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Measurement of cortical, nerve, and muscle excitability in early phase clinical drug development

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CHAPTER 2

TRANSCRANIAL MAGNETIC STIMULATION AS BIOMARKER OF EXCITABILITY IN DRUG DEVELOPMENT: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED, CROSS-OVER STUDY

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ABSTRACT

The purpose of this study was to investigate pharmacodynamic effects of drugs targeting cortical excitability using transcranial magnetic stimulation (TMS) combined with electromyography (EMG) and electroencephalography (EEG) in healthy subjects, to further develop TMS outcomes as biomarkers for proof-of-mechanism in early phase clinical drug development. Anti-epileptic drugs presumably modulate cortical excitability. Therefore, we studied effects of levetiracetam, valproic acid and lorazepam on cortical excitability in a double-blind, placebo-controlled, four-way cross-over study. In 16 healthy male subjects, single- and paired-pulse TMS-EMG/EEG measurements were performed pre-dose and 1.5, 7, and 24 hours post-dose. Treatment effects on motor-evoked potential (MEP), short (SICI) and long intra-cortical inhibition (LICI) and TMS-evoked potential (TEP) amplitudes, were analysed using a mixed model ANCOVA and cluster-based permutation analysis. We show that MEP amplitudes decreased after administration of levetiracetam (estimated difference (ED) -378.4 μV; 95% confidence interval (95%CI): -644.3 μV, -112.5 μV; p<0.01), valproic acid (ED -268.8 μV; 95%CI: -532.9 μV, -4.6 μV; p=0.047) and lorazepam (ED -330.7 μV; 95%CI: -595.6 μV, -65.8 μV; p=0.02) when compared with placebo. LICI was enhanced by levetiracetam (ED -60.3%; 95%CI: -87.1%, -33.5%; p<0.001) and lorazepam (ED -68.2%; 95%CI: -94.7%, -41.7%; p<0.001) at a 50 ms interstimulus interval. Levetiracetam increased TEP-component N45 (p=0.004) in a central cluster and decreased N100 (p<0.001) in a contralateral cluster.

In conclusion, this study shows that levetiracetam, valproic acid and lorazepam decrease cortical excitability, which can be detected using TMS-EMG/EEG in healthy subjects. These findings provide support for the use of TMS excitability measures as biomarkers to demonstrate pharmacodynamic effects of drugs that influence cortical excitability.

INTRODUCTION

Transcranial magnetic stimulation (TMS) is a non-invasive technique which can be used to investigate corticospinal excitability. Stimulation targeted at the motor cortex generates motor evoked potentials (MEP) and TMS-evoked potentials (TEP), that can be quantified by electromyography (EMG)[1] and electroencephalography (EEG),[2,3] respectively. TMS-EMG and TMS-EEG facilitate assessment of different measures of cortical excitability, using a single pulse (sp) and paired pulse (pp) stimulation paradigm, of which the latter facilitates assessment of intra-cortical inhibition.[4,5] This study is intended to broaden and deepen the knowledge about effects of anti-epileptic drugs (AEDs) on TMS-EMG/EEG outcomes, to further develop these outcomes as biomarkers for pharmacodynamic effects on cortical excitability. Although TMS-EMG has been widely used to assess the effects of drugs targeted at cortical excitability,[6] the number of research groups investigating pharmacological effects on TEPs is limited.[7–13] There is abundant space for further progress in replicating and extending the current knowledge about cortical excitability and in showing the value of TMS to measure biomarkers for pharmacodynamic effects in early phase drug development.[7] Before being able to use TMS-related outcomes in clinical drug development with new pharmacological targets, it is of importance to determine the sensitivity of the measurement to detect pharmacological effects in healthy subjects, and the typical effect size of regularly used drugs administered at a dose within the therapeutic range. A reliable biomarker is a valuable investigative tool in clinical drug development, particularly in the development of new pharmacological treatments for diseases with underlying pathology related to cortical excitability, such as epilepsy[14,15] and amyotrophic lateral sclerosis.[16] The goal would be to use TMS-EMG/EEG outcomes as biomarkers for proof-of-mechanism.

Therefore, the primary objective of this study was to evaluate effects of three commonly prescribed AEDs (levetiracetam, valproic acid, and lorazepam) on cortical excitability in a placebo-controlled, cross-over fashion in healthy subjects. These AEDs are expected to decrease cortical excitability with distinct mechanisms of action. The secondary objective was to evaluate intra- and inter-subject variability of cortical excitability measures.

MATERIALS AND METHODS

This study was approved by the Ethics Committee 'Stichting Beoordeling Ethiek Biomedisch Onderzoek', Assen, The Netherlands. The trial was executed in accordance to the Declaration of Helsinki at the Centre for Human Drug Research (CHDR), Leiden, The Netherlands, between September 2017 and February 2018. The study is registered in the Dutch Trial Registry (NTR) under NL6638.

SUBJECTS Subjects gave written informed consent. Healthy male subjects between 18 and 45 years were recruited using online advertisements and CHDR's subject database. Eligibility was confirmed by a medical screening up to 30 days before the first dose, consisting of evaluation of medical history, physical examination, electrocardiogram, blood chemistry, haematology, and urinalysis. Subjects with contra-indications according to the TMS safety questionnaire[17] were excluded, as well as subjects with an abnormal sleeping pattern, (history of) illicit drug or alcohol abuse or a positive test for such substances, nicotine use a month before dosing, or a resting motor threshold (rMT) of >83% of the maximum stimulator output (MSO). Use of medication was prohibited from 14 days prior to the first dose. Use of caffeine was prohibited from 24 hours before dosing.

EXPERIMENTAL DESIGN This is a randomized, double-blind, double-dummy, placebo-controlled, cross-over study. On four visits, subjects received a single dose of levetiracetam 2000 mg (Levetiracetam, oral solution 100mg/mL, Aurobindo) and placebo capsules; valproic acid 1000 mg (Depakine sugarfree oral solution 200 mg/5mL, Sanofi-aventis) and placebo capsules; lorazepam 2 mg (Lorazepam, 2 tablets of 1 mg, Apotex Europe BV) and placebo solution; or placebo solution and placebo capsules. Lorazepam tablets were encapsulated, and matching placebo capsules and solutions were produced. Drug doses were chosen within the therapeutic range. A Williams design was used to balance first-order carry-over effects.[18] The randomization of the treatment order was generated in SAS (version 9.4, SAS Institute Inc., Cary, USA) by a statistician uninvolved with data collection. The randomization remained blinded for all staff, apart from the statistician and the pharmacy preparing the medication. Subjects were enrolled by a blinded physician.

