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Long-interval intracortical inhibition as biomarker for epilepsy: a transcranial magnetic stimulation study

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Cortical excitability, as measured by transcranial magnetic stimulation combined with electromyography, is a potential biomarker for the diagnosis and follow-up of epilepsy. We report on long-interval intracortical inhibition data measured in four different centres in healthy controls ($n = 95$), subjects with refractory genetic generalized epilepsy ($n = 40$) and with refractory focal epilepsy ($n = 69$). Long-interval intracortical inhibition was measured by applying two supra-threshold stimuli with an interstimulus interval of 50, 100, 150, 200 and 250 ms and calculating the ratio between the response to the second (test stimulus) and to the first (conditioning stimulus). In all subjects, the median response ratio showed inhibition at all interstimulus intervals. Using a mixed linear-effects model, we compared the long-interval intracortical inhibition response ratios between the different subject types. We conducted two analyses; one including data from the four centres and one excluding data from Centre 2, as the methods in this centre differed from the others. In the first analysis, we found no differences in long-interval intracortical inhibition between the different subject types. In all subjects, the response ratios at interstimulus intervals 100 and 150 ms showed significantly more inhibition than the response ratios at 50, 200 and 250 ms. Our second analysis showed a significant interaction between interstimulus interval and subject type ($P = 0.0003$). Post hoc testing showed significant differences between controls and refractory focal epilepsy at interstimulus intervals of 100 ms ($P = 0.02$) and 200 ms ($P = 0.04$). There were no significant differences between controls and refractory generalized epilepsy groups or between the refractory generalized and focal epilepsy groups. Our results do not support the body of previous work that suggests that long-interval intracortical inhibition is significantly reduced in refractory focal and genetic generalized epilepsy. Results from the second analysis are even in sharper contrast with previous work, showing inhibition in refractory focal epilepsy at 200 ms instead of facilitation previously reported. Methodological differences, especially shorter intervals between the pulse pairs, may have contributed to our inability to reproduce previous findings. Based on our results, we suggest that long-interval intracortical inhibition as measured by transcranial magnetic stimulation and electromyography is unlikely to have clinical use as a biomarker of epilepsy.

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Introduction

Epilepsy is a paroxysmal neurological condition characterized by an enduring predisposition to generate epileptic seizures (Fisher *et al.*, 2014). The diagnosis is based on the clinical history, often supported by interictal or ictal epileptic discharges on the EEG (Rosenow *et al.*, 2015). These pathological changes in the EEG are paroxysmal and do not always occur during a short EEG recording (Smith, 2005). The diagnostic sensitivity of routine EEG for epilepsy is estimated at 17% in adults and at 58% in children (Bouma *et al.*, 2016). Sensitivity can be moderately increased by increasing recording time, or by using activation procedures such as sleep deprivation, hyperventilation or photic stimulation (Smith, 2005; Rosenow *et al.*, 2015). In about 70% of those diagnosed with epilepsy, seizures can be suppressed with anti-epileptic drugs (AEDs) (Brodie *et al.*, 2012), but finding the optimal AED type and dose for an individual patient can be a difficult and time-consuming process. The EEG does not provide a direct measure of seizure proneness and its use in the follow-up of epilepsy is therefore limited, spurring the search for a reliable biomarker of epilepsy activity to improve its management (Smith, 2005; Engel, 2008).

Increased cortical excitability resulting from an imbalance between excitatory and inhibitory activity is thought to play an important role in the pathophysiology of epilepsy (Schwartzkroin, 1994). Cortical excitability can be measured non-invasively using single or paired pulse transcranial magnetic stimulation (TMS) (Reutens and Berkovic, 1992). Single pulse protocols are used to assess the motor threshold, motor-evoked potential (MEP) amplitude and cortical silent period. With paired pulse protocols, short-interval intracortical inhibition, intracortical facilitation and long-interval intracortical inhibition (LICI) can be measured (Kobayashi and Pascual-Leone, 2003). Several studies showed increased cortical excitability in groups of drug-naïve subjects with generalized or focal epilepsy compared to healthy controls (for review see de Goede *et al.*, 2016). Four studies reported a decrease in excitability after successful treatment with AEDs but not after ineffective treatment (Badawy *et al.*, 2010a, 2013, 2013c, d).

Of the different variables measured with TMS, those measured with paired pulse protocols appear to hold the greatest potential as biomarkers for epilepsy for several reasons: first, they provide information about cortical excitability rather than integrated corticospinal excitability as is the case for single pulse measures (Ziemann *et al.*, 1996). Second, they can be expressed as dimensionless ratios, enabling comparison across different institutions. Third, paired pulse protocols appear to yield more reliable findings than single pulse protocols (de Goede *et al.*, 2016). Several studies consistently found facilitation at the short (2 and 5 ms) and long (250 and 300 ms) interstimulus intervals (ISIs), instead of inhibition as in healthy controls, providing evidence for cortical hyperexcitability in drug-naïve epilepsy (for review see de Goede *et al.*, 2016). Lastly, this difference between facilitation in drug-naïve epilepsy and inhibition in controls was larger at long than at short ISIs (Badawy *et al.*, 2007). Of the different TMS variables, LICI may thus be the most suitable as an epilepsy biomarker. LICI is measured by applying two supra-threshold stimuli with an ISI of 50–400 ms and calculating the ratio between the response to the second (test stimulus) and to the first (conditioning stimulus) (a variant consists of the ratio between the response to the test stimulus and to an unconditioned stimulus). A ratio with values < 1 indicates inhibition, while values > 1 indicate facilitation (Valls-Solé *et al.*, 1992). LICI is thought to be linked to GABA-B receptor mediated inhibition (Werhahn *et al.*, 1999; McDonnell *et al.*, 2006).

In epilepsy, LICI was mainly studied by one group of investigators who, in several studies, showed facilitation instead of inhibition at ISIs of 50, 150, 250 and 300 ms in groups of drug-naïve subjects with different types of genetic generalized epilepsy (Brodtmann *et al.*, 1999; Badawy *et al.*, 2007, 2010b, 2012, 2013, 2013b, c, 2014; Badawy and Jackson, 2012). In drug-naïve focal epilepsy, cortical excitability was consistently increased in the hemisphere ipsilateral to the epileptic focus, but not in the contralateral hemisphere, at ISIs 250 and 300 ms

(Badawy *et al.*, 2007, 2010*b*, 2012, 2013, 2013*a*, *b*, *d*, 2014, 2015; Badawy and Jackson, 2012). In successfully treated epilepsy, hyperexcitability normalized over time in seizure-free groups, becoming more similar to controls, but it remained increased in refractory groups (Badawy *et al.*, 2010*a*, 2013, 2013*c*, *d*). Two recent studies from other groups reported contrasting findings, however, the first found significantly lower cortical excitability in subjects with genetic generalized epilepsy on AEDs compared to a healthy control group and a drug-naïve generalized epilepsy group at ISIs between 200 and 250 ms. No significant differences were found between the drug-naïve epilepsy and control groups (Silbert *et al.*, 2015). The second study found inhibition at an ISI of 50 ms in poorly controlled epilepsy, but not in moderately controlled epilepsy or healthy controls. At an ISI of 200 ms, the groups with poorly and moderately controlled epilepsy both showed inhibition (more prominent in the poorly controlled group), whereas healthy controls did not. These results, however, were not significant after correction for multiple comparisons (Pawley *et al.*, 2017).

