Characterization of GABAB-receptor mediated neurotransmission in the human cortex by paired-pulse TMS-EEG

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ABSTRACT

GABAB-receptor (GABABR) mediated inhibition is important in regulating neuronal excitability. The paired-pulse transcranial magnetic stimulation (TMS) protocol of long-interval intracortical inhibition (LICI) likely reflects this GABABergic inhibition. However, this view is based on indirect evidence from electromyographic (EMG) studies. Here we combined paired-pulse TMS with simultaneous electroencephalography (paired-pulse TMS-EEG) and pharmacology to directly investigate mechanisms of LICI at the cortical level. We tested the effects of a conditioning stimulus (CS_{100}) applied 100ms prior to a test stimulus (TS) over primary motor cortex on TS-evoked EEG-potentials (TEPs). Healthy subjects were given a single oral dose of baclofen, a GABABR agonist, or diazepam, a positive modulator at GABAARs, in a placebo-controlled, pseudo-randomized double-blinded crossover study. LICI was quantified as the difference between paired-pulse TEPs (corrected for long-lasting EEG responses by the conditioning pulse) minus single-pulse TEPs. LICI at baseline (i.e. pre-drug intake) was characterized by decreased P25, N45, N100 and P180 and increased P70 TEP components. Baclofen resulted in a trend towards enhancement of LICI of the N45 and N100, and significantly enhanced LICI of the P180. In contrast, diazepam consistently suppressed LICI of late potentials (i.e. N100, P180), without having an effect on LICI of earlier (i.e. P25, N45 and P70) potentials. These findings demonstrate for the first time directly at the system level of the human cortex that GABABR-mediated cortical inhibition contributes to LICI, while GABAAR-mediated inhibition occludes LICI. Paired-pulse TMS-EEG allows investigating cortical GABABR-mediated inhibition more directly and specifically than hitherto possible, and may thus inform on network abnormalities caused by disordered inhibition, e.g. in patients with schizophrenia or epilepsy.

1. Introduction

Inhibitory neurotransmission mediated by γ-aminobutyric acid (GABA)-receptors is critical for physiological function of the cerebral cortex. Paired-pulse transcranial magnetic stimulation (TMS) offers the possibility to study GABAergic inhibition non-invasively in humans. The application of a conditioning stimulus 50-200ms prior to a test stimulus (TS) over the primary motor cortex (M1) suppresses the test motor evoked potential (MEP) in the target muscle with respect to an unconditioned MEP (Valls-Sole et al., 1992). This phenomenon is known as long-interval intracortical inhibition (LICI) and was shown to be associated with activation of GABAB-receptors (GABABRs) (McDonnell et al., 2006; Müller-Dahlhaus et al., 2008). The notion that LICI reflects GABABR-mediated inhibition is further supported by studies demonstrating that LICI suppresses GABAA-receptor (GABAAR)-mediated cortical inhibition [SICI: short-interval intracortical inhibition (Kujirai et al., 1993; Peurala et al., 2008; Ziemann et al., 1996a)], likely through activation of auto-inhibitory pre-synaptic GABABRs (Daskalakis et al., 2002; Sanger et al., 2001), in line with cellular experiments (Davies et al., 1990). However, evidence on the mechanisms of LICI is only indirect as it is derived from EMG recordings, which limits its potential as a diagnostic tool in a clinical setting.

Recently, first studies using a combination of TMS and electroencephalography (TMS-EEG) have started to directly investigate mechanisms of LICI at the cortical level (Daskalakis et al., 2008; Farzan et al., 2010; Fitzgerald et al., 2009; Rogasch et al., 2013). It was shown that a conditioning stimulus (LICI protocol) induced a reduction of the averaged TS-evoked EEG potential (TEP) recorded at the C3 electrode, which is located above the stimulated M1 (Daskalakis et al., 2008; Farzan et al., 2010; Fitzgerald et al., 2009; Rogasch et al., 2013). However, the physiological underpinnings and spatiotemporal characteristics of this effect remained unexplored.

By combining high-density TMS-EEG with pharmacology we were recently able to demonstrate (Premoli et al., 2014) that specific components of (single-pulse) TEPs, i.e. the negative potentials at around 45 (N45) and 100 (N100) milliseconds post stimulus (Lioumis et al., 2009; Rogasch et al., 2013), can be linked to GABAAR- and GABABR-mediated neurotransmission, respectively. In the current study, we extend these studies by applying *paired-pulse* TMS-EEG and pharmacology to characterize and study the spatiotemporal dynamics of LICI directly at the level of the cortex. Subjects received a single oral dose of placebo, baclofen (i.e., GABABR agonist), or diazepam (i.e., benzodiazepine and allosteric positive modulator of α1, α2, α3 and α5 subunit bearing GABAARs) in a randomized, placebo-controlled double-blinded crossover study. LICI was quantified as the difference between paired-pulse TEPs (corrected for long-lasting EEG responses by the conditioning pulse) minus single-pulse TEPs. LICI at baseline (i.e. pre-drug intake) was characterized by decreased P25, N45, N100 and P180 and increased P70 TEP components. Baclofen showed a trend towards enhancement of LICI of the N45 and N100, and significantly enhanced LICI of the P180. In contrast, diazepam consistently suppressed LICI of later potentials (i.e. N100, P180), without having an effect on LICI of earlier (i.e. P25, N45 and P70) potentials. Our data thus provide, for the first time, novel evidence at the cortical level that LICI reflects GABABR-mediated inhibition and suggest a tight control of GABABR-mediated inhibition by GABAAergic neurotransmission.

2. Material and Methods

2.1 Subjects

Nineteen male subjects aged 22-32 years (mean age, 26.4 ± 3.5 years) participated in this study after having given written informed consent. Female participants were excluded due to menstrual cycle related effects on cortical excitability, which can be a potential confound in TMS studies (Smith et al., 1999). All subjects were right-handed according to the Edinburgh Handedness Inventory (laterality score ≥75%) (Oldfield, 1971). Subjects underwent a physical examination before each experiment and were screened for contraindications to TMS (Rossi et al., 2009). Exclusion criteria included the presence of a history of neurological or psychiatric disease, use of CNS active drugs, abuse of any drugs (including nicotine and alcohol) or contraindications to the study medications (baclofen and diazepam). All subjects were naïve to the study medication. The study was approved by the local Ethics Committee of the Medical Faculty of the Eberhard-Karls University Tübingen, Germany.

2.2 Experimental design

Subjects participated in three pseudo-randomized experimental sessions, which were separated by at least one week to exclude carry-over effects. Subjects received either a single oral dose of baclofen (50mg Lioresal®, Novartis Pharma), diazepam (20mg Diazepam-ratiopharm®, ratiopharm GmbH), or placebo (P-Tabletten Lichtenstein). Baclofen is a specific GABABR agonist, while diazepam is a classical benzodiazepine binding at α1, α2, α3 and α5 subunit containing GABAARs. Drug doses and time points of post-drug measurements were chosen according to previous experiments that reported a significant modulation of TMS-EMG and TMS-EEG parameters for motor cortical inhibition 90 minutes after drug intake (Müller-Dahlhaus et al., 2008; Premoli et al., 2014), and in line with pharmacokinetic studies showing peak plasma concentrations 90 minutes after drug intake

(McDonnell et al., 2006; Shader et al., 1984). Thus, TMS-EEG recordings were conducted at baseline and 90 minutes after drug intake.

2.3 Transcranial magnetic stimulation (TMS) and electromyography (EMG)

Focal TMS of the hand area of left primary motor cortex (M1) was performed with a figure-of-eight coil (external diameter of each wing, 90mm) connected to a Magstim 200^2 magnetic stimulator (Magstim Company, Carmarthenshire, Wales, UK) with a monophasic current waveform. The optimal coil position over the hand area of left M1 for eliciting MEPs in the right *abductor pollicis brevis* muscle (APB) was determined as the site where TMS at a slightly suprathreshold intensity consistently produced the largest MEPs. MEP recordings were obtained by surface electromyography (EMG), using Ag-AgCl cup electrodes in a belly–tendon montage. The EMG raw signal was amplified and band-pass filtered (20Hz to 2kHz; D360 amplifier, Digitimer, Hertfordshire, UK) and digitized at an A/D rate of 10kHz per channel (CED Micro 1401; Cambridge Electronic Design, Cambridge, UK). The coil was held tangential to the scalp with the handle pointing backwards and 45 degrees away from the midline, thus activating the corticospinal system preferentially trans-synaptically via horizontal cortico-cortical connections (Di Lazzaro et al., 2008). Resting motor threshold (RMT) was determined using the relative frequency method (Groppa et al., 2012) and was defined as the minimum intensity (as a percentage of the maximum stimulator output [MSO]) that was sufficient to elicit MEPs larger than 50μV peak-to-peak amplitude in at least five out of ten subsequent trials. The position of the APB hotspot was marked with a felt pen on the EEG cap to ensure constant coil placement throughout the experiment.