Subjects remained fasted from minimally eight hours before until two hours after dosing. TMS-EMG-EEG measurements were performed before dosing and 1.5, 7 and 24 hours after dosing, based on the pharmacokinetic (PK) profile of the study drugs. The first post-dose measurement was performed around T_{max}, the second when an intermediate plasma concentration was expected, and the third at low concentrations. Measurements were performed at approximately the same clock time for all subjects, to minimize potential effects of diurnal variation on TMS outcomes. Samples for PK analysis were drawn directly before all TMS measurements and directly after the measurement at 1.5 hours post-dose. Additionally, samples were obtained at 0.5, and 3.5 hours post-dose. Between each study visit was a wash-out of at least seven days. There was a safety follow-up seven to ten days after the last dose.

TRANSCRANIAL MAGNETIC STIMULATION Sp and ppTMS were applied according to guidelines by Rossi and colleagues,[19] using a MagPro R30 with MagOption stimulator and a MCF-B65 butterfly coil (MagVenture GmbH, Hückelhoven, Germany).

Stimulation was performed at the motor hotspot of the dominant abductor digiti minimi (ADM) muscle as determined by the Edinburgh Handedness questionnaire.[20] The TMS coil was fixated using a frame at an angle of 45° from the midline, in direct contact with the EEG cap. At the start of each measurement, rMT was determined as the lowest stimulus intensity at which a minimum of 5 out of 10 TMS pulses elicited a MEP with a peak-to-peak amplitude of at least 50 μV.[21,22] Hereafter, 50 single pulses were applied at 120% rMT. This was followed by 50 paired pulses at different inter-stimulus intervals (ISI), namely 2, 5, 50, 100, 150, 200, 250 and 300 ms, applied in randomized order (total 400 paired pulses). Conditioning and test pulses were applied at 120% rMT, except for ISIs 2 and 5 ms, where conditioning pulses were applied at an intensity of 80% rMT. The duration between single pulses and pairs of paired pulses was randomized between 3.5 and 4.5 seconds.

EMG and EEG acquisition EMG and EEG were registered simultaneously during TMS stimulation using NeuroCenter software (Clinical Science Systems, Leiden, The Netherlands). EMG was recorded with Ag/AgCl surface electrodes (Blue Sensor N, AMBU, Denmark) on the ADM and corresponding tendon. TEPs were registered using a TMS-compatible 32-leads EEG cap (ANT Neuro, Enschede, The Netherlands) and EEG amplifier

(TMSi, Oldenzaal, The Netherlands). The ground electrode, used for both EEG and EMG, was located between CZ and Fpz. Electrode impedances were below 5 kΩ and signals were amplified at a frequency of 2048 Hz. During the TMS measurements, subjects received in-ear headphones with masking noise to minimize auditory evoked potentials.[23] Adapted noise, based on the frequencies of the TMS click, was played at an intensity individualized for each subject with a maximum volume of 95 dB. Masking of auditory components appears to be sufficient as represented by lateralized responses in the topographical plots even at late latencies,[24] see *Supplementary Figure 1*.

DATA PROCESSING AND ANALYSIS The following parameters were extracted from the TMS-EMG data: single pulse rMT (% of MSO) and mean peak-to-peak MEP amplitude (μV); long intra-cortical inhibition (LICI) at ISIs of 50-300 ms, defined as the percentage ratio of the mean MEP amplitude after the test pulse and the mean MEP amplitude after the conditioning pulse; short intra-cortical inhibition (SICI) at ISIs of 2 and 5 ms, defined as the percentage ratio of the mean MEP amplitude after the test pulse and the mean amplitude of the unconditioned spMEPs.

MEP amplitude, SICI and LICI were calculated using in-house written MATLAB (version R2015a, The Mathworks, Natick, USA) scripts. To correct for pre-existing muscle activation, responses were excluded if muscle activity was >50 μV in the 50 ms before each single or conditioning pulse.

TEPs were determined at all 32 EEG leads. EEG responses were analysed in common average montage and were baseline corrected by subtracting the average EEG amplitude 500-50 ms before applying the single or conditioning pulse. Individual EEG trials were defined from 100 ms before until 650 ms after each single or conditioning pulse. Principal component analysis (PCA) was used to reduce artefacts caused by TMS stimulation and muscle activation on the scalp.[25] The first four of 25 principal components were removed, after which individual trials were filtered (4th order Butterworth bandpass filter;1-35 Hz) and averaged over 50 repetitions to create the TEP for each EEG lead. Per condition (placebo, levetiracetam, valproic acid and lorazepam) TEP responses after artefact removal are shown for each individual subject in the *Supplementary Figures 2-5*.
After all data was collected, review of the blinded TMS-EMG data was performed as per standard operating procedure at CHDR. Measurements with technical errors were removed from analysis.

PK ANALYSIS Serum concentrations of levetiracetam were measured by a validated high-performance liquid chromatography diode array detection method at the ISO 15189 certified Clinical Pharmaceutical Laboratory of the Leiden University Medical Centre. Serum concentration of valproic acid were measured using an in vitro chemiluminescent microparticle immunoassay (CMIA) using an Abbott Architect system. The lower limit of quantification (LLOQ) was determined at 2.5 μg/mL for levetiracetam and 2 μg/mL for valproic acid. Serum lorazepam concentrations were analysed using an immunoassay at University Medical Centre Groningen. The LLOQ was 5.21 ng/mL. All assays were validated in accordance to the EMA bioanalytical method development guideline (all coefficients of variation (CV%) below 15%).

STATISTICAL ANALYSES Treatment effects were analysed up to 7 hours post-dose. We predefined that measurements performed at 24 hours after dosing would not be included due to the expected low drug concentrations at this time point but were measured for pharmacokinetic-pharmacodynamic (PKPD) modelling. Due to the exploratory nature of the study, no calculation of sample size was performed. Sample size was based on a previous pharmaco-TMS study in 15 healthy subjects.[10]

Statistical analysis of rMT, MEP and TEP endpoints was performed using a mixed model analysis of covariance (ANCOVA), with treatment, time, period, and treatment by time as fixed factors and subject, subject by treatment and subject by time as random factors, and the baseline measurement per period as covariate. Estimated differences between placebo and the AEDs were reported and statistical significance was defined at the 5% level. Analysis of effects on TMS-EMG endpoints was performed in SAS (version 9.4, SAS Institute Inc., Cary, USA).

