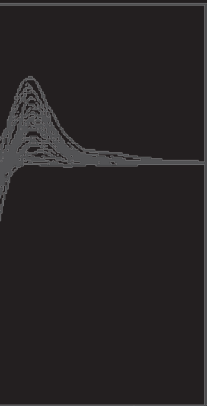


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SIGNAL TO SIGNATURE



TRANSCRANIAL MAGNETIC STIMULATION AS
A BIOMARKER OF DRUG-INDUCED CORTICAL
MODULATION IN EARLY-PHASE DEVELOPMENT

Kaye de Cuba

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CHAPTER I	Introduction – 7
CHAPTER II	Sustained pharmacodynamic effects of S-ketamine on cortical excitability and resting-state brain activity: a randomized, placebo-controlled trial – 19
CHAPTER III	Transcranial Magnetic Stimulation as a translational biomarker in early-phase anti-seizure medication development: a randomized, double-blind, placebo-controlled study in generalized epilepsy – 51
CHAPTER IV	TMS and EEG pharmacodynamic effects of a selective sphingosine-1-phosphate subtype 1 receptor agonist on cortical excitability in healthy subjects – 81
CHAPTER V	Differential cortical effects of synaptic versus extra-synaptic GABA _A receptor modulation with lorazepam and brexanolone: a randomized, double-blind, placebo-controlled, cross-over TMS and EEG trial – 105
CHAPTER VI	General discussion – 143
APPENDICES	Nederlandse samenvatting – 162 Resumen na Papiamento – 164 Portfolio – 166 Curriculum Vitae – 171 List of publications – 172 Acknowledgements – 174

CHAPTER I
INTRODUCTION

Cortical excitability refers to the electrical reactivity of neurons in the cerebral cortex in response to stimuli, whether electrical, magnetic, or pharmacological, and is a dynamic and tightly regulated feature of brain function.^{1,2} It reflects the net result of excitatory and inhibitory inputs within local neural circuits and plays a central role in shaping perception, cognition, and behavior. At its core lies the balance between excitatory glutamatergic and inhibitory gamma-aminobutyric acid (GABA) mediated neurotransmission commonly referred to as the excitation/inhibition (E/I) balance. Glutamate, the brain's principal excitatory neurotransmitter, facilitates neuronal firing via alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and N-methyl-D-aspartate receptor (NMDA) receptors, while GABA, the major inhibitory neurotransmitter, suppresses activity through GABA_A and GABA_B receptors (GABA_AR and GABA_BR).³ Beyond fast synaptic transmission, neuromodulatory systems such as cholinergic, serotonergic, and dopaminergic pathways fine-tune cortical excitability through indirect modulation of glutamatergic and GABAergic signaling and by shaping synaptic plasticity.⁴ Maintaining a stable E/I balance is essential for healthy brain function. Disruptions in cortical excitability have been implicated in a range of neurological and psychiatric disorders, including epilepsy, amyotrophic lateral sclerosis, schizophrenia, depression, and autism spectrum disorder.⁵⁻⁸ Understanding how disruptions in neurocircuitry relate to specific symptom profiles and underlying pathology may reveal new treatment targets, making cortical excitability an important focus for drug development.

The development of new treatments for neurological and psychiatric disorders remains an urgent and complex challenge. Many approved treatments offer only modest efficacy or are limited by side effects, leading to a trial-and-error approach in clinical practice. Despite major advances in neuroscience and molecular technologies, central nervous system (CNS) drug development continues to suffer from the lowest success rates across all therapeutic areas. For example, the likelihood of approval from Phase I to market approval is about 1 in 15 for CNS drugs (~6.5%), with attrition particularly high in Phase II due to lack of efficacy.⁹ These failures come at higher costs and longer development timelines for CNS programs compared to other therapeutic areas.⁹ Compounding this issue is the increasing global burden of CNS disorders. A systematic analysis from the Global Burden of Disease Study found that disorders affecting the nervous system were the leading cause of disability-adjusted life years (DALYs), accounting for approximately 443 million in 2021, an 18.2% increase since

1990, a burden that is expected to grow further with population aging.¹⁰ This further underscores the urgent need for more effective and efficient treatment development strategies.

One of the major contributors to this persistent attrition is the lack of precision pharmacology tools, specifically, reliable and translatable biomarkers. In early-phase drug development, particularly during first-in-human and proof-of-mechanism trials, the ability to demonstrate CNS penetration and pharmacodynamic (PD) effects is critical. However, most available biomarkers fall short. Techniques like cerebrospinal fluid sampling are invasive, while tools such as functional Magnetic Resonance Imaging (fMRI) are costly and logistically demanding. PD measures such as pharmaco-electroencephalography (PEEG) and neurocognitive tests are non-invasive, cheap and sensitive to drug effects, demonstrating CNS penetration and dose-response relationships,¹¹ but often lack the specificity to inform underlying mechanisms of neurocircuitry modulation. This leaves a critical gap: the field lacks biomarkers that can mechanistically link drug exposure to drug-specific modulation of human neural circuits or networks. The earlier such a biomarker can identify a lack of target engagement or specific pathway modulation, the greater the efficiency of the development pipeline. Early go/no-go decisions, informed by such translational biomarkers increase the likelihood of success in later phases.

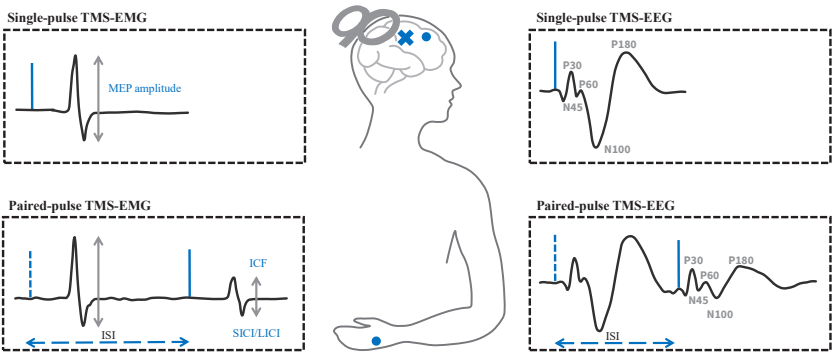
In this context, cortical excitability has emerged as a promising and translational biomarker for early-phase drug development targeting the CNS. Transcranial Magnetic Stimulation (TMS), a non-invasive brain stimulation technique first introduced by Barker et al., is widely used to assess cortical excitability *in vivo*.¹² TMS engages neuronal circuits by delivering brief magnetic pulses through a coil placed near the scalp, inducing localized electrical currents in the cortex.¹³ When applied over the motor cortex, this stimulation can elicit muscle responses, measurable with TMS combined with electromyography (TMS-EMG) as motor-evoked potentials (MEP), a readout of corticospinal excitability.¹⁴ In addition, combining TMS with electroencephalography (TMS-EEG) enables assessment of TMS-evoked potentials (TEP), a series of waveform components (e.g., N15, P30, N45) that reflect excitatory and inhibitory cortical processing across both motor and non-motor regions.¹⁵

Both TMS-EMG and TMS-EEG can be implemented using single- and paired-pulse paradigms, see **Figure 1**. Single pulses provide a general index of cortical reactivity,¹² while paired pulses offer mechanistic insight into intracortical inhibitory and facilitatory dynamics,¹³ reflecting the

functional status of key neurotransmitter systems. Paired-pulse paradigms can evaluate cortical inhibition and facilitation, based on the change in MEP amplitude following two pulses at specific interstimulus intervals (ISI), and can be categorized as short-interval intracortical inhibition (SICI), typically seen with pulse pairs between 1-5 ms apart, long-interval intracortical inhibition (LICI), between 50-300 ms, and intracortical facilitation (ICF), 7-20 ms.^{4,13,16,17} TMS-derived measures have also been shown to be sensitive to CNS-penetrant drugs⁴ and may aid in identifying effective target engagement.

FIGURE 1 Schematic overview of TMS-EMG and TMS-EEG.

Left: Transcranial magnetic stimulation (TMS) combined with electromyography (EMG) at the abductor digiti minimi to assess the single-pulse motor-evoked potential (MEP), as well as paired-pulse measures of short- and long-interval intracortical inhibition (SICI/LICI) and intracortical facilitation (ICF), which depend on the interstimulus interval (ISI). Right: TMS combined with electroencephalography (EEG) to capture cortical responses to TMS in the form of single- and paired-pulse TMS-evoked potentials (TEPs), characterized by distinct positive and negative deflections.



Adapted from: Barker et al. *The Lancet*. 1985; de Goede et al. *Clin Neurophysiol*. 2016.^{12,18}

Drugs that modulate cortical excitability can broadly be categorized into four classes based on their primary targets: 1) ion channels, 2) synaptic inhibition mediated by GABA, and 3) synaptic excitation mediated by glutamate,¹⁹ and 4) indirect modulation of GABA or glutamate (e.g. through sodium-potassium-chloride cotransporter 1 (NKCC1) or potassium-chloride co-transporter 2 (KCC2)). A series of studies have demonstrated that TMS-EMG is sensitive to PD effects across all three classes.⁴ Among single-pulse parameters, motor-evoked potential (MEP) amplitude and resting motor threshold (RMT), defined as the minimum stimulation intensity required to elicit at least five reproducible MEPs out of ten stimuli,²⁰ are particularly

sensitive to drugs targeting voltage-gated ion channels.⁴ Paired-pulse paradigms have been shown to reflect the activity of specific neurotransmitter systems. For example, short-interval intracortical inhibition (SICI) is enhanced by GABA_A-ergic agents such as benzodiazepines, long-interval intracortical inhibition (LICI) is potentiated by GABA_B agonists like baclofen, and intracortical facilitation (ICF) is primarily modulated by glutamatergic compounds.⁴ Moreover, pharmacological challenge studies using CNS active drugs with known mechanism of action (MOA) have begun to elucidate the neurophysiological basis of specific TEP components,²¹ with e.g. the N45 and N100 components being sensitive to GABAergic drugs and P60 to glutamate modulators. Therefore, there is also increasing scientific interest in TMS-EEG biomarkers to demonstrate drug-induced cortical modulation beyond the motor system.

In our previous work, we conducted the first validation study implementing both TMS-EMG and TMS-EEG in a proof-of-mechanism trial, demonstrating robust and distinct effects following administration of three widely prescribed, market-approved anti-seizure medications (ASM) with divergent MOA.²² More recently, exploratory studies with investigational compounds and novel mechanism of action, such as a potassium channel opener,²³ a selective alpha-5-GABA_AR antagonist,²⁴ and an AMPA-positive allosteric modulator,²⁵ have further supported the feasibility of using TMS-EMG and TMS-EEG to detect PD effects in early-phase CNS drug development. By providing objective and translatable measures of drug action on the neurocircuitry, these cortical excitability readouts can improve early go/no-go decision-making, support more precise dose selection for patient studies, and ultimately accelerate the development of novel therapies. Beyond PD assessment, TMS may also serve as a translational biomarker with potential to track clinical treatment response. For example, in epilepsy, cortical excitability is elevated across various epilepsy subtypes²⁶ and has been shown to normalize in patients who achieve seizure freedom following successful ASM treatment,²⁷ indicating its potential to serve as a surrogate efficacy biomarker.

Although further validation is required, cortical excitability has the potential to fulfil multiple regulatory-relevant roles in CNS drug development, owing to its pharmacological sensitivity and clinical relevance. According to the U.S. Food and Drug Administration (FDA)–National Institutes of Health (NIH) Biomarker Working Group, a biomarker is “a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an

exposure or intervention”,²⁸ encompassing neurophysiological measures such as cortical excitability. The FDA–NIH BEST (Biomarkers, EndpointS, and Other Tools) framework classifies biomarkers into seven categories: diagnostic, monitoring, predictive, prognostic, PD/response, safety, and susceptibility/risk.²⁸ Cortical excitability could be relevant to several of these categories, particularly diagnostic, predictive, and PD/response, but in this thesis the focus is on the latter: its application in early-phase (phase I–II) clinical trials.

Here, we propose a framework in which cortical excitability is used as a quantitative biomarker to index pharmacological and pathophysiological processes in the CNS. As summarized in **Table 1**, it can serve as a PD biomarker to demonstrate target engagement, support MOA characterization, aid patient stratification based on neurophysiological profiles, and act as a surrogate efficacy biomarker in disorders where E/I imbalance is a central pathophysiological feature. Ideally, a single biomarker could serve all four of these functions, making it a truly translatable biomarker capable of bridging the gap between molecular pharmacology and systems-level brain function, which can be leveraged in early proof-of-mechanism trials and can be advanced into proof-of-concept studies and efficacy trials.

TABLE 1 Application of cortical excitability biomarkers in early-phase drug development.

Category	Phase	Application of Cortical Excitability	Contextual Relevance
PD/Response biomarker	IA/B	Tracks early drug-induced changes in cortical excitability	Indicates CNS target engagement or pathway activation following intervention
MOA biomarker	IA/B	Captures compound-specific effects on neurocircuit-level modulation	Provides evidence of biological activity in the intended neural circuits; supports mechanistic claims
Surrogate efficacy endpoint	IB–III	Normalization of pathological cortical excitability patterns may correlate with symptom improvement	Potential surrogate for clinical efficacy
Patient stratification/identification biomarker	II/III	Identifies neurophysiological signatures of disrupted neurocircuitry linked to disease mechanisms	Supports patient selection, inclusion criteria, or enrichment strategies in proof-of-concept studies

Adapted from: Umbricht et al. *Eur Neuropsychopharmacol.* 2024.²⁹

While some gaps remain, the full potential of TMS as a translational biomarker is steadily unfolding. One of the main limitations is the high intra- and inter-subject variability in TMS-based measures.³⁰ Part of this variability can be mitigated through methodological refinements and standardization, for example limiting physiological factors that influence cortical excitability, such as variable sleep patterns,³¹ neuroactive substances like caffeine³² or nicotine,³³ and hormonal fluctuations.³⁴ Although some of these factors can be controlled in a clinical trial setting, others sources of physiological variability, such as circadian rhythms and ongoing brain oscillations,^{35–37} are more difficult to regulate. Employing cross-over designs, in which participants serve as their own control, can help mitigate some of this physiological variability. This further underscores the urgent need for standardization, as currently consensus on stimulation protocols or preprocessing pipelines, in particular for TMS-EEG, remain lacking,³⁸ thereby limiting reproducibility and comparability across studies. Also, TMS’s translational applicability is not yet fully established, as relatively few studies have extended early-phase PD findings in healthy volunteers to clinical populations, where TMS biomarkers could be used as surrogate efficacy biomarkers or aid in patient selection.

Aims and outline of this thesis

This thesis aims to address critical gaps in early-phase CNS drug development by investigating whether cortical excitability, measurable through TMS, can serve as a mechanistic and translational biomarker. By moving from signal to signature, this work explores whether TMS-based metrics can reveal distinct neurophysiological fingerprints of drugs with different MOA, thereby demonstrating the sensitivity of TMS to discriminate between pharmacological targets. Demonstrating the value of TMS-based measures in clinical settings helps build a biomarker-driven framework for early CNS drug development that bridges the gap to patient outcomes and advances tools for precision pharmacology.

To achieve this overarching goal, the thesis is structured around the following specific aims:

- To build a benchmark portfolio of drug-induced cortical signatures using standardized TMS-EMG and TMS-EEG protocols and study designs, characterizing the neurophysiological effects of established CNS-active compounds with known molecular targets.

- To evaluate the translatability of these drug-specific cortical excitability signatures from healthy volunteers to relevant patient populations
- To apply TMS as mechanistic biomarker for drugs with novel MOA.
- To evaluate whether TMS can distinguish between benchmark drugs and drugs with novel MOA.

To explore the value of TMS-derived measures as mechanistic biomarkers relative to conventional PEEG, determining whether TMS provides unique or complementary insights for PD profiling and early efficacy prediction. Based on the aims outlined, this thesis first incorporates a series of proof-of-mechanism trials, designed to systematically evaluate the potential of TMS-based measures as biomarkers in early-phase drug trials. Subsequently, evaluating both market-approved benchmark drugs and investigational compounds with novel MOA provides the opportunity to demonstrate PD responses and characterize drug-specific cortical excitability signatures. These signatures reflect engagement of distinct neurotransmitter systems and can help confirm or refine the compound's putative MOA. Extending these studies to patients allows us to address the translatability of cortical excitability signatures. The potential utility of TMS for patient stratification lies beyond the scope of this thesis, which focuses specifically on proof-of-mechanism trials intended to inform and de-risk future proof-of-concept studies. Together, these studies contribute to the development of a robust TMS-based biomarker platform capable of informing PD response, MOA, and potential surrogate efficacy, and thereby enhance decision making in early CNS drug development.

Chapter II addresses the sustained PD effects of S-ketamine in healthy volunteers, using both intravenous and oral formulations. This work expanded on earlier work profiling glutamatergic modulation.²⁵ By assessing cortical excitability over multiple days following a single dose, we aimed to determine whether TMS can capture the prolonged effects of ketamine, positioning it as a potential benchmark drug for novel rapid-acting antidepressants (RAAD). Additionally, comparing the two formulations allowed us to explore whether TMS could distinguish effects attributable to the parent compound versus active metabolites. **Chapter III** addresses the translatability of cortical excitability signatures from healthy participants to clinical populations by replicating a previously conducted healthy volunteer study with anti-seizure medications (ASM) in patients with generalized epilepsy. We selected levetiracetam as the benchmark compound, based on its robust effects in healthy volunteers.²² **Chapter**

IV shows the effects of an investigational ASM with a novel mechanism of action in healthy volunteers, now in a multiple dose setting, to examine cumulative PD effects over time. In **chapter V**, we directly compare the cortical signatures of two GABA_A-modulating drugs, a benzodiazepine used as a benchmark comparator, and an investigational compound with a novel MOA, aiming to demonstrate TMS's ability to differentiate mechanism-specific neurocircuit modulation. Finally, **Chapter VI** presents the general discussion, in which we revisit these aims, evaluate the extent to which they have been achieved, and provide recommendations for future research.

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CHAPTER II

**SUSTAINED PHARMACODYNAMIC
EFFECTS OF S-KETAMINE ON
CORTICAL EXCITABILITY AND
RESTING-STATE BRAIN ACTIVITY:
A RANDOMIZED, PLACEBO-
CONTROLLED TRIAL**

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ABSTRACT

This study aimed to investigate a set of pharmacodynamic biomarkers reflecting acute, delayed, and sustained central nervous system effects of S-ketamine, used as a tool compound for rapid-acting antidepressant activity, with the goal of informing biomarker strategies for delayed antidepressant effects. In this randomized, double-blind, double-dummy, placebo-controlled, 4-way crossover study in 16 healthy participants, we administered S-ketamine in a therapeutic intravenous dose (IV), a low and high oral dose, versus placebo. Measurements were conducted from baseline up to 7 days post-dose using Transcranial Magnetic Stimulation combined with electromyography (TMS-EMG) and encephalography (TMS-EEG), pharmacoelectroencephalography (PEEG), alongside drug and metabolite plasma concentrations. Outcomes were analyzed using mixed-effects analysis of covariance (ANCOVA) and cluster-based permutation testing. Post hoc concentration-effect relationships were explored. IV S-ketamine induced an acute reduction in motor-evoked potential (MEP) amplitude and sustained attenuation of long-interval intracortical inhibition (LICI₅₀), the latter showing a linear concentration-effect relationship with the parent compound. Acute TMS-evoked potential (TEP) modulation was observed across all treatments, whereas delayed effects occurred only after IV and high-dose oral. PEEG showed acute reductions in alpha, beta, and delta power (eyes closed) and sustained increases in delta power (eyes open) following IV and high-dose oral S-ketamine; with delta power exhibiting a less analyte-specific linear concentration-effect relationship. We provide evidence suggestive of delayed pharmacodynamic effects of S-ketamine in healthy participants sustained up to 7 days post-dose, using TMS and PEEG derived measures, which are markedly distinct from its acute effects and may be relevant to understand its antidepressant efficacy.

INTRODUCTION

Major Depressive Disorder (MDD) affects approximately 280 million people worldwide and remains a leading cause of disability.¹ Conventional monoaminergic antidepressants have limitations in terms of delayed onset, limited efficacy, and poor tolerability, that underscore the urgent need for rapidly acting pharmacological treatments with novel mechanisms to effectively address treatment-resistant depression (TRD).²

S-ketamine (SKET) is a non-competitive, non-selective, glutamate N-methyl-D-aspartate receptor (NMDAR) antagonist, originally approved as an anesthetic, but recently approved by the Food and Drug Administration (FDA) as monotherapy for TRD in an intranasal formulation (Spravato®). SKET produces a rapid onset of antidepressant effects; however, its broader clinical use in psychiatry is limited by transient sedation and psychomimetic adverse effects.³ Accordingly, the development of novel compounds that preserve the antidepressant efficacy associated with SKET while minimizing these untoward effects may offer safer and more scalable rapid-acting antidepressant strategies.

However, the mechanisms underlying SKET's antidepressant effects remain incompletely understood, and efforts to develop alternative rapid-acting antidepressant drugs (RAAD) are constrained by a lack of pharmacodynamic (PD) biomarkers. A biomarker platform capable of quantifying both therapeutic and central nervous system (CNS) mediated side effects in healthy participants would enable efficient early-phase screening of candidate compounds and dose selection. Importantly, optimizing such a platform requires a deeper understanding of SKET's underlying pharmacological effects and their temporal evolution.

Clinically, SKET's antidepressant effects typically emerge by 24 hours (H) post-dose,^{4,5} following its rapid systemic clearance, and persist for several weeks.³ These delayed effects are distinct from the acute concentration-dependent psychomimetic and CNS-depressant effects observed shortly after dosing.⁶ Results from a concurrently conducted study,⁷ comparing intravenous (IV) to orally administered SKET (PO-SKET) using a validated CNS test battery (NeuroCart), further support the hypothesis that SKET's acute effects up to 6H post-dose are primarily mediated by the parent compound through (direct) NMDAR antagonism.⁸ PO-SKET was associated with a significantly altered PD profile, consistent with differences in systemic exposure and metabolite-to-parent ratios (MPR) resulting from first-pass metabolism.⁹ Importantly, these differences

were primarily driven by lower parent-compound exposure rather than increased exposure to the active metabolite S-norketamine (SNOR).⁷ The same study further demonstrated the sensitivity of biomarkers such as saccadic peak velocity, adaptive tracking, body sway, and subjective drug effects for SKET's acute NMDAR-mediated adverse effects.

In contrast, SKET's sustained antidepressant efficacy may depend on glutamatergic modulation through mechanisms extending beyond initial NMDAR target engagement. These downstream processes potentially involve synaptic plasticity and network-level reorganization,¹⁰⁻¹⁵ for which PD biomarkers are currently lacking. In this context, Transcranial Magnetic Stimulation (TMS) and resting-state pharmaco-electroencephalography (PEEG) have emerged as potential methodologies for probing such longer-lasting neurophysiological changes. Previous studies have demonstrated decreased excitability¹⁶⁻¹⁸ and a shift in spectral power to the lower frequencies in TRD patients,^{19,20} suggesting potential relevance of these biomarkers to the disease pathology, and thus possibly to antidepressant efficacy. Although it remains unclear whether these measures can serve as translational biomarkers of delayed PD responses and subsequent clinical benefit, TMS and PEEG biomarkers were selected based on the a priori hypothesis that delayed antidepressant response to SKET is associated with sustained modulation of cortical excitability and oscillatory dynamics.

To date, TMS and PEEG have been applied predominantly to characterize the acute effects of ketamine,²¹⁻²³ but not its delayed effects. Nevertheless, TMS is of particular interest because of its demonstrated sensitivity to pharmacological modulation of glutamatergic neurotransmission through different mechanisms. For example, TMS has demonstrated sensitivity to other NMDAR antagonists, such as dextromethorphan and memantine, in healthy participants.²⁴ In addition, TMS has been shown to detect PD effects of the AMPA receptor positive allosteric modulator osavampator (TAK-653/NBI-1065845), a candidate RAAD, which also produced a psychostimulant-like profile on the NeuroCart.^{25,26} Together, these findings support the potential of TMS- and PEEG-derived measures as mechanistic biomarkers to investigate delayed glutamatergic effects relevant to antidepressant response.

Building on prior work by van Mechelen et al. characterizing the acute PD effects of SKET using the NeuroCart,⁷ the present study aims to evaluate TMS and PEEG as candidate biomarkers of corticospinal excitability, cortical excitability and resting-state brain, respectively, for not only acute ($\leq 6H$),

but also delayed (6–24H) and sustained (24H–7 days) CNS effects of SKET in healthy participants. Furthermore, by leveraging the pharmacokinetic divergence between IV- and PO-SKET, we explore whether differences in parent-compound and metabolite exposure relate to differences in the timing and nature of SKET's delayed PD effects.

METHODS

This study was approved by the Medical Ethics Committee, Foundation Beoordeling Ethiek Biomedisch Onderzoek (BEBO; ID: NL73916.056.20). The study was conducted in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonisation guidelines on Good Clinical Practice. Written informed consent was obtained from all participants before study participation. Centre for Human Drug Research (CHDR), Leiden, The Netherlands sponsored and conducted this study, from 12-Aug-2021 to 10-Mar-2022, registered in the Dutch Trial Registry (NTR, ID: 28424).

Participants

Male and female healthy participants, age 18-45 years, were selected following a medical screening (in- and exclusion criteria are listed in **Supplementary Materials**). Participants with an increased risk of TMS-related complications based on the TMS safety questionnaire,²⁷ or significant history or presence of neuropsychiatric illnesses were excluded.

Study Design

The study was a randomized, double-blind, double-dummy, placebo-controlled, 4-way crossover study (Schematic overview in **Supplementary Materials**). Participants were administered 1) single low-dose solution of 0.20 mg/kg PO-SKET + 40-minute placebo infusion, 2) single high-dose solution of 0.45 mg/kg PO-SKET + 40-minute placebo infusion, 3) 0.40 mg/kg 40-minute infusion of SKET (IV-SKET) + placebo PO solution, and 4) 40-minute placebo infusion + placebo PO solution, in randomized order. The randomization code was generated digitally by a study-independent unblinded statistician, participants were enrolled sequentially by the research physician and the medication was prepared by a study-independent unblinded pharmacist. Replacement participants received the same treatment allocation as the participant being replaced. Participants were admitted to the research center each study period on Day -1 to Day 2,

with a wash-out period of 14–21 days, and a return visit on Day 7. Details on safety and PK assessments are described elsewhere.⁷ PK samples were collected to measure SKET, SNOR and S-hydroxy-norketamine (SHNK) plasma concentrations. PEEG was performed pre-dose, and 2.5H, 6H, 24H and 7 days post-dose. TMS-EMG/-EEG was performed pre-dose and 4.5H, 24H and 7 days post-dose. PD effects were assessed over different time periods following SKET administration: effects measured at 2.5H, 4.5H, and 6H post-dose were regarded as acute; those at 24H as delayed; and those at 7 days as sustained.

Dose selection

A detailed description of the dose selection is also provided elsewhere.⁷ In summary, it was based on simulated SKET and SNOR concentrations from two population PK-models, developed with in-house data from IV-SKET administrations²⁸ and PO-SKET data from the literature.⁹ IV and PO administration routes were selected to maximize pharmacokinetic contrast between the parent compound and its metabolites, thereby facilitating evaluation of their differential contributions to SKET's delayed pharmacodynamic effects. An IV dose that demonstrated antidepressant efficacy in TRD⁵ and was expected to reach exposures associated with robust PD effects on the Neurocart test battery⁶ was selected: 0.40 mg/kg 40-minute IV. Two single PO doses were selected: 0.45 mg/kg, to target similar SNOR exposures as a multiple dose study being investigated for the treatment of TRD elsewhere²⁹ and 0.20 mg/kg, to ensure similar maximum plasma concentrations of SNOR as the selected IV dose. In order to align the expected time to maximum concentration (T_{max}), PO administrations were performed 15 minutes after the start of IV administration.

Procedures

Per the International Pharmacology-EEG Society (IPEG) guidelines,³⁰ PEEG was recorded with 5-min alternating periods of eyes closed and open every 64 seconds (s). TMS was applied per current guidelines.³⁰ The motor hotspot of the dominant abductor digiti minimi (ADM) muscle, assessed by the Edinburgh Handedness Questionnaire,³¹ was stimulated. The RMT was determined³² at the start of each measurement, after which single-pulse (SPTMS) and paired-pulse (PPTMS) protocols were applied. TMS-EMG and -EEG were recorded simultaneously during TMS stimulation. Data acquisition and processing was performed as described previously,³³ and a comprehensive explanation is provided in the **Supplementary Materials**. Endpoints for PEEG included total power per frequency band per electrode

and per eye state. TMS-EMG parameters included: mean single pulse peak-to-peak MEP amplitude (μV), paired-pulse short-interval intracortical inhibition as ISIs of 2 ms (SICI₂) and long-interval intracortical inhibition as ISIs of 50, 100 and 300 ms (LICI₅₀, LICI₁₀₀ and LICI₃₀₀).

