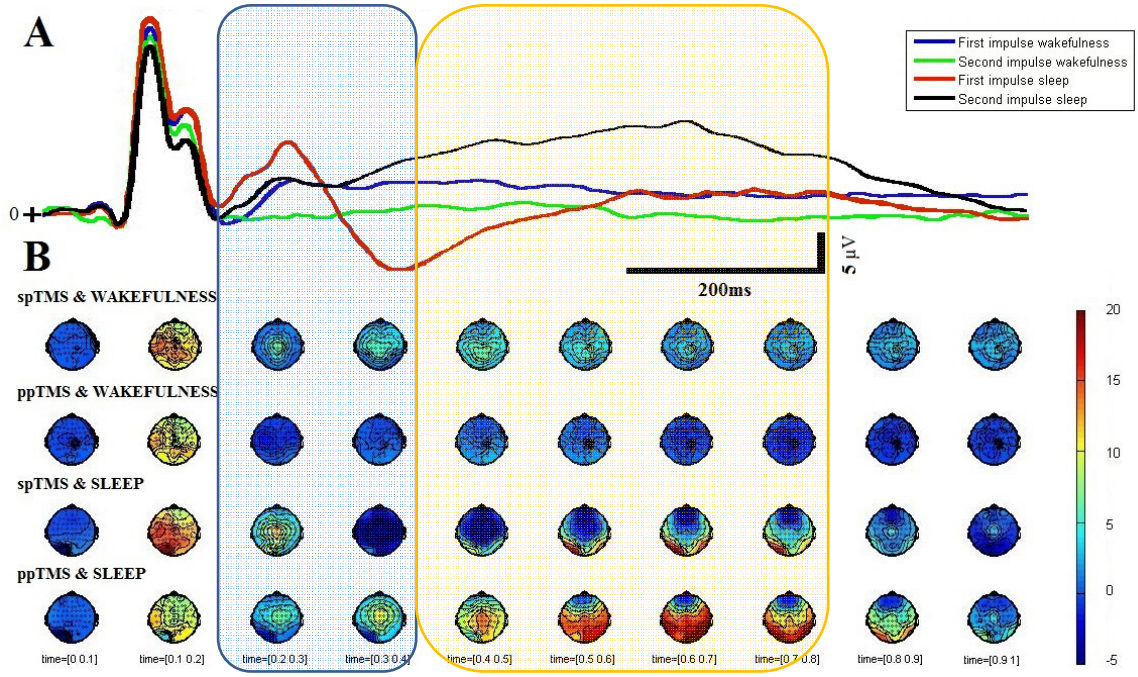

Figure 3. Examples of grand average, adjusted TEP recorded from all channels for four conditions (A). Blue EPs showing shifted (+100 ms) spTMS wakefulness condition, red showing shifted (+100 ms) spTMS NREM sleep condition, green showing subtracted (ppTMS – spTMS) wakefulness condition and black showing subtracted (ppTMS – spTMS) NREM sleep condition. (B) Plots of TMS-evoked subtracted ppTMS (ppTMS – spTMS) and shifted (+100 ms) spTMS brain potentials in wakefulness and NREM sleep conditions, data pooled over all subjects at different progressive time epochs after V1 stimulation, shown with 100 ms steps; plots between 0 to 1000 ms.

No main effect of arousal state or inter-stimulus interval was found ($p>0.05$ corrected for multiple comparisons). However, analysis revealed an interaction between the brain's state and the pulse identity (first or second TMS pulse) in the time range from 293 ms until 831 ms (expressed over all electrodes) ($p<0.05$ corrected for multiple comparisons). In the sleep state the second TMS impulse evoked a positive component starting from 300 ms and lasting untill 850 ms ($p<0.05$ corrected for multiple comparisons). This component is most strongly expressed in occipital area but travels through cortex.

The observed interaction indicates that the second impulse elicits a positive wave that is evident only in the sleep state. This effect can be seen in 6 out of 7 subjects (Figure 4). A post-hoc analysis confirmed that for the time window of 322 ms – 446 ms the second pulse created a stronger positivity than the first pulse ($p<0.05$ corrected for multiple comparisons) and that this occurred only in the sleep state ($p<0.05$ corrected for multiple comparisons).