Statistical analysis of TMS-EEG outcomes was performed using cluster-based permutation analysis (CBPA) incorporating all leads. CBPA was performed in Fieldtrip (Nijmegen, The Netherlands, downloaded 13-08-2015; http://fieldtrip.fcdonders.nl).[26] Comparison of drug versus placebo was performed using dependent samples t-tests, for each EEG

lead and time sample between 0-300 ms after the test pulse (both for sp- and ppTMS). To compensate for handedness, topographical plots of left-handed subjects were mirrored. Clusters were formed by t-values with a p-value <0.05, based on neighbouring leads (n≥2) and adjacent time samples. A permutation test (1500 times) was used to determine significance at the 5% level.[26] Additionally, we applied a Bonferroni correction (N=3) to compensate for multiple testing (three active conditions). Besides analysing the entire time sample of 300 ms after the test pulse, we applied the same analysis to time periods of interest (TOIs) around the TEP components (N15: 0-20 ms; P30: 20-40 ms; N45: 40-55 ms; P60: 55-80 ms; N100: 80-130 ms; P180: 130-230 ms).

For the purpose of evaluating repeatability, intra- and inter-subject variability were calculated, represented by CV%s. CV%s were calculated within the placebo visit, including measurements up to 7 hours post-dose, using estimates of covariance parameters produced by the mixed model analysis. The serum concentration of the AEDs was analysed using a non-compartmental analysis.

PKPD ANALYSIS Concentration-effect relationships between MEP amplitude and treatments (including all timepoints up to 24 hours post-dose) were investigated with non-linear mixed effects (NLME) modelling, using PK data linked to the closest available pharmacodynamic measurement in time. Tested PKPD-model structures included intercept (no effect), linear and non-linear (E_{max}) relationships, with additional inter-individual variability (IIV) and/or between-occasion variability (BOV) for the baseline parameter. Initial analysis was performed in R (version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria), where models were compared with an analysis of variance for nested models (p-value < 0.05) or with the Akaike Information Criterion (AIC, lowest value is favoured) for non-nested models. Treatments for which concentration-effect relationships other than intercept were selected, were also analysed using NONMEM (version 7.4, ICON Development Solutions, Hanover, USA), where models were compared based on drop in objective function value (DOFV>3.84, p<0.05) for one additional parameter for nested models or AIC for non-nested models.

NOMENCLATURE OF TARGETS AND LIGANDS Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to Pharmacology 2021/22.[27–29]

RESULTS

Seventeen subjects were enrolled in the study, of whom sixteen completed all study visits, for demographics see *Table* 1. One subject was excluded after one study visit, due to positive illicit drugs testing.

TABLE 1 Sample characteristics.

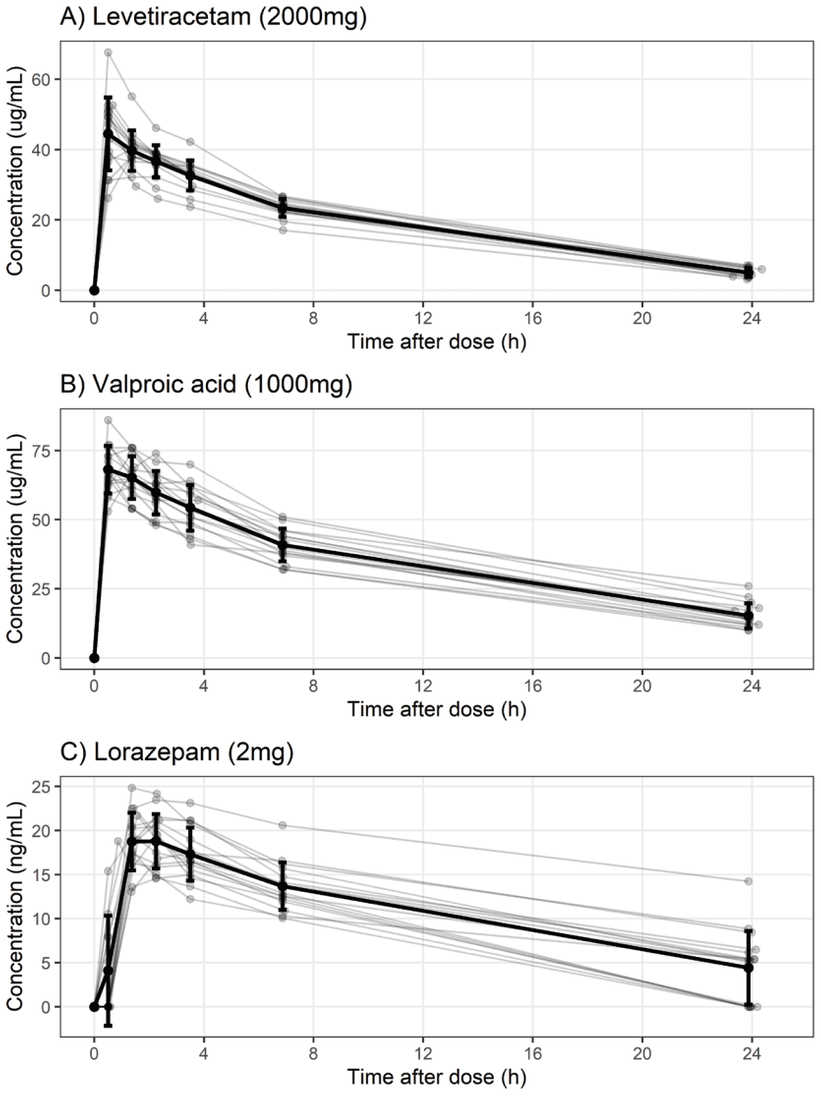
N=17	Mean	SD	Median	Range
Age (years)	25	6	24	20-44
Height (cm)	183	8	184	167-194
Weight (kg)	75	13	74	54-109
BMI (kg/m²)	22	4	21	19-32

BMI = *body mass index*; SD = *standard deviation*.

The AEDs and TMS measurements were well tolerated. Individual, and mean ± standard deviation serum concentrations of the AEDs are shown in *Figure* 1. Mean maximum concentrations (C_{max}) were 45.92 µg/mL (range 32.10 – 67.60) for levetiracetam, 70.69 µg/mL (range 58.00 – 86.00) for valproic acid and 19.79 ng/mL (range 14.99-24.84) for lorazepam. Mean serum concentrations per timepoint are also listed in *Supplementary Table* 1. Median T_{max} was 0.51 hours for levetiracetam (range 0.5-1.47 hours), and 0.53 hours for valproic acid (range 0.5-2.25 hours), corresponding to the first sampling point. The median T_{max} was 1.91 h (range 1.37-3.52 hours) for lorazepam, with 10 of 16 subjects showing a lag time of 30 minutes.