Statistical analysis

The sample size calculation was based on the MEP amplitude from Ruijs et al.,³⁴ with 16 participants providing 80% power to detect mean difference of -350 μV , assuming a standard deviation (SD) of 450 μV and a two-sided significance level of 0.05. Data for PEEG was log-transformed and back-transformed after analysis so the results could be interpreted as percentage change. Each PEEG and TMS-EMG parameter was analyzed with a mixed model analysis of covariance (ANCOVA) with treatment, time, period and treatment by time as fixed factors and participant, participant by treatment and participant by time as random factors and the (average) baseline measurement as covariate. To evaluate differences in acute, delayed and sustained PD effects, contrasts were calculated per treatment and post-dose timepoints within the model, e.g., low dose SKET: 0.20 mg/kg PO vs. placebo $\leq$ 6H for acute effects; 0.20 mg/kg PO vs. placebo at 24H for delayed effects; 0.20 mg/kg PO vs. placebo at 7 days for sustained effects. PEEG assessments for 2.5H and 6H post-dose were combined. We report the estimated difference (ED) of the least square mean (LSM), the 95%-confidence interval (95%-CI) and the p-value. Graphs of the change from baseline (CFB) of the LSM estimates over time by treatment are presented with 95%-CI as error bars. No correction for multiple testing is applied. TMS evoked potential (TEP) responses were analyzed across all EEG electrodes, for the full sample (0–300 ms) and at specific time periods of interest (TOI) around the characteristic TEP components³⁵ (N15: 0–20 ms; P30: 20–40 ms; N45: 40–55 ms; P60: 55–80 ms; N100: 80–130 ms; P180: 130–230 ms). To compare TEPs measured under placebo and active treatment conditions, nonparametric cluster-based permutation analysis was used.³⁶ Full methodological details, including model specifications and statistical parameters, are provided in the **Supplementary Materials**. Post hoc exploratory PK-PD analysis was performed on parameters demonstrating significant effects in the mixed effects model: MEP amplitude, LICI₅₀, and PEEG delta power (eyes open and closed). For the PK-PD analysis of the acute phase (up to 6.5 H post-dose), PD observations were matched with PK data from the closest PK sampling time. For the PK-PD analysis of PD endpoints collected at 24H and 7 days post-dose, the PD observations were matched with the participant's area under the concentration–time

curve from time zero to the last quantifiable concentration (AUC_{last}) on Day 1 of that treatment period. Parameters were modeled with an intercept only, a linear concentration–effect relationship, and a nonlinear concentration–effect relationship (E_{max}) in a mixed-effects model. A full description of the PK-PD analysis is provided in **Supplementary Materials**.

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 2021/22.³⁷

RESULTS

Demographics, PK and safety

Sixteen participants (eight males and eight females) completed the study. The median (MIN-MAX) age and Body Mass Index (BMI) were 25 (21–35) years and 21.9 (19.4–27.2) kg/m², respectively. Safety and PK results are reported elsewhere.⁷ Plasma-concentration over time curves for SKET, SNOR and SHNK, are summarized in **Figure 1**. Treatment emergent adverse events and PK parameters are provided in **Supplementary Materials**.

TMS-EMG

Acute and sustained effects were observed for 0.40 mg/kg IV-SKET on TMS-EMG, with a statistically significantly decreased MEP amplitude at 4.5H post-dose (ED: -430.17 μ V, 95%-CI: -806.74; -53.61, $p=0.03$) and attenuation of LICI₅₀ 7 days post-dose (ED: 57.83%, 95%-CI: 14.24; 101.43, $p=0.01$) compared with placebo, respectively (**Figure 2**). IV-SKET did not affect any other TMS-EMG parameters. Neither PO-SKET dose demonstrated acute, delayed nor sustained effects. For a complete overview of SP- and PPTMS-EMG results, see **Table S1**.

TMS-EEG

All treatments showed statistically significant acute (4.5H) effects, both 0.40 mg/kg IV-SKET and 0.45 mg/kg PO-SKET showed delayed effects (24H), and none of the treatments showed sustained effects (7 days). Overall, a pattern of two consistent clusters was observed. First, an acute effect at 4.5H post-dose was observed for both 0.20 mg/kg and 0.45 mg/kg PO-SKET, with significant clusters demonstrating an increased N45 component (more negative) and decreased P60 component (less positive) located

in the contralateral centro-parietal region, measured using PPTMS-EEG at long intervals (ISI 50, 100, and 300 ms) (**Figure 3A-D**). A comparable cluster was found as delayed effect measured 24H post-dose for 0.45 mg/kg PO-SKET (**Figure 3E**). Secondly, a delayed effect measured 24H post-dose was observed for 0.40 mg/kg IV-KET and 0.45 mg/kg PO-SKET, with significant clusters demonstrating an increased P180 component (more positive) located in the contralateral parieto-occipital region, measured using PPTMS at ISI 2 ms (**Figure 4**). For a complete overview of the significant TMS-EEG clusters and corresponding topo-plots see **Supplementary Materials** and **Figures S1-S3**.

pEEG

For a complete overview of the pEEG results, see **Tables S2-3**. In summary, 0.40 mg/kg IV-SKET and 0.45 mg/kg PO-SKET showed significant acute, delayed and sustained effects on pEEG. Most consistent were decreases in delta, alpha, and beta power in the eyes-closed condition up to 6H post-dose, and increases in delta power in the eyes-open condition in the parieto-occipital regions for both 0.40 mg/kg IV-SKET and 0.45 mg/kg PO-SKET up to 24H or 7 days post-dose (**Figure 5**).

PK-PD relationship

Significant negative linear concentration–effect relationships were observed for DPzO1C and DPzO2C for both SKET and SNOR up to 6H post-dose (acute phase), and a significant positive linear concentration–effect relationship was observed between LICI₅₀ and SKET at 7 days post-dose (sustained phase). Results are reported in **Table S4** and **Figure S4**. For a summary of all the PD results, see **Supplementary Materials**.

DISCUSSION

In this study, we evaluated TMS- and pEEG-derived measures as candidate biomarkers of acute ($\leq 6H$), delayed (6–24H), and sustained (24H–7 days) pharmacodynamic effects of SKET in healthy participants. We observed what appears to be a temporally distinct pattern of change in corticospinal excitability, cortical excitability and resting-state brain activity following SKET administration. Acute effects were detectable across all modalities, whereas delayed effects were primarily captured by TMS-EEG and pEEG, and sustained effects by TMS-EMG and pEEG. Acute reductions in cortical excitability and spectral power within the first 6H post-dose were followed

by delayed and sustained increases at 24H and 7 days. Concentration–effect relationships were observed between SKET concentrations for delta power in the acute phase, and for LIC_{I50} in the sustained phase.

S-ketamine's effects on corticospinal excitability

Corticospinal excitability was affected only by 0.40 mg/kg IV-SKET, with reduced MEP amplitudes 4.5H post-dose, followed by increased excitability reflected by attenuated LIC_{I50} (reduced inhibition) 7 days post-dose. Neither PO-SKET dose affected TMS-EMG endpoints at any post-dose time point. Post hoc analysis indicated a positive concentration–effect relationship between SKET exposure and LIC_{I50}, suggesting the observed effects are mediated by SKET rather than its metabolites. Previous TMS-EMG studies report divergent acute effects of SKET on TMS endpoints (RMT, MEP amplitude, and SIC_I)^{21,22} and comparisons with the literature are hampered by limited data and methodological heterogeneity, including differences in timing (peak-exposure vs. post peak-exposure), dose, study design, and compound (e.g., racemic vs. enantiomer-specific ketamine with differing NMDAR affinities).³⁸ Unlike prior studies assessing peak exposure effects during infusion, our measurements capture post peak-exposure effects (~4H post-infusion measured at ~15% of C_{max}). These findings suggest that the corticospinal effects observed here might reflect a later phase of SKET's PD profile that is distinct from the initial effects reported during infusion. This may reflect a transition from early glutamate-mediated hyperexcitability to a later compensatory increase in inhibitory tone characterized as post peak-exposure reduction in corticospinal excitability. The attenuation of LIC_I observed at 7 days post-dose may point toward involvement of longer-term neuroplastic processes. Preclinical studies have shown that reductions in intracortical inhibitory activity can facilitate synaptic plasticity, including long-term potentiation.^{39,40} Moreover, a human TMS study using paired associative stimulation, a paradigm thought to induce long-term potentiation– or long-term depression–like plasticity, demonstrated attenuation of LIC_I (less inhibition) following potentiation specific stimulation protocols.⁴¹ While highly speculative, these observations provide a potential framework for interpreting the delayed reduction in intracortical inhibition observed here, which may reflect induction of downstream NMDAR–dependent plasticity.³⁹

S-Ketamine's effects on cortical excitability

TMS-EEG analysis revealed significant clusters for all treatments at 4.5H post-dose, suggesting its high sensitivity to SKET's acute effects. Delayed

effects were observed only for 0.40 mg/kg IV-SKET and 0.45 mg/kg PO-SKET at 24H post-dose, but these effects were not sustained up to 7 days post-dose. Two consistent cluster patterns emerged, reflecting distinct acute and delayed effects, but without clear differentiation between administration routes. Acute effects involved modulation of early TEP components (N₄₅, P₆₀) (Figure 3), whereas delayed effects were characterized by increased P₁₈₀ amplitude (Figure 4). Direct comparison with previous TMS-EEG studies in healthy participants is limited by differences in analytical approaches.^{42,43} Nevertheless, early TEP modulation aligns with findings from other NMDAR antagonists, supporting sensitivity to glutamatergic modulation.⁴⁴ However, SKET's delayed modulation of the late TEP components cannot be readily attributed to glutamatergic modulation alone. Available evidence suggests that late TEP components are modulated by GABAergic rather than by antglutamatergic agents.⁴⁵ Previous studies have reported reductions in P₁₈₀ amplitude after administration of baclofen, a GABA_B receptor agonist, and levetiracetam, an SV2A ligand.^{34,46} Their effects contrast with the delayed increase in P₁₈₀ amplitude observed following SKET in the current study, which may suggest enhanced cortical excitability at 24H post-dose.

S-Ketamine's effects on spontaneous brain activity

Significant acute effects, and more limited delayed and sustained effects, on spectral power were observed for both 0.40 mg/kg IV-SKET and 0.45 mg/kg PO-SKET. Acute decreases in delta, alpha, and beta power were observed up to 6H post-dose, whereas delayed increases in delta power emerged at 24H and persisted up to 7 days. Effects observed for both IV- and PO-SKET aligned in terms of directionality, not clearly indicating differential effects attributable to active metabolites. Post hoc analysis showed negative linear concentration–effect relationships for delta power for both SKET and SNOR, suggesting both SKET and SNOR contributed to acute delta power changes, with a stronger contribution from SKET (Table S4). Direct comparison with the existing literature remains limited. Available studies using IV administered racemic ketamine or SKET in healthy participants have primality measured PEEG at peak concentrations, often reporting increased theta and gamma power,^{23,47,48} whereas the present study captured post peak-exposure effects, which may partly explain these discrepancies. After 24H post-dose, resting-state eyes-open recordings showed increased delta power over parieto-occipital regions, with unchanged alpha power and no frontal effects. Therefore, this pattern

may suggest a region-specific shift rather than a global change in arousal or vigilance. Notably, both TMS-EEG and PEEG effects were located in the parieto-occipital region. While spectral power changes cannot be directly mapped onto specific functional networks, this spatial convergence raises the possibility that posterior cortical networks are preferentially engaged by SKET at delayed time points. One potential interpretative framework is the posterior default mode network, as key nodes such as the posterior cingulate cortex and precuneus are located within these regions and have been shown to be modulated by ketamine in functional imaging studies.^{47,49-53} However, given the indirect nature of PEEG measures, this interpretation remains speculative.

Future perspectives

The acute decreases align with the transient CNS depressant effects reported by van Mechelen et al.,⁷ whereas the delayed and sustained increases may reflect downstream neurophysiological changes extending beyond initial NMDAR antagonism. Of note, these delayed and sustained increases align temporally with the onset of SKET's antidepressant effects at approximately 24H post-dose.³ The possible clinical implication of the present work lies not in demonstrating ketamine-specific delayed effects per se, but in identifying neurophysiological markers sensitive to delayed and sustained CNS modulation beyond acute drug exposure, considered to be a common and important characteristic of RAAD. In early-phase CNS drug development, the lack of mechanistically informed biomarkers that bridge acute pharmacology with delayed clinical outcomes remains a major challenge. The present study suggests that TMS-EMG, TMS-EEG, and PEEG capture distinct but complementary aspects of SKET's pharmacodynamic profile, rather than providing interchangeable measures. Individually, these endpoints offer only a partial view of drug effects; however, when combined, they provide a more comprehensive characterization across temporal and mechanistic domains. TMS-EEG appeared most sensitive to acute drug effects, whereas PEEG captured both acute and later changes in network-level brain activity, with these effects appearing less analyte-specific. TMS-EMG provided more selective evidence of corticospinal modulation by the parent compound, with MEP amplitude reflecting an acute effect and LIC₅₀ a sustained effect. Together, these findings highlight the importance of multimodal biomarker approaches, in which complementary measures are used to capture distinct phases of drug action and may support more informed endpoint selection in early-phase studies.

While the present study was not designed to optimize dosing for therapeutic efficacy, these biomarkers, especially combined, may support early go/no-go decisions and inform dose selection including dosing regimen (e.g. weekly or biweekly dosing) by identifying exposure levels and their associated delayed CNS effects, thereby helping to balance efficacy and tolerability in future clinical studies.

Building on a more robust foundation, future studies should then extend these approaches beyond healthy participants to patient populations, particularly individuals with TRD, to assess the translational relevance of the observed PD effects and whether they are predictive of antidepressant response. While studies in healthy participants allow controlled characterization of acute, delayed, and sustained neurophysiological responses to SKET in the absence of disease-related confounds, alterations in baseline cortical excitability, excitation/inhibition balance, and large-scale network organization in TRD may substantially influence both the magnitude and temporal profile of drug-induced effects.

Limitations

Implications of the present work should be interpreted in light of a few limitations. Both TMS and PEEG were not performed at peak SKET concentrations, precluding replication of previously reported acute-phase findings. Our primary focus was, however, to identify biomarkers of sustained antidepressant effects, emerging after SKET clearance. The cross-over design, while limiting follow-up to 7 days to reduce participant burden, was essential to meet study aims. Although relatively low sub-anesthetic doses were used, these reflect proven therapeutic dose levels, enabling identification of biomarkers related to the intended clinical effect in MDD. Exclusion of the glutamate-sensitive TMS-EMG biomarker ICF⁵⁴⁻⁵⁷ due to time constraints represents another limitation. Prior studies have suggested that the P180 may reflect residual auditory-evoked activity.^{58,59} Given our cross-over design and the use of continuous masking noise,⁶⁰ we believe auditory confounds were sufficiently controlled, and that the observed delayed effects on the late TEP components represent genuine neurophysiological effects. Furthermore, measurements across multiple days introduce day-to-day variability, which may have reduced statistical power and increased the risk of Type I errors, particularly at later time points. In addition, no correction for multiple comparisons was applied, further increasing the risk of Type I error; however, this approach was considered appropriate given the hypothesis-generating

and exploratory nature of the study, which aimed to identify candidate neurophysiological markers for future validation in adequately powered confirmatory studies. Despite these limitations, post hoc PK-PD analyses demonstrated concentration–effect relationships, including the later time points, suggesting that the observed effects are unlikely to be purely spurious. Nevertheless, these results should be interpreted with caution in light of the aforementioned limitations. Future work is needed to validate these findings through larger samples, increased statistical power, and replication using similar dosing regimens and time points. Denser post-dose sampling may further help distinguish true PD effects from between-day variability and strengthen confidence in TMS- and PEEG-derived biomarkers.

CONCLUSION

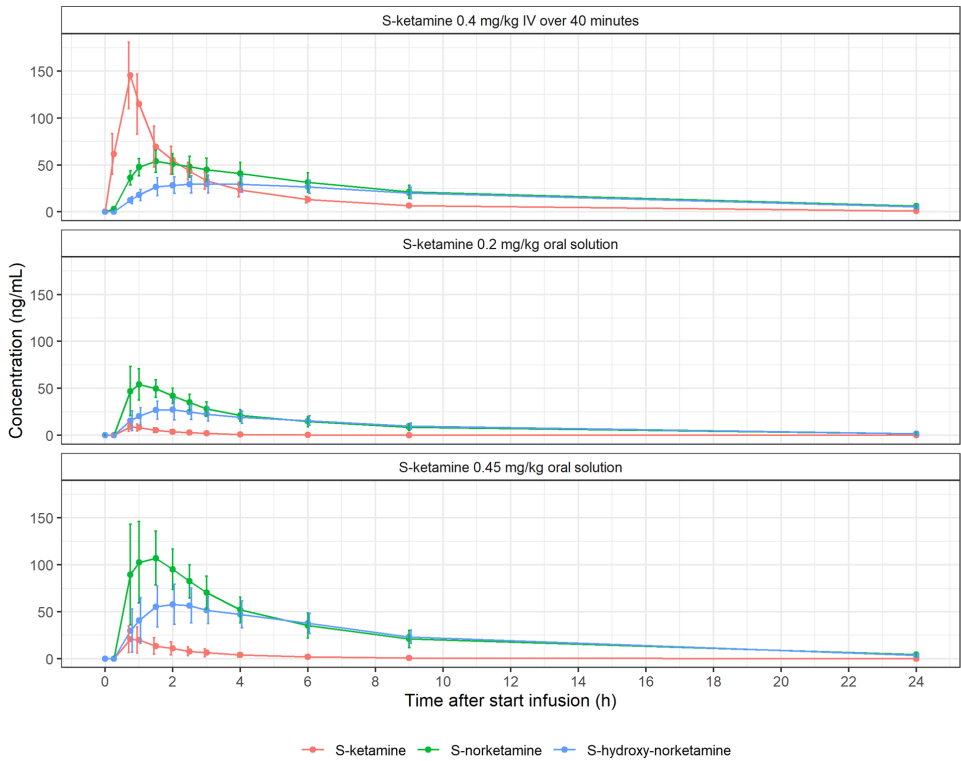
In this exploratory study, we provide evidence suggestive of delayed PD effects of SKET on cortical excitability and resting-state brain activity in healthy participants, extending beyond 24H post-dose and persisting for up to 7 days post-dose, when plasma exposure has fully dissipated. The effects emerging after 24H appeared distinct from the acute effects observed the first few hours after dosing, with a shift in directionality from reduced to increased cortical excitability and resting-state spectral power. While signal variability at the later timepoints warrants cautious interpretation, the consistency of this temporal pattern across modalities and concentration-effect relationships supports the presence of a delayed neurophysiological response. Notably, the emergence of increased brain activity around 24H post-dose coincides with the typical onset of SKET's antidepressant effects, suggesting potential relevance for understanding mechanisms underlying its clinical efficacy; however, the extent to which the neurophysiological changes observed here relate to therapeutic mechanisms or clinical response remains uncertain. Both 0.40 mg/kg IV-SKET and 0.45 mg/kg PO-SKET showed broadly similar delayed neurophysiological effects; however, LIC₅₀ outcomes appeared to be dependent on the parent compound concentration, whereas delta power was less analyte-specific. The combination of TMS and PEEG could serve as biomarkers of sustained antidepressant effects of non-selective NMDAR antagonists such as SKET, and possibly for novel rapid-acting glutamatergic antidepressants, although this remains to be confirmed in a TRD population.

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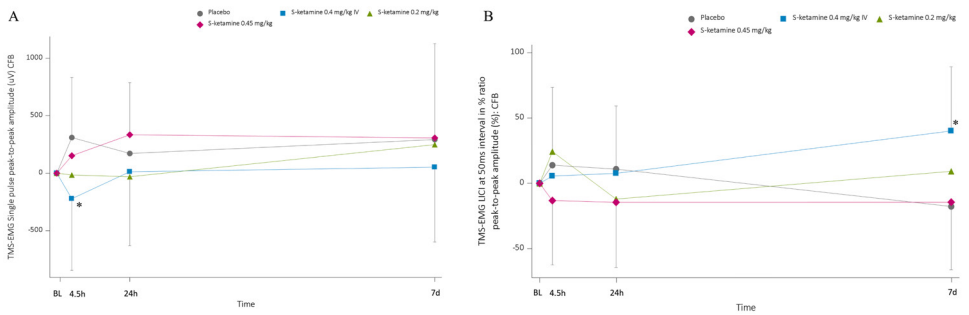
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FIGURE 1 Plasma-concentration (mean ± SD) over time curve.
Average S-ketamine (SKET; in red), S-norketamine (SNOR; in green) and S-hydroxy-norketamine (SHNK; in blue) plasma time-concentration profiles up to 24 hours post-dose for IV-SKET (0.40 mg/kg S-ketamine 40-min IV; top panel), HD-SKET (0.45 mg/kg S-ketamine PO; bottom panel), and LD-SKET (0.20 mg/kg S-ketamine PO; middle panel).



Abbreviations: H = hours; HD-SKET = high-dose oral S-ketamine (0.45 mg/kg S-ketamine PO); IV = intravenous; IV-SKET = 0.40 mg/kg S-ketamine 40-minute intravenous infusion; kg = kilogram; LD-SKET = low-dose oral S-ketamine (0.20 mg/kg S-ketamine PO); mg = milligram; mL = milliliters; ng = nanograms; PO = per os; SD = standard deviation.

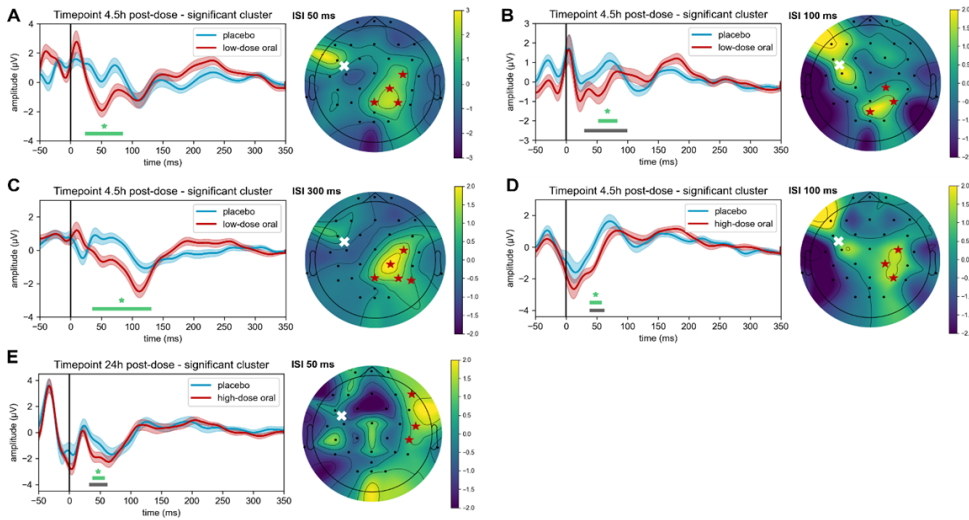
FIGURE 2 Summary graphs of Single-Pulse MEP amplitude and LIC150.
Change from baseline of the least square means (LSM) with 95% confidence interval (95%-CI) as error bars of the (A) motor-evoked potential (MEP) amplitude (μV) and (B) long-interval intracortical inhibition (LIC150) (%), using single- and paired-pulse transcranial magnetic stimulation, for all treatments. IV-SKET corresponds to 0.40 mg/kg S-ketamine 40-min IV (in blue), HD-SKET corresponds to 0.45 mg/kg S-ketamine PO (in pink) and LD-SKET corresponds to 0.20 mg/kg S-ketamine PO (in green). Significant results compared to placebo (in grey) are marked with an asterisk (*).



Abbreviations: 95%-CI = 95% confidence interval; BL = baseline; CFB = change from baseline; HD-SKET = high-dose oral S-ketamine (0.45 mg/kg S-ketamine PO); IV = intravenous; IV-SKET = 0.40 mg/kg S-ketamine 40-minute intravenous infusion; LD-SKET = low-dose oral S-ketamine (0.20 mg/kg S-ketamine PO); LIC150 = long-interval intracortical inhibition at interstimulus-interval of 50 ms; LSM = least square means; MEP = motor-evoked potential.

FIGURE 3 Significant early PPTMS-EEG clusters (N45 & P60) for PO-SKET.

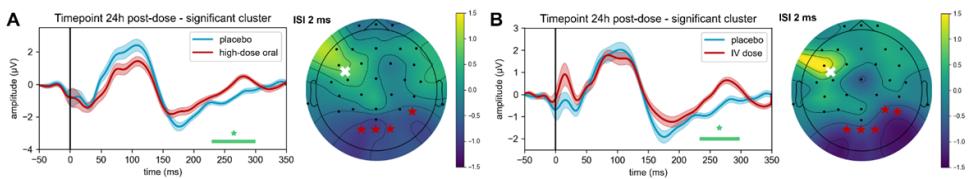
Overview of the significant clusters found when comparing TEPs of the placebo (in blue) and oral S-ketamine (in red) conditions, low-dose oral (0.20 mg/kg S-ketamine PO) or high-dose oral (0.45 mg/kg S-ketamine PO) for PPTMS-EEG (A) ISI 50 ms; low-dose; 4.5 hours post-dose, (B) ISI 100 ms; low-dose; 4.5 hours post-dose, (C) ISI 300 ms; low-dose; 4.5 hours post-dose, (D) ISI 100 ms; high-dose; 4.5 hours post-dose, (E) ISI 300 ms; high-dose; 24 hours post-dose. On the left side, the grand average (mean $\pm$ SEM) over all significant electrodes is presented. On the right side, the amplitude difference in topographical distribution (placebo – S-ketamine) at the time of the cluster is presented. The thick green bar represents the time window of significant differences, the thick grey bar the time window of the entire cluster (including non-significant parts), the white cross the stimulation site, and the black dots the electrode positions with the significant electrodes as red stars.



Abbreviations: ISI = interstimulus interval; ms = millisecond; PPTMS-EEG = paired pulse transcranial magnetic stimulation – encephalography; PO = per os; SEM = standard error of the mean; TEP = TMS evoked potential.

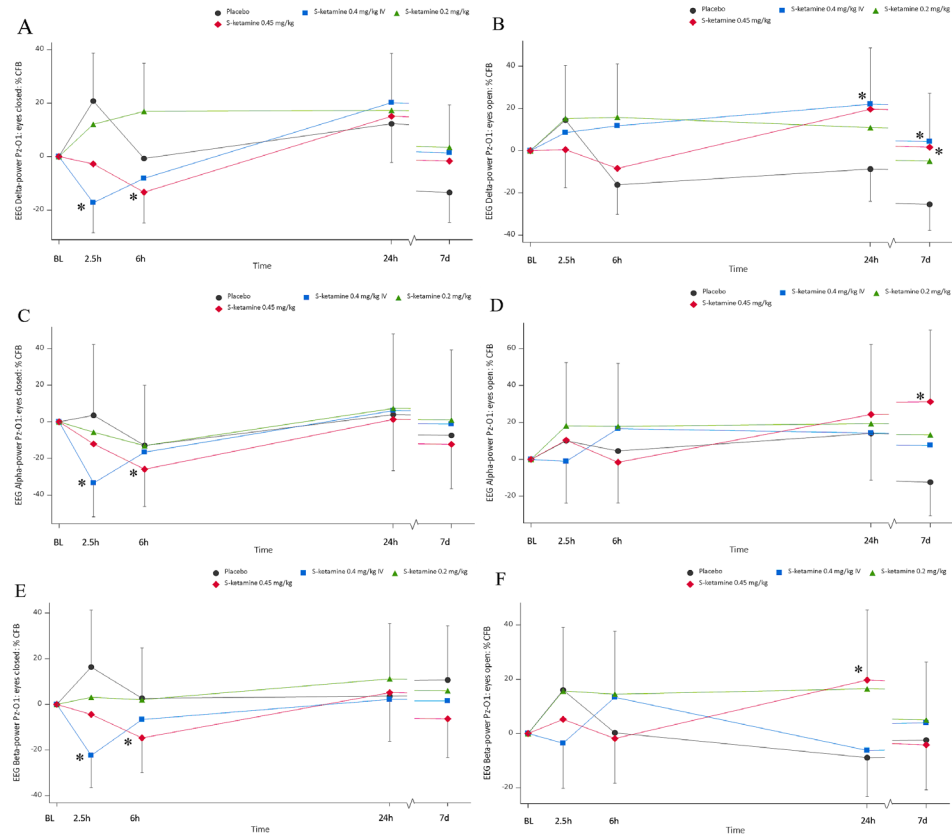
FIGURE 4 Significant late PPTMS-EEG clusters (P180) for IV-SKET and HD-SKET.

Overview of the significant clusters found at timepoint 24 hours post-dose when comparing TEPs of the placebo (in blue) and S-ketamine (in red) conditions for PPTMS-EEG at ISI 2 ms for (A) high-dose oral (0.45 mg/kg S-ketamine PO) or, and (B) IV dose (0.40 mg/kg S-ketamine 40-min IV). On the left side, the grand average (mean $\pm$ SEM) over all significant electrodes is presented. On the right side, the amplitude difference in topographical distribution (placebo – S-ketamine) at the time of the cluster is presented. The thick green bar represents the time window of significant differences, the white cross the stimulation site, and the black dots the electrode positions with the significant electrodes as red stars.