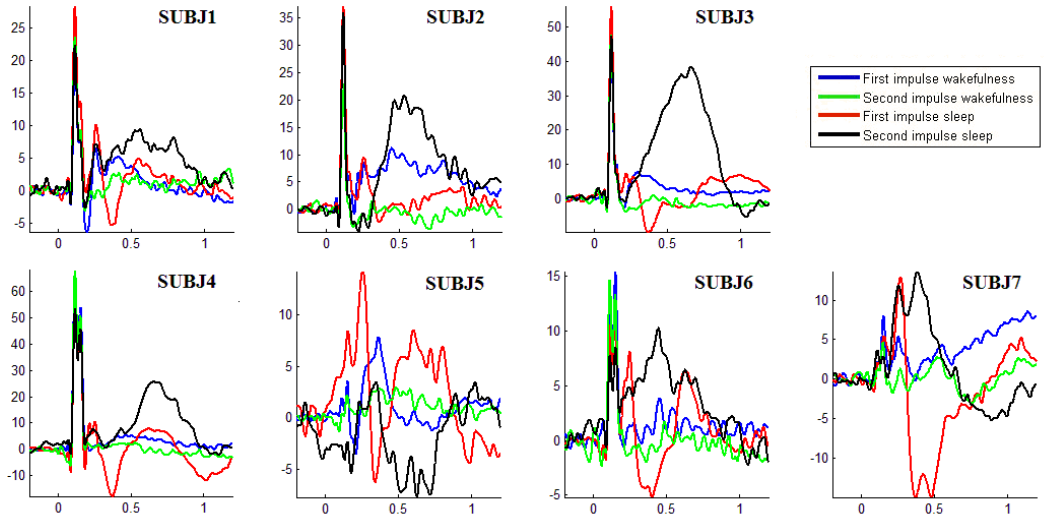

Figure 4. Examples of grand average, adjusted TEP recorded from all channels for four conditions shown for individual subjects. Blue EPs showing shifted (+100 ms) spTMS in wakefulness condition, red showing shifted (+100 ms) spTMS in NREM sleep condition, green showing subtracted (ppTMS – spTMS) in wakefulness condition and black showing subtracted (ppTMS – spTMS) in NREM sleep condition.

Therefore, in contrast to our hypothesis ppTMS in awake state did not increase slow negative processes in comparison to the spTMS. However, during sleep, the second pulse was followed by an increased positive effect that was qualitatively and quantitatively different from the effects found in the spTMS condition.

To summarize our empirical facts, we see that (i) slow potential data did not confirm the hypothesis about globally induced positivity induced by spTMS stimulation during NREM sleep, (ii) during NREM sleep spTMS and ppTMS evoked a negative peak at 290 ms, whereas spTMS had larger amplitude and longer latency, (iii) during NREM sleep ppTMS

triggered a slow TEP wave, which was expressed most strongly on the occipital area, (iv) qualitatively and quantitatively different processes take place when second TMS impulse with IPI of 100 ms is added during sleep.

Discussion

Although the underlying mechanisms responsible for slow wave activity are unclear, it has been previously shown (Massimini et al., 2007) that TMS can trigger individual slow waves (SW) similar to spontaneous SW (Huber et al., 2007). In order to do so high intensity TMS pulses (150–180 V/m on the cortical surface) were used during NREM sleep (Massimini et al., 2007). In order to avoid neck muscle contractions or other inconveniences for subjects, TMS was only used to stimulate sensorimotor cortex.

In the present work it was shown that SW can be triggered at much lower intensities (at 43 V/m) than it was previously thought possible (Massimini et al., 2007) when ppTMS is used. Importantly, the slow positive wave in response to single TMS pulse of such low intensity in sleep is negligible if not absent. Similarly to previous studies (Massimini et al., 2007) we show that TMS-triggering of SW is state-dependent and SW could only be triggered during sleep and not in waking state, where it disappeared completely. The characteristics (more than 500 ms and up to 20 microvolts, respectively) of the NREM sleep positive-waves compared to the roughly flat slow EEG activity over the same epoch in the awake state allow us to consider this signature as a robust sign of the unconscious state in the present context.

Moreover, lower intensity made it possible to stimulate occipital area without causing scalp muscle activation. We show that even though occipital gyri do not contribute much to spontaneous SW (Murphy et al., 2009), they can be still a source for TMS induced SW. The logical next step would be to use same stimulation parameters on different brain areas to see if the ppTMS evokes strong SW also in other areas. Changing stimulation intensity and IPI between the two pulses are also alternations that need to be considered in future studies in order to get a better understanding of the effect. Furthermore, the present results could be analyzed with other methods such as the time-frequency analysis as applied to EEG-TMS data (Rosanova et al., 2009; Aru, Korjus, Murd, & Bachmann, 2012).

We found that the ppTMS protocol with longer IPI (100ms) is a promising tool to investigate the neural basis of the conscious state and the possibilities of its objective assessment. We hypothesized that ppTMS in awake state is associated with an increase of slow negative TEP signatures compared to the NREM state that go beyond the simple sum of

the differences of two single pulse effects. In contrast to our hypothesis ppTMS in awake state did not augment slow negative processes. However, during sleep, the second TMS pulse brought about processes expressed by TEP components with positive polarity and reacted differently as compared to spTMS.

We also found that the N100 amplitude is higher in the conscious state than in sleep (similarly to Stamm, Aru, & Bachmann, 2011) while the negative peak at 290 ms cannot be observed during wakefulness; at the same time in sleep it is conspicuous and present both for spTMS and ppTMS conditions. However, spTMS negative peak has larger amplitude and begins later compared to ppTMS.