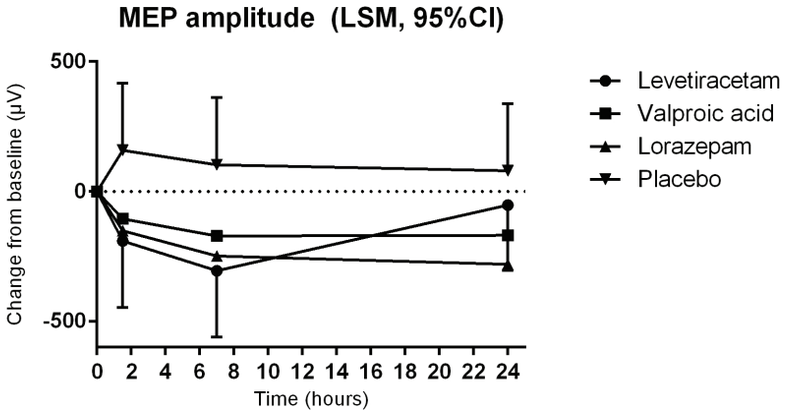
CORTICAL EXCITABILITY ASSESSED BY TMS-EMG In total, 192 measurements were recorded up to 7 hours post-dose. During blinded data review, six EMG recordings were excluded because of absence of MEPs (indicating there was no motor hotspot stimulation) or clipping of the EMG signal.

FIGURE 1 Individual and mean ± standard deviation (SD) serum concentrations of levetiracetam, valproic acid and lorazepam.



Estimated mean post-dose MEP amplitudes were 889.3 µV (placebo), 510.9 µV (levetiracetam), 620.5 µV (valproic acid) and 558.6 µV (lorazepam). All AEDs significantly decreased MEP amplitude after spTMS when compared to placebo, with an estimated difference of -378.4 µV (95%CI: -644.3, -112.5; p<0.01) for levetiracetam, -268.8 µV (95%CI: -532.9, -4.6; p=0.047) for valproic acid, and -330.7 µV (95%CI: -595.6, -65.8; p=0.02) for lorazepam, see *Figure* 2. Intra-subject CV% of MEP amplitude was 35%, inter-subject CV% 84%.

FIGURE 2 Change from baseline of the least square means (LSM) of the MEP amplitude (µV), using single pulse TMS, for levetiracetam, valproic acid, lorazepam and placebo.



Estimated mean post-dose rMT was 55.3%, 55.7%, 54.3% and 55.5% of MSO for placebo, levetiracetam, valproic acid and lorazepam, respectively. No significant treatment effects on rMT were detected when compared to placebo, with estimated differences of 0.4% for levetiracetam (95%CI: -1.1%, 1.9%; p=0.61), -1.0% for valproic acid (95%CI: -2.5%, 0.5%; p=0.19) and 0.2% for lorazepam (95% CI: -1.3%, 1.7%; p=0.78). Intra-subject CV% of rMT was 4%, inter-subject CV% 14%.

Levetiracetam and lorazepam both significantly enhanced LICI compared to placebo at ISI 50 ms (i.e. the percentage ratio decreased, indicating more intra-cortical inhibition). No significant effects on LICI were detected at the other ISIs, nor on SICI. Results and CV%s for SICI and LICI are listed in *Table* 2.

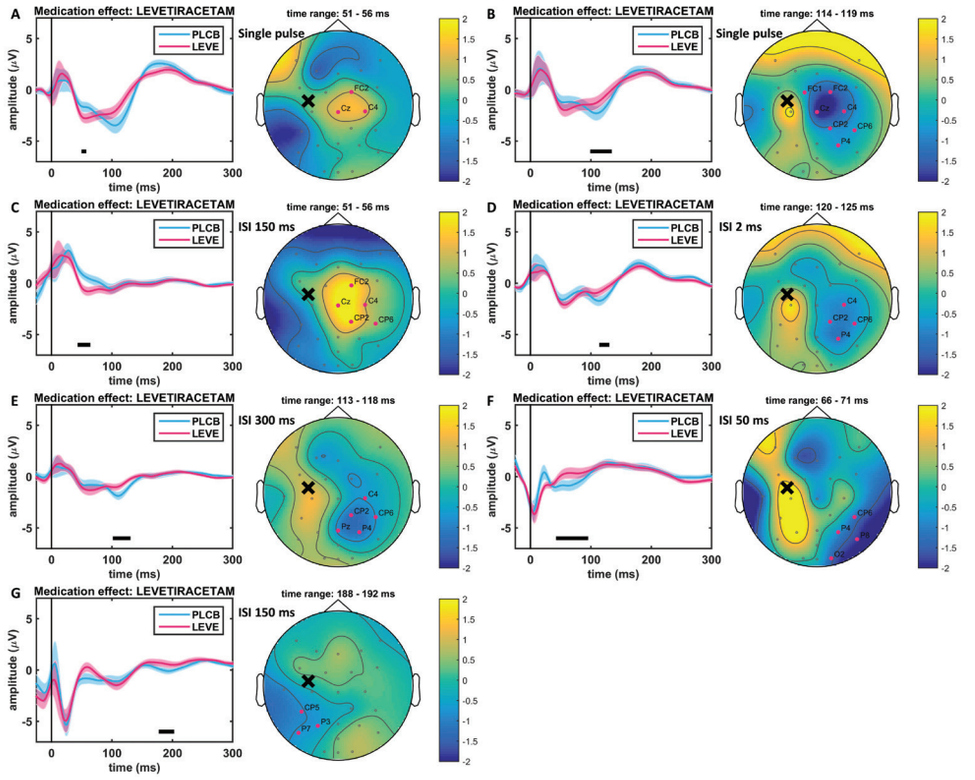
TABLE 2 Estimated mean (%) up to 7 hours of placebo, levetiracetam, valproic acid and lorazepam for long intra-cortical inhibition (LICI) and short intra-cortical inhibition (SICI) using paired-pulse TMS-EMG at 8 different interstimulus intervals (ISI). Estimated difference of placebo versus treatment (%), with 95% confidence interval (CI) and p-value. Intra-subject CV% (%) and inter-subject CV% (%) within the placebo occasion are listed.