Abbreviations: ISI = interstimulus interval; ms = millisecond; PPTMS-EEG = paired pulse transcranial magnetic stimulation – encephalography; PO = per os; SEM = standard error of the mean; TEP = TMS evoked potential.

FIGURE 5 Summary graphs of significant PEEG results. Change from baseline (%) of the least square means (LSM) with 95% confidence interval (95%-CI) as error bars of PEEG power in the frequency bands delta (δ): 1.5 < 6.0 Hz; alpha (α): 8.5 < 12.5 Hz; and beta (β): 12.5 < 30.0 Hz, for the parieto-occipital lead PzO1 in the eyes-closed condition (A, C, E), and the eyes-open condition (B, D, F) for all treatments at baseline, 2.5H+6H, 24H and 7 days post-dose. IV-SKET corresponds to 0.40 mg/kg S-ketamine 40-min IV (in blue), HD-SKET corresponds to 0.45 mg/kg S-ketamine PO (in pink) and LD-SKET corresponds to 0.20 mg/kg S-ketamine PO (in green). Significant results compared to placebo (in grey) are marked with an asterisk (*).



Abbreviations: 95%-CI = 95% confidence interval; BL = baseline; CFB = change from baseline, D = days; H = hours; HD-SKET = high-dose oral S-ketamine (0.45 mg/kg S-ketamine PO); IV = intravenous; IV-SKET = 0.40 mg/kg S-ketamine 40-minute intravenous infusion; LD-SKET = low-dose oral S-ketamine (0.20 mg/kg S-ketamine PO); LSM = least square means; PEEG = pharmacoelectroencephalography; PO = per os.

CHAPTER II

SUPPLEMENTARY MATERIALS

Full supplementary materials available at the publisher’s website:
<https://bpspubs.onlinelibrary.wiley.com/doi/10.1002/bcp.70649>
 Selected supplementary text, figures and tables are provided next.

TABLE S1 Summary of TMS-EMG results: all treatments versus placebo.

Estimated difference of IV-SKET (0.40 mg/kg S-ketamine 40-min IV), HD-SKET (0.45 mg/kg S-ketamine PO), and LD-SKET (0.20 mg/kg S-ketamine PO) versus placebo for single-pulse and paired-pulse TMS-EMG parameters, measured at the timepoints 4.5H (acute), 24H (delayed) and 7D (sustained) post-dose, with 95% confidence interval (95%-CI) and p-value. Significant p-values are indicated in bold.

Timepoint/ Treatment	Statistic	MEP (μV)	RMT (%)	SICI ₂ (%)	LICI ₅₀ (%)	LICI ₁₀₀ (%)	LICI ₃₀₀ (%)
4.5H: ACUTE							
IV-SKET	ED	-430.17	0.03	1.35	-8.33	-4.59	-17.58
	95%-CI	(-806.74, -53.61)	(-2.38, 2.45)	(-23.12, 25.81)	(-53.79, 37.12)	(-29.50, 20.32)	(-42.83, 7.67)
	p-value	p = 0.03	p = 0.98	p = 0.91	p = 0.72	p = 0.72	p = 0.17
HD-SKET	ED	-305.9	0.21	7.91	10.37	-11.35	-11.06
	95%-CI	(-670.42, 58.62)	(-2.21, 2.62)	(-16.10, 31.9)	(-34.10, 54.84)	(-35.78, 13.08)	(-35.39, 13.28)
	p-value	p = 0.10	p = 0.86	p = 0.52	p = 0.65	p = 0.36	p = 0.37
LD-SKET	ED	-167.62	0.08	-0.31	-27.12	-3.62	-2.13
	95%-CI	(-531.69, 196.44)	(-2.33, 2.49)	(-24.36, 23.75)	(-71.91, 17.67)	(-27.93, 20.69)	(-26.76, 22.50)
	p-value	p = 0.36	p = 0.95	p = 0.98	p = 0.23	p = 0.77	p = 0.86
24H: DELAYED							
IV-SKET	ED	-32.02	0.34	0.4	-3.24	14.5	7.06
	95%-CI	(-394.06, 330.08)	(-2.75, 2.08)	(-23.19, 23.98)	(-46.84, 40.36)	(-9.48, 38.48)	(-17.36, 31.49)
	p-value	p = 0.86	p = 0.78	p = 0.97	p = 0.88	p = 0.23	p = 0.57
HD-SKET	ED	-157.2	-0.1	10.34	-22.95	-2.37	-7.67
	95%-CI	(-515.22, 200.81)	(-2.51, 2.32)	(-13.24, 33.92)	(-66.54, 20.65)	(-26.37, 21.64)	(-31.62, 16.29)
	p-value	p = 0.39	p = 0.93	p = 0.39	p = 0.30	p = 0.85	p = 0.53
LD-SKET	ED	142.13	1.16	1.81	25.41	-0.28	-0.72
	95%-CI	(-222.63, 506.90)	(-1.29, 3.60)	(-22.35, 25.96)	(-70.15, 19.34)	(-24.63, 24.08)	(-25.47, 24.02)
	p-value	p = 0.44	p = 0.34	p = 0.88	p = 0.26	p = 0.98	p = 0.95
7D: SUSTAINED							
IV-SKET	ED	-112.53	-0.74	9.59	57.83	-5.93	6.79
	95%-CI	(-474.57, 249.51)	(-3.16, 1.67)	(-13.99, 33.18)	(14.24, 101.43)	(-29.91, 18.05)	(-17.64, 31.21)
	p-value	p = 0.54	p = 0.53	p = 0.42	p<0.01	p = 0.63	p = 0.58
HD-SKET	ED	1.94	0.81	6.29	26.97	9.86	20.44
	95%-CI	(-356.07, 359.96)	(-1.60, 3.22)	(-17.29, 29.88)	(-16.63, 70.57)	(-14.15, 33.86)	(-3.52, 44.39)
	p-value	p = 0.99	p = 0.49	p = 0.60	p = 0.22	p = 0.42	p = 0.09
LD-SKET	ED	29.49	1.56	3.68	3.31	-18.56	10.48
	95%-CI	(-335.28, 394.26)	(-0.89, 4.00)	(-20.47, 27.83)	(-41.43, 48.05)	(-42.91, 5.80)	(-14.26, 35.22)
	p-value	p = 0.87	p = 0.20	p = 0.76	p = 0.88	p = 0.13	p = 0.40

Abbreviations: HD-SKET = high-dose oral S-ketamine (0.45 mg/kg S-ketamine PO); IV = intravenous; IV-SKET = 0.40 mg/kg S-ketamine 40-minute intravenous infusion; LD-SKET = low-dose oral S-ketamine (0.20 mg/kg S-ketamine PO); LICI₅₀ = long-interval intracortical inhibition at interstimulus-interval of 50 ms; LICI₁₀₀ = long-interval intracortical inhibition at interstimulus-interval of 100 ms; LICI₃₀₀ = long-interval intracortical inhibition at interstimulus-interval of 300 ms; MEP = motor evoked potential; RMT = resting motor threshold; SICI₂ = short-interval intracortical inhibition at interstimulus-interval of 2 ms; TMS-EMG = transcranial magnetic stimulation combined with electromyography.

TABLE S2 Summary of PEEG results in the eyes-closed condition: all treatments versus placebo.

Estimated difference of IV-SKET (0.40 mg/kg S-ketamine 40-min IV), HD-SKET (0.45 mg/kg S-ketamine PO), and LD-SKET (0.20 mg/kg S-ketamine PO) versus placebo, measured at the timepoints 2.5H+6H combined (acute), 24H (delayed) and 7D (sustained) post-dose, for PEEG parameters in the eyes-closed condition reporting in %, with 95% confidence interval (95%-CI) and p-values of the total power (μV²), pEEG spectral power are reported per frequency band (delta (δ): 1.5 < 6.0 Hz; theta (θ): 6.0 < 8.5 Hz; alpha (α): 8.5 < 12.5 Hz; beta (β): 12.5 < 30.0 Hz; and gamma (γ): 30.0 < 40.0 Hz), and electrode of interest FZ-CZ, PZ-O1, and PZ-O2, and are abbreviated to e.g. δFZCZ for delta power in FZ-CZ. Statistically significant (p < 0.05) results are marked in bold.

Timepoint/ Treatment	Statistic	δFzCz	δPzO1	δPzO2	θFzCz	θPzO1	θPzO2	αFzCz	αPzO1	αPzO2	βFzCz	βPzO1	βPzO2	γFzCz	γPzO1	γPzO2
2.5H AND 6H: ACUTE																
IV-SKET	ED	-0.1	-0.2	-0.2	0.0	-0.2	-0.2	-0.1	-0.2	-0.3	-0.1	-0.2	-0.2	0.0	-0.1	-0.1
	95%-CI	(-23.4, 3.2)	(-32.8, -5.7)	(-36.4, -10.0)	(-19.4, 16.9)	(-34.0, 2.9)	(-35.5, 0.4)	(-28.6, 1.4)	(-33.1, -8.0)	(-37.2, -11.4)	(-20.2, 3.8)	(-32.1, -10.6)	(-32.5, -12.0)	(-13.7, 8.0)	(-25.5, 17.4)	(-28.1, 14.8)
HD-SKET	p-value	p=0.12	p<0.01	p<0.01	p=0.75	p=0.09	p=0.05	p=0.07	p<0.01	p<0.01	p=0.16	p<0.01	p<0.01	p=0.53	p=0.56	p=0.42
	ED	0.0	-0.2	-0.1	0.0	-0.2	-0.1	-0.1	-0.2	-0.1	-0.1	-0.2	-0.1	0.0	-0.1	-0.1
LD-SKET	95%-CI	(-17.3, 11.4)	(-29.2, -0.8)	(-24.9, 6.0)	(-14.1, 24.5)	(-34.5, 1.9)	(-27.9, 11.6)	(-20.9, 12.3)	(-27.2, -0.7)	(-26.0, 4.0)	(-20.3, 3.6)	(-27.7, -5.5)	(-23.3, -0.2)	(-9.0, 13.7)	(-28.9, 11.3)	(-26.4, 17.2)
	p-value	p=0.58	p=0.04	p=0.19	p=0.72	p=0.07	p=0.32	p=0.50	p=0.04	p=0.13	p=0.15	p<0.01	p=0.05	p=0.76	p=0.30	p=0.53
IV-SKET	ED	0.1	0.0	0.0	0.2	0.0	0.0	0.0	0.0	-0.1	0.0	-0.1	-0.1	0.0	-0.1	-0.1
	95%-CI	(-8.5, 23.4)	(-11.7, 23.8)	(-18.2, 15.5)	(-4.2, 38.9)	(-20.6, 23.7)	(-23.4, 18.7)	(-15.8, 19.5)	(-18.3, 11.4)	(-21.3, 10.7)	(-11.7, 14.7)	(-17.9, 7.3)	(-18.8, 5.7)	(-7.7, 15.4)	(-29.5, 10.2)	(-27.3, 15.5)
HD-SKET	p-value	p=0.42	p=0.60	p=0.74	p=0.12	p=0.94	p=0.66	p=0.97	p=0.55	p=0.42	p=0.93	p=0.35	p=0.25	p=0.57	p=0.27	p=0.46
	ED	0.0	0.1	0.0	-0.1	0.0	0.0	0.0	0.0	0.0	-0.1	0.0	0.1	0.0	0.1	0.2
LD-SKET	95%-CI	(-15.5, 19.8)	(-11.6, 29.5)	(-20.1, 17.2)	(-27.1, 12.3)	(-24.3, 24.0)	(-25.2, 21.9)	(-21.3, 20.6)	(-17.8, 26.9)	(-16.8, 31.7)	(-18.9, 10.9)	(-17.7, 18.3)	(-10.9, 33.8)	(-11.2, 15.6)	(-18.9, 53.4)	(-9.6, 69.6)
	p-value	p=0.95	p=0.49	p=0.73	p=0.36	p=0.80	p=0.70	p=0.81	p=0.85	p=0.70	p=0.51	p=0.88	p=0.55	p=0.85	p=0.50	p=0.18
IV-SKET	ED	0.1	0.0	-0.1	0.0	-0.1	-0.1	0.1	0.0	-0.1	0.1	0.0	-0.1	0.1	0.1	0.1
	95%-CI	(-8.3, 30.8)	(-15.5, 24.3)	(-22.8, 13.7)	(-17.2, 28.4)	(-27.6, 19.3)	(-27.5, 18.7)	(-10.0, 39.2)	(-21.8, 21.6)	(-32.6, 7.7)	(-10.0, 23.8)	(-15.5, 21.9)	(-21.2, 10.2)	(-6.6, 22.1)	(-21.9, 49.3)	(-23.5, 45.5)
HD-SKET	p-value	p=0.31	p=0.80	p=0.51	p=0.78	p=0.56	p=0.54	p=0.31	p=0.82	p=0.18	p=0.50	p=0.87	p=0.40	p=0.33	p=0.64	p=0.74
	ED	0.0	0.0	0.0	-0.1	0.0	-0.1	0.0	0.0	-0.1	0.0	0.1	-0.1	0.1	0.2	0.0
LD-SKET	95%-CI	(-17.7, 16.9)	(-13.7, 26.5)	(-20.5, 16.7)	(-30.1, 7.6)	(-24.0, 24.5)	(-33.0, 9.0)	(-16.0, 28.9)	(-16.8, 28.2)	(-31.4, 8.7)	(-14.2, 17.3)	(-10.3, 28.4)	(-20.1, 11.1)	(-7.6, 20.4)	(-13.9, 62.5)	(-23.5, 43.6)
	p-value	p=0.83	p=0.65	p=0.70	p=0.19	p=0.83	p=0.20	p=0.71	p=0.77	p=0.21	p=0.97	p=0.44	p=0.47	p=0.43	p=0.30	p=0.77

Timepoint/ Treatment		8FzCz	8PzO1	8PzO2	0FzCz	0PzO1	0PzO2	aFzCz	aPzO1	aPzO2	βFzCz	βPzO1	βPzO2	γFzCz	γPzO1	γPzO2
7D: SUSTAINED																
IV-SKET	ED	0.0	0.2	0.1	0.2	0.1	0.0	-0.1	0.1	0.0	-0.1	-0.1	-0.1	0.0	-0.1	-0.2
	95%-CI	(-12.5, 24.1)	(-3.2, 41.8)	(-12.4, 28.4)	(-5.5, 45.5)	(-12.5, 43.4)	(-19.6, 31.0)	(-30.0, 7.3)	(-14.1, 32.6)	(-21.3, 24.5)	(-21.0, 8.0)	(-23.5, 10.0)	(-24.7, 4.7)	(-15.0, 10.6)	(-35.2, 22.5)	(-44.8, 3.6)
HD-SKET	p-value	p=0.64	p=0.10	p=0.54	p=0.15	p=0.36	p=0.83	p=0.19	p=0.56	p=0.93	p=0.32	p=0.35	p=0.15	p=0.64	p=0.48	p=0.08
	ED	0.2	0.1	0.1	0.3	0.0	-0.1	0.1	-0.1	-0.1	0.0	-0.2	-0.1	0.0	-0.1	0.0
LD-SKET	95%-CI	(0.1, 42.7)	(-6.4, 37.8)	(-10.2, 32.3)	(6.4, 65.0)	(-22.2, 28.1)	(-26.4, 20.5)	(-8.4, 41.6)	(-24.0, 18.1)	(-28.5, 14.1)	(-13.1, 19.6)	(-29.5, 1.6)	(-27.4, 1.4)	(-9.9, 17.8)	(-36.1, 22.1)	(-29.6, 33.9)
	p-value	p=0.05	p=0.19	p=0.38	p=0.01	p=0.99	p=0.63	p=0.24	p=0.65	p=0.39	p=0.81	p=0.07	p=0.07	p=0.66	p=0.45	p=0.86
LD-SKET	ED	0.1	0.2	0.1	0.3	0.2	0.1	0.2	0.1	0.0	0.1	0.0	-0.1	0.1	0.0	0.1
	95%-CI	(-4.7, 35.2)	(-13.3, 44.6)	(-7.2, 36.1)	(5.9, 63.0)	(-6.5, 53.2)	(-24.1, 39.9)	(-12.5, 51.5)	(-12.1, 35.3)	(-24.3, 19.9)	(-5.8, 28.9)	(-20.0, 14.6)	(-20.4, 10.7)	(-4.0, 35.0)	(-24.5, 42.3)	(-23.3, 44.0)
LD-SKET	p-value	p=0.15	p=0.07	p=0.23	p=0.01	p=0.15	p=0.45	p=0.06	p=0.43	p=0.68	p=0.22	p=0.63	p=0.45	p=0.17	p=0.82	p=0.75

Abbreviations: HD-SKET=high-dose oral S-ketamine (0.45 mg/kg S-ketamine PO); IV=intravenous; LD-SKET = low-dose oral S-ketamine (0.20 mg/kg S-ketamine PO); pEEG = pharmaco-electroencephalography, PO = per os.

TABLE S3 Summary of pEEG results in the eyes-open condition: all treatments versus placebo. Estimated difference of IV-SKET (0.40 mg/kg S-ketamine 40-min IV), HD-SKET (0.45 mg/kg S-ketamine PO), and LD-SKET (0.20 mg/kg S-ketamine PO) versus placebo, measured at the timepoints 2.5H+6H combined (acute), 24H (delayed) and 7D (sustained) post-dose, for pEEG parameters in the eyes-open condition reporting in %, with 95% confidence interval (95%-CI) and p-values of the total power (μV²). pEEG spectral power are reported per frequency band (delta (δ): 1.5 < 6.0 Hz; theta (θ): 6.0 < 8.5 Hz; alpha (α): 8.5 < 12.5 Hz; beta (β): 12.5 < 30.0 Hz; and gamma (γ): 30.0 < 40.0 Hz), and electrode of interest FZ-CZ, PZ-O1, and PZ-O2, and are abbreviated to e.g. δFZCZ for delta power in FZ-CZ. Statistically significant (p < 0.05) results are marked in bold. Significant increases in power are highlighted in green. Significant decreases in power are highlighted in orange.

Timepoint/ Treatment		8FzCz	8PzO1	8PzO2	0FzCz	0PzO1	0PzO2	aFzCz	aPzO1	aPzO2	βFzCz	βPzO1	βPzO2	γFzCz	γPzO1	γPzO2
2.5H+6H: ACUTE																
IV-SKET	ED	0.0	0.1	0.0	0.0	0.2	0.2	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1
	95%-CI	(-12.7, 13.5)	(-11.7, 43.1)	(-18.8, 34.2)	(-13.5, 26.6)	(-9.8, 63.2)	(-11.5, 63.3)	(-15.5, 20.7)	(-27.0, 37.4)	(-25.2, 34.8)	(-9.1, 32.6)	(-22.7, 21.6)	(-16.5, 19.9)	(-13.2, 12.1)	(-23.4, 30.6)	(-21.4, 44.6)
HD-SKET	p-value	p=0.94	p=0.33	p=0.73	p=0.64	p=0.19	p=0.23	p=0.92	p=0.99	p=0.98	p=0.32	p=0.78	p=1.00	p=0.83	p=1.00	p=0.68
	ED	-0.1	0.0	0.1	0.1	0.0	0.1	0.0	0.0	0.1	0.1	-0.1	-0.1	0.0	-0.1	-0.1
HD-SKET	95%-CI	(-18.4, 7.4)	(-23.2, 24.8)	(-24.6, 24.8)	(-12.4, 28.5)	(-25.3, 35.1)	(-19.4, 48.9)	(-15.3, 22.1)	(-29.1, 33.2)	(-19.7, 44.8)	(-10.8, 30.3)	(-24.8, 18.0)	(-21.3, 13.1)	(-12.4, 13.1)	(-28.7, 20.4)	(-32.6, 23.9)
	p-value	p=0.34	p=0.86	p=0.81	p=0.54	p=0.98	p=0.55	p=0.85	p=0.86	p=0.61	p=0.43	p=0.60	p=0.52	p=0.94	p=0.57	p=0.56

Timepoint/ Treatment		8FzCz	8PzO1	8PzO2	0FzCz	0PzO1	0PzO2	aFzCz	aPzO1	aPzO2	βFzCz	βPzO1	βPzO2	γFzCz	γPzO1	γPzO2
24H: DELAYED																
LD-SKET	ED	0.0	0.2	0.1	0.0	0.2	0.2	0.0	0.1	0.1	0.1	0.1	0.0	0.0	-0.1	-0.1
	95%-CI	(-11.0, 27.8)	(2.2, 74.7)	(-15.0, 40.6)	(-14.2, 23.4)	(-9.0, 64.7)	(-11.0, 64.5)	(-13.6, 23.2)	(-19.8, 51.1)	(-19.8, 44.7)	(-4.9, 38.6)	(-14.9, 33.8)	(-13.7, 23.7)	(-12.7, 12.7)	(-32.6, 12.9)	(-31.0, 26.4)
HD-SKET	p-value	p=0.56	p=0.18	p=0.48	p=0.75	p=0.18	p=0.22	p=0.73	p=0.55	p=0.61	p=0.15	p=0.57	p=0.71	p=0.90	p=0.30	p=0.65
	ED	0.1	0.3	0.2	0.1	0.2	0.2	-0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.2
LD-SKET	95%-CI	(-11.0, 29.7)	(-0.7, 72.8)	(-18.6, 44.4)	(-8.5, 50.9)	(-11.7, 70.5)	(-17.3, 62.7)	(-25.7, 17.2)	(-29.7, 42.8)	(-30.8, 33.1)	(-16.4, 28.3)	(-20.8, 33.8)	(-7.5, 42.1)	(-12.4, 19.1)	(-28.5, 46.0)	(-20.7, 76.3)
	p-value	p=0.48	p=0.03	p=0.15	p=0.28	p=0.39	p=0.40	p=0.55	p=0.99	p=0.80	p=0.75	p=0.83	p=0.21	p=0.78	p=0.91	p=0.41
HD-SKET	ED	0.1	0.3	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.3	0.2	0.0	0.3	0.1
	95%-CI	(-12.0, 29.7)	(-0.7, 72.8)	(-18.6, 44.4)	(-8.5, 50.9)	(-11.7, 70.5)	(-17.3, 62.7)	(-25.7, 17.2)	(-29.7, 42.8)	(-30.8, 33.1)	(-16.4, 28.3)	(-20.8, 33.8)	(-7.5, 42.1)	(-12.4, 19.1)	(-28.5, 46.0)	(-20.7, 76.3)
LD-SKET	p-value	p=0.50	p=0.06	p=0.58	p=0.20	p=0.21	p=0.39	p=0.51	p=0.64	p=0.57	p=0.09	p=0.05	p=0.21	p=0.80	p=0.17	p=0.77
	ED	0.1	0.2	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.1	0.3	0.1	0.0	0.3	0.1
LD-SKET	95%-CI	(-12.1, 26.7)	(-7.1, 59.0)	(-18.8, 41.8)	(-20.7, 26.5)	(-21.1, 50.3)	(-23.1, 49.6)	(-19.3, 27.4)	(-26.7, 49.3)	(-27.4, 40.0)	(-13.1, 33.7)	(-17.7, 66.3)	(-7.6, 42.1)	(-18.0, 11.5)	(-5.5, 91.6)	(-25.7, 65.3)
	p-value	p=0.56	p=0.15	p=0.61	p=0.99	p=0.60	p=0.67	p=0.90	p=0.80	p=0.96	p=0.49	p=0.07	p=0.21	p=0.57	p=0.10	p=0.61

Timepoint/ Treatment		8FzCz	8PzO1	8PzO2	0FzCz	0PzO1	0PzO2	aFzCz	aPzO1	aPzO2	βFzCz	βPzO1	βPzO2	γFzCz	γPzO1	γPzO2
7D: SUSTAINED																
IV-SKET	ED	0.1	0.4	0.2	0.3	0.6	0.4	-0.1	0.2	0.3	0.1	0.1	0.1	0.0	-0.1	-0.1
	95%-CI	(-8.0, 32.0)	(7.0, 82.7)	(-9.3, 58.0)	(-14.4, 58.8)	(13.4, 115.3)	(2.8, 99.0)	(-25.0, 18.3)	(-13.8, 74.9)	(-5.1, 82.3)	(-8.7, 40.1)	(-18.0, 38.4)	(-14.8, 30.8)	(-11.0, 20.9)	(-34.8, 32.8)	(-42.2, 28.5)
HD-SKET	p-value	p=0.29	p=0.02	p=0.20	p=0.06	p=0.01	p=0.03	p=0.60	p=0.25	p=0.10	p=0.26	p=0.63	p=0.61	p=0.63	p=0.69	p=0.46
	ED	0.2	0.4	0.2	0.3	0.3	0.3	0.2	0.5	0.4	0.3	0.0	0.1	0.1	-0.2	-0.1
HD-SKET	95%-CI	(1.8, 48.9)	(3.7, 79.0)	(-8.9, 60.2)	(2.0, 66.1)	(-3.1, 85.4)	(-5.0, 85.4)	(-2.2, 57.0)	(4.6, 114.2)	(3.8, 101.4)	(1.1, 56.7)	(-24.7, 28.1)	(-15.5, 30.8)	(-8.2, 25.6)	(-46.7, 9.5)	(-41.4, 32.6)
	p-value	p=0.03	p=0.03	p=0.19	p=0.03	p=0.08	p=0.10	p=0.08	p=0.03	p=0.03	p=0.04	p=0.89	p=0.65	p=0.37	p=0.14	p=0.54
LD-SKET	ED	0.1	0.3	0.1	0.1	0.4	0.3	0.0	0.3	0.2	0.2	0.1	0.1	0.1	0.0	0.0
	95%-CI	(-10.3, 29.3)	(-2.5, 66.7)	(-15.5, 47.5)	(-15.1, 35.1)	(-16.8, 88.9)	(-6.5, 88.9)	(-16.8, 31.4)	(-9.3, 84.4)	(-10.0, 73.4)	(-3.8, 47.7)	(-17.2, 40.0)	(-9.8, 38.7)	(-9.4, 23.1)	(-27.7, 46.3)	(-31.4, 52.5)
LD-SKET	p-value	p=0.43	p=0.08	p=0.43	p=0.56	p=0.06	p=0.12	p=0.70	p=0.15	p=0.18	p=0.11	p=0.38	p=0.30	p=0.48	p=0.88	p=0.91

Abbreviations: HD-SKET = high-dose oral S-ketamine (0.45 mg/kg S-ketamine PO); IV = intravenous; LD-SKET = low-dose oral S-ketamine (0.20 mg/kg S-ketamine PO); pEEG = pharmaco-electroencephalography, PO = per os.

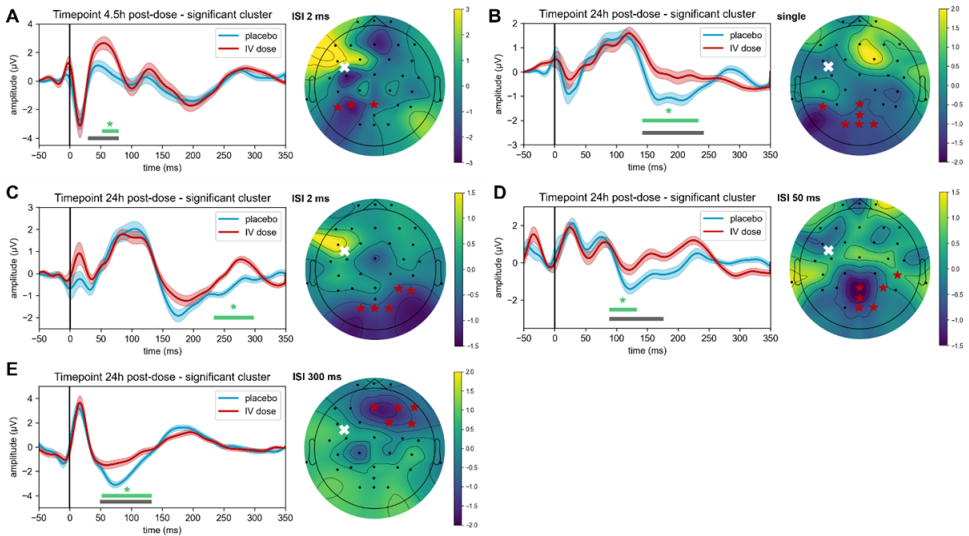
TABLE S4 Concentration-effect relationships pharmacodynamic parameters with significant treatment effects. Results of concentration-effect relationship analysis for PEEG delta power eyes-closed at channels PZ-O1 and PZ-O2, and LIC150. For the acute phase, data from plasma concentrations of S-ketamine, S-norketamine, S-hydroxynorketamine and placebo were included. For the sustained phase, the AUC_{last} from Day 1 was matched with the pharmacodynamic endpoints observed at 7 days post-dose. Fixed effects, relative standard error and the p-value compared to the intercept model are reported.