Paired-pulse TMS to test LICI was applied according to an established protocol (Valls-Sole et al., 1992), consisting of a conditioning stimulus applied 100ms (CS_{100}) before delivery of a TS. Stimulus intensities for CS_{100} and TS were 100% RMT in order to limit re-afferent somatosensory feedback, which can contaminate the EEG signal (Paus et al., 2001). During

the EEG registration 125 randomly intermixed single and paired TMS pulses each were applied over the left M1 APB hotspot at an inter-trial interval (ITI) of 5s on average (random ITI variation of 25% in order to reduce anticipation of the next trial).

2.4 High-density electroencephalography (EEG) recordings during TMS

TMS-evoked EEG potentials were recorded using a TMS-compatible EEG system (BrainAmp DC, BrainProducts GmbH, Munich, Germany), which prevents EEG amplifier saturation and allows continuous data recording during TMS. The EEG signal was digitized at a sampling frequency of 5kHz and continuously recorded by 62 electrodes mounted on an elastic cap according to the standard layout (BrainCap-Fast'n Easy 64Ch, Brain Products GmbH). Horizontal eye movements were recorded by placing an electrode outside the outer canthus of the eye, while an electrode placed below the right eye recorded vertical eye movements and blinks. The impedance of all electrodes was kept <5kΩ throughout the experiment.

During the TMS-EEG recordings subjects were seated in a comfortable reclining chair and were asked to stay awake with eyes open. To avoid contamination of the EEG signal by auditory potentials evoked by the click associated with current discharge through the TMS coil, a masking noise was applied to both ears by earphones (Massimini et al., 2005).

2.5 Data processing and analysis

EEG data preprocessing and TEP analysis were performed using the Fieldtrip open source MATLAB toolbox (www.ru.nl/fcdonders/fieldtrip/) (Oostenveld et al., 2011). The EEG signal was first re-referenced to the linked mastoids (channels TP9 and TP10) and down-sampled to 1kHz. Single- and paired-pulse trials were defined from continuously recorded EEG time series and segmented with respect to the TS and CS, respectively, such that each epoch included a 500ms pre-stimulus baseline and a 600ms post-stimulus recording. TMS artifacts (by single- and paired-pulses) were removed by applying a linear interpolation for 10ms

before and after the TMS pulses (Thut et al., 2011). Thereafter, each trial was linearly detrended and band-pass filtered between 2 and 80Hz. A 50Hz notch filter was applied to reduce line noise contamination. EEG trials were visually scrutinized and trials containing artifacts due to, e.g., eye movements or muscle activation were eliminated. The data from four subjects had to be excluded from final analysis because of excessive artifact contamination of the EEG traces. Thus, data reported was analyzed from 15 subjects.

Artifact-free EEG trials from 62 channels (see above) for single (averaged number of trials across subjects ± SEM before and after diazepam: 107 ± 3 and 99 ± 3; baclofen: 109 ± 3 and 108 ± 3; placebo: 104 ± 3 and 107 ± 4) and paired (averaged number of trials across subjects before and after diazepam: 105 ± 3 and 95 ± 3; baclofen: 110 ± 3 and 105 ± 4; placebo: 104 ± 3 and 105 ± 4) TMS pulses were baseline corrected by subtracting the mean amplitude during an interval between -500ms and -150ms before TMS onset (i.e. before CS [paired-pulse condition] and TS alone [single-pulse condition], respectively).

When registering EEG responses during paired-pulse TMS, a serious confound is the effect of late (i.e. > 100 ms) EEG responses evoked by CS_{100}, which superimpose early EEG responses evoked by TS (Daskalakis et al., 2008). To correct for such late CS_{100}-induced EEG responses we used a subtraction method: the average cortical evoked potential elicited by the TS alone (single-pulse TMS trials; **Figure 1A**), aligned to CS_{100} onset, was subtracted from the average cortical evoked potential elicited by the CS_{100}-TS pulse pair (**Figure 1B**) [(Daskalakis et al., 2008); paired-pulse corrected, **Figure 1C**]. To determine LICI, we then subtracted the average single-pulse TEP, aligned to TS onset, from the paired-pulse corrected TEP (**Figure 1D**). This procedure is justified, as both CS and TS intensity equaled 100%RMT. Throughout this work, TEPs elicited by paired-pulse TMS will refer to evoked potentials corrected for late EEG responses of CS_{100} (**Figure 1B**), while LICI will refer to the difference between single-pulse and paired-pulse corrected TEPs (**Figure 1D**).

Single and paired-pulse TEPs were calculated by averaging the EEG signal over all retained trials for each channel. In order to smooth the TEP, we filtered the data between 1 and 45 Hz. In accordance with the literature (Lioumis et al., 2009; Premoli et al., 2014), five TEP components were considered (non-overlapping time windows of interest [TOI] given in brackets; P, positive deflection; N, negative deflection): P25 (17 - 37 ms), N45 (38 - 57 ms), P70 (58 - 84 ms), N100 (85 - 150 ms) and P180 (160 - 230 ms) (**Figure 2A**). TOIs were chosen on the basis of the grand average of single- and paired-pulse TEPs, and were equal in both TMS (i.e., single- and paired-pulse) and across drug conditions. To evaluate the cortical effects of LICI, single- versus paired-pulse corrected TEPs were contrasted as described above for all TEP components. The effects of diazepam and baclofen, respectively, on LICI were investigated by evaluating changes of LICI between post- and pre-drug conditions for all TEP components.

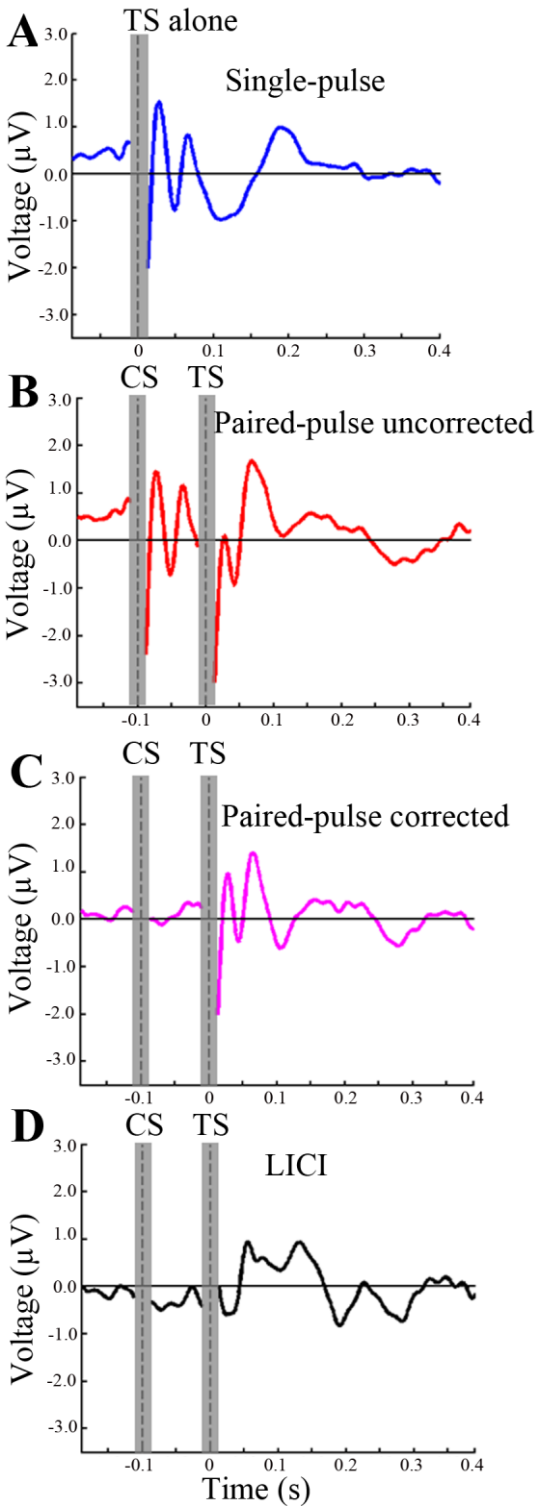

Figure 1. Determination of the EEG signature of long-interval intracortical inhibition (LICI).