To establish the true potential of LICI as a clinical biomarker of epilepsy, the promising findings need to be replicated and extended to larger groups. An ideal biomarker needs to provide consistent results across different centres. For it to be useful on an individual level, the interindividual variability should be low. A difference in excitability between refractory epilepsy and healthy controls would support the use of LICI to rapidly evaluate the effect of treatment with AEDs. We report on LICI data from healthy controls and subjects with refractory genetic generalized and focal epilepsy from four different centres in two different countries. Our results do not support the utility of LICI as a biomarker for epilepsy.

Materials and methods

Data were collected independently in four different tertiary referral centres, two each in the Netherlands and the UK, and retrospectively pooled. The centres were: (i) Medisch Spectrum Twente (Netherlands); (ii) Stichting Epilepsie Instellingen Nederland – SEIN (Netherlands); (iii) King's College London (UK); and (iv) University College London (UK).

The studies were performed in accordance with guidelines for TMS use in clinical practice and research (Rossi *et al.*, 2009). All study protocols were approved by the local ethics committees of each of the participating centres.

Participants

Informed written consent was provided by all participants. For those younger than 18 years, assent was also obtained from both parents. People with contra-indications to TMS other than epilepsy and pregnant females were excluded.

Centre 1

Healthy adults (aged 18 years or over) were recruited locally through advertisement at the University of Twente and the Medisch Spectrum Twente. People with a history of epilepsy, brain lesions or spinal cord surgery were excluded. Hand dominance was assessed with the Dutch Handedness Questionnaire (van Strien, 1992, 2003).

Centre 2

Healthy participants (aged 12 years or over) were recruited locally through digital and paper adverts. People with a neurological or psychiatric condition, including migraine or epilepsy, diabetes mellitus and people taking medication that could affect cortical excitability (such as psychoactive drugs and β-blockers) were excluded. Hand dominance was assessed with the Dutch version of the Edinburgh handedness questionnaire (Oldfield, 1971).

Centre 3

Adults with a clinical diagnosis of epilepsy were recruited via specialized neurology and epilepsy clinics at King's College Hospital, St Thomas' Hospital, St George's Hospital, London, Kent and Canterbury Hospital and Queen Elizabeth Hospital, Woolwich. For the control group, healthy adults without a personal or family history of neurological or psychiatric conditions were recruited through a local research volunteer's database and friends of participants. Those with epilepsy who had a neuropsychiatric condition other than epilepsy, non-epileptogenic seizures, an estimated IQ < 70 or who did not cooperate with the TMS procedures were excluded. In part of the cohort, hand dominance was assessed with the Edinburgh handedness questionnaire (Oldfield, 1971).

Centre 4

Adults with epilepsy were recruited through specialized epilepsy clinics and an inpatient unit at the National Hospital for Neurology and Neurosurgery (Queen Square and Chalfont sites). Participants with a clinical diagnosis of refractory genetic generalized or focal epilepsy were included. Hand dominance was assessed with the Edinburgh handedness questionnaire (Oldfield, 1971).

Data acquisition

In all centres, participants were seated in a comfortable chair with their hands in a relaxed position and their eyes open. The experimental set-up and stimulation protocol of each centre is summarized in Table 1.

Centre 1

TMS was performed with a Magstim Rapid[2] Stimulator (maximum stimulator output 1.5 T), and a figure-of-eight air-cooled 70 mm coil (The Magstim Company Limited). Biphasic TMS pulses were given to both motor hotspots of the abductor digiti minimi (ADM) muscle. Muscle activity was recorded using two surface Ag/AgCl electrodes placed in a belly-tendon montage. MEPs were recorded from the contralateral ADM muscle with a 72-channel Refa system (TMSi). Data were recorded with a sampling frequency of 5 kHz and stored for offline analysis. Measurements were conducted between 9 AM and 5 PM.

	Centre 1	Centre 2	Centre 3	Centre 4
Stimulator type	Magstim Rapid[2]	MagPro X100	Magstim BiStim[2]	Magstim BiStim[2]
Maximum stimulator output (T)	1.5	3.9	1.5	1.5
Magnetic pulse waveform	Biphasic	Biphasic	Monophasic	Monophasic
Coil type	Figure-of-eight	Round	Figure-of-eight	Figure-of-eight
Stimulation location	Motor hotspot	Vertex (Cz)	Motor hotspot	Motor hotspot
Target muscle	ADM	APB	FDI	APB
Intertrial interval	$\sim$4 s	1 s	4 s	5 s
Repetitions per ISI	50	6	10	10
Order of ISIs	Random	Increasing	Random	Random
Conditioning pulse intensity (%rMT)	120	110	120	110
Test pulse intensity (%rMT)	120	110	120	110
LICI response ratio	Mean(TR)/mean(CR)	Mean(TR)/mean (unconditioned MEP)	Mean(TR/CR)	Mean(TR)/mean (unconditioned MEP)
Time of day measurement	9 AM–5 PM	9 AM–4 PM	9 AM–5 PM	9 AM–5 PM

rMT = resting motor threshold.

Centre 2

TMS was performed with a MagPro X100 magnetic stimulator (maximum stimulator output 3.9 T), and a 12-cm diameter parabolic circular MMC-140 coil (Magventure). Biphasic TMS pulses were given on the vertex (Cz). MEPs were recorded bilaterally from the abductor pollicis brevis (APB) muscles with a Nicolet Viking EDX EMG system (Natus). Data were recorded with a sampling frequency of 4 kHz and stored for offline analysis. Measurements were conducted between 9 AM and 4 PM.