ACUTE PHASE (UP TO 6.5H)				
Analyte	Parameter	Baseline (RSE)	Slope (RSE)	p-value
S-ketamine	DPZO1C	11.76 (8.3%)	-0.019 (34.5%)	0.004
S-ketamine	DPZO2C	11.84 (0.1%)	-0.022 (24.6%)	<0.001
S-norketamine	DPZO1C	11.78 (8.2%)	-0.006 (43.9%)	0.037
S-norketamine	DPZO2C	11.84 (9.0%)	-0.006 (45.1%)	0.031
SUSTAINED PHASE (DAY 7)				
Analyte	Parameter	Baseline (RSE)	Slope (RSE)	p-value
S-ketamine	LIC150	111.51 (22.1%)	0.128 (48.0%)	0.042

Abbreviations: AUC_{last} = area under the concentration–time curve from time zero to the last quantifiable concentration; DPZO1C = delta power in PZ-O1 in eyes closed condition; DPZO2C = delta power in PZ-O2 in eyes closed condition; H = hours; PEEG = pharmacoelectroencephalography; RSE = relative standard error.

FIGURE S1 Significant TMS-EEG clusters for IV-SKET versus placebo.

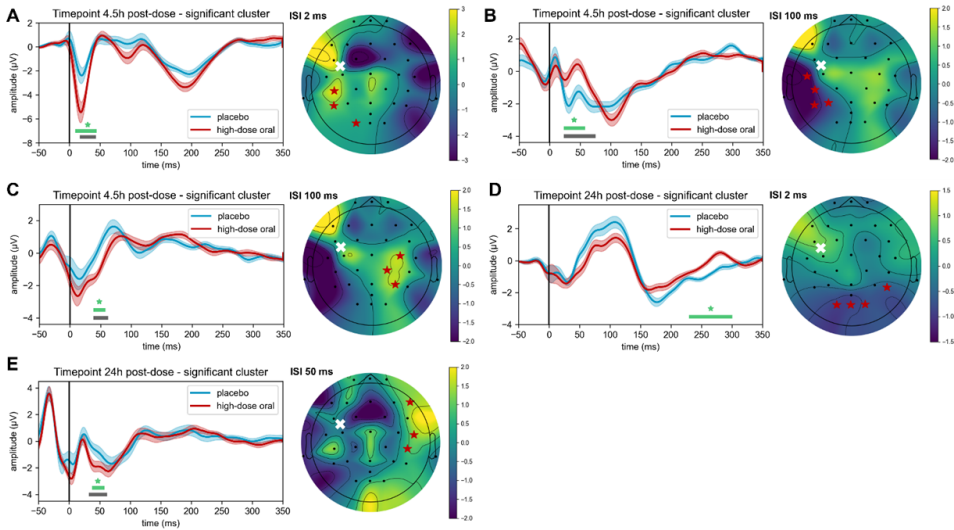
Overview of the significant cluster found when comparing TEPs of the placebo (in blue) and IV-SKET (0.40 mg/kg S-ketamine 40-min IV) (in red) conditions for A) PPTMS-EEG at ISI 2 ms at timepoint 4.5 hours post-dose, B) SPTMS-EEG at timepoint 24 hours post-dose, C) PPTMS-EEG at ISI 2 ms at timepoint 24 hours post-dose, D) PPTMS-EEG at ISI 50 ms at timepoint 24 hours post-dose, and E) ISI 300 ms at timepoint 24 hours post-dose. On the left side, the grand average (mean ± SEM) over all significant electrodes is presented. On the right side, the amplitude difference in topographical distribution (placebo – IV dose) at the time of the cluster is presented. The thick green bar represents the time window of significant differences, the thick grey bar the time window of the entire cluster (including non-significant parts), the white cross the stimulation site, and the black dots the electrodepositions with the significant electrodes as red stars.



Abbreviations: IV = intravenous; IV-SKET = 0.40 mg/kg S-ketamine 40-minute intravenous infusion; PPTMS-EEG = paired-pulse transcranial magnetic stimulation – encephalography; ISI = interstimulus interval; SEM = standard error of the mean; ms = millisecond.

FIGURE S2 Significant TMS-EEG clusters for HD-SKET versus placebo.

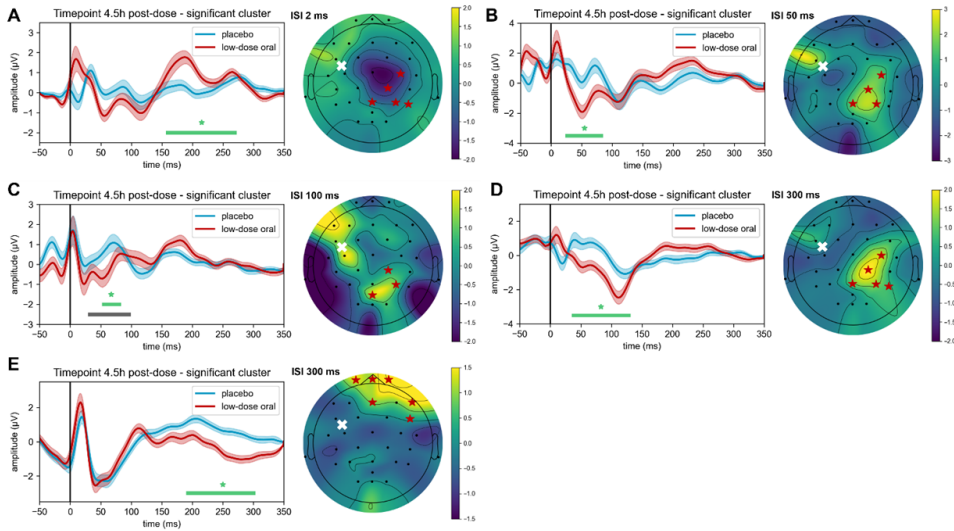
Overview of the significant cluster found when comparing TEPS of the placebo (in blue) and HD-SKET (high-dose oral 0.45 mg/kg S-ketamine) (in red) conditions for PPTMS-EEG A) ISI 2 ms at timepoint 4.5 hours post-dose, B-C) ISI 100 ms at timepoint 4.5 hours post-dose, D) ISI 2 ms at timepoint 24 hours post-dose, and E) ISI 50 ms at timepoint 24 hours post-dose. On the left side, the grand average (mean $\pm$ SEM) over all significant electrodes is presented. On the right side, the amplitude difference in topographical distribution (placebo – high-dose oral) at the time of the cluster is presented. The thick green bar represents the time window of significant differences, the thick grey bar the time window of the entire cluster (including non-significant parts), the white cross the stimulation site, and the black dots the electrodepositions with the significant electrodes as red stars.



Abbreviations: HD-SKET = high-dose oral S-ketamine (0.45 mg/kg S-ketamine PO); PO = per os; PPTMS-EEG = paired-pulse transcranial magnetic stimulation – encephalography; ISI = interstimulus interval; SEM = standard error of the mean; ms = millisecond.

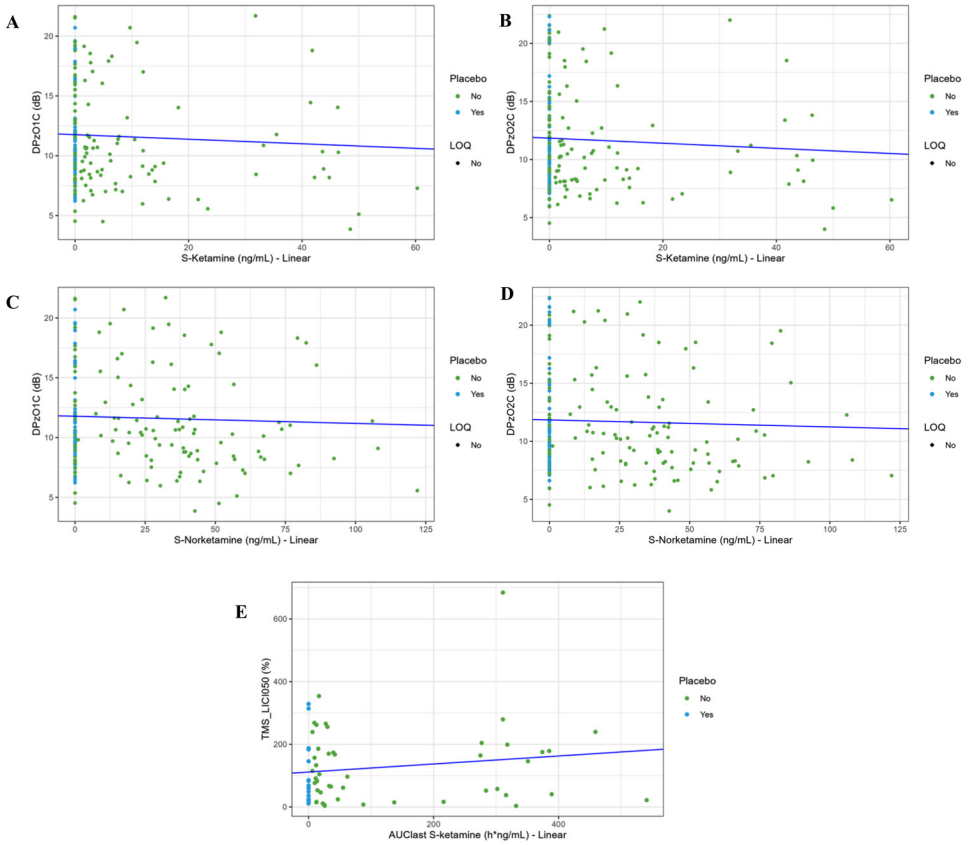
FIGURE S3 Significant TMS-EEG clusters for LD-SKET versus placebo.

Overview of all significant cluster found at timepoint 4.5 hours post-dose when comparing TEPS of the placebo (in blue) and LD-SKET (low-dose oral 0.20 mg/kg S-ketamine) (in red) conditions for PPTMS-EEG A) ISI 2 ms, B) ISI 50 ms, C) ISI 100 ms, and D-E) ISI 300 ms. On the left side, the grand average (mean $\pm$ SEM) over all significant electrodes is presented. On the right side, the amplitude difference in topographical distribution (placebo – low-dose oral) at the time of the cluster is presented. The thick green bar represents the time window of significant differences, the thick grey bar the time window of the entire cluster (including non-significant parts), the white cross the stimulation site, and the black dots the electrodepositions with the significant electrodes as red stars.



Abbreviations: LD-SKET = low-dose oral S-ketamine (0.20 mg/kg S-ketamine PO); PO = per os; PPTMS-EEG = paired-pulse transcranial magnetic stimulation – encephalography; ISI = interstimulus interval; SEM = standard error of the mean; ms = millisecond.

FIGURE S4 Concentration-effect relationship graphs. Graphical representation of the concentration-effect relationship between S-ketamine (A and B) and S-norketamine (C and D) and delta power in the eyes-closed condition at channels PZ-O1 (A and C) and PZ-O2 (C and D). S-ketamine AUC_{last}-effect relationship on Day 7 for LIC150 (E).



Abbreviations: AUC_{last} = area under the concentration–time curve from time zero to the last quantifiable concentration; dB = decibel; DPZ-O1C = delta power in PZ-O1 in eyes closed condition; DPZ-O2C = delta power in PZ-O2 in eyes closed condition; h = hours; LOQ = limit of quantification; PEEG = pharmaco-electroencephalography; RSE = relative standard error.

CHAPTER III

TRANSCRANIAL MAGNETIC STIMULATION AS A TRANSLATIONAL BIOMARKER IN EARLY-PHASE ANTI-SEIZURE MEDICATION DEVELOPMENT: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED STUDY IN GENERALIZED EPILEPSY

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ABSTRACT

One-third of epilepsy patients remain treatment-resistant, underscoring the need for novel anti-seizure medications (ASM) and reliable biomarkers of central target engagement. Cortical hyperexcitability is a hallmark of epilepsy, making excitability a valuable pharmacodynamic biomarker for early-phase drug development supporting go/no-go-decision-making. Building upon prior Transcranial Magnetic Stimulation (TMS) work in healthy participants, this study aimed to establish TMS-derived endpoints as translational biomarkers of ASM effects in patients. In a randomized, double-blind, placebo-controlled, two-way cross-over design, patients with generalized epilepsy on levetiracetam (500 mg twice daily; n=14) or valproic acid (≤ 1000 mg/day; levetiracetam-naïve, n=12) received a single oral dose of levetiracetam (2000 mg) or placebo. Participants were asked to refrain from their morning ASM dose to minimize background ASM levels while maintaining seizure protection. TMS combined with electromyography (EMG) and electroencephalography (EEG), and resting-state EEG were performed pre-dose, 1.5 hours, and 3 hours post-dose. Outcomes were analyzed using mixed-effects analysis of covariance or cluster-based permutation testing. Levetiracetam significantly reduced motor evoked potential (MEP) amplitude, enhanced long-interval intracortical inhibition (LICI), and increased frontal gamma and beta power, with larger effect sizes in levetiracetam-naïve patients. These findings were further supported by linear concentration–response relationships for these endpoints. TMS-EEG revealed group-specific modulation: early TMS-evoked potential components (P30/N45) in levetiracetam patients versus later components (N100/P180) in levetiracetam-naïve patients. These findings build upon prior results, demonstrating that TMS-based biomarkers are sensitive to levetiracetam's acute effects in epilepsy patients with MEP amplitude, LICI, and frontal beta/gamma power emerging as promising biomarkers, possible also for new ASM development.

INTRODUCTION

Epilepsy affects ~50 million people worldwide.¹ One-third of patients remain treatment-resistant,² highlighting the urgent need for novel, targeted therapeutic strategies. In anti-seizure medication (ASM) development, penetration of the active compound or its metabolite into the central nervous system (CNS) is essential. However, reliable mechanistic biomarkers that not only demonstrate target engagement but also provide proof of mechanism are lacking. This gap contributes to the high failure rate of drugs in development for neuropsychiatric disorders.^{3,4} Pharmacodynamic (PD) biomarkers that demonstrate direct target engagement are crucial to support go/no-go decision-making in early-stage development. Additionally, capturing distinct drug-induced modulation profiles on a neurocircuitry level may help identify and inform candidate compound selection within the ASM pipeline. Pharmacoelectroencephalography (PEEG) is a relatively easy and accessible method to measure the effects of pharmacological agents on resting-state brain activity. It is widely employed to quantify drug-induced CNS modulation, demonstrate target engagement, and characterize dose–response relationships.^{5,6} PEEG is sensitive to many drugs with diverse mechanisms of action (MOA),⁵ but might be less sensitive to effects that only emerge during active neurocircuitry engagement.⁴ Transcranial Magnetic Stimulation (TMS) is a noninvasive brain stimulation technique that engages neurocircuitry by inducing brief magnetic pulses in specific brain regions.⁷ Combined with electromyography (-EMG) and electroencephalography (-EEG), TMS assesses corticospinal and cortical excitability, respectively, and can probe mechanisms of enhanced or suppressed cortical excitability depending on the stimulation protocol.⁷ TMS-derived measures are sensitive to many CNS-penetrant drugs,⁸ including different classes of ASM, and support target engagement assessment. Motor-evoked potential (MEP) amplitude responds broadly to drugs with various mechanisms of action,⁹ while other TMS parameters to distinct neurophysiological pathways. For example, increases in motor thresholds are associated with voltage-gated sodium channels (VGSC) blockage, whereas intracortical inhibition reflects gamma-aminobutyric acid (GABA) mediated modulation and intracortical facilitation is linked to glutamatergic mechanisms.^{8,10} In addition to assessing general CNS drug activity, TMS-EMG/-EEG could provide valuable translational biomarkers that can reflect underlying mechanisms of cortical excitability, which has been shown to be increased in patients

with various epilepsy subtypes¹¹ and normalizes in those who achieve seizure freedom following ASM treatment.¹²

Building on previous findings in healthy participants, demonstrating a decrease in cortical excitability following administration of three different ASM classes,⁹ we aim to investigate whether similar effects on cortical excitability can be observed in patients with generalized epilepsy following single-dose levetiracetam administration, and whether these findings are translatable from healthy participants to a patient population. Furthermore, we aim to evaluate the potential of TMS-EMG/-EEG derived outcomes as a translational biomarkers compared to the established method of pEEG in early-phase development of ASM.

METHODS

Ethics

The Ethics Committee Stichting Beoordeling Ethiek Biomedisch Onderzoek, Assen, The Netherlands approved this study. The trial was executed in accordance with the Declaration of Helsinki at the Centre for Human Drug Research (CHDR), Leiden, The Netherlands, between July 2020 and November 2023. The study is registered in Dutch Trial Registry (NTR, ID: 25822). Written informed consent was obtained from all participants before study participation.

Participants

Male and female patients with well-controlled generalized epilepsy, confirmed by their neurologist, aged 18 to 70 years, and seizure-free for at least one year prior to dosing, were recruited using online advertisements, CHDR's trial participant database or were referred by their neurologist. Two groups were included: Group 1) patients on levetiracetam monotherapy (500 mg twice daily) maintenance therapy ('levetiracetam patients'), or Group 2) patients on valproic acid monotherapy (max 1000 mg/day; 'levetiracetam-naïve patients'). We considered it neither safe nor ethical to discontinue maintenance therapy in patients with epilepsy, and enrolling newly diagnosed, treatment-naïve patients in such a study poses logistical and ethical challenges. Therefore, we included patients receiving levetiracetam maintenance therapy, acknowledging that incomplete washout and potential physiological adaptations to chronic levetiracetam exposure could result in smaller effect sizes. To mitigate this, we added a comparison group of patients on valproic acid maintenance

therapy who were levetiracetam-naïve. Prior exposure to levetiracetam was permitted, provided it was not part of ongoing treatment at the time of study participation. Additionally, we only enrolled patients on low-dose maintenance treatment to increase the likelihood of identifying effects of our interventional treatment on cortical excitability. Eligibility was confirmed by a medical screening up to 42 days before the first dose (in- and exclusion criteria are listed in **Supplementary Materials**). Patients with contraindications according to the TMS safety questionnaire¹³ and a history of serious side effects related to levetiracetam administration were excluded. Use of medication other than levetiracetam or valproic acid monotherapy, as previously described, was prohibited for 14 days prior to the first dose.

Experimental design

This was a randomized, double-blind, two-way cross-over study to evaluate the effects of levetiracetam on cortical excitability in patients with generalized epilepsy, as measured by single- and paired-pulse TMS-EMG/-EEG and pEEG. During 2 visits, levetiracetam patients and levetiracetam-naïve patients received either a single oral dose of levetiracetam 2000 mg (Levetiracetam, 500 mg tablets, UCB Pharma SA) or placebo in a randomized order (**Figure 1**). The randomization code was generated in SAS (version 9.4, SAS Institute Inc., Cary, USA) by a statistician uninvolved with data collection. Randomization remained blinded for all staff, apart from the statistician and the pharmacy preparing the medication. Patients were asked to take the final dose of their maintenance therapy at 19:00 the evening before the study day and to withhold their morning dose on study days. Patients were admitted to the clinical research unit the morning of the study day. TMS and pEEG (twice) were performed at baseline, at 1.5 hours (H) (expected C_{max}) and 3H after dosing of levetiracetam or placebo. Patients already using levetiracetam received 500 mg of levetiracetam or placebo after all PD assessments were completed, while patients on valproic acid took their regular morning maintenance dose (**Figure 1**). Pharmacokinetic (PK) sample collection times matched the timing of the PD measurements. Serum concentrations of levetiracetam and valproic acid 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 Center. Safety assessments included monitoring adverse events (AEs), vital signs, electrocardiography (ECG), laboratory tests and physical examinations.

Dose selection

A single oral dose of 2000 mg of levetiracetam was safe and well tolerated in a prior study performed in healthy participants, showing effects on both TMS-EMG and TMS-EEG at plasma concentrations of 45.6 mg/L (range: 32.1 - 67.6 mg/L).⁹ Considering the therapeutic window of levetiracetam is 12–46 mg/L, a 2000 mg single dose was selected to target the upper end of the range to maximize the likelihood of detecting pharmacological effects.¹⁴

Pharmacodynamic measures

Data acquisition and processing was performed as described previously¹⁴ and is described in detail in **Supplementary Materials**. Single- and paired-pulse TMS were applied following the current guidelines¹⁵ using a MagPro R30 with a MagOption stimulator and an MCF-B65 butterfly coil (MagVenture GmbH, Hückelhoven, Germany). The motor hotspot of the dominant abductor digiti minimi muscle, identified using the Edinburgh Handedness questionnaire,¹⁶ was stimulated. Resting motor threshold (RMT), defined as the lowest stimulus intensity evoking 5/10 MEPs with a peak-to-peak amplitude of 50 μ V, was assessed at the beginning of each session. Stimulation consisted of 75 single pulses delivered at 120% of RMT, and 50 paired pulses at varying interstimulus intervals (ISIs)—2, 50, 100 and 300 ms—in a randomized sequence (totaling 200 paired pulses). Conditioning and test pulses were given at 120% RMT (conditioning pulse at 80% RMT for ISI 2 ms). TMS-EMG and TMS-EEG were recorded simultaneously. The TMS-evoked potential (TEP) response following the test pulse was analyzed for all 32 EEG leads, in the full-time sample of 0–300 ms and at specific time periods of interest (TOI) around the characteristic 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). TMS-EMG parameters included: RMT (% of the maximum stimulator output), mean single-pulse peak-to-peak MEP amplitude (μ V); paired-pulse short-interval intracortical (%) at ISI 2 ms (SICI₂), and long-interval intracortical (%) at ISIs of 50, 100 and 300 ms (LICI₅₀, LICI₁₀₀, and LICI₃₀₀). PEEG was recorded per the international pharmaco-EEG society (IPEG) guidelines,⁶ with 5-minute alternating periods of eyes open and closed every 64 s.¹⁷ PEEG data included: total power per frequency band (μ V²), per electrode, per eye state, e.g., alpha power in FZ-CZ with eyes open.

Statistical analysis

Using data from the previously conducted TRACE project,⁹ a sample of 16 participants per group was estimated to have 81% power to detect a mean difference of -380 μ V in TMS-EMG MEP amplitude, assuming a standard deviation of 500 μ V, through a paired t-test with a two-sided significance level of 0.05. This estimated sample size is likely conservative as we expect the effect in patients to move in the same direction and to be larger than in healthy participants. Therefore, we aimed to include at least 12 patients per group if enrolling 16 proved infeasible due to recruitment challenges. The serum concentration of levetiracetam and valproic acid was analyzed using a noncompartmental analysis. Repeatedly measured PD data were analyzed with a mixed model analysis of covariance (ANCOVA) with fixed factors treatment, period, group, time and their interactions, random factors participant, participant by treatment and participant by time and the average baseline value as covariate. Data for PEEG was log-transformed and back-transformed after analysis so the results could be interpreted as percentage change. The following contrasts are calculated within the models: All patients: 2000 mg levetiracetam vs. placebo; Group 1) Levetiracetam patients: 2000 mg levetiracetam vs. placebo; Group 2) Levetiracetam-naïve patients: 2000 mg levetiracetam vs. placebo, thus combining both post-dose timepoints. Results are reported relative to placebo as the estimated differences (ED) of the Least Square Means (LSM) for each contrast with 95% confidence interval (95%-CI) and p-values. Statistical significance was defined at the 5% level. Analysis of effects was performed in SAS (version 9.4, SAS Institute INC., Cary, NC, USA). Graphs are generated with the LSM of the analysis of the data as change from baseline, with 95%-CI as error bars. Statistical analysis of TMS-EEG outcomes was performed incorporating all 32 leads using a non-parametric cluster-based permutation analysis,¹⁷ a statistical method to analyze spatiotemporal dynamics of multidimensional TMS-EEG data without being affected by multiple comparisons. Comparison of 2000 mg levetiracetam vs. placebo was performed in all patients combined, and for Group 1 and Group 2 separately. Each ISI was analyzed individually and to compensate for handedness, topographical plots of left-handed patients were mirrored. A linear mixed effect model was applied with treatment, time, and treatment by time as fixed factors and participant, participant by treatment and participant by time as random factors and the average

baseline measurement as covariate. Dependent samples t-tests were used to compare the TEPs measured between two treatment conditions, for each electrode position and each time sample between 0–300 ms. Clusters were formed by t-values with a p-value < 0.05, based on neighboring leads ($n \geq 2$) and adjacent time samples. A permutation test (1500 times) was used to determine significance at the 5% level and p-values of significant clusters are reported. Besides analyzing the entire time sample of 300 ms after the test pulse, we applied the same analysis to TOI around the TEP components. As the TOI are based on TEP morphology measured at CZ, and as we also examined TEP morphology in other leads, they may differ from the characteristic TEP components. Therefore, we also included full-time samples.

PK/PD analysis

Exploratory PK/PD analysis was performed on parameters demonstrating significant effects of levetiracetam in the mixed effects model. PK/PD analysis was performed with pooled data from all included patients. Covariate analysis was used to explore differences between the two patient groups. A full technical description of the PK/PD-analysis is provided in **Supplementary Materials**.

RESULTS

A total of 26 patients were enrolled (levetiracetam patients: $n=14$, levetiracetam-naïve patients: $n=12$), of whom 23 completed all study visits (Levetiracetam patients: $n=13$, levetiracetam-naïve patients: $n=10$). Participant flow and demographics are presented in **Supplementary Materials** (CONSORT diagram). Levetiracetam and TMS procedures were well tolerated without any severe or serious adverse events (AEs). The total incidence of AEs is reported in **Supplementary Materials**. One participant in the levetiracetam group experienced a brief absence seizure one day prior to dosing on the second study occasion (six days after receiving levetiracetam on the first occasion) and was subsequently withdrawn from the study as a precautionary measure. Overall mean levetiracetam concentrations reached a maximum of 53.66 mg/L (range 35.9–72.5) in levetiracetam patients and 50.37 mg/L (range 37.6–77.2) in levetiracetam-naïve patients with a median $T_{\max}$ 1.5 h after levetiracetam administration. Mean serum concentrations of levetiracetam and valproic acid over time are listed in **Table S1**.

Corticospinal excitability assessed by TMS-EMG

Levetiracetam significantly decreased single-pulse TMS-EMG MEP amplitude in all patients (ED: $-382.4 \mu\text{V}$; 95%-CI: -580.3 , -184.4 ; $p=0.0009$) and in levetiracetam-naïve patients (ED: $-548.1 \mu\text{V}$; 95%-CI: -845.5 , -250.8 ; $p=0.0013$). For paired-pulse TMS-EMG, levetiracetam significantly enhanced LIC₅₀ (i.e., the percentage ratio decreased, indicating more intracortical inhibition) in all patients (ED: -98.8% ; 95%-CI: -153.9 , -43.6 ; $p=0.0015$) and levetiracetam-naïve patients (ED: -130.7% ; 95%-CI: -213.4 , -48.0 ; $p=0.0038$). Patients already on levetiracetam showed a trend towards a decreased MEP amplitude (ED: $-216.6 \mu\text{V}$; 95%-CI: -473.7 , 40.5 ; $p=0.0925$) and LIC₅₀ (ED: -66.8% ; 95%-CI: -140.3 , 6.6 ; $p=0.0716$). Levetiracetam only significantly enhanced LIC₁₀₀ in patients already on levetiracetam (ED: -7.1% ; 95%-CI: -12.7 , -1.5 ; $p=0.0155$). No significant effects were detected for RMT, SIC₂ or LIC₃₀₀. For an overview of all single and paired-pulse TMS-EMG parameters, see **Figure 2** and **Table S2**.

Resting-state EEG power spectral analysis

A breakdown of all frequency band effects across regions and conditions is provided in **Table S3**. In summary, spectral power analysis revealed an increase in PEEG frontal gamma power in both groups and an increase in frontal beta power with a more pronounced effect in levetiracetam-naïve patients (**Figure 3**).

Concentration/response relationship

For TMS, a significant negative linear proportional relationship was found for both MEP amplitude (baseline: $956.18 \mu\text{V}$, slope: $-0.0059 \text{ mg/L}^{-1}$, p-value < 0.001, **Figure 4A**) and LIC₅₀ (baseline: 133.64% , slope: $-0.0123 \text{ mg/L}^{-1}$, p-value < 0.001, **Figure 4B**) without significant covariates, meaning there were no significant differences identified between the groups. No significant relationship for LIC₁₀₀ was identified, therefore, differences between groups were not explored. For βFzCzC , a significant positive linear proportional relationship was identified (baseline: $10.15 \mu\text{V}^2$, slope: 0.0084 mg/L^{-1} , p-value < 0.001) with a significant negative covariate effect on the slope parameter ($-0.0056 \text{ mg/L}^{-1}$), indicating a smaller effect in levetiracetam patients (slope: 0.0028 mg/L^{-1}) compared to levetiracetam-naïve patients (**Figure 4C**). For γFzCzC , a significant positive linear proportional relationship was identified (baseline: $0.6 \mu\text{V}^2$, slope: 0.0064 mg/L^{-1} , p-value < 0.001) with a significant positive covariate effect on the

baseline parameter ($0.357 \mu V^2$), indicating a higher baseline in levetiracetam patients (baseline: $0.957 \mu V^2$) compared to levetiracetam-naïve patients (**Figure 4D**).