Paired-pulse TEPs (B) were corrected for late (>100ms) CS-evoked EEG responses, which overlap with early TS-evoked EEG potentials in the paired pulse condition, by subtracting EEG responses evoked by TS alone (A) from paired-pulse TEPs (B) aligned to the time of CS

(paired-pulse corrected TEPs, C). The effect of CS on TS-evoked TEPs, i.e. LICI (D), was obtained by subtracting single-pulse TEPs (A) from paired-pulse corrected TEPs (C) aligned to the time of TS. Note that both CS and TS were delivered at an intensity of 100% RMT, justifying this approach. Data are grand-averaged over all channels and the three drug conditions at baseline (i.e. before drug intake). Vertical dashed lines indicate times of TMS pulses. The shaded grey bars depict the part of the EEG trace (± 10ms), which was linearly interpolated to remove TMS-induced artifacts.

2.6 Statistics

To assess whether RMT measurements were consistent and reproducible at baseline across the three drug conditions a one way repeated measures ANOVA with the within-subject factor DRUG (3 levels: diazepam, baclofen and placebo) was employed. Cronbach's alpha was used to assess test-retest reliability for every TEP component of single- and paired-pulse TEPs, respectively.

To analyze (i) LICI and (ii) drug-induced changes in LICI, multiple dependent sample *t*-tests [(i) paired- vs. single-pulse; (ii) LICI post- vs. LICI pre-drug intake] were separately applied for the different conditions and TOIs. To correct for multiple comparisons, we adopted a cluster based permutation analysis (Maris and Oostenveld, 2007) as implemented in FieldTrip (http://fieldtrip.fcdonders.nl/). That is, a paired *t*-test comparing the different conditions was performed for each electrode at each time bin within the five different TOIs. T-values exceeding an a priori threshold of $p < 0.01$ were clustered based on adjacent time bins and neighboring electrodes. Cluster-level statistics were calculated by taking the sum of the *t*-values within every cluster. The statistical comparisons were done with respect to the maximum values of summed *t*-values. By means of a permutation test, i.e. randomizing data across (i) paired vs. single-pulse and (ii) LICI post- vs. LICI pre-drug conditions and rerunning the statistical test 1500 times, we obtained a reference distribution of the maximum

of summed cluster t-values to evaluate the statistic of the actual data. Clusters in the original data set were considered to be significant at an α-level of 5% if less than 5% of the permutations used to construct the reference distribution yielded a maximum cluster-level statistic larger than the cluster-level value observed in the original data. The reported *p*-values were further Bonferroni-corrected for TOIs (n = 5) and conditions (n = 2) [(i) paired vs. single-pulse; (ii) post- vs. pre-drug], which is reflected by a corrected alpha-value of $\alpha = 0.05/10$. All data are presented as means ± SEM if not indicated otherwise.

3. Results

Experimental procedures and study drugs were generally well tolerated except for mild to moderate sedation and dizziness, which did not affect the capability of the subjects to fully comply with the requirements of this study. Part of the data from the single-pulse TMS trials has been published previously (cf. Experiment 2 in Premoli et al., 2014).

3.1 Differences between paired- and single-pulse TMS-evoked potentials (TEPs) before drug intake (LICI at baseline)

First, we evaluated reliability of single-pulse TEPs, and the difference between paired- and single-pulse TEPs (i.e., LICI; cf. Material and Methods, Fig. 1), at baseline, i.e. pre-drug intake. Grand averages of single-pulse TEPs and LICI were calculated separately for the three pre-drug conditions (**Figure 2A-B**). The amplitudes of the single-pulse P25, N45, P70, N100 and P180 TEP components, and LICI of these TEP components, were then extracted from the grand averages of all channels, for each subject. Cronbach's alpha values > 0.7 (Farzan et al., 2010; Neuper et al., 2005) revealed a high level of reproducibility for all single-pulse TEPs and LICI of TEP components across drug conditions (**Figures 2C-D**), except for LICI of the P25 which showed a low level of reliability (Cronbach's alpha values < 0.7). RMT was not significantly different between drug conditions at baseline before drug intake (diazepam: 44.6 ± 1.3% MSO; baclofen: 44.1 ± 1.3% MSO; placebo: 44.8 ± 1.2% MSO; $F_{(2,28)}$= 1.12, p = 0.34) and thus cannot account for any of the observed drug-induced changes of LICI (see below).

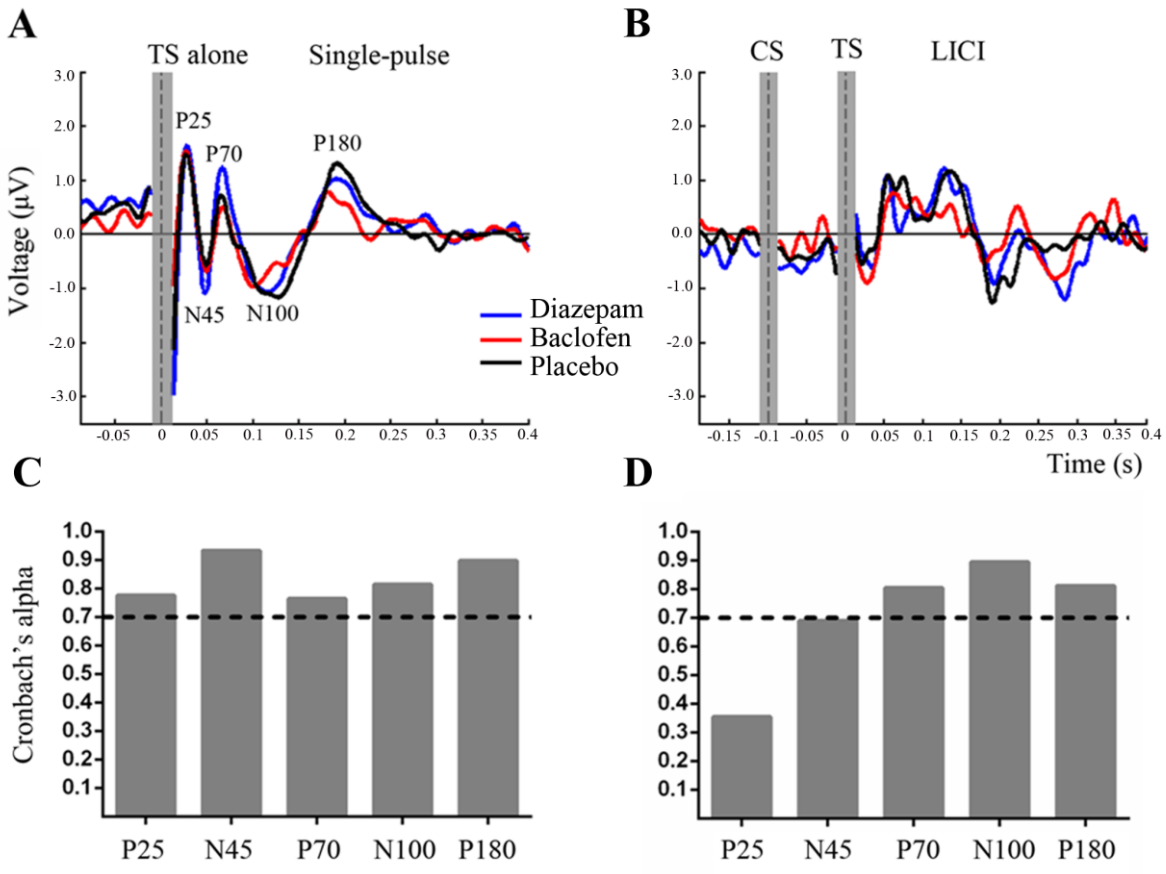

Figure 2. Reproducibility Test-retest reliability of single-pulse TEPs and LICI (paired-minus single-pulse TEPs) at baseline before drug intake.

Grand-average (over all electrodes and artifact-free trials) of single-pulse TEPs (A) and LICI (paired- minus single-pulse TEPs; B) calculated separately before intake of diazepam (blue line), baclofen (red line) and placebo (black line). Vertical dashed lines indicate times of TMS pulses. The shaded grey bars depict the part of the EEG trace (± 10ms), which was linearly interpolated to remove TMS-induced artifacts. (C and D) Histograms illustrate reproducibility (i.e. test-retest reliability) of single-pulse TEPs (C) and LICI (D) across drug conditions at baseline for each TEP and LICI component (i.e. P25, N45, P70, N100 and P180 amplitudes), respectively. Cronbach's alpha > 0.70 indicates a high level of reliability across drug conditions at baseline.