ISI (ms)		Estimated mean relative amplitude of conditioned pulse to unconditioned pulse (%)	Estimated difference with placebo (%) (95% CI), p-value	Intra-subject CV%	Inter-subject CV%
2	Placebo	35.7		50%	58%
	Levetiracetam	42.4	6.7 (-6.5, 20.0), p= 0.31		
	Valproic Acid	48.5	12.8 (-0.4, 26.0), p= 0.06		
	Lorazepam	47.4	11.7, (-1.5, 24.9), p= 0.08		
5	Placebo	74.0		45%	48%
	Levetiracetam	78.5	4.5 (-20.9, 29.9), p= 0.72		
	Valproic Acid	88.7	14.7 (-10.4, 39.8), p=0.24		
	Lorazepam	90.4	16.4 (-9.1, 41.8), p= 0.20		
50	Placebo	102.9		85%	103%
	Levetiracetam	42.6	-60.3 (-87.1, -33.5), p<.001		
	Valproic Acid	78.0	-24.9 (-51.2, 1.4), p= 0.06		
	Lorazepam	34.7	-68.2 (-94.7, -41.7), p <.001		
100	Placebo	9.9		134%	172%
	Levetiracetam	7.3	-2.6 (-10.9, 5.6), p= 0.52		
	Valproic Acid	8.9	-1.0 (-9.2, 7.2), p= 0.81		
	Lorazepam	4.9	-5.0 (-13.3, 3.2), p= 0.22		
150	Placebo	19.9		92%	121%
	Levetiracetam	21.0	1.1 (-11.2, 13.5), p= 0.86		
	Valproic Acid	18.1	-1.8 (-14.0, 10.4), p= 0.77		
	Lorazepam	14.7	-5.2 (-17.6, 7.2), p= 0.40		
200	Placebo	64.4		38%	60%
	Levetiracetam	70.6	6.2 (-10.3, 22.8), p= 0.45		
	Valproic Acid	63.4	-1.0 (-17.6, 15.6), p= 0.91		
	Lorazepam	56.8	-7.6 (-24.1, 8.8), p= 0.36		
250	Placebo	64.7		47%	45%
	Levetiracetam	73.8	9.0 (-8.5, 26.6), p=0.31		
	Valproic Acid	77.7	13.0 (-4.3, 30.3), p= 0.14		
	Lorazepam	78.9	14.2 (-3.3, 31.6), p= 0.11		
300	Placebo	55.3		34%	49%
	Levetiracetam	54.4	-0.9 (-12.7, 10.9), p= 0.88		
	Valproic Acid	52.9	-2.4 (-14.0, 9.3), p= 0.68		
	Lorazepam	66.6	11.3 (-0.4, 23.1), p= 0.06		

CORTICAL EXCITABILITY ASSESSED BY TMS-EEG Of 192 TMS-EEG recordings, three were excluded after blinded data review of the EMG data, because of absence of MEPs.

For spTMS, levetiracetam significantly increased the N45 compared to placebo (p=0.004) in a centrally located cluster (*Figure* 3A). Furthermore, levetiracetam significantly decreased the N100 in a contralateral centro-parietal cluster (p<0.001) (*Figure* 3B).

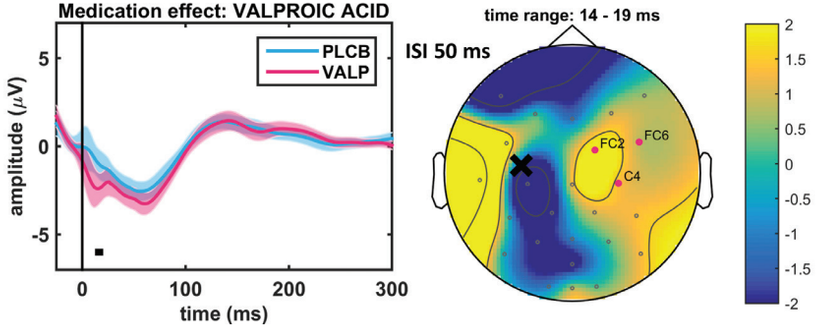
FIGURE 3 Significant clusters found using CBPA of TEPs, comparing placebo (PLCB; in blue) to levetiracetam (LEVE; in red). A) single pulse (N45 cluster), B) single pulse (N100 cluster), C) ISI 150 ms (N45/P60 cluster), D) ISI 2 ms (N100 cluster), E) ISI 300 ms (N100 cluster), F) ISI 50 ms (N45/P60/N100 cluster), and G) ISI 150 ms (P180 cluster). For each cluster the grand average (mean ± standard error of the mean (SEM)) over all significant electrodes is presented, as well as the difference in topographical distribution at the time of the cluster. The colors of the topographical plot of the cortex show the increase or decrease of amplitude (μV) of the response. The black cross represents the stimulation site, the red dots significant electrodes and the thick black bar below the average TEP response corresponds to the time interval with significant differences.



In line with the results for spTMS, levetiracetam significantly increased the N45 and P60 in a similar centro-parietal cluster at ISI 150 ms (p<0.001 and p=0.004, respectively) (*Figure* 3C). In addition, we found that levetiracetam significantly decreased N100 clusters at ISIs 2 and 300 ms (p=0.003 and p=0.003, respectively) (*Figure* 3D and 3E), these clusters are comparable to the N100 cluster found using spTMS. Furthermore, we found a significant N45, P60 and N100 cluster (p=0.004, p<0.001 and p=0.004, respectively) at ISI 50 ms (*Figure* 3F). A significant P180 cluster (p=0.006) was detected at ISI 150 ms (*Figure* 3G).

Valproic acid significantly increased the N15 amplitude (p=0.005) at ISI 50 ms in a contralateral cluster (*Figure* 4). Lorazepam significantly decreased the N100 (p=0.001) at ISI 300 ms in a contralateral parietal cluster (*Figure* 5).

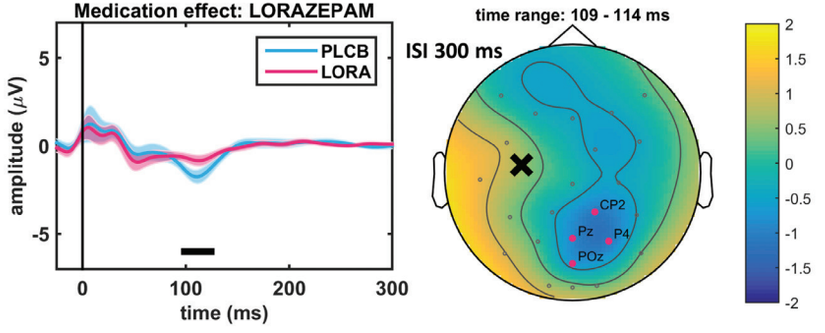
FIGURE 4 Significant N15 cluster comparing paired pulse TEPs of placebo (PLCB; in blue) with valproic acid (VALP; in red) for ISI 50 ms. The grand average (mean ± (SEM)) over all significant electrodes is presented, as well as the difference in topographical distribution at the time of the cluster. The colors of the topographical plot of the cortex show the increase or decrease of amplitude (μV) of the response. The black cross represents the stimulation site, the red dots significant electrodes and the thick black bar below the average TEP response corresponds to the time interval with significant differences.