Centre 3

TMS was performed using two Magstim 200[2] stimulators connected via a BiStim module, and a figure-of-eight 90 mm coil (The Magstim Company Limited). Monophasic TMS pulses were given to both motor hotspots of the first dorsal interosseous (FDI) muscle. MEPs were recorded from the contralateral FDI muscle with a CED1902 EMG amplifier and CED 1401 Signal 3.13 software (Cambridge Electronic Design) using a sampling rate of 15 kHz, a bandwidth of 10–5000 Hz, a gain of 1000 (ranging from -5 to 5 V), and traces recorded on Signal 3.13 software (CED 1401) and stored for offline analysis. Measurements were conducted between 9 AM and 5 PM.

Centre 4

Hardware and software for TMS-EMG data collection were the same as in Centre 3. A figure-of-eight 70 mm D70 alpha coil was used (The Magstim Company Limited). Monophasic TMS pulses were given to the dominant motor hotspot of the APB muscle. MEPs were recorded with a sampling frequency of 2 kHz and stored for offline analysis. Recordings were obtained between 9 AM and 5 PM.

Estimation of the resting motor threshold

For the three centres using a figure-of-eight coil, the resting motor threshold was determined by applying single pulses to the ADM (Centre 1), FDI (Centre 3) or APB (Centre 4) motor hotspots. The hotspot was defined as the location were the largest MEPs were induced when the TMS coil was placed tangentially with the handle pointed backwards and laterally at an angle of 45° from the midline. Stimulation commenced at 30% of maximum stimulator output and increased in 5% increments until a MEP was seen. One per cent changes in intensity were then used to find the threshold, defined as the minimum stimulus intensity, which produced a MEP with a peak-to-peak amplitude $>50\,\mu$V ($>100\,\mu$V in Centre 4) in 50% or more of 10 trials in the fully relaxed target muscle (Rossini *et al.*, 1994). Relaxation of the target muscle was monitored by continuous visual observation of the EMG.

The approach was different in Centre 2, where the resting motor threshold was approximated using a single pulse stimulus-response curve, with the coil on the vertex. Stimulation started at 20% of stimulator output with 5% stepwise increments until there was a consistent twitch in the hand contralateral to the stimulated hemisphere in 50% or more of eight trials (approximated resting motor threshold). Then, a semi-automated, in-house designed scanning protocol (created in Matlab®) (version 7.5.0 R2007b The MathWorks Inc., Natick, MA, USA) was used to automatically deliver stimuli with a fixed intertrial interval of 2 s, and eight stimuli at each intensity. Scanning started at a stimulator output value of 10–12% below the approximated resting motor threshold and increased in 2% steps until a reproducible MEP ($>200\,\mu$V) was seen after every stimulus (corresponding to 110–120% resting motor threshold). The resting motor threshold was defined as the lowest stimulus intensity eliciting a visible twitch in any hand muscle in 50% or more of eight stimuli (Varnava *et al.*, 2011).

Assessment of long-interval intracortical inhibition

Centre 1

Paired pulse stimulation was applied with the coil over the ADM motor hotspot. Both pulses were given at 120% of the resting motor threshold with ISIs between 50 to 300 ms, with 50 ms increments (six intervals). Stimulation was repeated 50 times for each ISI. The stimulus pairs were given randomly with $\sim$4 s (range 3.5–4.5 s) between stimulus pairs (intertrial interval). To calculate the LICI for each ISI, the ratio was

taken between the mean peak-to-peak amplitude of the responses to the second (test) stimuli (test response, TR), and the mean peak-to-peak amplitude of the responses to the first (conditioning) stimuli (conditioning response, CR): mean(TR)/mean(CR).

Centre 2

Paired pulse stimulation was applied with the coil over the vertex. Both pulses were given at 110% of the resting motor threshold, with increasing ISIs between 50 and 400 ms, with 25 ms increments (15 intervals). Stimulation was repeated six times for each ISI. The stimulus pairs were given in a fixed increasing order with an intertrial interval of 1 s. An unconditioned stimulus was given six times immediately before the start of the paired pulse stimulation protocol. To calculate the LICI for each ISI, the mean peak-to-peak amplitude in response to the conditioned, second stimuli (TR) was divided by the mean peak-to-peak amplitude in response to the unconditioned stimuli: mean (TR)/mean (unconditioned MEP).

Centre 3

Paired pulse stimulation was applied with the coil over the FDI motor hotspot. Both paired pulses were given at 120% of the resting motor threshold. Four ISIs were tested: 50, 150, 200 and 250 ms. Stimulation was repeated 10 times for each ISI. The stimulus pairs were given in random order with an intertrial interval of 4 s. To calculate the LICI for each ISI, the ratios were taken between the peak-to-peak amplitudes of the responses to the second test stimuli (TR), and the peak-to-peak amplitudes of the responses to the first conditioning stimuli (CR). Then the mean over all ratios was taken: mean(TR/CR).

Centre 4

Paired pulse stimulation was applied with the coil over the APB motor hotspot. Both pulses were given at 110% of the resting motor threshold. Five ISIs were tested: 50, 100, 150, 200 and 250 ms. Stimulation was repeated 10 times for each ISI. The stimulus pairs were given in a random order with a 5 s intertrial interval. LICI was calculated in the same way as in Centre 2.

Data analysis

Each centre provided the following individual data for analysis: age, gender, hand dominance (if available), epilepsy diagnosis, including whether epilepsy was refractory to treatment with AEDs (defined as at least one seizure in the year preceding the TMS measurement), number of different AEDs, side of seizure focus (in case of focal epilepsy), and the mean LICI for each ISI. Only LICI values of healthy controls and people with refractory epilepsy (generalized or focal) were included in the analysis. Based on previous reports, cortical excitability as measured by LICI remains elevated in those who are refractory to pharmacological treatment, whereas it normalizes (returns to the levels seen in controls) in those who become seizure-free. The refractory epilepsy group is, therefore, the most interesting to assess in this context. For controls and genetic generalized epilepsy, we only analysed the LICI when stimulating the dominant hemisphere (left hemisphere for right-hand dominance). When hand dominance was unknown and in ambidextrous participants, we analysed the LICI when stimulating the left hemisphere. For focal epilepsy, we included the LICI when stimulating the hemisphere ipsilateral to the epileptic focus in the analysis, or the dominant/left hemisphere when epileptic foci were bilateral. We included the LICI measured at ISIs of 50, 100, 150, 200, and 250 ms.