As previously described by our group (Premoli et al., 2014), the grand-average EEG response evoked by single-pulse TMS of the left M1 is characterized by the most prominent TEP components P25, N45, P70, N100 and P180, each with a distinct topographical distribution (**Figure 3A-B**). Compared to single-pulse TEPs, topographical surface voltage maps of paired-pulse TEPs (**Figure 3A,C**) showed a marked reduction of all TEP components except for the P70, which was significantly increased in the stimulated hemisphere. Quantification of LICI, i.e. the difference paired- minus single-pulse TEPs (**Figure 3D**), by means of a non-parametric, cluster-corrected permutation analysis in 5 non-overlapping TOIs showed that the amplitudes of the P25 (single-pulse: 3.8 ± 0.6μV, paired-pulse: 2.4 ± 0.5μV; p = 0.003), N45 (single-pulse: -2.2 ± 0.4μV, paired-pulse: -1.7 ± 0.4μV; p = 0.004), N100 (single-pulse: -3.2 ± 0.5μV, paired-pulse: -1.1 ± 0.3μV; p = 0.002) and P180 (single-pulse: 2.4 ± 0.4μV, paired-pulse: 1.2 ± 0.2μV; p = 0.004) potentials were significantly reduced. In contrast, the amplitude of the P70 potential was significantly increased in the paired- vs. single-pulse TMS condition (single-pulse: 2.3 ± 0.7μV, paired-pulse: 3.4 ± 0.7μV; p = 0.004) (**Figure 3A, F**). Topographical plots of LICI showed that modulation of single-pulse TEPs by CS_{100} was expressed at the site of the TEP components (**Figure 3E**).

Investigation of LICI at the level of individual subjects revealed that most of the subjects showed a decrease in P25, N45, N100 and P180 potentials, and an increase in the P70 potential, in the paired- vs. single-pulse TMS condition, but few subjects showed opposite effects compared to the group on average (**Figure 4**).

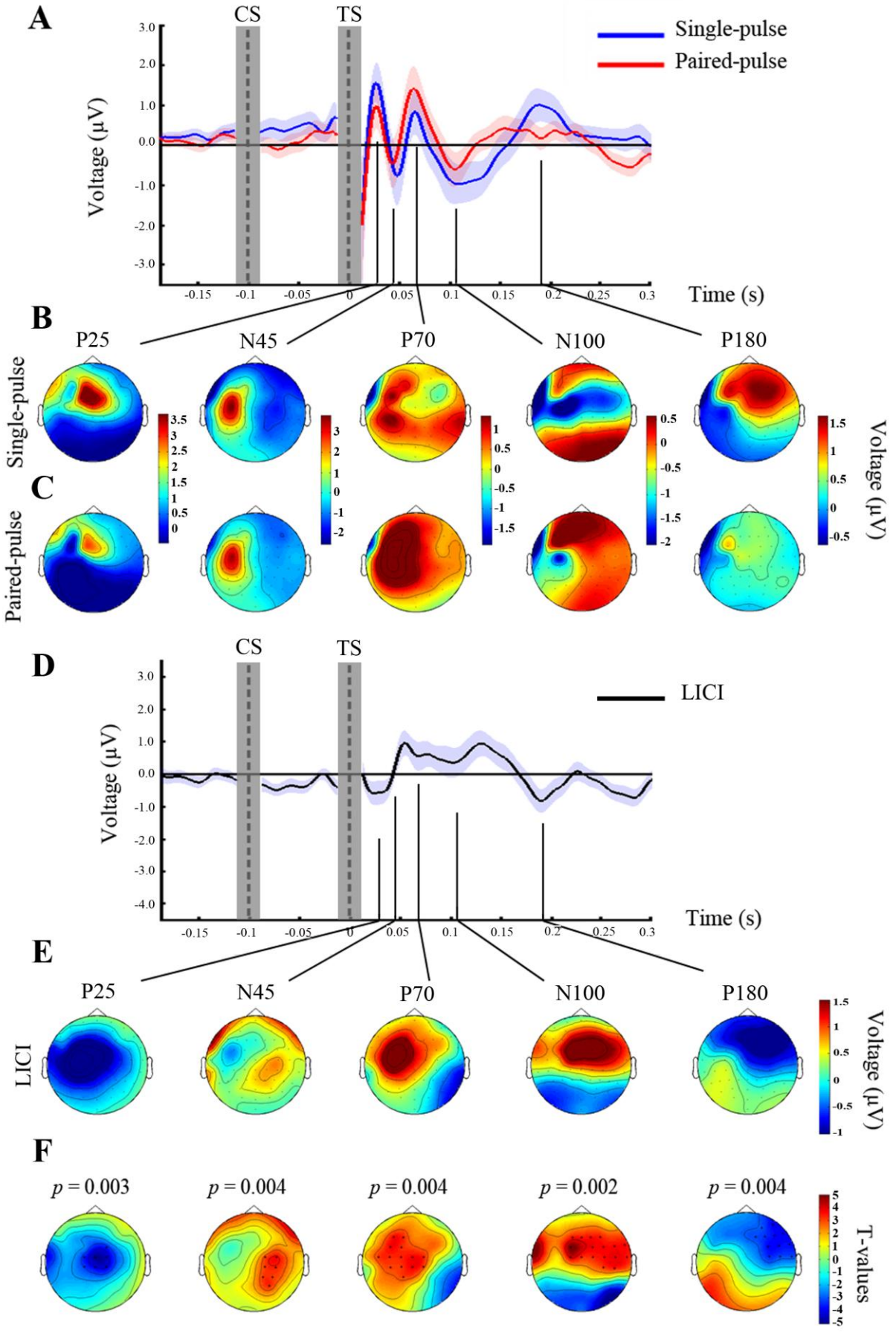
A
CS
TS
Single-pulse
Paired-pulse
Voltage (μV)
Time (s)
B
C
P25
N45
P70
N100
P180
Single-pulse
Paired-pulse
Voltage (μV)
D
CS
TS
LICI
Voltage (μV)
Time (s)
E
P25
N45
P70
N100
P180
LICI
Voltage (μV)
F
p = 0.003
p = 0.004
p = 0.004
p = 0.002
p = 0.004
T-values

Figure 3. Spatiotemporal expression of LICI at baseline before drug intake.

(A) Grand-averaged TEPs at baseline before drug intake (over all electrodes, artifact-free trials and drug conditions) induced by left M1 single- (blue) and paired- (red) pulse stimulation. Note that TS-evoked EEG responses in the paired-pulse condition were corrected for late (i.e. >100ms) CS-evoked EEG responses (cf. Fig. 1C and section 2.5). The vertical dashed lines indicate the times of CS (paired-pulse TMS) and TS (single- and paired-pulse TMS), respectively. The shaded grey bars depict the parts of the EEG trace (± 10ms), which were linearly interpolated to remove the TMS-induced artifacts. Blue and red shades represent ± 1 SEM of grand-averaged TEP curves. (B) and (C) illustrate topographical distributions of surface voltages for the most pronounced TEP components (P25, N45, P70, N100, P180; grand-average over all drug conditions) elicited by single- (B) and paired- (C) pulse stimulation. Maps are scaled and color-coded individually according to their respective maximum (red) and minimum (blue) voltages. (D) LICI (grand-averaged over all drug conditions) as determined by subtraction of paired- minus single-pulse TEPs (cf. Fig. 1D). The grey shade represents ± 1 SEM. (E) illustrates the topographical distribution of LICI for each TEP component, whilst (F) shows t-statistic maps of TEP amplitude differences (paired- minus single-pulse TEPs) for these components (P25, N45, P70, N100, P180). Asterisks in (F) indicate channels, which showed significant differences between paired- and single-pulse TEP amplitudes. Blue colors indicate a decrease in positivity, while red colors reflect either a decrease in negativity or an increase in positivity.