CONCENTRATION-EFFECT RELATIONSHIP OF MEP AMPLITUDE

A concentration-effect relationship between MEP amplitude and levetiracetam could be characterized with a proportional effect on baseline, described with an E_{max}-equation (p<0.001 compared to intercept only). IIV and BOV on the baseline parameter was log-normally distributed and statistically preferred over either IIV or BOV alone, although shrinkage for BOV was moderate (25% and 33%). Parameters were estimated with small uncertainty (i.e. relative standard error, RSE<50%), except for EC50 which was estimated with an RSE of 90.38%. Inclusion of IIV for EC50 or E_{max} did not improve the model in terms of OFV nor RSE%. Residual unexplained variability (RUV) was best described with a proportional error structure. Model parameters from analysis in NONMEM are listed in *Supplementary Table* 2 and a model simulation for the typical individual is shown in *Supplementary Figure* 6. Based on available data, no concentration-effect relationships could be found for valproic acid and lorazepam on MEP amplitude.

FIGURE 5 Significant N100 cluster comparing paired-pulse TEPs of placebo (PLCB; in blue) with lorazepam (LORA; in red) for ISI 300 ms. The grand average (mean ± SEM) over all significant electrodes is presented, as well as the difference in topographical distribution at the time of the cluster. The colors of the topographical plot of the cortex show the increase or decrease of amplitude (μV) of the response. The black cross represents the stimulation site, the red dots significant electrodes and the thick black bar below the average TEP response corresponds to the time interval with significant differences.



DISCUSSION

In this study, the effects of three AEDs on cortical excitability were assessed using sp- and ppTMS-EMG and TMS-EEG. All drugs decreased cortical excitability. Levetiracetam, valproic acid and lorazepam all significantly decreased MEP amplitude. Additionally, levetiracetam and lorazepam enhanced LICI at ISI 50 ms. Levetiracetam affected the amplitude of TEP components N45 and N100 in EEG clusters after spTMS. The mechanism through which these drugs inhibit cortical excitability

differ, which is reflected by the distinguishing fingerprints that were detected on TEP components. This finding gives new insights into pharmacological effects on TEPs, in addition to the existing literature.[30] In this discussion, we compared our results to placebo-controlled trials, to facilitate accurate comparison to our results.

TMS AS A PHARMACODYNAMIC BIOMARKER We assessed the variability of TMS-EMG and the feasibility of TMS-EMG/EEG for the purpose of using it in early phase clinical drug development. The main goal would be to use TMS-EMG/EEG outcomes as pharmacodynamic biomarkers for proof-of-mechanism of novel compounds that modulate cortical excitability. In our opinion, TMS-EMG and TMS-EEG are suitable to be used for this purpose. This is supported by our observed significant effects of single doses of three AEDs with different mechanisms of action, in a sample size that is typically used in early phase proof-of-mechanism-like drug studies. Moreover, TMS-EMG/EEG embodies certain qualities that are favourable to pharmacodynamic biomarkers: the method is non-invasive and relatively quick to perform, which allows for multiple measurements at different drug concentrations.

Although it should be noted that the variability of the outcome parameters is relatively high, the effect size was large enough to generate significant results in a small number of subjects. Therefore, we consider TMS-EMG/EEG outcomes as useful biomarkers for proof-of-mechanism of new compounds. In our opinion TMS-EMG can be used in Phase I dose escalation study designs, to evaluate target engagement and to aid in dose finding for further studies. Because the inter-subject variability of TMS-EMG was higher than the intra-subject variability, we would propose to use TMS to demonstrate pharmacological effects in a cross-over rather than a parallel study design.

As an exploratory outcome of this study, we have evaluated the concentration-effect relationship between the study drugs and MEP amplitude. A significant PKPD relationship was detected for levetiracetam, but not for valproic acid and lorazepam. It should be noted that the design of our study was not ideal for the assessment of PKPD relationships, because the concentration range observed in this study is relatively small and the number of post-dose measurements is limited. This is also demonstrated by the high uncertainty around the estimated EC50 parameter for levetiracetam. Whether TMS-EMG/EEG, despite the high variability of the outcomes, can be used for evaluation of concentration-effect relations therefore remains to be confirmed in future studies. Administration of multiple dose levels of the same compound can inform this concentration-effect relationship across a wider range of concentrations which would lower the parameter uncertainty currently observed in the model.

EFFECTS OF LEVETIRACETAM Levetiracetam targets synaptic vesical glycoprotein SV2A, which decreases central neurotransmitter release[31] and therefore theoretically decreases cortical excitability. We showed a significant decrease of MEP amplitude induced by levetiracetam, indicating reduced excitability, in line with previously reported results.[32] Other studies showed a non-significant decrease of MEP amplitude after administration of levetiracetam,[33,34] and brivaracetam, an AED with a closely related mechanism of action.[7]

With use of CBPA of spTEPs, our study demonstrated that levetiracetam increased the amplitude of the N45 component in a central cluster and decreased the N100 amplitude contralateral to the stimulation site. The decrease in N100 is consistent with changes caused by brivaracetam.[7] The increase in N45 is also in line with literature.[10] We found the effect in a contralateral cluster, whereas the N45 component showed widespread negativity in the study by Premoli *et al.*, with the maximum effect in the ipsilateral hemisphere.[10]

To our knowledge, our study is the first to evaluate effects of levetiracetam on paired pulse TMS-EEG. Interestingly, the effect we observe on the N100 cluster following spTMS, is very similar in shape and localization to the significant N100 clusters detected at ISIs 2 and 300 ms.

There is substantial evidence that the N45 component represents γ-aminobutyric acid-A ($GABA_A$) mediated inhibition, whereas $GABA_B$ receptor activity is reflected by the N100 component.[11] Our findings on the N45 component may therefore provide further indication that levetiracetam indirectly affects $GABA_A$ergic inhibition.[10,35] The effect of levetiracetam on N100 in the contralateral hemisphere may be caused by inhibition of cortico-cortical connections, as previously suggested for brivaracetam.[7]

EFFECTS OF VALPROIC ACID The anti-epileptic mechanism of action of valproic acid has not been completely clarified. It induces inhibition through the increase of GABA availability. Furthermore, valproic acid blocks voltage-gated sodium channels, affects neuronal potassium and calcium regulation, and inhibits N-methyl-D-aspartate (NMDA) transmission.[36]

To our knowledge this is the first study to report that valproic acid decreased MEP amplitude in healthy volunteers. A previous study did not report an effect on MEP amplitude, but this study was not placebo-controlled.[37] The effect on MEP amplitude confirms that valproic acid decreases cortical excitability, as can be expected based on the mechanism of action.