Assessment of cortical excitability by TMS-EEG

All TMS-EEG results and corresponding topoplots can be found in **Supplementary Materials**. Two distinct patterns emerged, differentiating levetiracetam patients (Group 1) from levetiracetam-naïve patients (Group 2). When combining all patients, similar clusters were detected as in the individual groups. In levetiracetam patients, we observed modulation of the early TEP components. The single pulse TEP revealed a parieto-occipital cluster with a significantly more negative P30 component at the 1.5H post-dose timepoint (**Figure 5A**). A similar, more centrally located cluster was seen for the paired pulse TEP at ISIs 2 and 100 ms (**Figure 5B & 5C**). This pattern was not observed at the 3H post-dose timepoint. In levetiracetam-naïve patients, we observed modulation of the late TEP components. The paired pulse TEP response at ISI 2 ms showed a contralateral centro-parietal cluster with a significantly less negative N100 component at 1.5H post-dose (**Figure 5D**). A similar cluster was found at 3H post-dose for paired pulse TMS at ISIs 2 and 50 ms (**Figure 5E & 5F**). A contralateral frontal cluster with a significantly more positive P180 component was observed for paired pulse TEP at ISIs 2 and 50 ms at 1.5H post-dose (**Figure 5G & 5H**).

DISCUSSION

We demonstrated a single dose of levetiracetam 2000 mg decreased cortical excitability in patients with well-controlled generalized epilepsy using TMS. This effect was most pronounced in levetiracetam-naïve patients (on valproic acid monotherapy), reflected by a greater reduction in MEP amplitude and stronger enhancement of LICI₅₀, in line with previous findings in healthy participants.⁹ In addition, PEEG showed increased frontal gamma power in both groups, suggesting its potential as a sensitive biomarker of levetiracetam's pharmacodynamic activity. Frontal beta power was also increased, particularly in levetiracetam-naïve patients. Exploratory PK/PD analysis demonstrated a linear concentration-response relationship for these biomarkers. Furthermore, TEP analysis showed group-dependent modulation for both single- and paired-pulse paradigms following levetiracetam 2000 mg: levetiracetam patients exhibited changes

in early TEP components (P30/N45), whereas levetiracetam-naïve patients showed modulation of later components (N100/P180). Levetiracetam plasma concentrations were within the therapeutic range¹⁸ aligning with previous findings in healthy participants,⁹ and slightly higher in the levetiracetam monotherapy group, as they had not fully cleared the previous evening's dose.

MEP amplitude and LICI are sensitive TMS-EMG biomarkers for levetiracetam activity

The marked decrease in MEP amplitude and enhancement of LICI₅₀, largest in levetiracetam-naïve patients and supported by linear concentration-response relationships, align with effects observed in healthy participants ($-378.4 \mu V$ MEP amplitude reduction and -60.3% enhancement of LICI₅₀).⁹ We observed stronger effects on LICI₅₀ in levetiracetam-naïve patients compared to those on levetiracetam monotherapy, possibly due to incomplete levetiracetam washout and/or compensatory mechanisms at play in patients chronically exposed to levetiracetam. In contrast, LICI₁₀₀ was only enhanced in levetiracetam monotherapy patients, possibly due to a floor effect in the valproic acid monotherapy (levetiracetam-naïve) subgroup, as this group exhibited very small LICI₁₀₀ values (**Table S2**).

Besides the study by Ruijs et al., healthy participant studies evaluating levetiracetam's effects on cortical excitability are limited to three double-blind, placebo-controlled, cross-over studies administering single doses of 2000–3000 mg to 6–15 participants.^{19–21} Two studies evaluated MEP recruitment curves, demonstrating dose- and intensity-dependent reductions in MEP amplitude.^{20,21} While LICI was not evaluated, levetiracetam had mixed effects on RMT, and SICI and ICF were largely unaffected.^{19–21} In drug-naïve epilepsy patients, initiation of ASM reversed cortical hyperexcitability, with full normalization in treatment responders, irrespective of seizure type or drug.^{12,22,23} Interestingly, a three-year longitudinal study in idiopathic generalized epilepsy demonstrated divergent trajectories of TMS markers between responders and non-responders. Patients with refractory epilepsy exhibited progressive loss of SICI and LICI, reflecting impaired GABAergic neurotransmission and resulting hyperexcitability, whereas seizure-free patients maintained or regained normal levels of inhibition.¹² Together, our findings support MEP amplitude and LICI as potential biomarkers of levetiracetam's activity, and possibly also other ASM.

Frontal beta and gamma power emerge as PEEG biomarkers for levetiracetam activity

Frontal gamma power was consistently increased across all patient groups, supporting its potential as a sensitive biomarker of levetiracetam's pharmacodynamic activity. PK/PD analysis confirmed a linear concentration-response relationship, with higher gamma power in chronically exposed patients compared to levetiracetam-naïve patients. Frontal beta power also increased, particularly in levetiracetam-naïve patients, with PK/PD analysis confirming smaller effects in chronically exposed patients. Our findings align with previous studies in healthy participants reporting beta and gamma power enhancement following single doses of levetiracetam.²⁴ In drug-naïve patients, however, levetiracetam not only increased alpha and beta power but also reduced delta and theta power,²⁵ findings which we did not replicate, possibly because our population was not naïve to ASM therapy. Compared to healthy participants, patients with generalized epilepsy exhibit increased power in lower frequency bands (delta, theta, and lower alpha); however, these changes did not correlate with treatment responsiveness.²⁶ Taken together, current and previous data suggest that while beta and gamma power reliably reflect levetiracetam's CNS activity, they are unlikely to serve as biomarkers of its antiepileptic efficacy.

Distinct TEP modulation patterns observed for levetiracetam monotherapy and naïve patients

TEP analysis revealed group-specific modulation, with levetiracetam monotherapy patients showing suppression of early components (P30/N45 more negative) in centro-parieto-occipital regions, while levetiracetam-naïve patients demonstrated enhancement of the late components (N100 less negative and P180 more positive) in the contralateral centro-parietal and frontal regions. To date, no TMS-EEG studies have examined levetiracetam in epilepsy patients, limiting direct comparisons. The observation that significant clusters appear in parietal and occipital regions despite stimulation over the motor cortex is consistent with the known propagation of TMS-evoked activity across cortical networks. TMS-EEG studies have repeatedly demonstrated that stimulation of the motor cortex produces not only local responses but also distributed activity across connected cortical regions, reflecting the spread of activation through functional and anatomical networks.²⁷ In epilepsy, alterations in thalamocortical and

cortico-cortical connectivity may further influence these propagation patterns. Therefore, parietal or occipital cluster involvement likely reflects network-level responses to stimulation rather than purely local effects at the stimulation site.

In healthy participants, levetiracetam has been shown to increase the amplitude of the N45 single-pulse TEP component^{9,19} consistent with our findings. Although our early cluster was more closely related to the P30 than the N45 TOI, it showed similarities in both direction and cluster location. By contrast, we did not replicate the effects on the single-pulse N100 and P180 components reported in previous studies in levetiracetam patients.^{9,19} This discrepancy could reflect smaller effect sizes resulting from incomplete levetiracetam washout or physiological adaptations following chronic exposure. Alternatively, it may be related to methodological differences, as Ruijs et al. combined the 1.5H and 7H post-dose measurements, whereas we analyzed these time points separately. Differences between studies might also reflect variability in topographical cluster location, which can affect TEP morphology.

Instead, we did observe N100 and P180 modulation for the paired-pulse paradigms in levetiracetam-naïve patients, aligning with the healthy participant findings by Ruijs et al.⁹ As de Goede et al. demonstrated comparable spatiotemporal dynamics for single- and paired-pulse TEPs, this may suggest that modulation of specific TEP components and the topographical distribution of clusters may be comparable across paradigms.²⁷ The relationship between drug-induced modulation of specific TEP components, particularly in paired-pulse paradigms, and mechanistic underpinnings of the TEP signal remains unclear. Previous studies suggest that increased N45 and decreased N100 are indicative of GABAergic effects.¹⁰ Therefore, our findings indicate that levetiracetam's antiepileptic properties may be mediated, at least in part, by indirect modulation of GABAergic neurons. The lack of N45 modulation in valproic acid-treated (levetiracetam-naïve) patients may reflect its sodium channel-blocking properties, which preferentially affect early TEP components.¹⁰ The observed paired-pulse N100 and P180 effects resemble those reported for baclofen,²⁸ also suggesting GABAergic involvement. Interestingly, TEP analysis also demonstrated involvement of the N100 and P180 components in epilepsy, with increased amplitudes compared to healthy controls, irrespective of seizure type.²⁹ Taken together, these results highlight levetiracetam's modulatory profile across early and late TEP components and suggest that attenuation of late components may serve as a biomarker for its antiepileptic efficacy.

Unraveling levetiracetam's putative neurocircuitry effects

Although the exact basis of its antiepileptic efficacy remains to be elucidated, levetiracetam's primary MOA is thought to involve binding to Synaptic Vesicle Glycoprotein 2A (SV2A),³⁰ through which it inhibits presynaptic calcium channels,³¹ thereby reducing neurotransmitter release. Unlike classical anticonvulsants, levetiracetam does not inhibit voltage-dependent sodium channels or bind directly to GABAergic or glutamatergic receptors.³² However, *in vitro* studies suggest that levetiracetam might indirectly influence GABAergic neurotransmission by suppressing the effects of GABA-negative allosteric modulators, such as Zn²⁺ and β -carbolines.³³ This interpretation is further supported by our findings on LIC1, frontal beta power, and the N45 and N100 TEP components, which are all markers of GABAergic drug effects.^{5,8,10}

All modalities in this study showed acute effects after a single dose of levetiracetam 2000 mg in patients with generalized epilepsy, particularly in levetiracetam-naïve patients, consistent with previous findings in healthy participants.⁹ These results support using these biomarkers to guide early-phase development and translate evidence of target engagement from healthy participants to patients, positioning levetiracetam as a tool compound for such translational approaches. While MEP amplitude proved to be a sensitive biomarker, being modulated by multiple ASM with distinct mechanisms of action,⁹ LIC1 may serve as a biomarker of antiepileptic efficacy as loss of LIC1 is related to poor seizure control or treatment response, but needs to be confirmed in larger, more heterogeneous cohorts.

Limitations

Recruitment was limited by criteria balancing patient safety and signal detection, resulting in smaller subgroups and reduced statistical power. This may have prevented significance for MEP amplitude and LIC1₅₀ in the levetiracetam monotherapy subgroup, given the small sample size and limited effect size. Although most primary pharmacodynamic effects were detected in the full sample, the relatively small number of participants in subgroup analyses may have reduced statistical power and the results of this study should be interpreted with caution until confirmed in larger cohorts. Furthermore, we only included patients with well-controlled generalized epilepsy for safety reasons and because they exhibit prominent bilateral excitability abnormalities,³⁴ which facilitates standardized stimulation but limits extrapolation to focal epilepsy. Detailed syndromic

classification of generalized epilepsy was not available for all participants. Although the magnitude of neurophysiological effects may differ between epilepsy syndromes, generalized epilepsies share key features such as cortical hyperexcitability and reduced inhibition.³⁵ Moreover, because our analyses focused on within-subject pharmacodynamic changes, rather than between-syndrome comparisons, the absence of detailed syndromic classification is unlikely to have materially affected interpretation of the PD effects observed. Nevertheless, the findings should be interpreted with caution when extrapolating the results to specific generalized epilepsy syndromes. Extending this approach to more heterogeneous and treatment-resistant epilepsy populations represents an important next step for future research. The limited concentration range investigated with a single dose level should be noted. In addition, potential effects of incomplete washout of levetiracetam and valproic acid, as well as tolerance or compensatory mechanisms arising from chronic exposure, cannot be excluded. Baseline plasma levels of levetiracetam (~6 mg/L) and valproic acid (~40 mg/L) were at the lower end of their respective therapeutic ranges: 12–46 mg/L for levetiracetam and 50–100 mg/L for valproic acid.¹⁸ Moreover, levetiracetam naïvety and background ASM therapy were coupled at the group level, as levetiracetam-naïve participants were treated with valproic acid, which may partly contribute to observed between-group differences. Furthermore, although carryover effects cannot be formally excluded in the two-period cross-over design, the 7-day washout and adjustment for period and pre-dose baseline values in the statistical analysis make meaningful residual levetiracetam effects unlikely.

Finally, several technical aspects warrant consideration. TMS-EEG was recorded with a 32-channel system, providing lower spatial resolution than the 64- or 128-channel caps used in other studies. Principal component analysis (PCA) was applied for artifact removal; while effective,³⁶ it differs from other methods³⁷ and highlights the lack of consensus on a gold standard.³⁸ Importantly, we used a placebo-controlled, baseline-adjusted design, which is preferred over pre/post-dose comparisons but limits direct comparability. Due to the hypothesis-generating and exploratory nature of the study, aimed to identify potential neurophysiological signatures of levetiracetam effects rather than to provide definitive confirmatory evidence, no multiple testing corrections were applied; replication in larger, diverse cohorts is essential to solidify translational relevance.

CONCLUSION

This follow-up study demonstrates decreased cortical excitability after a single dose of levetiracetam in well-controlled generalized epilepsy patients, with greater effects in levetiracetam-naïve individuals. Findings align with our earlier results in healthy participants and underscore the translational value of TMS as a proof-of-mechanism and proof-of-concept biomarker for early-phase ASM development. TMS-based measures could help fill the gap in reliable biomarkers that demonstrate target engagement in healthy participants, while remaining translatable to patients. Moreover, TMS offers complementary insights into the neural circuitry underlying ASM effects. Future studies in larger, more diverse populations are needed to establish the role of these biomarkers in translational drug development.

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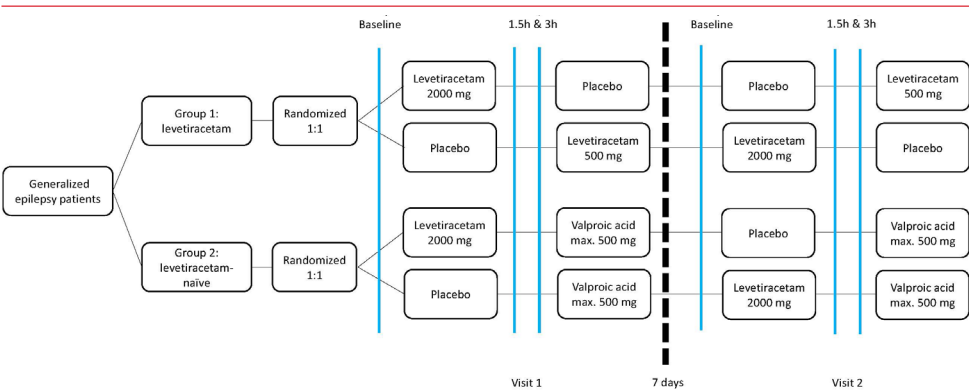
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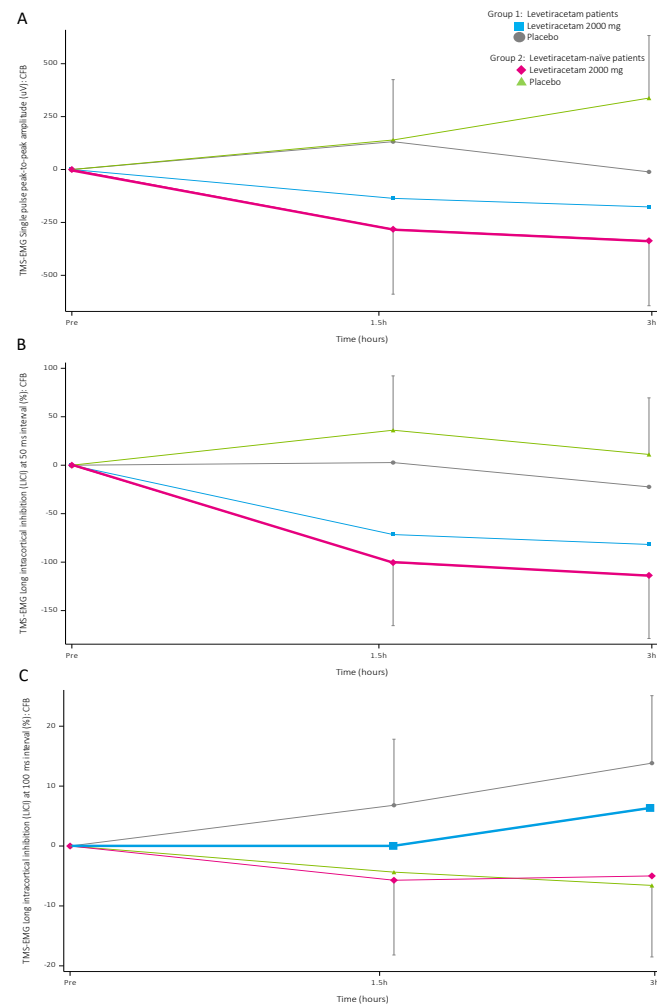
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FIGURE I Schematic overview of study design. Schematic overview of study design with different subgroups, treatment allocations and timing of the pharmacodynamic measures (blue lines). Pharmacokinetic samples were time-matched to the TMS and PEEG measures. Patients on levetiracetam maintenance therapy were administered levetiracetam 500 mg or placebo following the final pharmacodynamic measurements (+3h), depending on the randomization sequence. Patients on valproic acid maintenance therapy (levetiracetam-naïve), took their usual morning dose following the final pharmacodynamic measurements (+3h), irrespective of randomization sequence. The two treatment visits were separated by 7 days (dashed line).



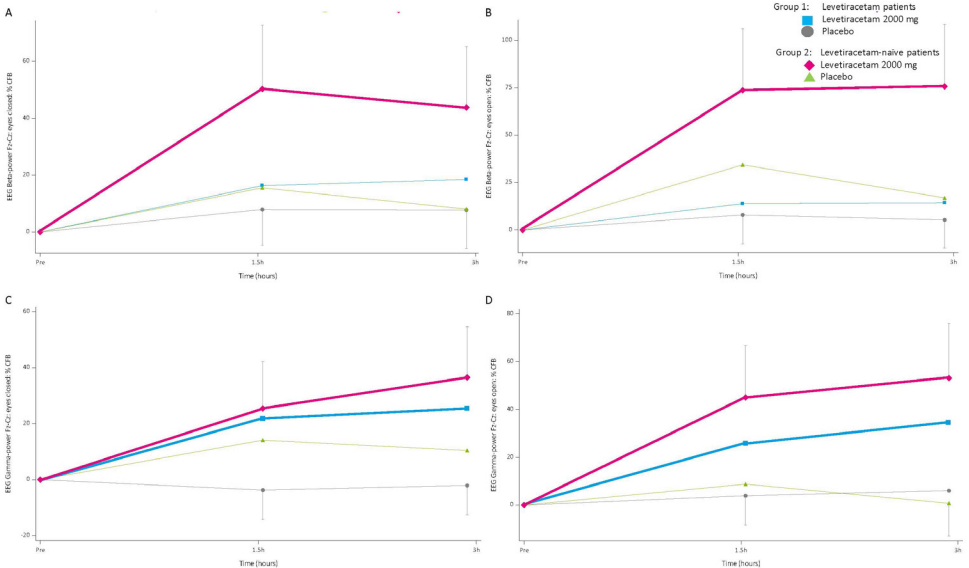
Abbreviations: h = hour; mg = milligram; MAX. = maximum; PEEG = pharmaco-electroencephalogram; TMS = Transcranial Magnetic Stimulation.

FIGURE 2 Summary graph MEP amplitude (A), LIC_{I50} (B) and LIC_{I100} (C) – change from baseline. Change from baseline of the least square means (LSM) with 95% confidence interval (95%-CI) as error bars of the motor-evoked potential (MEP) amplitude (2A), long-interval intracortical inhibition at interstimulus interval of 50 ms (2B) and 100 ms (2C) using single-pulse transcranial magnetic stimulation, for levetiracetam 2000 mg and placebo, presented for patients on levetiracetam monotherapy and valproic acid monotherapy separately. Significant contrasts are indicated with thick lines. The MEP amplitude was significantly reduced for patients on valproic acid monotherapy (levetiracetam-naïve), and when combining all patients (2A). LIC_{I50} was significantly reduced (more inhibition) for patients on valproic acid monotherapy (levetiracetam-naïve), and when combining all patients (2B). LIC_{I100} was significantly reduced (more inhibition) for patients on levetiracetam monotherapy (2C).



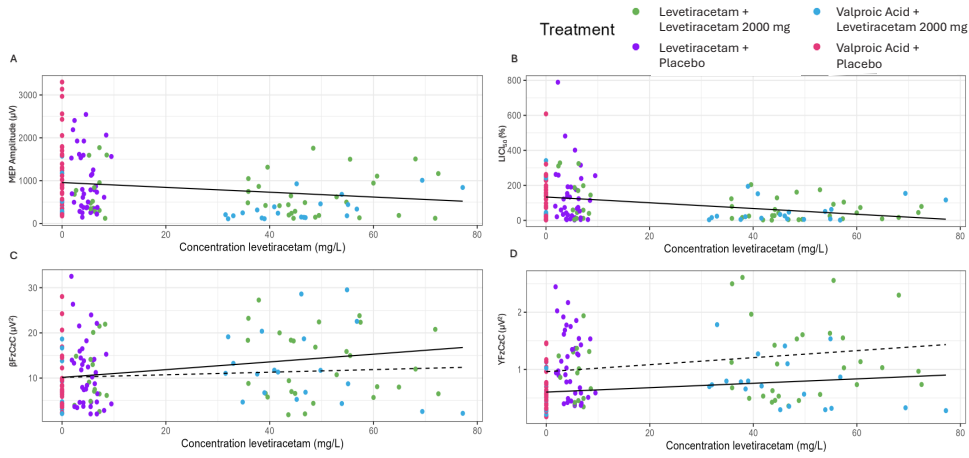
Abbreviations: CFB = change from baseline; LIC_{I50} = long-interval intracortical inhibition at interstimulus interval 50 ms; LIC_{I100} = long-interval intracortical inhibition at interstimulus interval 100 ms; LSM = least square means; 95%-CI = 95% confidence interval; MEP = motor-evoked potential; mg = milligram.

FIGURE 3 Summary graph of PEEG frontal beta (A-B) and gamma power (C-D)– change from baseline. Change from baseline of the least square means (LSM) with 95% confidence interval (95%-CI) as error bars of beta power (β : 12.5 < 30.0 Hz) with eyes closed (3A) and eyes open (3B), and gamma power (γ : 30.0 < 40.0 Hz) with eyes closed (3C) and eyes open (3D), measured in FZ-CZ, for levetiracetam 2000 mg and placebo, presented for patients on levetiracetam monotherapy and valproic acid monotherapy separately. Significant contrasts are indicated with thick lines. Frontal beta power was significantly increased for patients on valproic acid monotherapy (levetiracetam-naïve), and when combining all patients, for both eye conditions (3A-3B). Frontal gamma power was significantly increased for both patient groups separately, and when combining all patients, for both eye conditions (3C-3D).



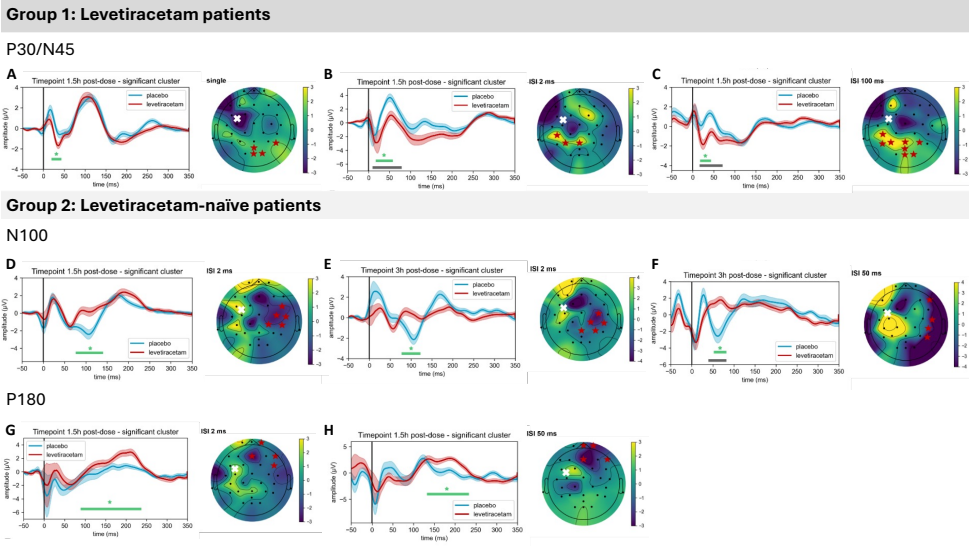
Abbreviations: CFB = change from baseline; FZ-CZ = frontocentral leads; LSM = least square means; 95%-CI = 95% confidence interval; mg = milligram; PEEG = pharmaco-electroencephalogram.

FIGURE 4 Concentration-response relationship for significant TMS-EMG and PEEG parameters. Concentration-response relationship graphs demonstrating linear proportional relationships for levetiracetam concentrations and MEP amplitude (4A), LIC₅₀ (4B), beta power (β : 12.5 < 30.0 Hz) in FZ-CZ with eyes closed (4C) and gamma power (γ : 30.0 < 40.0 Hz) in FZ-CZ with eyes closed (4D). Solid lines represent model predictions, points represent observed data. For subfigures 4C and 4D, the solid lines represent the model predictions of the reference population (levetiracetam monotherapy) and the dashed lines represent the model predictions with the slope and baseline covariates, respectively.



Abbreviations: FZ-CZ = frontocentral leads; LIC₅₀ = long-interval intracortical inhibition at interstimulus interval of 50 ms; MEP = motor evoked potential; mL = milliliter; mg = milligram; ng = nanogram; PEEG = pharmaco-electroencephalogram; TMS-EMG = transcranial magnetic stimulation – electromyography.

FIGURE 5 Grand average and topographical distribution of significant paired-pulse TMS-EEG clusters. Overview of the consistent patterns of significant clusters found when comparing TEPs of levetiracetam 2000 mg (in red) compared to the placebo (in blue) for the two different patient groups separately. The first row shows modulation of the early TEP components (P30/N45) by levetiracetam 2000 mg in patients already on levetiracetam monotherapy (5A-5C). The second and third row show modulation of the late TEP components (N100 and P180) by levetiracetam 2000 mg in patients on valproic acid monotherapy (levetiracetam-naïve) (5D-5H). On the left side, the grand average (mean $\pm$ SEM) TEP following the test pulse over all significant electrodes is presented. On the right side, the amplitude difference in topographical distribution (placebo – levetiracetam) at the time of the cluster is presented. The thick green bar represents the time window of significant differences, the thick grey bar the time window of the entire cluster (including non-significant parts), the white cross the stimulation site, and the black dots the electrode positions with the significant electrodes as red stars.



Abbreviations: H = hours; ISI = interstimulus interval; mg = milligram; ms = millisecond; SEM = standard error of the mean; TEP = transcranial magnetic stimulation evoked potential; TMS-EEG = transcranial magnetic stimulation combined with encephalography.

CHAPTER III

SUPPLEMENTARY MATERIALS

Full supplementary materials available at the publisher's website:
<https://ascpt.onlinelibrary.wiley.com/doi/10.1002/cpt.70327>
Selected supplementary text, figures and tables are provided next.

TABLE S1 Plasma pharmacokinetic parameters for levetiracetam and valproic acid in patients on levetiracetam and levetiracetam naïve patients.

Descriptions: Summary of PK parameters in plasma following administration of levetiracetam 2000 mg QD. Parameters are reported by N, mean (SD), CV% and median (MIN, MAX).

Timepoint (H)	Concentration in blood (mg/L)	Levetiracetam		Valproic acid		
		Group 1: Levetiracetam patients		Group 2: Levetiracetam-naïve patients	Group 2: Levetiracetam-naïve patients	
		Levetiracetam + Levetiracetam 2000 mg (N=14)	Levetiracetam + Placebo (N=13)	Valproic acid + Levetiracetam 2000 mg (N=10)	Valproic acid + Levetiracetam 2000 mg (N=10)	Valproic acid + Placebo (N=12)
0	Mean (SD)	6.0 (1.8)	6.1 (1.9)	-	43.8 (21.8)	45.0 (17.9)
	CV%	29.7	30.3	-	49.9	39.8
	Median (MIN, MAX)	6.1 (2.4-8.6)	6.3 (3.3-9.5)	-	39.0 (8.3-84.2)	44.1 (4.6-71.2)
1.5	Mean (SD)	53.7 (11.2)	4.5 (1.5)	50.4 (12.2)	44.9 (25.1)	42.4 (19.0)
	CV%	20.9	34.1	24.2	56	44.9
	Median (MIN, MAX)	50.8 (35.9-72.5)	4.2 (2.1-6.7)	51.8 (37.6-77.2)	36.6 (5.9-84.5)	40.9 (4.8-74.5)
3	Mean (SD)	46.4 (9.5)	3.9 (1.4)	42.7 (11.4)	43.9 (26.6)	44.5 (23.2)
	CV%	20.5	34.9	26.6	60.6	52.1
	Median (MIN, MAX)	43.9 (35.8-64.9)	3.90 (1.8-6.0)	43.4 (31.5-69.4)	34.7 (7.0-90.4)	38.9 (3.5-85.7)

Abbreviations: QD = once daily; N = number of participants; SD = standard deviation; CV% = coefficient of variation; MIN = minimum; MAX = maximum.