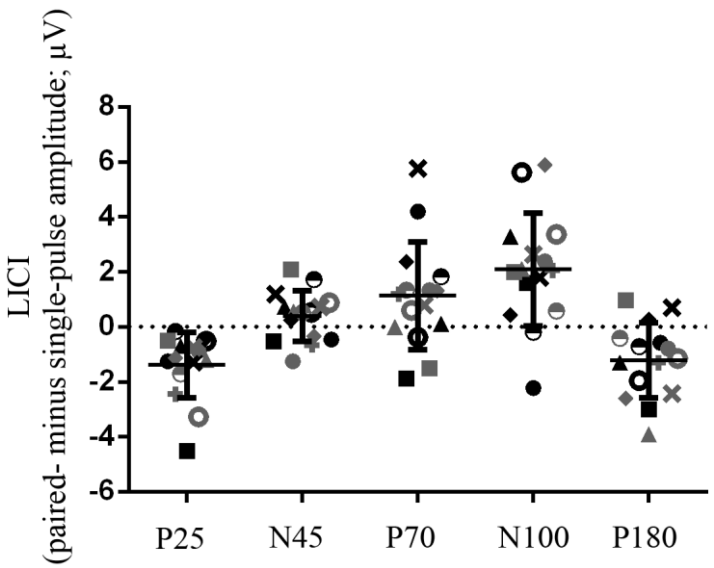

Figure 4. LICI at baseline before drug intake (single subject data).

Scatter plot of individual LICI of TEP components, i.e. amplitude modulations of the P25, N45, P70, N100 and P180 potentials by CS_{100} (paired- minus single-pulse TEPs, cf. Fig. 3D) at baseline before drug intake. For each TEP component amplitudes were extracted from those channels showing a significant difference between the paired- and single-pulse condition (cf. Fig. 3F). Each subject is represented by a specific shape and color. Horizontal bars represent group averages ± 1 SEM. Note that positive values for the negative TEP components (N45, N100) indicate a decrease in negativity in the paired- vs. single-pulse condition.

As we have shown previously, the N100 component of single-pulse TEPs reflects GABABR-mediated neurotransmission (Premoli et al., 2014). Thus, the TS in the paired-pulse condition was delivered at a time of (CS_{100}-induced) enhanced GABABR activity. LICI of the P25 (i.e. the averaged difference paired- minus single-pulse TEP amplitudes over channels which showed significant modulation of the P25, cf. Fig. 3F) correlated directly with the N100 amplitude in the single-pulse condition (Spearman correlation, $r^2 = 0.34$, $p = 0.02$, data extracted from those channels which were significantly modulated by baclofen, cf. Fig.

7C, Premoli et al., 2014) (**Figure 5**), suggesting that LICI of the P25 was GABABR-mediated. In contrast, none of the CS_{100}-induced modulations of any of the other TEP components correlated with the N100 amplitude in the single-pulse condition ($p > 0.05$).

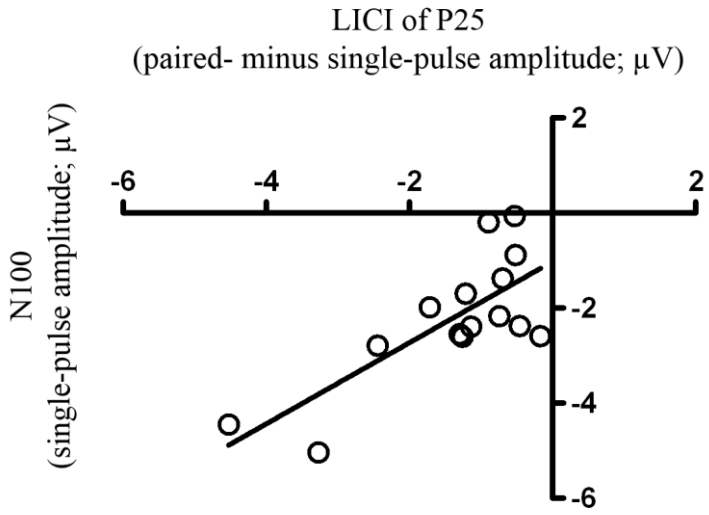

Figure 5. Correlation between LICI of the P25 TEP component and single-pulse N100 amplitude.

A significant positive correlation (Spearman correlation, $r^2 = 0.34$, $p = 0.02$) was found between suppression of the P25 amplitude by CS_{100} in the paired-pulse condition (paired- minus single-pulse TEP amplitude, i.e. LICI; x-axis) and the N100 amplitude in the single-pulse condition as an index of GABABR-mediated inhibition (y-axis; extracted from the channels which were significantly modulated by baclofen, cf. Fig. 7C, Premoli et al., 2014).

3.2 Modulation of LICI by diazepam and baclofen

To evaluate drug effects on LICI (i.e. the difference between paired- minus single-pulse TEPs), we compared LICI post- vs. pre-drug by using a cluster-based permutation analysis (computed over all channels) in the 5 non-overlapping TOIs, corresponding to the P25, N45, P70, N100 and P180 potentials. Diazepam occluded LICI of the N100 over contralateral,

fronto-central sites (pre: 1.4 ± 0.3 μV, post: 0.1 ± 0.2μV; average over channels significantly modulated by diazepam [cf. Fig. 6D], p = 0.003) and even turned LICI of the P180 into facilitation over central areas (pre: -0.4 ± 0.3μV, post: 0.8 ± 0.2μV; average over channels significantly modulated by diazepam [cf. Fig. 6D], p = 0.003) (**Figure 6A-D**). In contrast, the GABABR agonist baclofen showed a trend towards a significant enhancement of LICI of the N45 in the contralateral (pre: 0.2 ± 0.3μV, post: 0.7 ± 0.2μV; average over channels significantly modulated by baclofen [cf. Fig. 7D], p = 0.01) and of LICI of the N100 in the ipsilateral hemisphere (pre: -0.9 ± 0.5μV, post: 0.2 ± 0.6μV; average over channels significantly modulated by baclofen [cf. Fig. 7D], p = 0.009). Baclofen significantly increased LICI of the P180 in frontal and right-central regions (pre: -0.5 ± 0.3μV, post: -1.3 ± 0.3μV; average over channels significantly modulated by baclofen [cf. Fig. 7D], p = 0.001) (**Figure 7A-D**). There were no significant differences pre vs. post placebo in any of the five TOIs (p > 0.005, corrected for multiple comparisons; data not shown).

For diazepam, these results were confirmed by a cluster-corrected analysis between post-drug conditions. Compared to placebo, diazepam showed a trend towards a decrease of LICI of the N100 (p = 0.01, channels significantly modulated: 'C4', 'C6', 'CP4', 'CP6' and 'TP8') and a decrease of LICI of the P180 (p = 0.001, channels significantly modulated: 'FT7', 'F7', 'F5', 'F3', 'F1', 'Fz', 'FC5', 'FC3', 'FC1', 'FC2', 'C1', 'Cz', 'C2', 'C4', 'CP2' and 'CPz'). In contrast, post-drug comparisons between baclofen and placebo did not show any significant differences (p > 0.005, corrected for multiple comparisons).