To the best of our knowledge, no previous studies using TMS-EEG were performed to investigate the effect of valproic acid in healthy volunteers. Using TMS-EEG, we detected a significant N15 cluster at ISI 50 ms. Interestingly, considering the proposed mechanisms of action of the drug, our results indicate that valproic acid does not induce the same effect on the N45 and N100 components as lorazepam and other positive allosteric modulators (PAM) of $GABA_A$ receptors.[11] The effect also does not bear resemblance to the effect of sodium channel blockers, such as lamotrigine which increased N45 and decreased P180,[10] nor NMDA-receptor antagonists, such as dextromethorphan which increases the N45 component.[38] The effects of valproic acid on TEPs will need to be repeated to confirm if the effects on N15 can be reproduced.

EFFECTS OF LORAZEPAM Lorazepam is a $GABA_A$ receptor PAM and stimulates GABAergic inhibition.[39] Our study demonstrated a decrease in MEP amplitude by lorazepam, in line with previous findings on stimulus response curves,[40,41] indicating reduced excitability. LICI at ISI 50 ms was enhanced by lorazepam, which is associated with $GABA_B$ receptor mediated inhibition,[42] similar to the N100 component. No effect on SICI was detected, corresponding to results of other studies.[40,43]

Previous studies using spTMS showed effects of other $GABA_A$-PAMs on N45 and N100, leading to the hypothesis that the N45 component is correlated to $GABA_A$ receptor mediated inhibition.[11] It is therefore unexpected that we did not replicate these findings with lorazepam, which may be explained by a smaller number of subjects and relatively large variability in the measurements, indicating that our study is possibly underpowered for demonstrating this effect. In our study, lorazepam did induce a significant cluster with a decrease of N100 using ppTMS (ISI 300 ms).

RECOMMENDED STATISTICAL ANALYSIS IN PLACEBO CONTROLLED TMS TRIALS Previous studies often assessed drug effects on cortical excitability by comparing pre-dose and post-dose outcomes in treatment and placebo condition separately. However, in a placebo-controlled trial, a more appropriate analysis would be to compare the treatment effect to placebo.[44] In the current study, an ANCOVA was used, because it can provide a comparison between treatment and placebo, using the baseline measurement per period as covariate. This analysis takes into account the inter-subject variability by introducing a random subject effect, while the intra-subject variability is given by the residual error term. Time effects, such as diurnal variation, are taken into account by including time effect in the model, while the subject by time interaction allows for different time effect between subjects. Similarly, the subject by treatment interaction included in the model allows for different treatment effect across subjects. Finally, potential pre-treatment differences are corrected by including the baseline as covariate. Due to these advantages over pre-dose versus post-dose comparison, we strongly recommend direct comparison of treatment versus placebo in future placebo-controlled pharmaco-TMS studies.

POSSIBLE LIMITATIONS The use of a 32-lead EEG cap, as opposed to 64-leads, could have impacted results and could explain why certain treatment-induced EEG clusters detected in previous studies were not confirmed in this study. A cluster, consisting of a minimum of three leads with significant signal changes in the same direction, covers a relatively larger area using 32-lead EEG and therefore needs to be more extensive than with 64-lead EEG.

Single trial PCA was applied to reduce artefacts caused by TMS stimulation and muscle activation on the scalp. PCA has shown to be an effective method to reduce both artefacts simultaneously, as seen for example in subjects 4, 5 and 8 in the *Supplementary Figures* 7-10. However, in others (e.g. subjects 2, 6 and 11) the final TEP is still contaminated by residual artefacts. Since no consensus has been reached within the TMS-EEG community on a common 'gold standard' analysis approach,

numerous alternative artefact rejection methods exist with each their own advantages and disadvantages.[45] As the final TEP is most likely largely influenced by the applied preprocessing pipeline,[46] we chose to use PCA making our results comparable with previous own findings, although this method may not always perform optimally.

Although not a limitation on itself, it should be noted that the stimulation intensity was adjusted prior to each TMS session, based on the rMT at the start of each measurement. This is in contrast to some previous studies, such as a study analysing levetiracetam effects on TMS-EEG.[10] We chose this approach to make sure that stimulation intensity was always related to the rMT, and that changes in rMT (e.g., due to drug effects) would not lead to subthreshold stimulation. Importantly, we did not observe a significant change in rMT and therefore this should not have impacted the comparison of the results of our study to previous studies.

CONCLUSIONS

The aim of this study was to show the value of TMS-EMG and TMS-EEG in determining effects of drugs targeting cortical excitability, for the purpose of developing these measurements as pharmacodynamic biomarkers for use in early phase clinical drug development. Pharmacodynamic effects on TMS-EMG have been intensively studied, but studies that assess drug effects on TMS-EEG are limited. Therefore, we investigated the sensitivity of TMS-EMG/EEG to detect effects of three commonly prescribed AEDs on cortical excitability in a double-blind, placebo-controlled, four-way cross-over study in healthy subjects. Our study shows that a single doses of levetiracetam, valproic acid and lorazepam decrease cortical excitability, as expected from anti-epileptic drugs. These findings support the development of TMS-EMG and TMS-EEG as a suitable biomarkers for proof-of-mechanism of new treatments in the early clinical phase.

SUPPLEMENTARY TABLE 1 **Mean serum concentrations of levetiracetam, valproic acid and lorazepam at the scheduled sampling times.**