TABLE S2 Summary of results for single and paired pulse TMS-EMG.

Estimated post-dose means of single pulse and paired pulse TMS-EMG parameters after administration of levetiracetam in all patients, levetiracetam patients and levetiracetam-naïve patients. The analysis includes all post-dose timepoints (both 1.5H and 3H post-dose) and reported as the estimated difference of levetiracetam vs. placebo with 95% confidence interval (95%-CI) and p-value.

		Estimated mean; Estimated difference with placebo (95%-CI), p-value		
Parameter		All patients	Levetiracetam patients	Levetiracetam-naïve patients
MEP (µV)	Placebo	1000.1	910.9	1089.3
	Levetiracetam 2000 mg	617.7	694.2	541.2
	Levetiracetam 2000 mg vs. Placebo	-382.4 (-580.3, -184.4) p=0.0009	-216.6 (-473.7, 40.5) p=0.0925	-548.1 (-845.5, -250.8) p=0.0013
RMT	Placebo	56.8	56.4	57.1
	Levetiracetam 2000 mg	57.0	57.0	56.9
	Levetiracetam 2000 mg vs. Placebo	0.2 (-1.2, 1.6) p=0.7989	0.5 (-1.4, 2.4) p=0.5762	-0.2 (-2.3, 1.9) p=0.8711
SICI ₂ (%)	Placebo	39.2	39.7	38.6
	Levetiracetam 2000 mg	41.8	41.1	42.6
	Levetiracetam 2000 mg vs. Placebo	2.6 (-4.1, 9.4) p=0.4166	1.3 (-7.4, 10.1) p=0.7450	3.9 (-6.3, 14.2) p=0.4251
LICI ₅₀ (%)	Placebo	137.6	120.9	154.3
	Levetiracetam 2000 mg	38.9	54.1	23.7
	Levetiracetam 2000 mg vs. Placebo	-98.8 (-153.9, -43.6) p=0.0015	-66.8 (-140.3, 6.6) p=0.0716	-130.7 (-213.4, -48.0) p=0.0038
LICI ₁₀₀ (%)	Placebo	14.0	21.9	6.1
	Levetiracetam 2000 mg	10.5	14.8	6.2
	Levetiracetam 2000 mg vs. Placebo	-3.5 (-7.6, 0.6) p=0.0868	-7.1 (-12.7, -1.5) p=0.0155	0.1 (-5.7, 6.0) p=0.9676
LICI ₃₀₀ (%)	Placebo	72.7	72.3	73.1
	Levetiracetam 2000 mg	57.2	55.6	58.8
	Levetiracetam 2000 mg vs. Placebo	-15.5 (-33.7, 2.8) p=0.0927	-16.7 (-40.9, 7.6) p=0.1668	-14.2 (-41.7, 13.2) p=0.2937

Abbreviations: TMS-EMG = transcranial magnetic stimulation - electromyography; MEP = motor evoked potential; RMT = resting motor threshold; MSO = maximum stimulator output; SICI₂ = short-interval intracortical inhibition at interstimulus-interval of 2 ms; LICI₅₀ = long-interval intracortical inhibition at interstimulus-interval of 50 ms; LICI₁₀₀ = long-interval intracortical inhibition at interstimulus-interval of 100 ms; LICI₃₀₀ = long-interval intracortical inhibition at interstimulus-interval of 300 ms; vs. = versus.

TABLE S3 Summary of Results for PEEG.

Estimated difference of levetiracetam vs. placebo of PEEG parameters in %, with 95% confidence interval (95%-ci) and p-values of the total power ($(\mu V)^2$), per frequency band, per lead, per eye state. The analysis includes all post-dose timepoints (both 1.5h and 3h post-dose). Frequency per band: delta (δ): 1.5 < 6.0 Hz; theta (θ) 6.0 < 8.5 Hz; alpha (α): 8.5 < 12.5 Hz; beta (β): 12.5 < 30.0 Hz; and gamma (γ): 30.0 < 40.0 Hz, and Statistically significant (p<0.05) results are marked in bold. Significant increases in power are highlighted in green.

Levetiracetam 2000 mg vs. Placebo						
ED, (95%-CI), p-value						
	Eyes Closed			Eyes Open		
Frequency/ Lead	All patients:	Levetiracetam	Levetiracetam- naïve patients	All patients	Levetiracetam	Levetiracetam- naïve patients
δFzCz	7.5% (-5.5%, 22.3%) p=0.2573	14.7% (-3.4%, 36.1%) p=0.1104	0.7% (-17.0%, 22.3%) p=0.9389	8.9% (-5.4%, 25.4%) p=0.2195	-3.8% (-19.8%, 15.4%) p=0.6608	23.3% (-0.6%, 52.8%) p=0.0558
δPzO1	7.6% (-3.9%, 20.5%) p=0.1855	7.4% (-7.5%, 24.7%) p=0.3224	7.8% (-9.0%, 27.8%) p=0.3595	10.8% (-2.5%, 25.8%) p=0.1090	17.0% (-0.8%, 38.0%) p=0.0608	4.9% (-13.7%, 27.4%) p=0.6144
δPzO2	9.0% (-2.0%, 21.3%) p=0.1060	17.3% (1.8%, 35.2%) p=0.0292	1.4% (-13.6%, 18.9%) p=0.8610	13.3% (-4.8%, 34.8%) p=0.1494	32.4% (5.5%, 66.1%) p=0.0186	-3.1% (-25.6%, 26.3%) p=0.8059
θFzCz	11.5% (-4.5%, 30.3%) p=0.1594	7.3% (-12.7%, 31.7%) p=0.4854	16.0% (-8.3%, 46.6%) p=0.2030	18.2% (-3.1%, 44.4%) p=0.0951	4.4% (-19.4%, 35.2%) p=0.7323	33.9% (-1.2%, 81.6%) p=0.0590
θPzO1	9.2% (0.5%, 18.6%) p=0.0384	5.4% (-5.6%, 17.7%) p=0.3311	13.1% (-0.1%, 28.1%) p=0.0516	26.6% (5.5%, 52.0%) p=0.0144	23.6% (-2.4%, 56.5%) p=0.0755	29.7% (-1.9%, 71.4%) p=0.0659
θPzO2	9.9% (-0.7%, 21.7%) p=0.0664	2.6% (-10.4%, 17.5%) p=0.6977	17.8% (1.2%, 37.2%) p=0.0362	27.9% (4.5%, 56.7%) p=0.0204	27.1% (-2.3%, 65.4%) p=0.0705	28.7% (-5.5%, 75.3%) p=0.1024
αFzCz	-7.2% (-20.1%, 7.8%) p=0.3152	-1.5% (-19.1%, 19.9%) p=0.8774	-12.5% (-30.2%, 9.6%) p=0.2319	-1.5% (-19.3%, 20.1%) p=0.8734	2.8% (-20.5%, 32.8%) p=0.8273	-5.6% (-30.3%, 27.8%) p=0.6956
αPzO1	-10.2% (-23.1%, 4.9%) p=0.1645	5.0% (-14.7%, 29.3%) p=0.6295	-23.2% (-39.0%, -3.4%) p=0.0262	27.5% (-5.1%, 71.3%) p=0.1016	42.9% (-2.8%, 110.1%) p=0.0678	13.7% (-27.3%, 78.0%) p=0.5560
αPzO2	-12.1% (-26.0%, 4.4%) p=0.1342	6.7% (-15.2%, 34.3%) p=0.5632	-27.7% (-44.0%, -6.5%) p=0.0155	29.3% (-7.0%, 79.9%) p=0.1190	41.0% (-8.4%, 117.0%) p=0.1113	18.6% (-28.1%, 95.6%) p=0.4846
βFzCz	19.7% (9.2%, 31.2%) p=0.0006	8.9% (-3.5%, 22.9%) p=0.1574	31.6% (14.6%, 51.1%) p=0.0005	22.2% (9.2%, 36.6%) p=0.0013	7.1% (-7.3%, 23.7%) p=0.3333	39.4% (17.5%, 65.4%) p=0.0006
βPzO1	-0.9% (-13.1%, 13.0%) p=0.8858	1.0% (-15.1%, 20.2%) p=0.9053	-2.8% (-20.2%, 18.4%) p=0.7680	19.7% (3.2%, 38.9%) p=0.0200	21.5% (0.1%, 47.5%) p=0.0490	18.0% (-5.9%, 47.9%) p=0.1435
βPzO2	-2.9% (-13.1%, 8.4%) p=0.5837	-2.5% (-15.8%, 13.0%) p=0.7284	-3.4% (-18.1%, 13.9%) p=0.6711	16.4% (0.3%, 35.1%) p=0.0463	18.0% (-2.8%, 43.1%) p=0.0901	14.8% (-8.6%, 44.2%) p=0.2212
γFzCz	21.8% (11.3%, 33.2%) p=0.0002	27.2% (12.8%, 43.4%) p=0.0004	16.6% (1.8%, 33.5%) p=0.0286	32.8% (18.9%, 48.3%) p<0.0001	23.9% (7.2%, 43.1%) p=0.0057	42.3% (20.3%, 68.4%) p=0.0003

Levetiracetam 2000 mg vs. Placebo						
ED, (95%-CI), p-value						
	Eyes Closed			Eyes Open		
Frequency/ Lead	All patients:	Levetiracetam	Levetiracetam- naïve patients	All patients	Levetiracetam	Levetiracetam- naïve patients
γPzO1	4.6% (-13.5%, 26.6%) p=0.6245	28.3% (0.0%, 64.6%) p=0.0497	-14.6% (-36.1%, 14.0%) p=0.2678	33.6% (0.8%, 77.0%) p=0.0446	41.7% (-1.8%, 104.4%) p=0.0607	25.9% (-17.9%, 93.2%) p=0.2695
γPzO2	6.4% (-11.5%, 28.0%) p=0.4896	11.8% (-12.6%, 43.0%) p=0.3573	1.4% (-23.1%, 33.7%) p=0.9200	24.1% (-0.3%, 54.5%) p=0.0532	10.1% (-17.2%, 46.3%) p=0.4895	39.9% (0.1%, 95.4%) p=0.0491

Abbreviations: EEG = electroencephalography; vs. = versus.

CHAPTER IV

**TMS AND EEG PHARMACODYNAMIC
EFFECTS OF A SELECTIVE
SPHINGOSINE-1-PHOSPHATE SUBTYPE
1 RECEPTOR AGONIST ON CORTICAL
EXCITABILITY IN HEALTHY SUBJECTS**

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ABSTRACT

Current anti-epileptic drugs lack efficacy, cause many side-effects and one-third of all patients are treatment resistant. Drugs targeting the sphingosine-1-phosphate receptor (S1PR) show potential anticonvulsant effects in animal models and decrease cortical excitability in patients with multiple sclerosis, but available compounds alter lymphocyte trafficking and cause immunosuppression, limiting their clinical anti-epileptic potential. TRV045 is a selective sphingosine-1-phosphate subtype 1 receptor (S1PR1) agonist without effects on lymphocyte trafficking, demonstrating efficacy in animal models of epilepsy, with potential to target abnormal cortical excitability. This randomized, double-blind, placebo-controlled, two-way cross-over, multiple dose study evaluated the effects of TRV045 on cortical excitability in healthy male adults, measured by pharmacoelectroencephalography (EEG) and transcranial magnetic stimulation (TMS). Subjects received TRV045 250mg or placebo, once daily for 4 days, in randomized order. Endpoints were analyzed with a mixed effects model analysis of covariance.

Twenty-five of the twenty-seven subjects completed the study. There was a significant increase in alpha power with eyes open after treatment with TRV045 on Day 1, increasing after 4 days of dosing. Less pronounced significant effects in beta, gamma and delta power were observed after 4 days. For TMS-Electromyography (EMG) there was a non-significant decreased post-dose single pulse peak-to-peak amplitude on Day 1 only, and there were no effects on paired-pulse parameters. Several significant TMS-EEG clusters were seen after 4 days of dosing.

These findings show that TRV045 has central nervous system activity with evolving effects following repeated dosing. These data support further studies to elucidate the mechanism of action of TRV045 and its potential anti-epileptic effects.

INTRODUCTION

Epilepsy is a central nervous system (CNS) disorder with abnormal brain activity causing unprovoked seizures. It affects approximately 45 million people, often requiring life-long treatment.¹ To date no disease-modifying therapies have been registered for the treatment of epilepsy. One third of the patients are therapy-resistant under current anti-seizure medications (ASM) due to lack of efficacy or dose-limiting side effects.² There is an unmet need for effective ASM with a different mechanism of action (MOA) from existing therapies. Sphingosine-1-phosphate subtype 1 receptors (S1PR1) are highly expressed in the CNS and regulate neuroinflammation,³⁻⁵ a process involved in epileptogenesis. S1PR1s also influence neuronal function through the regulation of neurotransmitters such as glutamate.⁵⁻⁸ Fingolimod, a market-approved drug for multiple sclerosis, is a nonselective sphingosine-1-phosphate receptor (S1PR) modulator with high potency for S1PR1, but also shows activity at S1PR subtypes 3-5. Fingolimod demonstrated anti-epileptic effects in a diverse range of preclinical epilepsy models, including the lithium-pilocarpine rat model,⁹ pentylenetetrazol-induced kindling mouse model,¹⁰ and supra-hippocampal kainate mouse model.¹¹ However, it also causes lymphopenia, which contributes to its efficacy in multiple sclerosis (MS), but makes it unfavourable for lifelong treatment of epilepsy. TRV045 is a potent and selective S1PR1 agonist that has not shown lymphopenia in animal and early human studies. In the context of the National Institute of Neurological Disorders and Stroke (NINDS) Epilepsy Therapy Screening Program, TRV045 was demonstrated to reduce seizures in the corneal-kindled mouse model and the rat maximal electroshock seizure model of acute epilepsy.¹² Additionally, TRV045 reduced seizure burden and increased seizure freedom in the rat post-kainic acid spontaneous recurrent seizure model of temporal lobe epilepsy,¹² and elevated the threshold for seizure induction, i.e., time to first myoclonic twitch of whole body and time to onset of generalized clonus, caused by the intravenous infusion of the chemoconvulsant pentylenetetrazol (Trevena, Inc., data on file), supporting the development of TRV045 as an ASM. Before studying TRV045's efficacy in patients with epilepsy, we aimed to demonstrate CNS penetration and CNS-mediated pharmacodynamic (PD) effects in healthy participants. Both quantitative resting-state pharmacoelectroencephalography (EEG)¹³ and Transcranial Magnetic Stimulation-Electromyography-Electroencepha-

lography (TMS-EMG/-EEG)¹⁴ have proven their usefulness as PD biomarkers for CNS target engagement in early-phase drug trials. Additionally, these biomarkers have translational value, as both patients with generalized and focal epilepsy have epileptic activity on EEG and increased cortical excitability as measured with TMS.¹⁵ Moreover, ASM with different MOA modulate pharmaco-EEG and TMS-EMG/-EEG signals differently, making both methods interesting tools for drug profiling novel therapeutic agents.^{13,14,16-18} The aim of this study was to measure CNS-mediated PD effects of SIPRI modulation by TRVO45 with pharmaco-EEG and TMS-EMG/-EEG in male healthy participants to demonstrate CNS penetrance and to profile its drug effects, which could provide more information on the potential anti-epileptic effects of TRVO45.

MATERIAL AND METHODS

This study was approved by the Medical Ethics Committee, Foundation Beoordeling Ethiek Biomedisch Onderzoek (BEBO) and performed according to International Conference on Harmonisation guidelines on Good Clinical Practice. Written informed consent was obtained from all participants before study participation. The study was sponsored by Trevena, Inc. and conducted at the Centre for Human Drug Research, Leiden, The Netherlands, from 13-Feb-2023 to 05-Jun-2023. The study was registered in the European Union Clinical Trials Information System (EU trial number 2022-502638-17-00). Healthy males, age 18-55 years, were selected following a medical screening. A complete overview of inclusion and exclusion criteria is available in **Supplementary Materials**. Only males were included in the study to exclude potential menstrual cycle-related confounding effects on cortical excitability.¹⁹ Subjects with an increased risk of infection or TMS-related complications, based on the TMS safety questionnaire,²⁰ were excluded. Individuals with a resting motor threshold (RMT) higher than 75% of the maximum stimulator output (MSO) were excluded, as stimulation at 120% of this value would be very close to the MSO.

The study consisted of a randomized, double-blind, placebo-controlled, two-period cross-over design with multiple dosing (**Figure 1**). Participants were admitted to the research center on Day -1 for 5 days during each study period, with a wash-out period of 1 week between visits, which is appropriate as earlier pharmacokinetic (PK) evaluation at the top dose of 250 mg demonstrated a half-life ranging from 14.9-19.7 hours

(H) after single dose administration, and ranging from 10.9H-14.8H after multiple dose administration for 7 days (Trevena, Inc, data on file). The randomization was balanced to minimize first-order carry-over effects between visit 1 and 2. Safety assessments were performed, consisting of vital signs, electrocardiogram, physical and neurological examination, Columbia Suicide Severity Rating Scale, and laboratory assessments. Pharmaco-EEG and TMS-EMG/-EEG, were performed pre-dose and 4H post-dose (expected T_{max}) on Day 1 and Day 4.

The fed dose of TRVO45 250 mg (**Figure 2**) administered once daily for 4 days was selected based on the PK data observed after multiple dose administration over 7 days as described above. These data suggest that steady state would be expected to have been reached around Day 4 of dosing. Based on the plasma concentrations seen at this dose, and the preclinical plasma protein binding data, it was estimated that administration of 250 mg for 4 days should achieve unbound plasma concentration consistent with the effective unbound concentrations observed in nonclinical seizure models (6-20 ng/mL; Trevena, Inc., data on file). Protein binding for TRVO45 was measured in mouse, rat, dog, monkey and human plasma using high throughput dialysis methods, and is estimated at 99.84% in mice and 98.39% in humans. PK samples were collected pre-dose and 1H, 2H, 4H, 6H, and 8H post-dose on Day 1 and 4. On Day 2 and 3, only pre-dose samples were collected. TRVO45 plasma concentrations were measured by a validated high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS), with the coefficient of variance (CV) and lower limit of quantitation of 7.7 % and 1.00 ng/mL respectively (data on file).

Pharmaco-EEG was recorded per International Pharmaco-EEG Society (IPEG) guidelines, with 5 minutes (min) alternating periods of eyes open and closed every 64 seconds (s).²¹ TMS was applied per current guidelines,²⁰ using a MagPro R30 with MagOption stimulator and an MCF-B65 butterfly coil (2 × 75 mm; both MagVenture GmbH, Hueckelhoven, Germany), targeting the motor hotspot of the dominant abductor digiti minimi (ADM) muscle, identified by the Edinburgh Handedness Questionnaire.²² The coil was placed tangentially at a 45° angle to the skull midline. The RMT was determined,²³ followed by 75 single TMS pulses (SPTMS) at 120% RMT with intervals of 3.5-4.5 seconds. The paired-pulse TMS (PPTMS) included 75 pairs of pulses in randomized order with inter-stimulus intervals (ISI) of 2, 15, 100 and 300 milliseconds (ms), using 120% RMT (80% for ISI 2 and 15 ms conditioning pulses). EEG was continuously recorded using a 40-channel

recording system (Refa-40, TMSi B.V., the Netherlands) with electrodes placed according to the international 10-20 system (32-lead cap, TMSi B.V., the Netherlands), replacing A1 and A2 with M1 and M2 (at the mastoids)). Scalp electrode impedance was kept below 5k Ω and ground electrode at AFz. Signals were recorded from 21 or 32 electrodes sampled at 1024 Hz for pharmaco-EEG and 2048 Hz for TMS. Electrooculograms (EOG) were recorded to detect ocular artefacts, and ADM muscle activity was recorded using Ag/AgCl electrodes in a belly-tendon montage. Pharmaco-EEG data included: total power per frequency band, per electrode, per eye state, e.g., alpha-power in FZ-CZ with eyes open. TMS-EMG parameters included: mean single pulse peak-to-peak motor evoked potential (MEP) amplitude (μ V); paired pulse short-interval intracortical inhibition at ISIs of 2 ms (SICI₂), intracortical facilitation at ISI of 15 ms (ICF₁₅) and long-interval intracortical inhibition at ISIs of 100 and 300 ms (LICI₁₀₀ and LICI₃₀₀). Adapted noise masked auditory evoked potentials.²⁴ Principal component analysis (PCA) was applied for artifact removal.²⁵ A comprehensive explanation of the pharmaco-EEG and TMS-EMG/-EEG data synthesis is provided in the **Supplementary Materials**.

The sample size calculation was based on the MEP amplitude from Ruijs et al.,¹⁴ with 24 subjects providing 80% power to detect a -300 μ V difference, assuming a standard deviation of 500 μ V and a 2-sided significance level of 0.05. The study was double-blinded, with subjects and investigators blinded for treatment allocation. Randomization was generated digitally using SAS 9.4 by an independent statistician, with subjects enrolled sequentially (1-27) by the research physician. Medication was prepared by an unblinded pharmacist. Pharmaco-EEG data was log-transformed for analysis and back-transformed for interpretation as percentage change. Treatment effects were analyzed using analysis of covariance (ANCOVA) with treatment, time, period and their interactions as fixed factors, and subject-related factors as random effects. Baseline measurement served as covariate. The Kenward-Roger approximation estimated degrees of freedom, and parameters were estimated with restricted maximum likelihood.²⁶ Variance components were used for random effects, and treatment effect were reported with 95% confidence interval (CI), least square means (LSM), and p-value. Graphs display LSM estimates and change from baseline. The TMS-evoked potential (TEP) response following the test pulse was analyzed for all EEG leads, in the full-time sample of 0-300 ms and at specific periods of interest (TOI) around the characteristic 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).²⁷ To compare treatment and placebo single and paired-pulse TEPs, cluster-based permutation analysis was used,²⁸ a nonparametric method suited for multidimensional TMS-EEG data. Dependent samples t-tests were used for electrode and time comparisons, clustering t-values with $p < 0.05$. Significant clusters were determined using permutation testing (1500 permutations), and results were reported if less than 5% of the summed t-values obtained by permutation were larger than the cluster value observed in the original data. P-values of significant clusters are reported.

An exploratory post-hoc analysis was performed on the PK/PD relationship of TMS-EMG and pharmaco-EEG parameters that showed most promise for a PK/PD-relationship based on the results from the planned statistical analysis, namely alpha-power in FZ-CZ, PZ-O1, and PZ-O2 in the eyes open condition, and MEP amplitude. A detailed description of the analysis can be found in the **Supplementary Materials**. In summary, each parameter was modeled with an intercept only and with an additive and proportional effect, being a linear or non-linear ($E_{\max}$) concentration-effect relationship, using a mixed effects model. The linear and non-linear relationships were compared with the intercept-only model with an analysis of variance. The fit of the additive versus proportional and linear versus non-linear relationship were compared based on the Akaike Information Criterion (AIC), in which the model with the lowest AIC or a p-value of < 0.05 was selected.

RESULTS

Twenty-five out of twenty-seven subjects completed all study periods with two early terminations for reasons unrelated to safety or tolerability. Demographics are given in **Supplementary Materials**. TRV045 was well tolerated without any severe or serious adverse events (AES), or AE related discontinuations, and all AES resolved. Overall, the incidence of AES was similar between groups, except for headache, fatigue, and dizziness, which were reported more often during treatment with TRV045 (**Supplementary Materials**). Mean ($\pm$ standard deviation (SD)) TRV045 plasma concentrations increased over time and peaked at approximately 6h at concentrations of 690.5 ± 138.13 ng/mL on Day 1 and 893.80 ± 209.09 ng/mL on Day 4, corresponding with an estimated unbound concentration of 11.1 ng/mL and 14.4 ng/mL respectively, being within the range of efficacious unbound concentrations estimated in nonclinical seizure models.

For an overview of all pharmacology-EEG results, see **Table 1**. When comparing TRV045 vs. placebo for the overall treatment period, TRV045 significantly increased alpha-power in the parieto-occipital regions (PZ-O1 estimated difference (ED): 29.0%, 95%-CI: 6.5%;56.2%; PZ-O2 ED: 28.2%, 95%-CI: 7.4%;53.0%) and increased beta-power in the frontocentral region (FZ-CZ ED: 18.5%, 95%-CI: 2.1%;37.6%) with eyes open. There was a non-significant decrease in delta-power in the frontocentral region with eyes closed. Effects increased after multiple days of dosing. On Day 1, only alpha-power was significantly increased with eyes open, with a more localized effect (PZ-O2 ED: 26.9%, 95%-CI: 1.3%;58.9%). Also, delta-power was significantly decreased in the frontocentral region with eyes closed (FZ-CZ ED: 14.7%, 95%-CI: -25.4%;-2.4%). On Day 4, not only was alpha-power increased in all leads with eyes open (FZ-CZ ED: 40.7%, 95%-CI: 6.8%;85.3%; PZ-O1 ED: 38.8%, 95%-CI: 11.2%;73.1%; PZ-O2 ED: 26.6%, 95%-CI: 0.9%;58.9%), but beta-power and gamma-power were also increased in the frontocentral region (respectively FZ-CZ ED: 23.1%, 95%-CI: 3.0%;47.1%; and FZ-CZ ED: 21.3%, 95%-CI: 1.5%;45.1%). In addition to delta-power being significantly decreased in the frontocentral region with eyes closed (FZ-CZ ED: -14.0%, 95%-CI: -24.9%;-1.4%), delta-power also decreased in the parieto-occipital region with eyes open (PZ-O2 ED: -15.2%, 95%-CI: -27.6%;-0.5%).

For an overview all SP- and PPTMS-EMG parameters, see **Table 2**. The SPTMS-EMG MEP amplitude did not significantly change after administration of TRV045 in the overall treatment period (ED: 130.850 μ V, 95%-CI: -433.4;171.7), but there was a trend towards a decrease in MEP amplitude post-dose on Day 1 (ED: -304.136 μ V, 95%-CI: -688.2;79.9) (**Figure 3**). This trend was not observed post-dose on Day 4, nor when combining Day 1+4 treatment effects. There were no statistically significant effects of TRV045 on RMT, or on PPTMS-EMG parameters SICI₂, ICF₁₅, LIC₁₀₀ or LIC₃₀₀. A complete overview of the TMS-EEG results and corresponding topo-plots can be found in **Figure 4** and **Supplementary Materials**. No significant SPTMS-EEG clusters were found after TRV045 treatment. For PPTMS-EEG, two significant clusters were observed on Day 4, one pre-dose and one post-dose, both at an ISI of 300 ms and in the ipsilateral centro-parietal region. Pre-dose, we observed a significantly more negative P30 TEP amplitude in this cluster, but this difference was not significant when analyzing the entire time period. Post-dose, we observed a significantly more positive P60 TEP amplitude in this cluster; however, this difference was not significant when analyzing the entire time period.

For an overview of the PK/PD relationship analysis, see **Supplementary Materials**. We did not find a significant concentration-effect relationship for alpha-power in any of the brain regions (FZ-CZ, PZ-O1, PZ-O2). The MEP amplitude demonstrated a proportional linear concentration-effect relationship ($p < 0.0001$), see **Figure 5**, with an estimated baseline of 847 μ V (relative standard error (RSE): 0.15%) and a coefficient of -0.0004 (ng/mL)⁻¹ (RSE: 22.1%).

DISCUSSION

This study evaluated PD effects of SIPRI modulation by TRV045 on spontaneous electrical brain activity and cortical excitability, measured by pharmacology-EEG and TMS-EMG/-EEG. TRV045 significantly increased power in the higher frequency (alpha, beta and gamma) bands and decreased power in the lower (delta) frequencies in various brain regions, with an increasing effect over multiple days of dosing. The data provides pharmacodynamic evidence of CNS penetrance and CNS activity of TRV045, without accompanying clinical signs of sedation, as the incidence of somnolence was comparable between groups (**Supplementary Materials**). The TEAEs headache, fatigue, and dizziness were reported more frequently in the TRV045 group, which are common side-effects for CNS active drugs and are in line with the results from the first-in-human studies (Trevena, Inc., data on file). A non-significant decrease in SPTMS-EMG MEP amplitude was observed post-dose on Day 1, possibly indicating an acute decrease in cortical excitability following initial dosing with TRV045, which, however, was not present on Day 4. Two significant PPTMS-EEG clusters were found at ISI of 300 ms in the ipsilateral centro-parietal region after 4 days of dosing with TRV045, with a more negative P30 TEP amplitude pre-dose and a more positive post-dose P60 TEP amplitude, but these differences were not confirmed after analyzing the entire time period. The PK/PD relationship analysis identified a proportional linear effect for TRV045 plasma concentrations and MEP amplitude but could not establish a concentration-effect relationship for alpha-power with eyes open. While there was a statistically significant treatment effect for these parameters, a possible explanation for not detecting a relationship may be the limited number of datapoints with measurable TRV045 concentrations available per subject and dosing interval. This limitation could have made the analysis less suitable for identifying PK/PD relationships. Additionally, the

limited amount of data prevented us from studying underlying dynamics such as rhythms, placebo effects, or indirect (i.e., delayed) effects, which may be necessary for distinguishing the actual pharmacodynamic effects, especially regarding the changes observed in pharmaco-EEG.