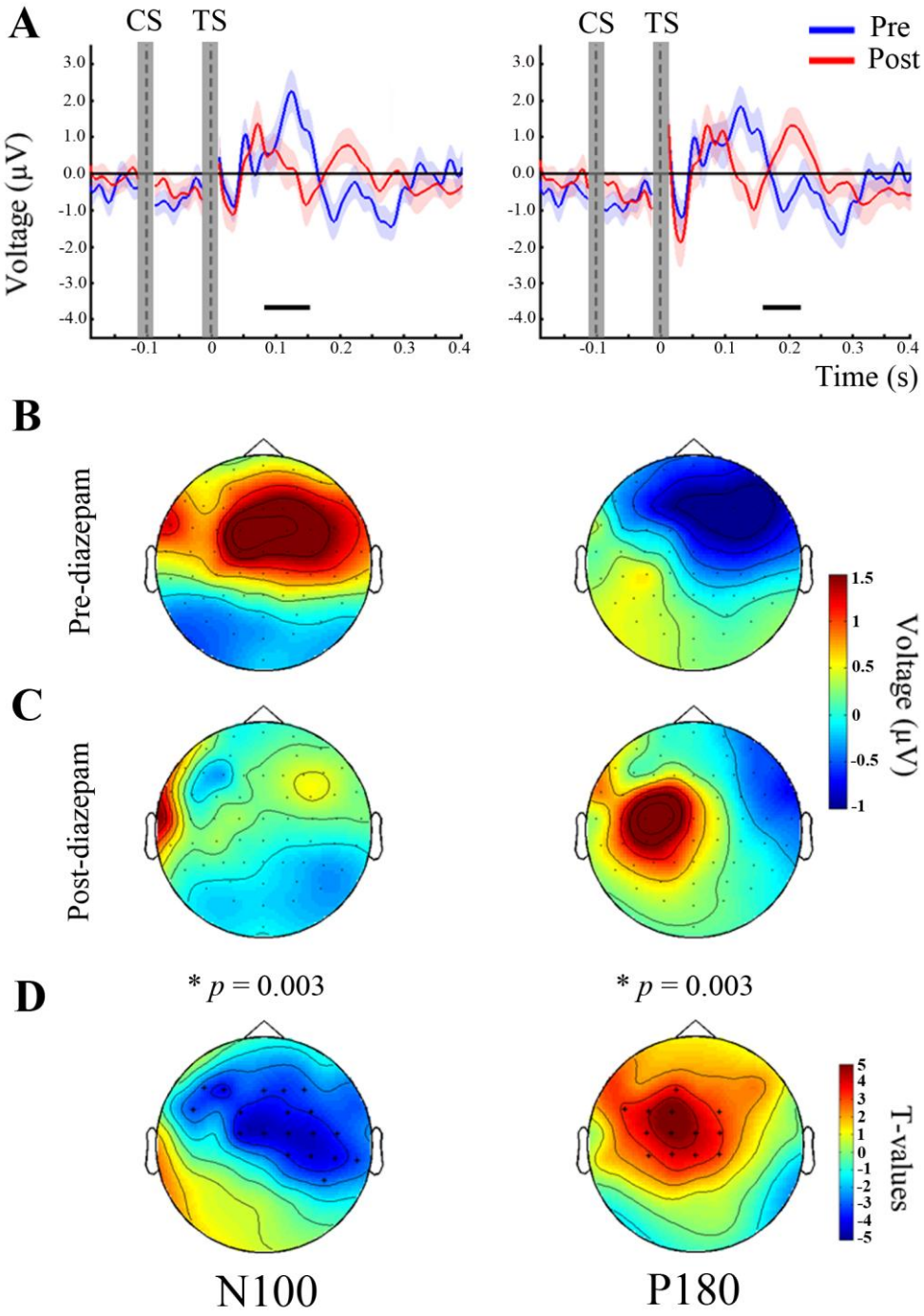

Figure 6. Diazepam effects on LICI.

LICI (i.e., paired- minus single-pulse TEPs, cf. Fig. 3D) was obtained before (Pre, blue) and after (Post, red) intake of diazepam (A). LICI curves are averaged across channels, which showed significant drug-induced effects as indicated by asterisks in (D). (B-C) Topography of LICI before (B) and after (C) diazepam intake. Blue colors indicate a decrease in positivity, while red colors reflect either a decrease in negativity or an increase in positivity. Black bars

in (A) represent TOIs of significant drug-induced changes in LICI corresponding to the N100 and P180 potentials. Plots in (D) show t-statistic maps of drug-induced changes in LICI (post-minus pre-drug intake) for the N100 and P180 TEP components. Red indicates a decrease of 'negative' LICI (i.e. the suppressive effect of CS_{100} on P180 was decreased by diazepam), whilst blue indicates a decrease of 'positive' LICI (i.e. the suppressive effect of CS_{100} on N100 is decreased by diazepam). Note that diazepam suppressed LICI of the N100 and P180 potentials.

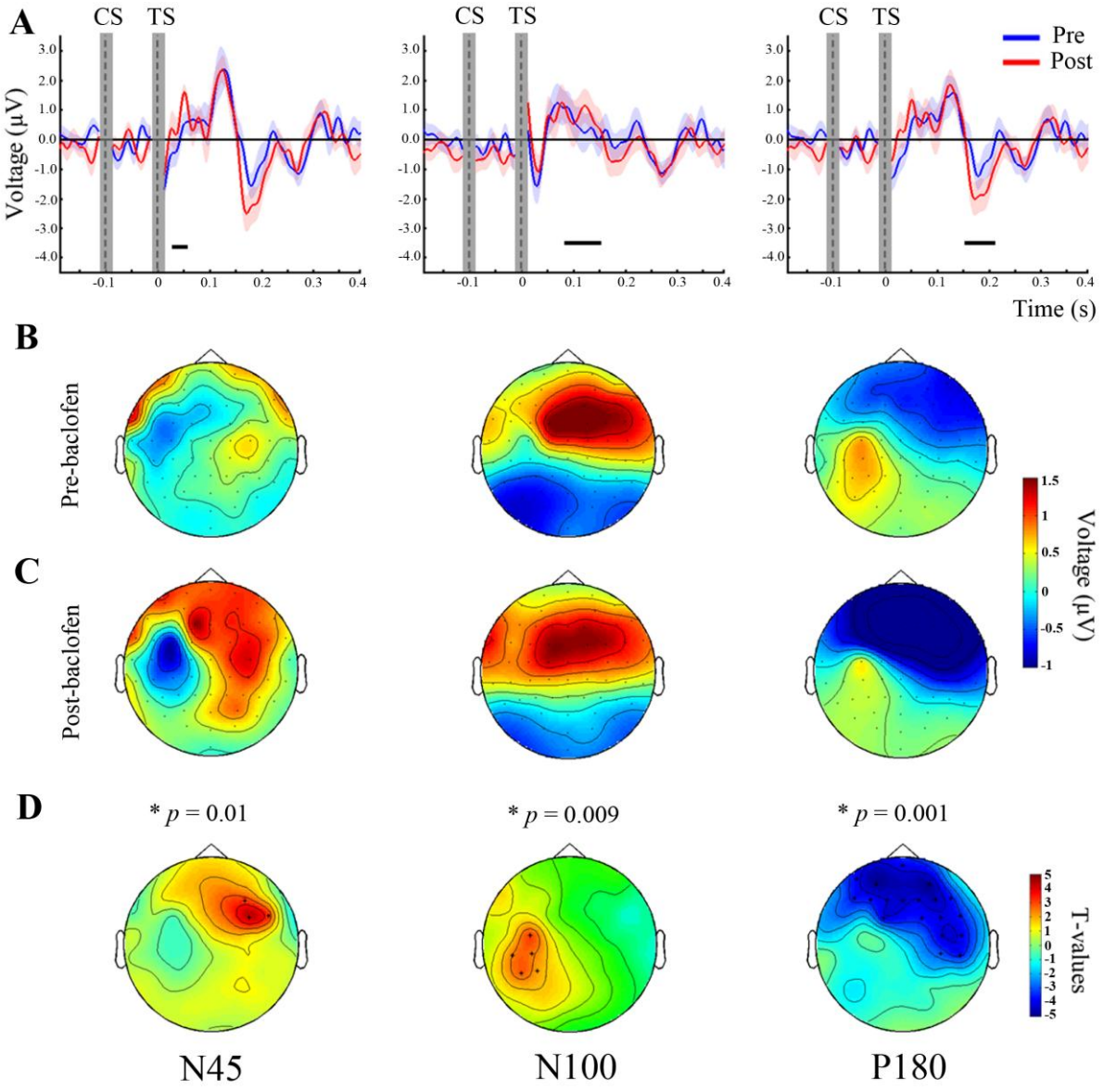

Figure 7. Baclofen effects on LICI.

LICI (i.e. paired- minus single-pulse TEPs, cf. Fig. 3D) was obtained before (Pre, blue) and after (Post, red) intake of baclofen (A). LICI curves are averaged across channels, which showed significant drug-induced effects as indicated by asterisks in (D). Topography of LICI before (B) and after (C) baclofen intake. Blue colors indicate a decrease in positivity, while red colors reflect either a decrease in negativity or an increase in positivity. Black bars in (A) represent TOIs of significant drug-induced changes in LICI corresponding to the N45, N100 and P180 potentials. Plots in (D) show t-statistic maps of drug-induced changes in LICI (post- minus pre-drug intake) for the N45, N100 and P180 TEP components. Red indicates an increase of 'positive' LICI (i.e. the suppressive effect of CS_{100} on N45 and N100 were enhanced by baclofen), whilst blue indicates an increase of 'negative' LICI (i.e. the suppressive effects of CS_{100} on P180 were enhanced by baclofen).

Investigation of drug-induced changes in LICI at the level of individual subjects revealed moderate inter-individual variability with most of the subjects showing a suppression of LICI of the N100 and P180 potentials after diazepam intake (**Figure 8A),** and an enhancement of LICI of the N45, N100 and P180 potentials after baclofen intake (**Figure 8B**), but some subjects showed opposite effects as the group on average.