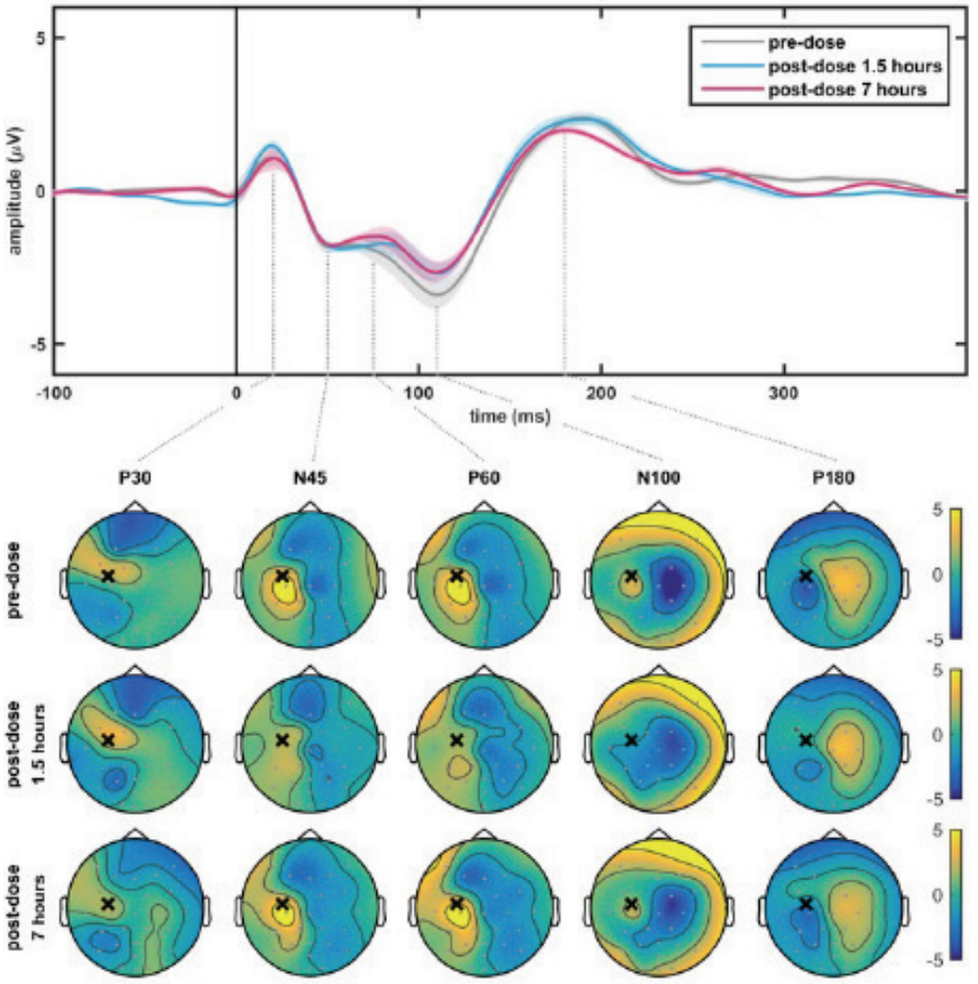
Treatment	Time after dosing	Mean serum concentration	SD	Median
Levetiracetam (2000mg)	0	0 µg/mL	0	0
	30 min	44.45 µg/mL	10.37	46.25
	1 h 22 min	39.68 µg/mL	5.76	39.4
	2 h 15 min	36.64 µg/mL	4.6	37.95
	3 h 30 m	32.61 µg/mL	4.28	32.95
	6 h 52 min	23.37 µg/mL	2.53	23.5
	23 h 52 min	5.03 µg/mL	1.28	4.75
Valproic acid (1000mg)	0	0 µg/mL	0	0
	30 min	68.12 µg/mL	8.59	66.5
	1 h 22 min	65.25 µg/mL	7.73	65
	2 h 15 min	59.81 µg/mL	7.84	60
	3 h 30 m	54.31 µg/mL	8.26	54
	6 h 52 min	40.88 µg/mL	5.88	40.5
	23 h 52 min	15.25 µg/mL	4.52	14
Lorazepam (2mg)	0	0 ng/mL	0	0
	30 min	4.11 ng/mL	6.24	0
	1 h 22 min	18.75 ng/mL	3.27	18.76
	2 h 15 min	18.79 ng/mL	3.09	19.2
	3 h 30 m	17.32 ng/mL	3.01	16.56
	6 h 52 min	13.69 ng/mL	2.69	13.35
	23 h 52 min	4.42 ng/mL	4.17	5.31

SUPPLEMENTARY TABLE 2 PKPD-model parameters to describe the proportional effect of levetiracetam on MEP amplitude.

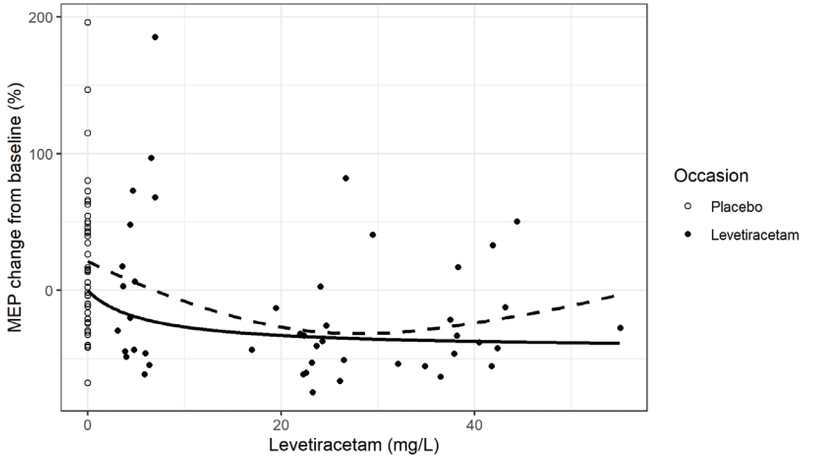
Parameter	Estimate	RSE(%)
POPULATION PARAMETERS		
Baseline (μV)	633.5	18.35
EC_{50} (mg/L)	6.069	90.38
E_{max} (%)	-43.35	26.35
VARIABILITY ON BASELINE (VARIANCE)		
IIV	0.1287	41.13
BOV	0.4290	35.25
RESIDUAL UNEXPLAINED VARIABILITY (RUV, σ^2)		
Proportional error	0.1246	20.97

RSE = relative standard error, EC50 = concentration at which 50% of the maximum effect is achieved, E_{max} = maximum effect, IIV = inter-individual variability, BOV = between-occasion variability.

SUPPLEMENTARY FIGURE 1 Average single pulse TEP and topographical plots of the characteristic TEP components at pre-dose (in grey and top row) and post-dose 1.5 hours (in blue and middle row) and 7 hours (in red and bottom row) for the placebo condition. Masking of auditory components appears to be sufficient as represented by lateralized responses in the topographical plots even at late latencies. Each TEP is the average over all subjects (mean ± SEM) at electrodes CZ, C4, FC2, CP2, CP6 and P4 (similar to significant clusters found for levetiracetam). The topographical plots show the distribution of the P30, N45, P60, N100 and P180 components, where the black cross represents the stimulation site and the grey dots the 32 electrodes. The colours of the topographical plot of the cortex show a positive (in yellow) or negative (in blue) amplitude (μV) of the response.



SUPPLEMENTARY FIGURE 6 PKPD-relationship between levetiracetam and MEP amplitude change from baseline. Measured data is shown as dots. The solid line shows the E_{max}-model simulation for a typical individual and the dotted line is a smooth curve (loess-regression, span width = 1).



MEP = *motor-evoked potential*

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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