To our knowledge, there is no literature available on the effects of SIPRI modulators on EEG spectral power in animals or humans. A systematic review aiming to profile EEG effects of ASM with different MOAs provides an overview of studies performed in epilepsy patients and healthy participants. The most common findings were EEG slowing, with increased delta and theta activity and decreased activity in higher frequency ranges.¹⁶ EEG slowing, in particular the attenuation of the posterior dominant alpha rhythm, correlates with cognitive impairment, a frequently reported AES of ASM in healthy participants studies with ion channel blockers, including gabapentin, carbamazepine and phenytoin.²⁹⁻³¹ Interestingly, milacemide, a glycine prodrug with anti-convulsant effects by increasing gamma-aminobutyric acid (GABA) and endogenous glycine levels, showed an effect opposite to other ASM, but in line with our study. It reduced delta power and increased alpha and beta power at lower dosages, while improving cognitive functions.³² This might be explained by milacemide's MOA, as glycine is a co-agonist of glutamate for activation of N-methyl-D-aspartate (NMDA) receptors^{33,34} and might have an (indirect) effect on glutamate neurotransmission. This, together with the results from SIPRI animal models⁵⁻⁸ and a TMS-EMG study performed in relapsing-remitting multiple sclerosis (RRMS) patients showing that fingolimod decreased ICF,³⁵ a marker which is mainly mediated by glutamate,³⁶⁻³⁸ further support the hypothesis that SIPRI modulators may influence glutamatergic neurotransmission and thereby reduce cortical excitability. Thus, one can hypothesize that the observed pattern of EEG changes after TRV045 treatment may be a direct or indirect effect of reduced glutamate activity and that TRV045 may demonstrate anti-convulsant activity without accompanying sedative side effects.

Although TRV045 did not significantly affect TMS-EMG parameters, there was a trend towards a decrease in MEP amplitude after the first dose with an effect size (**Figure 3**) comparable to those observed after single dose of other ASM,¹⁴ possibly indicating an early decrease in cortical excitability following a single dose of TRV045. Moreover, we found a proportional linear concentration-effect relationship between TRV045 plasma concentrations and MEP amplitude. This relationship indicates that we may have had to achieve higher concentrations on Day 1, in order to find this

effect in the planned statistical analysis too. A reduction of -400 μ V could theoretically be achieved at a TRV045 concentration of 1181 ng/mL for the typical individual, which is almost twice the mean concentration observed on Day 1. However, in this light, it remains unclear why such an effect was not observed on Day 4, while plasma concentrations were higher than on Day 1. Focusing on Day 1, it should also be considered that we had smaller statistical power to demonstrate significant effects after a single dose of TRV045, as we only performed 1 post-dose measurement on Day 1, while the study was powered on 3 post-dose measurements across 4 days of dosing. This in contrast to our previous study, in which we performed 2 post-dose measurements after a single dose of ASM.¹⁴ The MEP amplitude observed pre-dose on Day 4 was comparable to the baseline MEP amplitude and decreased again post-dose on Day 4, but with a smaller effect size than on Day 1, indicating a potentially changing effect that is not only driven by plasma concentrations. Surprisingly, we did not find any significant effects of TRV045 on PPTMS-EMG parameters, although this was expected based on its hypothesized MOA, as ICF is thought to reflect excitatory transmission by glutamate.³⁶⁻³⁸ The study conducted in patients with RRMS reported a decrease in ICF at ISI of 9 ms after 60 days of fingolimod treatment.³⁵ The most important differences between the fingolimod study and the current study are that we evaluated healthy participants and only measured ICF at ISI 15 ms, which is a longer interval than the ISIs evaluated in the fingolimod study. Although not significant, we did observe a decrease in ICF on Day 4 consistent with the fingolimod results, which raises the question whether measuring a broader range of ISIs would have allowed us to demonstrate a significant effect of TRV045 on cortical excitability. On the other hand, one should consider the possibility of time effects in the fingolimod study, such as disease progression, as a possible confounder when interpreting the results, as that study was conducted in a patient population, with a treatment duration of 60 days and without a control group.

TRV045 had significant effects on the PPTMS-EEG at ISI of 300 ms in the ipsilateral centro-parietal region on Day 4. Pre-dose on Day 4, TRV045 resulted in a significantly more negative P30 TEP amplitude and post-dose a more positive P60 TEP amplitude (**Figure 4** and **Supplementary Materials**). In particular, the Day 4 pre-dose cluster was unexpected given that TRV045 plasma concentration was lowest at this timepoint compared to the other post-dose timepoints (**Supplementary Materials**). This, together with the fact that we did not find significant clusters on Day 1,

indicates that the observed effect is not an acute effect. However, the lack of significant differences observed for SPTMS-EEG and other PPTMS-EEG ISIs, does raise the question whether the significant differences observed at ISI 300 ms are true pharmacological effects or a type I error (false positive). There are no previous TMS-EEG studies investigating the effects of SIPR modulators, and overall, there is limited data available of pharmacological effects on PPTMS-EEG TEP, making it difficult to directly relate our outcomes to the current literature. To our knowledge, there have only been two studies investigating pharmacological effects on PPTMS-EEG TEP in healthy volunteers.^{14,39} In these studies, ion channel blockers levetiracetam and valproic acid, and GABAergic drugs lorazepam, diazepam and baclofen were investigated. Levetiracetam, lorazepam, baclofen and diazepam all affected the paired pulse TEP at ISIs > 100 ms,^{14,39} but only affected later TEP components (N100 and P180), whereas TRV045 modulated earlier TEP components (P30 and P60). There is some evidence hinting that the P60 TEP component of the PPTMS-EEG might be mediated by glutamate/GABA_B interaction, as a conditioning pulse delivered at a short ISI (SICI 2 ms) attenuated the test pulse P60 TEP component, and at longer intervals (ICF 10 ms and LICI 100 ms) enhanced the P60 component.^{39,40} However, as TEPs represent the sum of excitatory and inhibitory neural activity at a given time following the TMS pulse, it is difficult to translate the observed effects to an increase or decrease in cortical excitability, let alone an isolated mechanism of action.

Differences in effects between Day 1 and 4, as observed with pharmac-EEG and TMS-EMG/-EEG, may provide insights into the evolving effects of TRV045 with repeated dosing. There is no literature available on whether the initial dose effects of other ASM on cortical excitability, such as described previously for TMS-EMG/-EEG,¹⁴ persists with repeated dosing, or evolve over time. Thus, it is hard to fully contextualize these observations relative to what is known about ASM and their effects on EEG and TMS. The brain is a complex system of excitatory and inhibitory pathways, and drugs influencing cortical excitability could very well have different initial and repeat dose effects. Based on the observed PD effects in this study, as well as all the currently available data, it could be hypothesized that the observed effects are due to glutamatergic modulation by TRV045. Whether this will also translate to anti-epileptic effects will need to be investigated in a future study.

This study has several limitations. Firstly, as drug effects changed over time based on the pharmac-EEG signals, combining PD measurements

on Day 1 and 4 for TMS in the analysis, does not provide the same information as performing multiple measurements on a single day. As we performed one post-dose TMS measurement per day, there was lower statistical power to detect a signal on each separate day. We had to limit the number of post-dose assessments per day due to the dense schedule of assessments, and thus timed the PD measurements to coincide with maximum drug exposure to measure maximum PD effects, assuming a direct concentration-effect relationship. Additionally, our power calculation was based on a single dose study in which all post-dose measurements were performed on the same day,¹⁴ and measuring TMS on different days introduced between-day-variability in the current study, thus increasing the SD of the difference. Although we used baseline correction to minimize variability as much as possible, our model does not correct for day-to-day variability, which also negatively impacts the statistical power. Therefore, we might have been underpowered to demonstrate a significant effect on the MEP amplitude. It is also possible that with a higher TRV045 exposures, we would have reached a larger, and significant effect, but as 250 mg was the highest dose administered in humans in a multiple-dose fashion, and pharmacokinetics were not dose proportional at this dose level and exposures at higher doses would not provide further increases in plasma exposure, we could not increase the dose or exposure any higher for this study. Another limitation is that C_{max} was reached slightly later than anticipated (6H) while PD measures were performed at 4H post-dose, although the impact is limited because exposures were not much different at these timepoints (plasma concentration mean $\pm$ SD: Day 1 + 4H 591.8 $\pm$ 177.5 ng/mL vs. Day 1 + 6H 652.1 $\pm$ 149.2 ng/mL and Day 4 + 4H 834.9 $\pm$ 228.0 ng/mL vs. Day 4 + 6H 849.3 $\pm$ 218.2 ng/mL). Furthermore, only males were included in the study to exclude potential menstrual cycle-related confounding effects on cortical excitability,¹⁹ which limits the translatability of the findings to females. Although the family-wise error rate was controlled using cluster-based permutation analysis, no further correction for multiple testing was performed in our study when comparing TRV045 to placebo, which is considered valid because of the exploratory nature of the study. Lastly, the cross-over nature of the study could be a limitation, but based on TRV045's half-life, a wash-out period of 1 week should be sufficient to prevent PK carry-over effects. Our statistical model corrects for possible differences between the treatment periods. Furthermore, because we implemented a balanced for first carry-over effects randomization, delayed PD carry-over effects are not expected to confound our results.

CONCLUSION

With this study we demonstrated that TRV045, a S1PR1 agonist, is a CNS active compound, with an incremental effect on the pharmaco-EEG signal after multiple days of dosing with a distinct pattern that differs from registered ASM. There appears to be an acute decrease in corticospinal excitability, but the reduction in MEP amplitude observed post-dose on Day 1 was not significant and was not sustained after multiple days of dosing. This is the first study describing the effects of a S1PR1 modulator on pharmaco-EEG and TMS-EMG/-EEG. Further evaluation is required to elucidate the physiological underpinnings of TRV045's observed PD effects and whether these translate into anti-epileptic effects.

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TABLE 1 Summary of Results for Pharmaco-EEG. Estimated difference of TRV045 250 mg vs. placebo of pharmaco-EEG parameters in %, with 95% confidence interval (95%-CI) and p-values of the total power ((μV)²), per frequency band, per lead, per eye state. Frequency per band: alpha (α): 8.5 < 12.5 Hz; beta (β): 12.5 < 30.0 Hz; delta (δ): 1.5 < 6.0 Hz; gamma (γ): 30.0 < 40.0 Hz; and theta (θ) 6.0 < 8.5 Hz. Statistically significant (p<0.05) results are marked in bold. Significant increases in power are highlighted in green. Significant decreases in power are highlighted in yellow.

TRV045 250 MG VS. PLACEBO							
Eyes Closed				Eyes Open			
Overall	Individual Contrasts			Overall	Individual Contrasts		
All post-dose timepoints*	Day 1 post-dose	Day 1+4 post-dose	Day 4 post-dose	All post-dose timepoints*	Day 1 post-dose	Day 1+4 post-dose	Day 4 post-dose
EEG ALPHA-POWER FZ-CZ							
8.8	-9.3	1.4	13.3	27.2	-5.7	15.2	40.7
(-4.3, 23.7)	(-23.4, 7.5)	(-11.8, 16.5)	(-4.6, 34.4)	(-0.5, 62.6)	(-28.4, 24.2)	(-10.5, 48.4)	(6.8, 85.3)
p = 0.1840	p = 0.2552	p = 0.8411	p = 0.1502	p = 0.0543	p = 0.6697	p = 0.2609	p = 0.0164
EEG ALPHA-POWER PZ-OI							
1.0	-4.3	0.8	6.2	29.0	10.6	23.9	38.8
(-11.9, 15.9)	(-19.8, 14.1)	(-13.0, 16.8)	(-11.0, 26.7)	(6.5, 56.2)	(-11.4, 37.9)	(1.5, 51.1)	(11.2, 73.1)
p = 0.8784	p = 0.6178	p = 0.9150	p = 0.5009	p = 0.0113	p = 0.3645	p = 0.0358	p = 0.0047
EEG ALPHA-POWER PZ-O2							
-1.1	-2.8	0.1	3.1	28.2	26.9	26.8	26.6
(-13.6, 13.2)	(-19.0, 16.6)	(-13.6, 16.1)	(-14.3, 24.0)	(7.4, 53.0)	(1.3, 58.9)	(4.9, 53.2)	(0.9, 58.9)
p = 0.8632	p = 0.7565	p = 0.9871	p = 0.7400	p = 0.0079	p = 0.0386	p = 0.0158	p = 0.0418
EEG BETA-POWER FZ-CZ							
10.4	5.8	7.9	10.1	18.5	3.6	12.9	23.1
(-4.9, 28.1)	(-11.0, 25.8)	(-7.6, 26.1)	(-7.5, 31.1)	(2.1, 37.6)	(-13.3, 23.8)	(-3.5, 32.0)	(3.0, 47.1)
p = 0.1846	p = 0.5149	p = 0.3227	p = 0.2722	p = 0.0270	p = 0.6943	p = 0.1242	p = 0.0235
EEG BETA-POWER PZ-OI							
9.1	6.5	8.2	9.8	8.9	-0.5	5.7	12.3
(-8.6, 30.2)	(-13.2, 30.8)	(-10.0, 30.0)	(-10.5, 34.8)	(-10.7, 32.7)	(-20.7, 24.9)	(-13.9, 29.8)	(-10.5, 41.0)
p = 0.3198	p = 0.5375	p = 0.3897	p = 0.3611	p = 0.3765	p = 0.9636	p = 0.5771	p = 0.3044
EEG BETA-POWER PZ-O2							
7.7	8.8	6.0	3.3	13.3	6.4	8.2	10.0
(-8.4, 26.5)	(-9.6, 31.0)	(-10.4, 25.4)	(-14.3, 24.5)	(-6.7, 37.6)	(-14.4, 32.4)	(-11.4, 32.2)	(-11.7, 36.9)
p = 0.3544	p = 0.3642	p = 0.4821	p = 0.7281	p = 0.1941	p = 0.5645	p = 0.4248	p = 0.3852
EEG DELTA-POWER FZ-CZ							
-6.3	-14.7	-14.3	-14.0	-6.6	-5.8	-9.6	-13.2
(-15.0, 3.2)	(-25.4, -2.4)	(-23.1, -4.6)	(-24.9, -1.4)	(-17.9, 6.3)	(-20.1, 11.2)	(-21.1, 3.7)	(-26.5, 2.5)
p = 0.1771	p = 0.0214	p = 0.0061	p = 0.0306	p = 0.2639	p = 0.4710	p = 0.1391	p = 0.0919
EEG DELTA-POWER PZ-OI							
-2.2	-2.4	-2.6	-2.8	-7.1	-6.7	-9.4	-12.1
(-10.6, 7.0)	(-15.0, 12.0)	(-12.1, 8.0)	(-15.4, 11.7)	(-18.2, 5.5)	(-20.3, 9.3)	(-20.8, 3.7)	(-24.9, 3.0)
p = 0.6077	p = 0.7233	p = 0.6031	p = 0.6845	p = 0.2403	p = 0.3842	p = 0.1451	p = 0.1091

TRV045 250 MG VS. PLACEBO							
Eyes Closed				Eyes Open			
Overall	Individual Contrasts			Overall	Individual Contrasts		
All post-dose timepoints*	Day 1 post-dose	Day 1+4 post-dose	Day 4 post-dose	All post-dose timepoints*	Day 1 post-dose	Day 1+4 post-dose	Day 4 post-dose
EEG DELTA-POWER PZ-O2							
-1.9	4.9	-1.1	-6.7	-6.0	0.2	-7.8	-15.2
(-8.9, 5.5)	(-8.7, 20.5)	(-9.9, 8.6)	(-18.9, 7.3)	(-16.2, 5.5)	(-14.4, 17.2)	(-18.7, 4.6)	(-27.6, -0.5)
p = 0.5837	p = 0.4956	p = 0.8138	p = 0.3265	p = 0.2699	p = 0.9838	p = 0.1949	p = 0.0432
EEG GAMMA-POWER FZ-CZ							
4.7	-11.1	0.0	12.5	12.4	-3.9	8.0	21.3
(-6.1, 16.8)	(-24.3, 4.5)	(-11.6, 13.2)	(-4.5, 32.4)	(-0.2, 26.6)	(-19.6, 14.9)	(-5.7, 23.6)	(1.5, 45.1)
p = 0.3914	p = 0.1508	p = 0.9996	p = 0.1554	p = 0.0529	p = 0.6573	p = 0.2583	p = 0.0343
EEG GAMMA-POWER PZ-O1							
13.7	7.8	13.3	19.1	11.9	-1.1	10.4	23.1
(-18.4, 58.4)	(-25.8, 56.5)	(-19.6, 59.5)	(-18.0, 72.9)	(-21.8, 60.1)	(-34.2, 48.9)	(-23.8, 59.9)	(-18.2, 85.3)
p = 0.4299	p = 0.6859	p = 0.4589	p = 0.3482	p = 0.5160	p = 0.9580	p = 0.5848	p = 0.3072
EEG GAMMA-POWER PZ-O2							
13.1	5.2	7.0	8.7	20.5	11.8	12.6	13.5
(-13.2, 47.4)	(-21.1, 40.3)	(-18.4, 40.2)	(-18.6, 45.2)	(-8.5, 58.7)	(-17.3, 51.2)	(-15.1, 49.4)	(-16.2, 53.7)
p = 0.3440	p = 0.7198	p = 0.6117	p = 0.5590	p = 0.1746	p = 0.4568	p = 0.3936	p = 0.4035
EEG THETA-POWER FZ-CZ							
-0.1	-9.9	-9.2	-8.5	14.4	3.0	4.5	6.0
(-13.2, 15.0)	(-24.3, 7.2)	(-21.9, 5.4)	(-23.3, 9.0)	(-2.7, 34.6)	(-14.7, 24.4)	(-11.7, 23.7)	(-12.3, 28.1)
p = 0.9872	p = 0.2327	p = 0.1961	p = 0.3128	p = 0.0983	p = 0.7529	p = 0.5974	p = 0.5371
EEG THETA-POWER PZ-O1							
4.0	0.0	2.0	4.1	13.0	2.7	5.1	7.6
(-9.4, 19.5)	(-17.0, 20.5)	(-12.3, 18.7)	(-13.6, 25.4)	(-5.6, 35.3)	(-17.3, 27.4)	(-13.0, 26.9)	(-13.3, 33.5)
p = 0.5600	p = 0.9998	p = 0.7901	p = 0.6704	p = 0.1712	p = 0.8058	p = 0.5913	p = 0.4985
EEG THETA-POWER PZ-O2							
1.8	3.3	0.8	-1.6	14.9	17.2	12.0	7.1
(-13.7, 20.2)	(-16.5, 27.8)	(-15.6, 20.5)	(-20.6, 22.0)	(-4.4, 38.1)	(-6.5, 46.9)	(-7.8, 36.0)	(-14.7, 34.4)
p = 0.8218	p = 0.7620	p = 0.9268	p = 0.8807	p = 0.1285	p = 0.1645	p = 0.2402	p = 0.5484

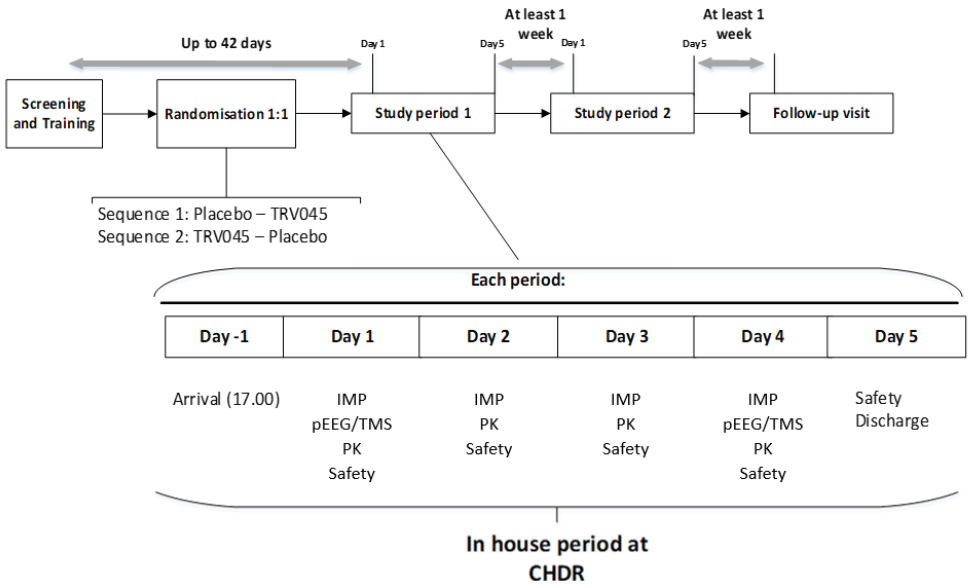
Abbreviations: EEG = electroencephalography; vs. = versus.
* All post-dose timepoints include: Day 1 +4H post-dose, Day 4 pre-dose, Day 4 +4H post-dose.

TABLE 2 Summary of results for TMS-EMG.
Estimated difference of TRV045 250 mg vs. placebo of single pulse and paired pulse TMS-EMG parameters, with 95% confidence interval (95%-CI) and p-value.

TRV045 250 MG VS. PLACEBO				
Overall		Individual Contrasts		
	All post-dose timepoints*	Day 1 post-dose	Day 1+4 post-dose	Day 4 post-dose
SINGLE PULSE TMS-EMG PARAMETERS				
MEP amplitude (µV)	-130.9	-304.1	-178.9	-53.7
	(-433.4, 171.7)	(-688.2, 79.9)	(-502.5, 144.7)	(-437.8, 330.4)
	p = 0.3793	p = 0.1182	p = 0.2678	p = 0.7802
RMT (%)	-0.7	0.8	-0.1	-1.0
	(-1.9, 0.5)	(-1.2, 2.7)	(-1.5, 1.3)	(-2.9, 0.9)
	p = 0.2531	p = 0.4261	p = 0.8797	p = 0.3100
PAIRED PULSE TMS-EMG PARAMETERS				
SICI ₂ (%)	-3.7	8.7	-1.8	-12.3
	(-16.2, 8.8)	(-8.9, 26.3)	(-15.8, 12.1)	(-30.1, 5.4)
	p = 0.5435	p = 0.3273	p = 0.7920	p = 0.1688
ICF ₁₅ (%)	-17.1	19.5	-4.2	-27.9
	(-51.0, 16.7)	(-24.2, 63.2)	(-40.8, 32.3)	(-71.6, 15.8)
	p = 0.3052	p = 0.3737	p = 0.8160	p = 0.2052
LICI ₁₀₀ (%)	4.4	-0.3	1.4	3.1
	(-5.3, 14.)	(-12.2, 11.6)	(-8.8, 11.7)	(-8.8, 5.0)
	p = 0.3614	p = 0.9613	p = 0.7816	p = 0.6035
LICI ₃₀₀ (%)	3.4	5.5	4.2	3.0
	(-3.5, 10.3)	(-7.3, 18.2)	(-4.4, 12.9)	(-9.7, 15.8)
	p = 0.3131	p = 0.3953	p = 0.3270	p = 0.6383

Abbreviations: TMS-EMG = transcranial magnetic stimulation - electromyography; vs. = versus; MEP = motor evoked; RMT = resting motor threshold; SICI₂ = short-interval intracortical inhibition at interstimulus-interval of 2 ms; ICF₁₅ = intracortical facilitation at interstimulus-interval of 15 ms; LICI₁₀₀ = long-interval intracortical inhibition at interstimulus-interval of 100 ms; LICI₃₀₀ = long-interval intracortical inhibition at interstimulus-interval of 300 ms.
* All post-dose timepoints include: Day 1 +4H post-dose, Day 4 pre-dose, Day 4 +4H post-dose.

FIGURE 1 Study Design. Schematic detail of treatment periods. Each subject has two identical treatment periods, in which treatments are administered in randomized order.



Abbreviations: IMP = investigational medicinal product; pEEG = PharmacoeEG; TMS = Transcranial magnetic stimulation; PK = pharmacokinetics blood sample.

FIGURE 2 Chemical Name and Structure. International Union of Pure and Applied Chemistry (IUPAC) chemical name and structural formula of TRV045.

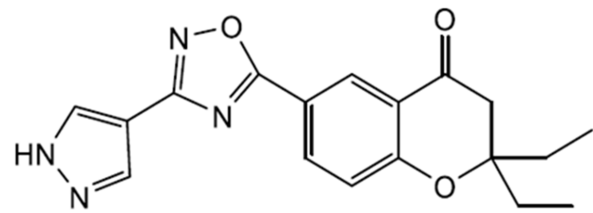
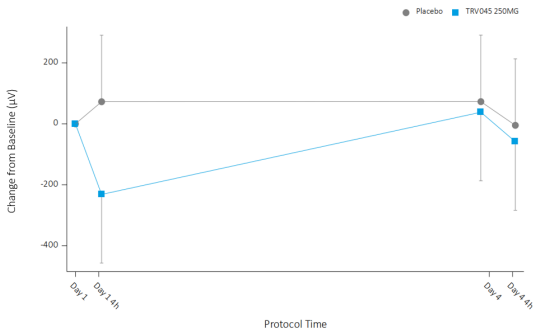
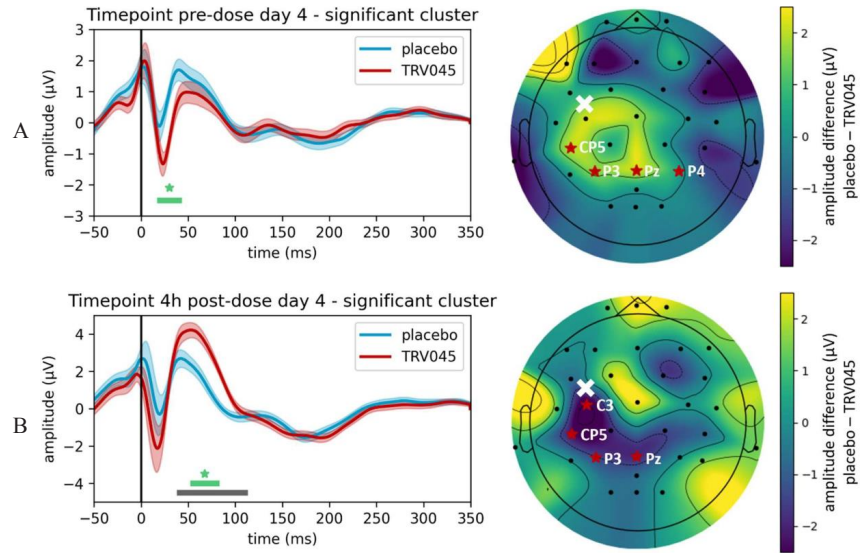


FIGURE 3 Peak-to-peak MEP Amplitude (LSM, 95%-CI). Change from baseline of the least square means (LSM) with 95% confidence interval (95%-CI) as error bars of the motor-evoked potential (MEP) amplitude (μV), using single pulse transcranial magnetic stimulation, for TRV045 and placebo.



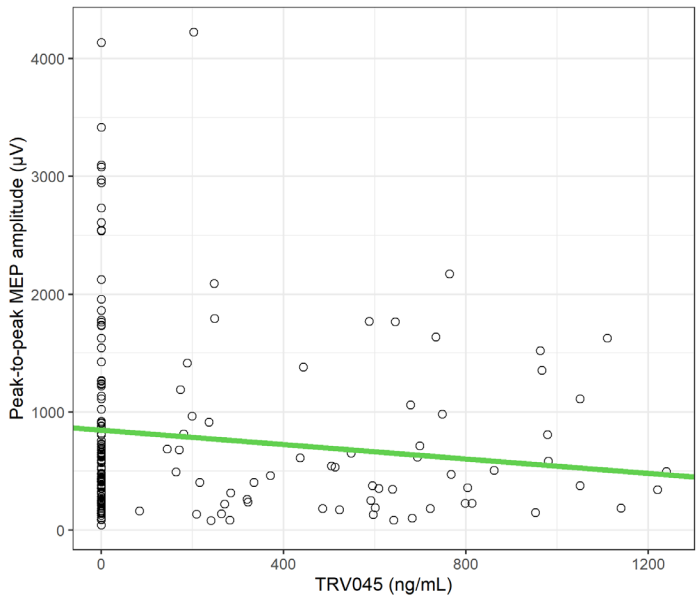
Abbreviations: MEP = motor-evoked potential; LSM = least square means; 95%-CI = 95% confidence interval.