Statistical analysis

Data analysis was done in R® (R Core Team, 2015). As the material and stimulation protocols differed between the centres, we expected not only large interindividual variability of LICI values, but also a large variability between the centres. This intercentre variability limits the comparison of data between the centres and means that data cannot simply be pooled for analysis. Linear mixed-effects models (lme4 package, Linear Mixed Effects version 4) (Bates et al., 2015) are the best way to deal with such datasets, as they allow for correction of systematic variability. We accounted for the heterogeneity of LICI values across subjects and centres by defining them as effects with a random intercept, thus instructing the model to correct for any systematic differences between the subjects (interindividual variability) and centres (intercentre variability). We then analysed the influence of two possible fixed effects on LICI: (i) the subject type (three levels: controls, refractory genetic generalized epilepsy and refractory focal epilepsy); and (ii) the ISIs (five levels: 50, 100, 150, 200 and 250 ms). LICI response ratios were log transformed to better approximate normality (Supplementary Fig. 1). To optimize our model, we checked the normality of the model residual.

We ran a type II analysis of variance. Wald chi-square tests were used for fixed effects in linear mixed-effects models. For post hoc tests we used the Lsmean package (Lsmean version 2.20-23) (Searle et al., 1980) where P-values were considered as significant at $P < 0.05$ and adjusted for the number of comparisons performed (Tukey method).

Results

Participants

Centre 1

Twenty-five healthy subjects were included. Four were excluded from the analyses: in two, stimulation was not possible at an intensity of 120% of the resting motor threshold, in one the session was terminated prematurely as the subject felt unwell, and in one, LICI data were not available from the dominant hemisphere. Twenty-one healthy individuals were included in the analysis (six males, mean age 28.6 years, range 20–49 years, three left-handed) (Fig. 1).

Centre 2

Thirty-eight controls were included; one was excluded due to non-specific EEG abnormalities. Data of 37 controls were included in the analysis (11 males, mean age 38.1 years, range 15–62 years, four left-handed, one ambidextrous) (Fig. 1).
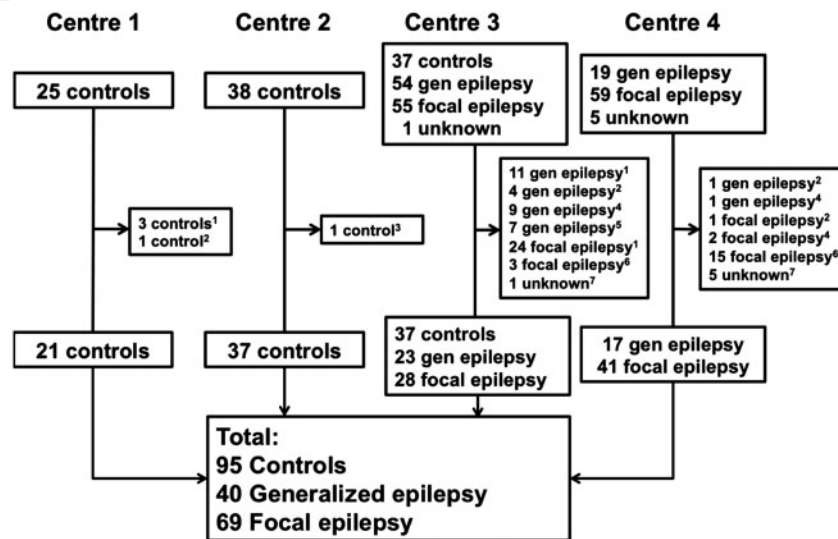
Figure 1 Included and excluded subjects per centre. Exclusion criteria: [1]No LICI data; [2]No LICI data for the dominant hemisphere; [3]Aspecific abnormalities on the EEG; [4]No treatment with AEDs; [5]Not refractory; [6]No LICI data for the hemisphere ipsilateral to the epileptic focus; and [7]Type of epilepsy unknown.

Centre 3

Thirty-seven controls and 110 subjects with epilepsy were included (54 with genetic generalized epilepsy, 55 with focal epilepsy and one with an unclear diagnosis). All controls were included in the analysis (19 males, mean age 30.2 years, range 18–52 years, four left-handed, one ambidextrous). Of the 54 subjects with generalized epilepsy, 31 were excluded from the analysis: in 11, LICI data were not collected (for reasons including too high motor threshold or discomfort during stimulation), in four, LICI data were not available from the dominant hemisphere, nine were not taking AEDs at the time of the experiment, and seven were not considered refractory. Thus, 23 subjects with refractory genetic generalized epilepsy were included in the analysis (10 males, mean age 30.1 years, range 18–54 years, hand dominance known in 12, of these two were left-handed, AEDs: median 1, range 1–4). In 24 of the 55 with focal epilepsy, LICI data were not collected. In three, LICI data were only available for the hemisphere contralateral to the epileptic focus. Thus, 28 subjects with refractory focal epilepsy were included in the analysis (12 males, mean age 39.4 years, range 21–66 years, AEDs: median 1, range 1–3) (Fig. 1). The hand dominance of this group was unknown.

Centre 4

Nineteen participants with genetic generalized epilepsy were included. One had to be excluded from the analysis as no AEDs were used and one because LICI data from the dominant hemisphere were not available. Seventeen subjects with genetic generalized epilepsy were included in the analysis (eight males, mean age 34.4 years, range 20–51 years, one left-handed, two ambidextrous, AEDs: median 3, range 1–5). Fifty-nine with focal epilepsy were included but

15 were excluded as LICI data from the hemisphere ipsilateral to the epileptic focus were not available. In one participant with a bilateral focus, LICI data from the dominant hemisphere were unavailable. Two participants were not taking AEDs, leaving 41 with refractory focal epilepsy for analysis (18 males, mean age 39.7 years, range 18–61 years, four left-handed, AEDs: median 2, range 1–6) (Fig. 1). A further five participants were included, but as the epilepsy diagnosis was unclear they were excluded from the current analysis.

In total, we included data from 204 subjects in the analysis, 40 with refractory generalized epilepsy, 69 with refractory focal epilepsy and 95 healthy controls (Fig. 1). Part of the data from this cohort were previously reported in other studies [Centre 1: de Goede and van Putten (2017), Centre 3: Chowdhury *et al.* (2015); Pawley *et al.* (2017) and Centre 4 (including patients with Dravet Syndrome): Stern *et al.* (2016, 2017)].