These findings might be explained by bistability of neural networks observed during the NREM sleep (Steriade, Timofeev, & Grenier, 2001). Bistability means that considerable part of neurons of the specific part of the brain can undergo alternations between being hyperpolarized (down-state), where virtually all neocortical neurons are silent, and being depolarized (up-state), which is associated with massive neuronal excitation. It has been shown (Constantinople & Bruno, 2011) that one of the characteristics of wakefulness is absence of prolonged periods of synaptic hyperpolarization, while in order to be awake one needs to have persistent up-like state. Underlying neuronal changes occur globally, whereas some local regions could be still active during sleep (Nir et al., 2011) or could go briefly "offline" even during wakefulness (Vyazovskiy et al., 2011). Such evidence implies that, although sleep is often considered a global phenomenon, it may be best understood in relation to activities of local circuits.

Therefore, changes in neocortical excitability are not random but likely depend on background changes in neocortical excitation (i.e. spontaneous neuronal activity at the population level) and result in producing SW (Steriade, Timofeev, & Grenier, 2001). For example, if corticocortical connections are damaged, synchronization of the slow oscillations is disrupted (Amzica & Steriade, 1995). Moreover, this excitability is likely organized into hierarchically nested oscillations of various frequencies, providing a precise temporal framework for information processing in the brain (Buzsáki, 2006).

A positive wave followed by a negative deflection has previously been associated with a loss of information capacity (Massimini et al., 2007). In our case it could mean that negative peak at 290 ms might arise as a result of a long-lasting period of neural hyperpolarization and this hyperpolarization is enough to inhibit the potential for spreading. Therefore, if neurons are in down-state, there is neither adequate level of information processing in the brain

associated with effective connectivity between different brain regions nor wide-range information integration and therefore no consciousness.

This however does not explain why in one case (ppTMS) SW activity is evoked and in other case (spTMS) not, although both evoke a negative peak at 290ms. To better understand how two pulses interact compared to spTMS, we substracted spTMS TEP from ppTMS TEP and spTMS condition was shifted by +100 ms so that spTMS onset was aligned with second ppTMS pulse onset. If comparing results without taking first impulse out (i.e., subtracting it) we might end up with: 1) missing hidden effects or/and 2) simply seeing a simple sum of the differences of two single pulse effects. This subtraction allowed comparing processes evoked by the first and the second TMS pulse more precisely and revealed additional differences. Results further confirmed the fact that second pulse elicits a conspicuous positive wave that is evident only in the sleep state and it was qualitatively and quantitatively different from the outcome of the first pulse.

The positive part of the slow wave is usually considered to index the start of the up-state in the underlying cortex (Massimini et al., 2007; Crunelli & Hughes, 2010). While a single weak TMS pulse is followed only by a down-state, this local hyperpolarization can be overcome to evoke a full-blown slow wave when either TMS with strong intensity (Massimini et al., 2007) or the paired pulse TMS as in our current study is used. This implies that the weak-intensity ppTMS will lead to somewhat similar effects to strong intensity spTMS. However, important differences exist. Most crucially, the strong intensity spTMS to the sensorimotor area evokes a strong negative deflection (Massimini et al., 2007) which has a much higher amplitude than the negative deflection evoked by our ppTMS. It seems that the ppTMS mainly boosts the positive part of the wave, starting from roughly 300 ms after the onset of the second TMS pulse. The implications of this finding are to be determined by further research.

Conclusion

In conclusion, ability to evoke slow waves noninvasively and nonpharmacologically is important for several reasons. Slow waves are involved in learning tasks (Huber et al., 2007), in memory consolidation (Steriade & Timofeev, 2003), synaptic (Tononi & Cirelli, Sleep function and synaptic homeostasis, 2006) and metabolic homeostasis (Xie et al., 2013), restorative function of sleep (Walsh et al., 2006). Triggering SW-s can also deepen the sleep (Huber et al., 2008) and is positively correlated with post-sleep performance improvement

(Huber, Ghilardi, Massimini, & Tononi, 2004). All this puts ground to methodology, which could be used to develop a diagnostic tool. The findings presented in this work add the important fact that SW can be evoked on relatively low intensities and also from the visual cortex, given the present double stimulation paradigm is used. Further research needs to show if these findings generalize to stimulation of other brain areas and whether other inter-pulse-intervals might be even more effective than the 100 ms one used in this study.

Moreover, the present study extends and supports the results found in previous state dependent studies (Ferrarelli et al., 2010; Massimini et al., 2010; Massimini et al., 2005; Rosanova, Gosseries, Casarotto, Boly, & Casali, 2012; Stamm, Aru, & Bachmann, 2011). Comparative characteristics of spTMS and ppTMS evoked potentials could be considered as a robust sign of the unconscious state. Therefore, it may be hoped that it is possible to develop a signature of the conscious state consisting in a TEP pattern combining enhanced N100, elimination of the negative peak at 290 ms, and the absence of a positive wave of P400-900. This could emerge as one of the important future vistas of consciousness research.

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