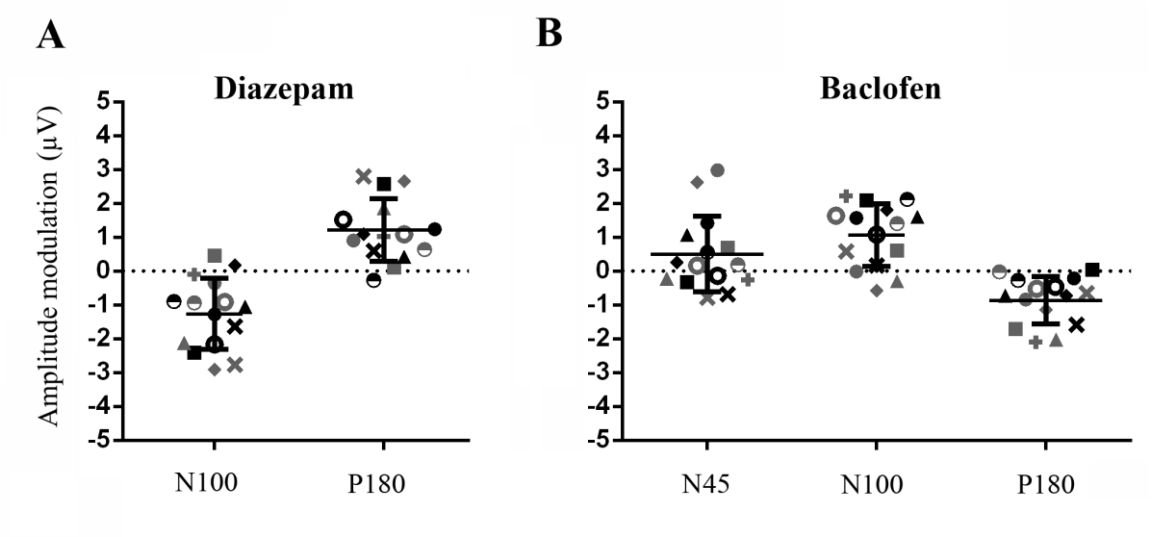

Figure 8. Drug effects on LICI (single subject data).

Scatter plots of diazepam (A) and baclofen (B) effects on LICI (post minus pre drug intake) of the significant TEP components (A: N100 and P180; B: N45, N100 and P180; cf. Fig. 6 and 7). LICI was extracted from those channels, which were significantly modulated by diazepam (cf. Fig. 6D) and baclofen (cf. Fig. 7D), respectively. Each subject is represented by a specific shape and color. Horizontal bars represent group average ± 1 SEM.

3.3 Relationship between LICI at baseline and drug-induced changes of LICI

To further investigate the cortical mechanisms of LICI, we performed correlation analyses between LICI and drug-induced changes of LICI. For diazepam we found a significant inverse correlation between LICI of the N100 (**Figure 9A**) and P180 (**Figure 9B**) at baseline and the diazepam-induced change in LICI of these potentials (Spearman $r^2 = 0.3$, $p = 0.03$ and Spearman $r^2 = 0.8$, $p < 0.001$, respectively), indicating that the diazepam-induced suppression of LICI of the N100 and P180 was highest in those individuals who showed the highest LICI of these potentials at baseline. In contrast, for baclofen we did not find significant correlations between LICI of the N45, N100 or P180 at baseline and the baclofen-induced change in LICI of these potentials (Spearman correlation, $p > 0.05$), suggesting independent underlying

mechanisms. In line with this notion, LICI of the N100 was expressed over the right hemisphere (i.e. contralateral to the stimulation; cf. Fig. 3E-F), whereas the baclofen-induced increase in LICI of the N100 was expressed ipsilateral to the stimulation (cf. Fig. 7D).

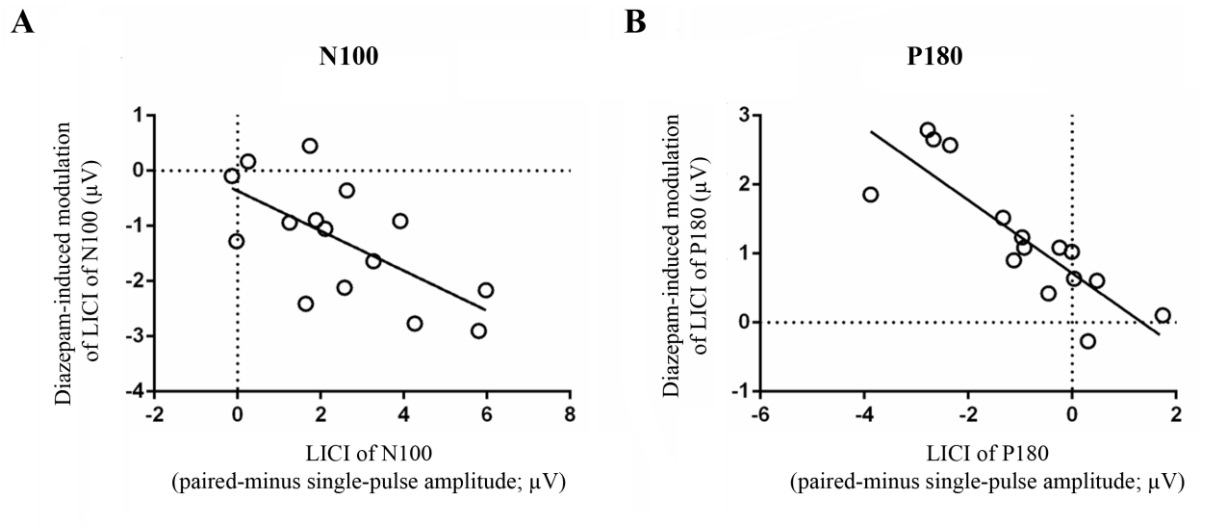

Figure 9. Correlation between LICI of the N100 and P180 potentials (at baseline) and the diazepam-induced change in LICI of these potentials.

(A) A significant inverse correlation was found between suppression of the N100 amplitude by CS_{100} in the paired-pulse condition (i.e. LICI of the N100; x-axis; data averaged across channels, which showed significant LICI of the N100, cf. Fig. 3F) and the diazepam-induced suppression of LICI of the N100 potential (y-axis; data averaged across channels, which showed significant diazepam-induced changes of LICI of the N100, cf. Fig. 6D). (B) A significant inverse correlation was found between suppression of the P180 amplitude by CS_{100} in the paired-pulse condition (i.e. LICI of the P180; x-axis; data averaged across channels, which showed significant LICI of the P180, cf. Fig. 3F) and the diazepam-induced suppression of LICI of the P180 potential (y-axis; data averaged across channels, which showed significant diazepam-induced changes of LICI of the P180, cf. Fig. 6D).

4. Discussion

In this study, by applying paired-pulse TMS (LICI protocol) in conjunction with simultaneous high-density EEG and pharmacology we provide novel evidence for inhibitory neurotransmission mediated by GABABRs in the human cortex.

4.1 EEG signatures of LICI at baseline (comparison between paired- and single-pulse TEPs)

Evidence from TMS-EMG experiments suggests that whilst LICI at short interstimulus intervals (ISI) of around 50ms may include spinal inhibitory mechanisms, LICI at longer ISIs of 100-200ms is predominantly cortical in nature (Di Lazzaro et al., 2002; Nakamura et al., 1997). Pharmacological studies further showed that LICI at an ISI of 100ms is associated with GABABR-activity (McDonnell et al., 2006; Müller-Dahlhaus et al., 2008). This notion is supported by the finding that SICI, another paired-pulse TMS paradigm likely representing GABAAR-mediated inhibition (Di Lazzaro et al., 2000; Di Lazzaro et al., 1998; Ilic et al., 2002; Kujirai et al., 1993; Peurala et al., 2008; Ziemann et al., 1996a, b) is suppressed in the presence of LICI, which is thought to derive from activation of presynaptic GABABRs (Cash et al., 2010; Müller-Dahlhaus et al., 2008; Sanger et al., 2001). In this study, LICI (quantified as paired- minus single-pulse TEPs) showed a suppression of the P25, N45, N100 and P180 potentials, whereas the P70 was increased (cf. Fig.3).