Long-interval intracortical inhibition recovery curves

For all subject types, the response ratios for each ISI are shown in Fig. 2 and Table 2. As expected, we found inhibition (median LICI value <1) in healthy controls at all analysed ISIs (50, 100, 150, 200 and 250 ms) (Fig. 2 and Supplementary Fig. 2). Unexpectedly, the median LICI was also <1 in refractory genetic generalized epilepsy (all ISIs except 50 ms) and refractory focal epilepsy (all ISIs). The mean LICI also showed inhibition in the controls, except in Centre 2 (all ISIs), in Centre 1 at ISI 50 ms and in Centre 3 at ISI 250 ms (Table 2). In the refractory epilepsy groups, the mean LICI was also <1 at most ISIs, except for genetic generalized epilepsy at ISI 50 ms and focal epilepsy in
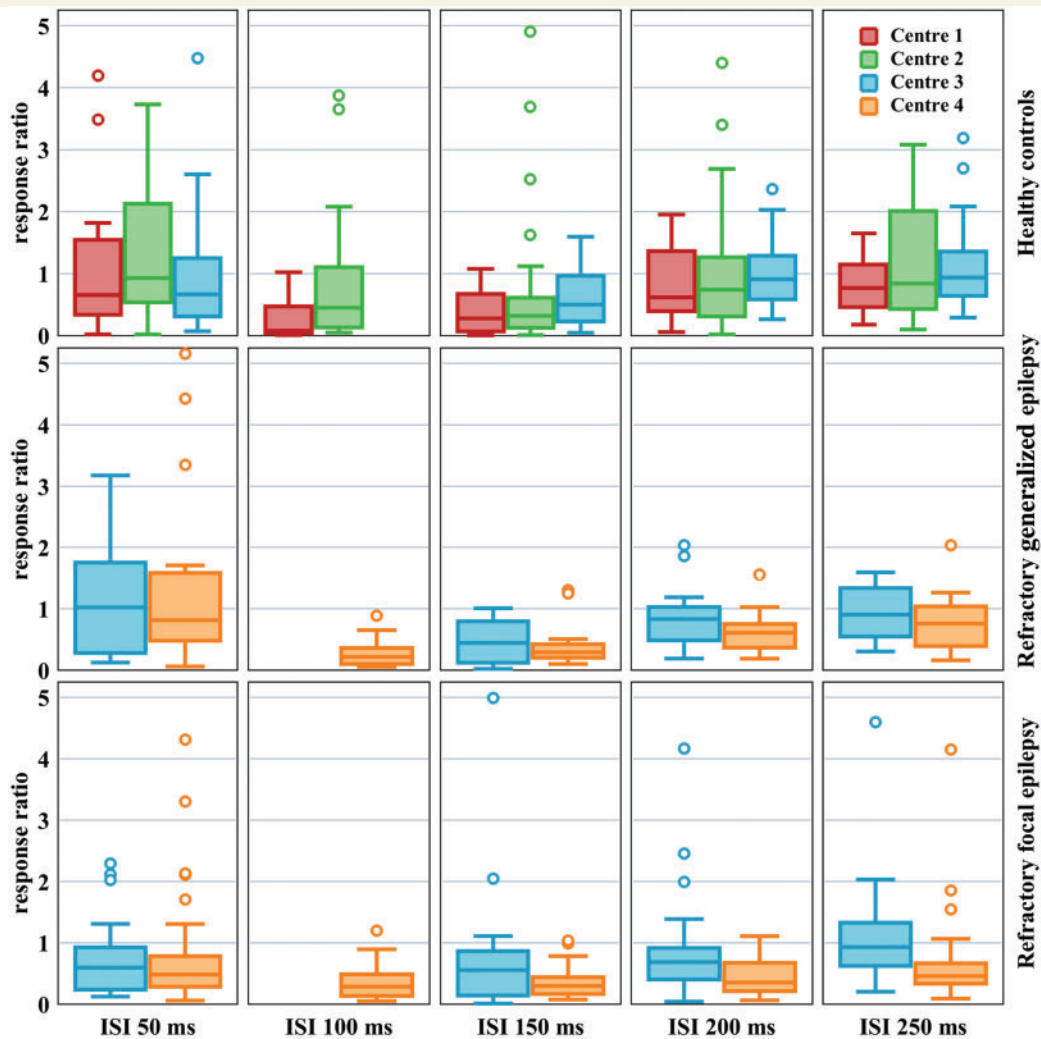
Figure 2 LICI at each ISI per centre and subject type. The boxplots show the median ± 25th percentiles; the whiskers show 1.5 times the interquartile range (IQR); the dots show outliers outside the whiskers. All outliers were included in the analysis but several are outside the boundaries of the y-axis of the figure: healthy controls – Centre 1: LICI = 7.00 (ISI 50 ms); Centre 2: LICI = 5.37, 5.42, 18.50, 24.20, 45.24 (ISI 50 ms), 5.56, 6.02, 7.37 (ISI 100 ms), 10.23, 10.31 (ISI 150 ms), 6.15, 9.68 (ISI 200 ms), 6.11, 6.61 (ISI 250 ms). Generalized epilepsy – Centre 3: LICI = 8.86 (ISI 50 ms). Focal epilepsy – Centre 3: LICI = 6.73 (ISI 250 ms).

Table 2 LICI (mean ± SD) at each ISI per centre and subject type

Subject type	Centre	ISI 50 ms	ISI 100 ms	ISI 150 ms	ISI 200 ms	ISI 250 ms
Controls	1	1.32 ± 1.69	0.28 ± 0.30	0.39 ± 0.36	0.81 ± 0.55	0.82 ± 0.40
	2	3.76 ± 8.74	1.14 ± 1.80	1.15 ± 2.43	1.32 ± 1.91	1.37 ± 1.50
	3	0.88 ± 0.84	-	0.58 ± 0.42	1.00 ± 0.52	1.06 ± 0.63
Generalized epilepsy	3	1.39 ± 1.84	-	0.45 ± 0.34	0.82 ± 0.47	0.91 ± 0.42
	4	1.37 ± 1.51	0.27 ± 0.23	0.40 ± 0.35	0.63 ± 0.34	0.78 ± 0.49
Focal epilepsy	3	0.73 ± 0.60	-	0.70 ± 0.95	0.88 ± 0.85	1.27 ± 1.36
	4	0.78 ± 0.89	0.33 ± 0.25	0.34 ± 0.24	0.46 ± 0.28	0.64 ± 0.66

For controls and refractory genetic generalized epilepsy LICI measured when stimulating the dominant hemisphere is shown, for refractory focal epilepsy when stimulating the ipsilateral hemisphere.

Centre 3 at ISI 250 ms (Table 2). The linear mixed-effects model showed that there was no significant interaction between subject type and LICI at any of the ISIs. We found a main effect of interaction between ISI and LICI ($P < 0.001$). *Post hoc* tests revealed that LICI at ISIs 100 and 150 ms showed significantly more inhibition compared to LICI at ISIs 50, 200 and 250 ms for all three subject types (Fig. 3). The methods of Centre 2 differ the most
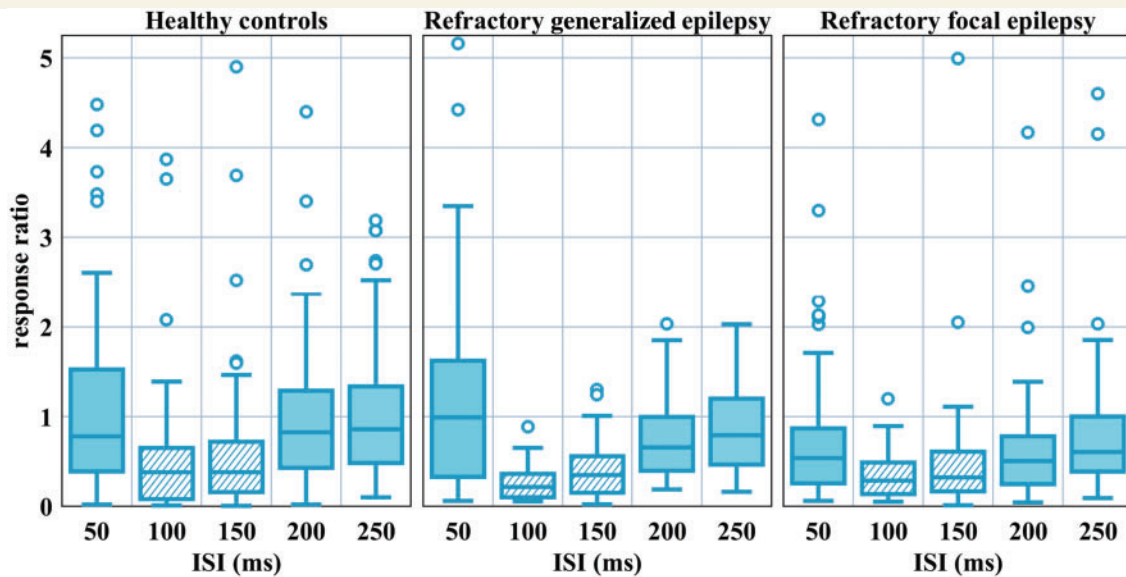
Figure 3 LICI at all ISIs per subject type pooled across centres. The boxplots show the median ± 25th percentiles; the whiskers show 1.5 times the IQR. LICI at ISIs 100 and 150 ms (diagonal pattern) shows significantly more inhibition than LICI at ISIs 50, 200 and 250 ms for all subject types.

from those used in the other centres, as it was the only site to use a round coil and stimulation on the vertex. It also used the shortest intertrial interval and the lowest number of repetitions per ISI. The statistical model takes this methodological heterogeneity into account but as Centre 2 also seems to have less consistent results and more outliers than Centres 1 and 3 and other studies in the field (Valls-Solé *et al.*, 1992; Wassermann *et al.*, 1996; Cash *et al.*, 2010; Caux-Dedeystère *et al.*, 2015; Silbert *et al.*, 2015), we re-ran the statistical analysis without Centre 2 data. In contrast with the first analysis, this showed a significant interaction between ISI and subject groups (*P* = 0.0003). *Post hoc* testing revealed a small, yet significant difference between controls and refractory focal epilepsy at ISIs of 100 ms (*P* = 0.02) and 200 ms (*P* = 0.04). There were no significant differences between the control and refractory generalized epilepsy groups or between the refractory generalized and focal epilepsy groups (Fig. 4). At an ISI 100 ms, the LICI of both controls and refractory focal epilepsy was <1, indicating inhibition. At an ISI of 200 ms, there was neither inhibition nor facilitation in the controls (LICI ~1) but inhibition in the focal epilepsy group (LICI <1).

Discussion

Our analysis of long-interval paired pulse TMS data collected in four different centres do not support previous promising findings (Badawy *et al.*, 2010a, 2013, 2013c, d). Our first analysis, including data from all centres, showed no significant differences in LICI between those with refractory genetic generalized epilepsy, subjects with refractory focal epilepsy and healthy controls. We observed

a statistically significant difference between the ISIs of 50, 200 and 250 ms on one hand, and ISIs 100 and 150 ms on the other hand for all subjects. Inhibition was measured at all five ISIs in all subject types but it was significantly stronger at ISIs 100 and 150 ms. The results from our second analysis without Centre 2 data, clearly contrast with previous findings (Badawy *et al.*, 2010a, 2013, 2013c, d). First, we find inhibition in the refractory epilepsy groups instead of facilitation (LICI >1) reported previously. Second, the differences in our sample are only found between refractory focal epilepsy and controls and not in generalized refractory epilepsy. Third, the differences are found at other ISIs (100 and 200 ms instead of 50, 150 and 250 ms) than previously reported (Badawy *et al.*, 2010a, 2013, 2013c, d). Lastly, the differences between controls and refractory focal epilepsy are much smaller in our sample than in previous reports (Badawy *et al.*, 2010a, 2013, 2013c, d).