Importantly, paired-pulse TEPs (i.e. TS-evoked potentials recorded in the presence of CS_{100}) were corrected for long-lasting (i.e. >100ms) EEG responses evoked by CS_{100} alone (cf. Fig.1) by subtracting TEPs evoked by an *unconditioned* single pulse (TS alone) from TEPs evoked by the pulse pair CS_{100}-TS, aligned to CS_{100} [see Material and Methods, and Fig.1C, paired-pulse corrected TEPs]. However, this subtraction does not take into account a possible modulation of late (i.e. >100ms) CS_{100}-evoked EEG responses by TS in the paired-

pulse condition. Thus, in turn, LICI, which was calculated by subtraction of TEPs evoked by an *unconditioned* single pulse (TS alone) from the paired-pulse corrected TEPs, aligned to TS [see Material and Methods, and Fig.1D, LICI] may also reflect this $TS \rightarrow CS_{100}$ interaction in addition to the $CS_{100} \rightarrow TS$ interaction. In other words, LICI represents the superimposition of CS_{100}-induced modulation of TS-evoked TEPs (the "pure" LICI we want to study) and a putative TS-induced modulation of CS_{100}-evoked late (i.e. >100ms) TEP components. Unfortunately, there is no way to disentangle these two interactions. To determine the $TS \rightarrow CS_{100}$ interaction, it needs a CS_{100}, which impacts on the subsequent TS (interaction $CS_{100} \rightarrow TS$). Thus, the interaction $TS \rightarrow CS_{100}$ cannot be studied without a concurrent interaction $CS_{100} \rightarrow TS$, and vice versa. Therefore LICI will always represent a superimposition of CS_{100}-induced modulation of TS-evoked TEPs and a possible modulation of late CS_{100}-evoked TEP components by a *CS_{100}-modulated* TS. Of note, this line of arguments also applies to LICI as obtained by TMS-EMG: the MEP inhibition is a result of this putatively bidirectional interaction of late CS_{100} effects and TS effects in the stimulated cortex.

Prior studies from our group indicated that the N100 potential reflects GABABR-mediated inhibition as it is selectively enhanced at the site of the stimulated M1 after intake of baclofen (Premoli et al., 2014). Thus, TS in the paired-pulse TMS condition in the present study was delivered at a time of increased GABABR-mediated inhibition in the stimulated cortex. Accordingly, LICI of the P25 correlated directly with the amplitude of the N100 potential in the single-pulse condition (cf. Fig.5). As the P25 potential is linked to the MEP amplitude elicited in the target muscle (Ferreri et al., 2011; Mäki and Ilmoniemi, 2010), LICI of the P25 may represent a marker of GABABergic inhibition of cortico-spinal neurons in the stimulated M1. Of note, this effect was present in all subjects tested (cf. Fig.4 and 5), whereas

LICI of later TEP components (i.e., N45, P70, N100, P180) showed higher inter-individual variability with some subjects showing opposite LICI effects as the group on average.

Topographical plots of LICI of later TEP components (i.e. N45, P70, N100, P180) demonstrated that it was expressed at the same sites as these potentials in the single-pulse condition (cf. Fig. 3E). These data suggest that LICI not only inhibits cortico-spinal excitability as reflected by a reduction of the P25 potential (see above), but also inhibits the cortico-cortical (incl. cortico-thalamo-cortical) spread of evoked neural activity, especially to the contralateral hemisphere. In contrast, LICI increased the P70 ipsilaterally to the stimulation. The reason for this effect is unclear, but may involve arrival of re-afferent or cutaneous sensory potentials from TMS-evoked finger movement as the underlying drivers of the P70 (Paus et al., 2001; Rogasch et al., 2013) in a disinhibited cortex in the stimulated hemisphere at around 160-180ms after CS_{100}. TMS-EMG experiments demonstrated a late cortical disinhibition at CS-TS intervals of around 180ms (Cash et al., 2010; Cash et al., 2011).

Our results are in general agreement with a previous study (Fitzgerald et al., 2009), which found long-lasting suppression of paired-pulse TEPs at an ISI of 100ms at the stimulation site (i.e. the C3 electrode over the stimulated M1). However, this study used a time-integrated measure of the mean TS-evoked EEG activity (i.e. the area under the rectified TEP curve) and paired-pulse TEPs were not corrected for long-lasting EEG effects of CS. More recent work from the same group focused on single TEP components and showed a differential modulation of early (P30, P60) and late (N100) potentials as a function of both CS and TS intensities (Rogasch et al., 2013). However, either CS or TS were given at an intensity to yield, on average, 1mV MEPs in the target small hand muscle when given alone. In contrast, in our study we applied both CS and TS at an intensity of 100% RMT and corrected

for long-lasting EEG effects of CS (cf. Fig.1 and see Material and Methods). Despite these methodological differences both the previous (Rogasch et al., 2013) and our study suggest a differential physiological role of early vs. late TEPs which may prove of relevance for the interpretation of TMS-EEG data in a clinical diagnostic setting.

4.2 Drug effects on LICI

If our hypothesis is correct that LICI represents GABABergic neurotransmission, then baclofen, a specific agonist at the GABABR should increase LICI. Indeed, we found that baclofen showed a trend towards enhancement of LICI of the N45 and N100 potentials, and significantly enhanced LICI of the P180 potential. Of note, the baclofen effect on LICI of the N100 was expressed ipsilateral, whilst the baclofen effect on LICI of the P180 was expressed contralateral to the stimulation. The mechanisms of baclofen-induced enhancement of LICI of these potentials are not clear, but may involve enhanced GABABergic neurotransmission in local inhibitory networks at the site of the stimulation, enhanced local GABABergic control of transcallosal inputs to the contralateral hemisphere (Irlbacher et al., 2007; Palmer et al., 2013), or a cross-talk between GABABRs and GABAARs as recently described within the thalamus (Connelly et al., 2013), resulting in disrupted thalamo-cortical communication important for propagation of later TEPs from the stimulated to the non-stimulated hemisphere. Future studies, including source modeling of TEPs, may help in elucidating the generation, propagation and modulation of TEPs by conditioning stimuli, and drugs.

In contrast, there was no effect of baclofen on LICI of the P25, even though LICI of the P25 at baseline (i.e. before drug intake) correlated directly with the amplitude of the N100 potential in the single-pulse condition [which can be considered as a marker of GABABR-mediated inhibition (Premoli et al., 2014)], suggesting an underlying GABABR-mediated mechanism. The reason for this discrepancy is not clear, but may be due to the low reliability

of LICI measurements of the P25 as suggested by a low Cronbach's alpha-value for test-retest reliability (cf. Fig. 2D). Prior TMS-EMG experiments have shown increased LICI under baclofen, i.e. baclofen led to a stronger suppression of the test MEP in the presence of CS_{100} as compared to the drug-naïve situation (McDonnell et al., 2006; Müller-Dahlhaus et al., 2008), and it was shown that the P25 can be linked to the MEP amplitude elicited in the target muscle (Ferreri et al., 2011; Mäki and Ilmoniemi, 2010). Taken together, these data suggest that the P25 may indeed only partially reflect the neural circuits underlying generation of the MEP, and point towards an additional spinal GABABergic inhibitory control of the MEP. Whatever the exact underlying mechanisms, our findings corroborate the notion that LICI as determined by TMS-EEG offers higher selectivity in testing *cortical* GABABergic neurotransmission than can be derived indirectly by TMS-EMG.

Diazepam consistently suppressed LICI of later potentials (i.e. N100, P180), without having an effect on LICI of earlier (i.e. P25, N45 and P70) potentials. In addition, correlation analyses showed that the diazepam-induced suppression of LICI of the N100 and P180 potential was highest in those individuals who showed the strongest LICI of these potentials at baseline. These findings again suggest a close link between GABAAR- and GABABR-mediated control of cortico-cortical (incl. cortico-thalamo-cortical) propagation of TS-evoked activity. GABAAR-mediated inhibition has been shown to control excitatory transcallosal pathways to contralateral GABAergic interneurons acting through GABABRs (Irlbacher et al., 2007; Palmer et al., 2013). Likewise, GABAAR-mediated recurrent inhibition of thalamic reticular nucleus cells results in suppression of intrathalamic GABABergic neurotransmission, and hence modulation of intrathalamic oscillations important for thalamo-cortical communication (Huguenard and Prince, 1994).

4.3 Conclusion

We here demonstrated, for the first time, directly at the system level of the human cortex that GABABR-mediated cortical inhibition contributes to LICI. Data further suggest a tight control of GABABR-mediated inhibition by GABAAergic neurotransmission. Paired-pulse TMS-EEG allows high-resolved characterization of the spatiotemporal dynamics of LICI, more directly than hitherto possible with other techniques such as TMS-EMG. Our findings provide important evidence on the mechanisms of LICI and help to build a solid basis for interpretation of paired-pulse TMS-EEG data in patients with impaired GABAergic cortical control such as in schizophrenia or epilepsy (Hasan et al., 2012; Kimiskidis et al., 2014; Lewis et al., 2005).

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