One of the main methodological differences between our study protocols and that of previous studies is the intertrial interval, which ranged between 1 and 5 s in our studies but was 15 s in previous studies (Brodtmann *et al.*, 1999; Badawy *et al.*, 2007, 2010a, b, 2012, 2013, 2013a, b, c, d, 2014, 2015; Badawy and Jackson, 2012). Another study of LICI in epilepsy, also using an intertrial interval of 5 s did not show a difference between the healthy control and drug-naïve epilepsy groups (Silbert *et al.*, 2015). Several studies have shown that the MEP amplitude is influenced by the intertrial interval but there are no studies assessing the influence of the intertrial interval on LICI. The optimal intertrial interval to obtain reproducible single pulse MEPs is probably between 10 and 20 s (Möller *et al.*, 2009; Julkunen *et al.*, 2012; Vaseghi *et al.*, 2015; Pellicciari *et al.*, 2016), although other studies show that stimulus-
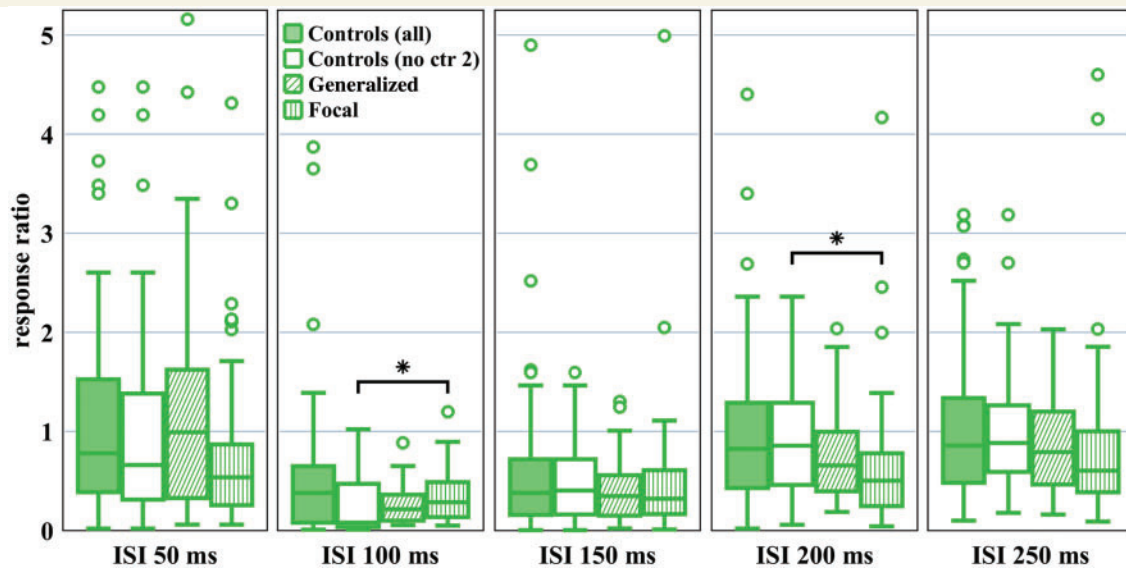
Figure 4 LICI at all ISIs per subject type pooled across Centres 1–4. The boxplots show the median ± 25th percentiles; the whiskers show 1.5 times the IQR. *Significant difference between controls (open boxes, without Centre 2) and refractory focal epilepsy at ISIs of 100 ms (P = 0.02) and 200 ms (P = 0.04). There were no significant differences between the control (open boxes, without Centre 2) and refractory generalized epilepsy group or between the refractory generalized and focal epilepsy groups. There were no differences between the groups when data from all centres were included in the analysis (filled boxes).

response curves can be obtained reliably using shorter intertrial intervals (Pearce *et al.*, 2013; Mathias *et al.*, 2014). Other LICI studies, mostly in relatively small cohorts, used random intertrial intervals between 4 and 15 s, but showed variability similar to our cohort (Valls-Solé *et al.*, 1992; Kujirai *et al.*, 1993; Wassermann *et al.*, 1996; Sanger *et al.*, 2001; Vallence *et al.*, 2017). Interestingly, Centre 2, with the shortest intertrial interval (1 s) shows mean LICI > 1 at all ISIs, but median LICI < 1 at all ISIs, which may be due to several extreme outliers and speculatively a cumulative effect of the paired pulses. The relatively short intertrial intervals in all centres may thus have contributed to our inability to reproduce previous findings obtained with an interstimulus interval of 15 s (Badawy *et al.*, 2010*a*, 2013, 2013*c*, *d*). A recent study, however, that also used an intertrial interval of 15 s showed facilitation at ISIs of 50, 80, 110 and 140 ms in a group of 20 healthy volunteers (Bolden *et al.*, 2017). Future studies are warranted to quantify the influence of intertrial interval on paired pulse TMS protocols.

Another explanation for the different results could be the participant cohorts. We only included data obtained from the dominant hemisphere in controls and generalized epilepsy and the ipsilateral hemisphere in focal epilepsy (i.e. the side of the seizure focus). In Centre 4 it was standard practise to stimulate the dominant hemisphere only, leading to the exclusion of those in whom the dominant hemisphere was not the ipsilateral hemisphere. Including the results of both hemispheres in our multi-centre analysis would have led to missing data for cases in which only one hemisphere was measured. Alternatively, we could have estimated a mean of both hemispheres measured,

which could have introduced a bias. Our choice lead to several exclusions, especially from Centre 4, but we feel that this was the best way to deal with this issue. Even if some bias were introduced this way, we would expect the large differences between controls and subjects reported in other studies to have been visible in our large sample. We included a large number of people with all types of refractory generalized and focal epilepsy as well as healthy controls, while previous studies often report on relatively small samples (~20 participants) of people with specific epileptic syndromes (Badawy *et al.*, 2010*b*, 2012, 2013*a*, *b*, *c*, 2014) or AEDs (Silbert *et al.*, 2015). Our retrospective study design did not allow us to go into such detail, limiting the comparison with these studies. It should be noted that the participant cohorts reported in previous studies appear to overlap, potentially leading to multiple publication bias and overestimation of the consistency of these findings (Brigo *et al.*, 2012; Badawy *et al.*, 2017; Bauer *et al.*, 2017).

Comparing TMS data across centres is challenging as variables such as the resting motor threshold depend directly on the equipment used. LICI, however, is expressed as a response ratio, and thus is dimensionless. It is, therefore, theoretically better suited for comparison between centres than the resting motor threshold. We report on data collected from multiple centres and although it provides a large body of data, it is limited by its retrospective set-up and the different equipment and stimulation protocols used. Clear methodological guidelines for LICI stimulation are currently lacking, as there is insufficient data to define the most robust LICI protocol in terms of stimulator, stimulation intensity, coil type, stimulation site, target muscle,

number of repetitions and intertrial interval. Some of the methodological differences are less likely to hamper the direct comparison between centres. For example, in three of the centres a figure-of-eight coil was used and stimulation was applied to the motor hotspot, while in one of the centres (Centre 2) a round coil was used and stimulation was applied on the vertex. The coil design (circular or figure-of-eight) affects the size and depth of the cortical region that is stimulated by the TMS pulse, and may result in different LICI responses. While several studies compared different coils for shorter interstimulus intervals (Shimizu *et al.*, 1999; Cantello *et al.*, 2000; Badawy *et al.*, 2011; Fleming *et al.*, 2012), only one study directly compared these different coils for LICI in a small sample ($n = 8$) (Valzania *et al.*, 1994). The LICI responses obtained at stimulation intensities of 110 and 120% resting motor threshold with either a circular or a figure-of-eight coil were similar in this study.

Other methodological differences are more likely to hamper direct comparison between centres. Centres 2 and 4 applied two pulses with an intensity of 110% resting motor threshold while the other centres applied two pulses at 120% of the resting motor threshold. This was previously shown to yield small differences in the LICI response (Valls-Solé *et al.*, 1992), and may have contributed to the weaker mean LICI (>1) seen in Centre 2. Despite the difference in stimulation intensity used in Centres 3 and 4, the mean LICI is <1 in both refractory epilepsy groups at ISIs 100, 150 and 200 ms (Table 2), contrasting with previous studies showing facilitation at these ISIs in refractory epilepsy (Badawy *et al.*, 2010a, 2013, 2013c, d). In our refractory epilepsy groups, facilitation is only seen at 50 ms in genetic generalized epilepsy (Centres 3 and 4) and at 250 ms in focal epilepsy (Centre 3, Table 2). Two centres (Centres 1 and 2) used biphasic pulses for stimulation. Biphasic and monophasic pulses preferentially excite partly different sets of cortical axons when using the same coil orientation (Groppa *et al.*, 2012), potentially contributing to different response patterns. The number of repetitions and stimulation sequence (random or fixed) also differed, being relatively low in all centres, except in Centre 1, where each ISI was repeated 50 times. Recent studies suggest the use of 20–30 repetitions for single pulse and short-interval paired-pulse protocols (Chang *et al.*, 2016; Goldsworthy *et al.*, 2016), although 10 repetitions are commonly used in studies of epilepsy (Badawy *et al.*, 2010b, 2012, 2013a, b, c, 2014). All these methodological differences result in a net intercentre variability, which limits the direct comparison of LICI outcomes from different centres. We accounted for intercentre variability by using a linear mixed-effects model, and although this is a methodological robust solution, it cannot replace a prospective multi-centre study in which all centres use exactly the same materials and methods. Despite the different methods, however, the results of Centres 1, 3 and 4 (and 2) are consistent, making the difference with the results of previous studies all the more striking. The

individual studies that constituted our sample were set up as prospective TMS trials and part of the data were previously reported [Centre 1: de Goede and van Putten (2017), Centre 3: Pawley *et al.* (2017) and Centre 4 (including patients with Dravet syndrome): Stern *et al.* (2017)]. In none of these studies were previous promising results replicated (Badawy *et al.*, 2010a, 2013, 2013c, d), spurring the current retrospective multi-centre analysis. Only one centre (Centre 3) in our study included all three participant types. Despite the same protocol and equipment being used, there was no significant difference between LICI measured in refractory generalized epilepsy, refractory focal epilepsy and control groups in this centre (see also Pawley *et al.*, 2017).

The LICI response ratios were not normally distributed in our sample. This is in line with one previous study of LICI (Silbert *et al.*, 2015). Another study, reporting on a large cohort of healthy subjects, showed that resting motor threshold and short-interval intracortical inhibition do not follow a normal distribution (Wassermann, 2002). We suggest that statistical analyses of TMS variables should be done on log-transformed data. Furthermore, responses at different ISIs should be treated as repeated measurements, warranting corrections for multiple comparisons when several ISIs are measured in the same participants (Pawley *et al.*, 2017).

Some authors consistently report low interindividual variability of LICI (Badawy *et al.*, 2010b, 2012, 2013, 2013a, c, d, 2014, 2015; Badawy and Jackson, 2012); however, others reported much higher variability of TMS responses (Valls-Solé *et al.*, 1992; Wassermann *et al.*, 1996; Nakamura *et al.*, 1997; Sanger *et al.*, 2001; Wassermann, 2002; Lang *et al.*, 2011; Du *et al.*, 2014). In our cohort, the response ratio to long-interval paired pulse stimulation varied between individuals from strong inhibition to facilitation supporting some previous studies (Valls-Solé *et al.*, 1992; Bolden *et al.*, 2017). LICI variability was shown to be linked to the time of day and sleep status (Lang *et al.*, 2011), neuropsychological profile (Bolden *et al.*, 2017) and to age and hemispheric dominance (Vallence *et al.*, 2017). While some TMS variables were shown to vary according to the menstrual cycle (Smith *et al.*, 2002; Inghilleri *et al.*, 2004; Hattemer *et al.*, 2007), no data are available for LICI. These and probably other unknown factors contribute to the large interindividual variability of LICI and need to be adequately accounted for. One previous study accounted for the interindividual variability by using a mixed model analysis, similar to our approach (Silbert *et al.*, 2015).

An ideal biomarker for epilepsy diagnosis and management should show a difference in cortical excitability between drug-naïve subjects with epilepsy and healthy controls. To be of clinical use for the management of epilepsy, it needs to show normalization of cortical excitability soon after treatment initiation in those who become seizure-free, but not in those with refractory seizures. In the latter group, cortical excitability should remain different

from controls despite treatment indicating active epilepsy with (refractory) seizures. Our analysis (both with and without Centre 2), however, shows the opposite effect. A good biomarker should also have low interindividual variability, so that it can also be used to assess disease activity on an individual level. In our analysis, we only included subjects with refractory seizures and controls. Based on our findings showing both no significant differences between subjects with refractory epilepsy and controls, and a variable response to long-interval paired pulse stimulation, we argue that the use of LICI measured with TMS-EMG as a biomarker for epilepsy is limited and that a prospective trial is urgently needed to confirm this finding. Recent studies show that combining TMS with EEG may provide a more direct method to assess cortical excitability and underlying processes (Ilmoniemi et al., 1997; Ilmoniemi and Kicić, 2010). There is only one report of paired pulse TMS-EEG in epilepsy (Kimiskidis et al., 2017). Using feature selection methods combined with a Bayesian classifier, this study found a cross-validated diagnostic accuracy of 0.92 for differentiating genetic generalized epilepsy from healthy controls and 0.80 for differentiating responders from non-responders, suggesting that paired pulse TMS-EEG may be useful for diagnosis and the assessment of disease severity (Kimiskidis et al., 2017). Further research is needed to extend and confirm these findings. TMS-EEG may also help to reveal the mechanisms underlying the difference in LICI at ISIs of 100 and 150 ms and LICI at ISIs 50, 200 and 250 ms that we found in all groups of our cohort.

Conclusion

In this retrospective study, we could not replicate the difference in LICI measured with TMS-EMG between people with refractory genetic generalized and focal epilepsy and healthy controls consistently reported in previous studies. Methodological differences, especially shorter intertrial intervals, may have contributed to our inability to replicate previous findings. Further studies are needed to assess the influence of the length of the intertrial interval on LICI and to establish guidelines for LICI stimulation protocols. Based on our findings, LICI measured with TMS-EMG is unlikely to be useful as a biomarker in the clinical management of epilepsy. Future prospective multi-centre trials are needed to confirm this finding.

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Conflicts of interest

S.M.S. has been consulted by, or received fees for lectures, or institutional support, from GSK, Vitaflo, Nutricia, Eisai and UCB Pharma. M.J.A.M.v.P. is co-founder of Clinical Science Systems, the Netherlands. J.W.S. has been consulted by and received fees for lectures from GSK, Eisai and UCB Pharma. All other authors declare no conflicts of interest.

Supplementary material

Supplementary material is available at *Brain* online.

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