FIGURE 4 Grand average and topographical distribution of significant pPTMS-EEG clusters. Overview of the significant cluster (A) P30 ($p = 0.02$) and (B) P60 ($p < 0.01$) found pre-dose on day 4 when comparing TEPs of the placebo (in blue) and TRV045 (in red) conditions for PPTMS-EEG LIC1300. On the left side, the grand average (mean $\pm$ SEM) over all significant electrodes is presented. On the right side, the difference in topographical distribution (placebo – TRV045) at the time of the cluster is presented. The thick green bar represents the time window of significant differences, the white cross the stimulation site, and the black dots the electrode positions with the significant electrodes as red stars.



Abbreviations: PPTMS-EEG = paired pulse transcranial magnetic stimulation – encephalography; LIC1300 = long-interval intracortical inhibition at interstimulus-interval of 300 ms; SEM = standard error of the mean; ms = millisecond.

FIGURE 5 PK/PD relationship analysis of peak-to-peak MEP Amplitude.
PK/PD relationship plot for peak-to-peak MEP amplitude and model derived parameters.



Abbreviations: PK = pharmacokinetic; PD = pharmacodynamic; MEP = motor evoked potential.

CHAPTER IV

SUPPLEMENTARY MATERIALS

Full supplementary materials available at the publisher’s website:
<https://ascpt.onlinelibrary.wiley.com/doi/10.1002/cpt.3521>

CHAPTER V

**DIFFERENTIAL CORTICAL EFFECTS OF
SYNAPTIC VERSUS EXTRASYNAPTIC
GABA_A RECEPTOR MODULATION
WITH LORAZEPAM AND
BREXANOLONE: A RANDOMIZED,
DOUBLE-BLIND, PLACEBO-
CONTROLLED, CROSS-OVER TMS AND
EEG TRIAL**

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ABSTRACT

GABA-kines are a diverse class of compounds that modulate gamma-aminobutyric acid (GABA) mediated inhibition, yet how their distinct receptor mechanisms translate into neurocircuit-level effects remains unclear. This double-blind, placebo-controlled, 4-way crossover study leveraged the benzodiazepine (BZD) lorazepam and neuroactive steroid (NAS) brexanolone to explore how synaptic versus combined synaptic+extrasynaptic GABA_A receptor modulation differentially impact brain circuitry, using a multimodal neurophysiology approach combining transcranial magnetic stimulation (TMS) with electromyography (TMS-EMG) and electroencephalography (TMS-EEG), and resting-state pharmaco-electroencephalography (PEEG). Participants received brexanolone (30 or 90 mcg/kg/h 3-hour infusion), lorazepam (2 mg oral), or matching placebo. Outcomes were analyzed using plasma drug concentrations and mixed models for repeated measures or cluster-based permutation testing. Brexanolone dose-dependently increased beta and theta power, enhanced long-interval intracortical inhibition at interstimulus interval of 100 ms (LICI₁₀₀), and modulated early TMS-evoked potentials (TEP) in contralateral centroparietal regions. Lorazepam increased beta power, suppressed theta power, and modulated later TEPs in frontocentral areas. Frontal theta power, LICI₁₀₀ and specific TEP modulation distinguished brexanolone from lorazepam and may serve as NAS-specific biomarkers. Beta power increases correlated with drug exposure for both compounds, suggesting it reflects general GABAergic activity. This first head-to-head comparison of a NAS and BZD in humans demonstrates mechanistically distinct neurocircuit effects, with NAS appearing to engage both synaptic and extrasynaptic GABA_A receptors. These findings underscore the value of multimodal neurophysiology in characterizing CNS drug mechanisms.

INTRODUCTION

Psychiatric conditions are a leading cause of disability worldwide, with an estimated 970 million people living with a current mental health disorder.¹ Disturbances in the brain's excitation-inhibition (E/I) balance are thought to underlie many psychiatric disorders.²⁻⁴ Targeting gamma-aminobutyric acid (GABA) receptor signaling, which primarily mediates inhibitory neurotransmission, represents an attractive therapeutic approach for novel drug development, given its role in regulating E/I balance. Pharmacological agents that enhance GABA signaling (GABAkines) are widely prescribed to manage psychiatric symptoms. GABAkines are typically classified by their binding sites on the GABA-gated type A chloride channel receptors (GABA_AR), which determine their divergent physiological effects. This class includes benzodiazepines (BZD), non-benzodiazepine hypnotics (Z-DRUGS), neuroactive steroids (NAS), barbiturates, ethanol, and anesthetic agents. Understanding how these agents differentially modulate E/I neurocircuitry may inform more personalized treatment strategies.

GABA_AR are pentameric, transmembrane, ion-gated channels assembled in various subunit combinations across diverse neurocircuits.⁵ BZDs bind at the interface of α - and γ -subunits on synaptic GABA_AR (BZD-binding site), whereas NAS have a unique binding domain between α - and β -subunits present on both synaptic and extrasynaptic GABA_AR (NAS-binding site).^{5,6} While BZD primarily enhance phasic inhibitory synaptic currents, NAS modulate both phasic and tonic components of inhibition, with a particularly strong effect on tonic inhibition.⁷ This raises the question of how differences in mechanism of action (MOA) between BZD and NAS, and thus synaptic versus combined synaptic+extrasynaptic inhibition, differentially modulate neurocircuitry. Extensive clinical experience with the BZD lorazepam and placebo-controlled studies of the NAS brexanolone⁸ (a synthetic form of allopregnanolone) support their use as model compounds to investigate this question. The use of multiple assessments of neurophysiological readouts in vivo may be a powerful approach to understand the nuanced differences between these distinct MOAs. Indeed, changes in resting-state electroencephalography following pharmacological intervention (PEEG) is a well-established translational tool that has been shown to be sensitive to novel GABAkines in rodents^{9,10} as well as in humans¹¹ (brexanolone data on file). Non-invasive neurostimulation using transcranial magnetic stimulation (TMS) is a promising approach to investigate E/I balance in response to distinct GABAergic

pharmacology, as it can elucidate changes in cortical E/I mechanisms depending on the stimulation protocol.¹² When combined with EEG and/or electromyography (EMG), TMS can quantify both cortical excitability and effects of CNS-penetrant drugs¹³ by constructing an “input-output” relationship to assess pharmacological modulation of neurocircuits.¹⁴ Similar to PEEG, TMS has been used to explore synaptic GABA-mediated CNS inhibition,^{13,15–17} including studies with lorazepam.^{18–20} However, the effects of combined synaptic+extrasynaptic GABA_AR modulation by NAS remain unclear. The aim of the current study was to explore the differential pharmacodynamic (PD) effects of two distinct classes of GABA_Akinines, the NAS brexanolone and the BZD lorazepam, leveraging both passive (PEEG) and active (TMS-EMG/-EEG) measures of neurocircuitry modulation. The primary hypothesis was that cortical activation, measured by PEEG and TMS, would significantly differ between brexanolone and lorazepam due to addition of extrasynaptic GABA_AR modulation by brexanolone. Knowledge gained from these experiments may clarify how synaptic versus synaptic+extrasynaptic inhibition, through their distinct effects on phasic and tonic brain inhibition, differentially impact neurocircuitry.

MATERIAL AND METHODS

This study was approved by the Medical Ethics Committee, Foundation Beoordeling Ethiek Biomedisch Onderzoek (BEBO) and performed according to the International Conference on Harmonisation guidelines on Good Clinical Practice. The study was sponsored by Sage Therapeutics, Inc. and conducted at the Centre for Human Drug Research (CHDR), Leiden, The Netherlands, from 22-May-2023 to 09-Oct-2023, and registered in the European Union Clinical Trials Information System (EU trial number 2022-503019-40-00).

Participants

Healthy males, aged 18–65 years, were included after obtaining written informed consent and a medical screening. Participants with a significant history or presence of psychiatric or neurologic disease or condition were excluded, as well as participants with an increased risk of TMS-related complications, based on the TMS safety questionnaire.²¹ A complete overview of inclusion and exclusion criteria is available in **Supplementary Materials**.

Study design

The study consisted of a randomized, double-blind, double-dummy, placebo-controlled, active-comparator, four-period cross-over design. Participants received single low (30 mcg/kg/h) and high (90 mcg/kg/h) dose of brexanolone via 3-hour (H) intravenous (IV) infusion, oral (PO) lorazepam (2 mg), and matching placebo (**Figure 1**). Randomization was generated digitally by an independent statistician using SAS® 9.4 for Windows or newer (SAS Institute Inc.) and participants were assigned to one of 4 treatment sequences. Medication was prepared by an unblinded pharmacist and subjects were enrolled sequentially by the blinded research physician. Participants were admitted on Day -1 and stayed for approximately 24H during each study period, with a 7–12 day washout period. TMS-EMG/-EEG was performed at baseline, 2H and 20 minutes (M) and 4H post-dose. PEEG was performed twice at baseline and 1H, 2H, 3H, 3H30M, 4H, and 5H post-dose. Pharmacokinetic (PK) sample collections were time-matched with the PD measurements, and plasma concentrations for brexanolone and lorazepam were measured by a validated high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Dose Selection

For brexanolone, a 3-hour infusion was given to allow adequate time to reach steady-state, expected at ~2H20M. Dose-selection was based on PK simulations using brexanolone data from previous clinical studies. To enable concentration-effect relationships analysis, neuro compartment exposures following low-dose brexanolone at the 1st post-dose TMS (+2H20M) and high-dose brexanolone at the 2nd post-dose TMS (+4H) were matched to ensure sufficient data was generated at adequately high exposures. A high dose of 90 mcg/kg/h of brexanolone was selected to maximize target occupancy within the limits of available safety data, which corresponded with a low dose of 30 mcg/kg/h based on the PK simulation. Lorazepam was selected as the BZD comparator due to its well-characterized PD effects in previous PEEG and TMS studies,^{11,17} and a 2 mg PO dose has been shown to produce clinically relevant exposure levels for these modalities.^{11,17} Lorazepam was administered at the start of the infusion to align peak exposures of both drugs towards the end of the infusion, as lorazepam's T_{max} occurs ~2H post-dose, similar to when brexanolone reaches steady-state.

Procedures

TMS and PEEG data acquisition and processing were performed as described previously^{17,22} and are described in detail in **Supplementary Materials**. In summary, PEEG was recorded per International Pharmacoe-EEG Society (IPEG) guidelines²³ and consisted of 5 minutes of resting-state per eye state (open and closed), with alternating eye states every 64 seconds. TMS was applied per current guidelines,²⁴ stimulating the motor hotspot of the dominant abductor digiti minimi (ADM) muscle, as assessed by the Edinburgh Handedness Questionnaire.²⁵ The resting motor threshold (RMT) was determined²⁶ at the start of each measurement, after which single (SPTMS) and paired pulse (PPTMS) protocols were applied. Paired pulses were delivered at inter-stimulus intervals (ISI) of 2, 15, and 100 ms, measuring short-interval intracortical inhibition (SICI₂), intracortical facilitation (ICF₁₅) and long-interval intracortical inhibition (LICI₁₀₀), respectively. During PEEG and TMS, EEG was continuously recorded using a 40-channel recording system (Refa-40, TMSi B.V., the Netherlands).

Statistical Analysis

Given the exploratory nature of this study, we based our sample size calculation on the effects of lorazepam on the MEP amplitude reported by Ruijs et al.,¹⁷ with 24 subjects providing 80% power to detect a $-300\mu\text{V}$ difference, assuming a $500\mu\text{V}$ standard deviation (SD) and 0.05 two-sided significance level. TMS-EMG and PEEG parameters were analyzed with a mixed model for repeated measures (MMRM) with treatment, time, period, sequence, and treatment-by-time interaction as fixed factors, subjects as random factors, and the (average) baseline measurement per period as covariate. Data were log-transformed and back-transformed after analysis so the results could be reported in original scale, with exception of the RMT. Pair-wise treatment differences were calculated for low- and high-dose brexanolone versus placebo and versus lorazepam, and for lorazepam versus placebo. This was done for the overall treatment effects and for each post-dose time point separately, based on the Least Square Means (LSM) estimates of the change from baseline (CFB) and standardized error (SE) for each treatment derived from the MMRM. Results are reported as the LSM difference with two-sided 95% confidence interval (CI) and p-values for RMT, and the LSM ratio with two-sided 95%-CI and p-values for all the other parameters. All statistical tests are conducted using a

two-sided alpha of 0.05, and all reported p-values are nominal. Unless specified otherwise, all results are reported as baseline-corrected relative to placebo. As effects were most consistent in the eyes-closed condition and generally aligned with eyes-open results, PEEG data are presented for the eyes-closed condition only. As the results for low and broadband beta power were comparable, only broadband beta power outcomes are reported. TMS-evoked potential (TEP) responses were analyzed across all 32 EEG channels, both over the full post-stimulus window (0–300 ms) and within predefined time periods of interest (TOI) corresponding to the characteristic TEP components.²⁶ To evaluate the spatiotemporal dynamics of the multidimensional TMS-EEG data while controlling for the multiple-comparisons problem, non-parametric cluster-based permutation testing²⁷ was employed to compare single- and paired-pulse TEPs following active treatment versus placebo, and brexanolone versus lorazepam, using baseline as covariate. Clusters in the original data, based on neighboring electrodes ($n \geq 2$), were considered significant if less than 5% of the summed t-values obtained by permutation were larger than the cluster value observed in the original data. P-values of significant clusters are reported.

PK/PD analysis

Two existing Population Pharmacokinetic (POP-PK) models were used to develop the PK/PD models, one for brexanolone²⁷ and one for lorazepam.²⁸ Observations for MEP amplitude, RMT, SICI₂, ICF₁₅, and LICI₁₀₀, as well as beta and theta power at FZ-CZ with eyes closed were investigated. The concentration-effect relationship for TMS-EMG and PEEG parameters was evaluated using linear and exponential models, and are reported as estimates, residual standard error (RSE) and confidence intervals of the concentration slope. For a complete summary of the PK/PD modelling methods, see **Supplementary Materials**.

RESULTS

Demographics, safety and PK

A total of 29 participants were enrolled, with 24 participants receiving all treatments: 24 placebo, 25 low- and high-dose brexanolone, and 28 lorazepam. In total, 5 participants discontinued, with 4 drug-related discontinuations: 1 participant after the low-dose brexanolone period due

to hypoxia, and 3 participants after the lorazepam period due to hypoxia (n=2) and urticaria (n=1). Participants had a mean age of 35.8 years ($\pm$ SD: 16.7), and most participants were white (72.4%) and right-handed (96.6%). Demographics are provided in **Supplementary Materials**. Brexanolone was safe and well tolerated, with no serious treatment emerging adverse events (TEAEs) being reported. TEAEs are provided in **Supplementary Materials**. Plasma concentrations peaked at a median $T_{\max}$ of 3H after infusion start for both low-dose (range: 1.71-3.04H) and high-dose brexanolone (range: 3.00-3.04H) (See **Figure S1A**), and at 1.6H for lorazepam (range: 0.79-5.06H) (See **Figure S1B**), with a mean ($\pm$ SD) $C_{\max}$ of 28.3 ng/mL ($\pm$ 3.6) for low-dose and 85.8 ng/mL ($\pm$ 10.4) for high-dose brexanolone, and 19.3 ng/mL ($\pm$ 2.9) for lorazepam. PK parameters of brexanolone and lorazepam are provided in **Supplementary Materials**.

Changes in PEEG theta power differentiate brexanolone from lorazepam

We first evaluated the differential effects of brexanolone and lorazepam on PEEG features. Brexanolone dose-dependently increased power across all frequency bands (**Table S1** and **Supplementary Materials**). The overall treatment effect, including all post-dose timepoints, showed a significant increase in frontal beta power for both low-dose and high-dose brexanolone (**Figure 2** and **S2**). At the high-dose, significant increases in frontal theta (**Figure 2** and **S2**) and gamma power, and parieto-occipital beta power also emerged. At individual post-dose timepoints, most statistically significant effects for brexanolone were observed at +1H and +2H, showing an increased power across all frequency bands, which dissipated after +3.5H. Similar to brexanolone, lorazepam significantly increased frontal beta and gamma power. In contrast to brexanolone, however, lorazepam reduced lower frequency band power, with generalized attenuation of theta and alpha power. Moreover, lorazepam also decreased beta power in the parieto-occipital region. This effect persisted across all timepoints, with most statistically significant effects for lorazepam observed at +5H. Direct comparison of brexanolone and lorazepam confirmed our observations, with increased power in the lower frequency bands (delta, theta and alpha), and parieto-occipital beta power following both doses of brexanolone. Frontal gamma power was decreased for both dose levels of brexanolone relative to lorazepam, driven by a larger effect size of lorazepam on frontal gamma power.

Brexanolone, but not lorazepam, decreases corticospinal excitability assessed with TMS-EMG

Brexanolone, but not lorazepam, significantly increased RMT at +4H following high-dose administration relative to placebo (**Figure 3A**). Brexanolone did not affect MEP amplitude (**Figure 3B**), whereas lorazepam showed a non-significant trend toward reduced MEP amplitude (**Figure 3B**). For PPTMS-EMG measures, brexanolone produced a dose-dependent enhancement of $LICI_{100}$, with smaller ratios indicating greater inhibition. Significant treatment effects were observed for both low- and high-dose brexanolone (**Figure 3C**). In contrast, lorazepam did not affect pptMS-EMG measures. We next directly compared brexanolone and lorazepam and found that both doses of brexanolone significantly increased the MEP amplitude, a significant effect that which was driven by the decreased MEP amplitude elicited by lorazepam. Compared to lorazepam, brexanolone also significantly increased the RMT. Only high-dose brexanolone showed significant enhancement of $LICI_{100}$. We did not observe any significant effects for $SICI_2$ and ICF_{15} for any of the comparisons (see **Table S2** for a complete overview of comparisons).

Concentration-effect relationship

Beta power was the most sensitive to drug concentration, with a linear exposure-response relationships for both brexanolone and lorazepam (**Figure 4** and **Supplementary Materials**). Theta power also demonstrated a concentration-effect relationship for both brexanolone and lorazepam, with an inverse linear relationship for lorazepam (**Figure 4** and **Supplementary Materials**). In contrast, concentration-effect relationships were not identified for the TMS endpoints.

Exploratory TMS-EEG analysis identifies distinct cortical activation patterns between brexanolone and lorazepam

Among the many clusters that were identified, two distinct patterns were observed showing consistent significant effects for multiple stimulation paradigms (SP- and PPTMS) and post-dose timepoints (+2H20M and +4H). Brexanolone modulated early TEP components (including the N_{15} , P_{30} , N_{45} and P_{60}) in the contralateral centroparietal region (**Figure 5A-B**), whereas lorazepam modulated later TEP components (including the N_{100} and P_{180}) in the contralateral frontocentral region (**Figure 5C-D**).

Brexanolone showed a significant early SPTMS-EEG cluster located centroparietal in the contralateral hemisphere at +2H20M for both low- and high-dose brexanolone, with the low-dose cluster including the N15 and P30 TEP components (Figure 5A) and the high-dose cluster including the N15, P30 and N45 TEP components (Figure 5B). A similar SPTMS cluster was observed for low-dose brexanolone at +4H. In addition, a comparable PPTMS cluster was observed at ISI 15 ms for low-dose brexanolone at +2H20M. Lorazepam showed a significant late PPTMS cluster at ISIs 2 and 15 ms located frontocentral in the contralateral hemisphere at both +2H20M and +4H (Figure 5C), including the N100 TEP component. Furthermore, a very late PPTMS cluster at ISIs 2 and 15 ms was found located frontocentral in the contralateral hemisphere at both +2H20M and +4H (Figure 5D), including the P180 TEP component. A similar very late SPTMS cluster was found for lorazepam at +4H. When comparing brexanolone's effects to lorazepam's, a similar pattern of clusters was identified as in the placebo comparisons (Figure 5E-F). A complete overview of the significant TMS-EEG clusters and corresponding topoplots per treatment can be found in **Supplementary Materials**.

DISCUSSION

This study provides the first head-to-head comparison of the NAS brexanolone and the BZD lorazepam, highlighting their distinct PD profiles on passive and active measures of neurocircuitry modulation in healthy male volunteers. Our findings indicate a differential impact on cortical activity between lorazepam and brexanolone, with distinct PD profiles on PEEG and TMS biomarkers (Figure 6), likely driven by the unique MOA of GABA_AR activation that involves combined phasic and tonic inhibition. Brexanolone's effects on PEEG and TMS-EMG clearly differentiated from lorazepam and placebo, with dose-dependent effects on theta power and LIC_{I100}, suggesting these paradigms are most sensitive to GABA_AR modulation through NAS activity at the studied doses. Additionally, specific modulation and topographical location of the clusters also differentiated between brexanolone and lorazepam, potentially reflecting engagement of distinct neurocircuitry.

Distinct NAS modulation of pEEG power

Brexanolone demonstrated a unique PEEG profile of increased delta, theta and alpha power relative to both lorazepam and placebo, consistent with prior preclinical findings for allopregnanolone and other NAS across

species. Despite conflicting reports on theta power,^{10,29,30} Antonoudiou et al. reported enhanced high-theta oscillations in the rodent basolateral amygdala following administration of the NAS SGE-516, mediated by tonic inhibition via extrasynaptic GABA_AR, a mechanism distinct from phasic inhibition by synaptic-preferring GABA_AR. In contrast, we found lorazepam decreased theta and alpha power while enhancing beta power, a commonly reported effect of synaptic-preferring GABA_AR agonists such as BZD and Z-DRUGS.^{11,31-33} This is further supported by PK/PD analysis, demonstrating opposing effects on theta power for brexanolone (direct linear) and lorazepam (inverse), but a direct linear relationship for beta power for both compounds. These findings support beta power as a biomarker of GABAergic activity and suggests theta power may serve as a specific biomarker for target engagement of extra-synaptic GABA_AR at clinically relevant and therapeutic dosages.

NAS-specific modulation decreases corticospinal excitability

We observed a profile of decreased cortical excitability following brexanolone, characterized by increased RMT and LIC_{I100}, distinct from both lorazepam and placebo. This finding is consistent with previous studies showing that synaptic preferring GABA_AR modulators like lorazepam and other BZD do not influence RMT.^{13,17} Interestingly, increases in RMT and LIC_{I100} have been described following the GABA_BR agonist baclofen,³⁴ resembling the effects observed with brexanolone. Historically, the "GABA_BR hypothesis"¹³ has attributed LIC_{I100} enhancement to GABA_BR specific mediated inhibition (e.g., baclofen, tiagabine).³⁴⁻³⁶ Instead, our findings point towards a role for tonic inhibition as a result of NAS' potentiation of extrasynaptic GABA_AR, supported by the similarity in RMT and LIC_{I100} effects observed for brexanolone and baclofen. We found no effects on MEP amplitude for brexanolone or lorazepam relative to placebo; however, the MEP amplitude did differentiate between the two drugs. This significant effect was driven by a trend towards a reduced MEP amplitude following lorazepam treatment, which is consistent with previous work.¹⁷ As we aimed to characterize PD effects across a broad range of ISIs, based on the hypothesis that different ISIs would capture distinct forms of inhibition (synaptic vs. synaptic + extrasynaptic), we only selected the inhibitory ISIs of 2 ms and 100 ms, at which maximal inhibition is typically observed.^{37,38} Previous studies evaluating effects of lorazepam on cortical excitability have also used alternative stimulation parameters and endpoints, such as SI₁MV (the stimulator output required to evoke ~1 mV peak-to-peak MEPS),^{18,20,39} rather than a fixed percentage

of RMT. To reduce operational burden and limit overall TMS duration, we limited the number of ISIs and used the fixed-percentage RMT approach, as it is easier to standardize within a clinical trial setting and yields more consistent stimulation intensities across participants and sessions. However, these methodological differences could have contributed to the fact that we did not observe the previously reported effects of lorazepam and other GABA_A-PAMS on SIC1 and LIC1.^{13,17} PK/PD analysis did not confirm a concentration-effect relationship for the TMS-EMG endpoints, possibly limited by the observed moderate to high intra-subject variability. PK/PD analyses of CNS-driven PD effects are particularly challenging as peripheral PK and central PD measures may not align directly, potentially introducing additional variability. Unlike PEEG, TMS-EMG captures PD signals involving both central and peripheral components of the nervous system, with synaptic noise at the spinal level further contributing to variability.

NAS cortical activation patterns identified with TMS-EEG

TMS-EEG analyses revealed clusters with distinct TEP modulation and topographical distribution for brexanolone and lorazepam. Brexanolone predominantly modulated early TEP components (N15, P30, N45, P60) in the centroparietal cortex, while lorazepam affected later components (N100, P180) in the frontocentral cortex. How pharmacological modulation of the specific TEP components relate to mechanistic underpinnings of the TEP signal, particularly for paired pulse paradigms, remains unclear. This highlighting the value of exploratory work in which assessing the effects of drugs with novel mechanisms of action, and comparing them to existing datasets, may help elucidate the mechanisms that generate TEPs. Previous studies suggest that modulation of the single-pulse N45 TEP component reflects GABA_AR activity, as its amplitude is increased by alprazolam, diazepam, and zolpidem, GABA_AR positive allosteric modulators (PAM) acting at the BZD-binding site.⁴⁰ Later TEP components, in particular the N100, are proposed to reflect GABA_BR-mediated tonic inhibition, as they are increased by baclofen.¹⁶ Consistent with these findings, high-dose brexanolone also increased the single-pulse N45 component. Brexanolone's preferential modulation of the N45 component, and early TEP components in general, without effecting the later TEP components suggests it enhances tonic inhibition through a mechanism distinct from GABA_BR modulation. In contrast, lorazepam did not modulate the N45 TEP component in the current study, nor in our previous HV study,¹⁷ possibly due to differences in GABA_AR receptor

subunit affinity and/or potency at the BZD-binding site of lorazepam and other GABA_AR-PAMS. Additionally, we found that lorazepam primarily modulated late TEP components following paired pulse paradigms at short ISIs (2 and 15 ms), e.g. enhancement of the N100 amplitude at ISI 2 ms (**Supplementary Materials**). These findings are consistent with those by Ruijs et al., although they also observed a decreased N100 amplitude at ISI 300 ms,¹⁷ an ISI not included in the current study.

Previous studies suggest the paired pulse N100 TEP component might be a sensitive biomarker for differential GABA_AR inhibition. Premoli et al. studied the pharmacological effects of diazepam and baclofen on the TEP components at ISI 2 of ms.^{41,42} They found that both the N100 and P180 TEP components of the test pulse were suppressed by the conditioning pulse and that diazepam attenuated the suppression of the N100, whereas baclofen enhanced it.⁴¹ However, differences in the spatial distribution of significant clusters, which can alter TEP morphology, and differences in TEP preprocessing (baseline-uncorrected vs. baseline-corrected) limit direct comparability across studies. The spatial distribution of brexanolone's and lorazepam's effects may reflect differential engagement of synaptic versus extrasynaptic GABA_AR populations across distinct brain regions. Although the regional and cellular distribution of GABA_AR subunits is complex and not fully characterized, there is evidence that NAS enhanced tonic inhibition through δ -subunit-containing extrasynaptic GABA_AR, typically co-assembled with $\alpha 4$ -subunits. GABA_AR containing these subunits are known to be involved in robust tonic GABAergic inhibition and are highly expressed in areas such as the thalamus and hippocampus, particularly in thalamic relay neurons ($\alpha 4\beta 3\delta$ isoform) and dentate granule cells ($\alpha 4\beta 2\delta$ isoform), projecting somatosensory information from the thalamus to the cerebral cortex.⁴²⁻⁴⁵ Therefore, brexanolone's effects in the parietooccipital region may reflect reduced relay or impaired cortical integration of sensory input following motor hotspot stimulation due to inhibition of thalamocortical pathways. The $\alpha 1\beta 2\gamma 2$ isoform, which is the predominant GABA_AR subtype in the human prefrontal cortex,⁴⁶ is benzodiazepine-sensitive due to it containing the $\gamma 2$ subunit, which could explain lorazepam's preferential modulation of frontal cortical regions. The clear differentiation between brexanolone and lorazepam across the multiple neurophysiological endpoints indicate distinct mechanisms by which they modulate GABA_AR, and supports the potential value of this multimodal approach for compound differentiation and dose selection in early-phase drug development. When

referencing findings from previous studies, the NAS brexanolone exhibits features that are both similar to and distinct from the GABA_BR agonist baclofen. At synaptic GABA_AR, GABA binding induces transient chloride influx, resulting in fast, short-lasting (phasic) inhibition.⁵ In contrast, extrasynaptic GABA_AR respond to low ambient GABA concentrations and remain open for extended periods, mediating tonic inhibition through sustained hyperpolarization.⁴⁷ GABA_BR also mediate slow and prolonged inhibiting, but through G-protein-coupled receptors and downstream signaling cascades.⁴⁸ Thus, brexanolone's dual activity at both synaptic and extrasynaptic GABA_AR likely contributes to a distinct neurophysiological signature among GABAergic modulators. A clearer understanding of how specific neurocircuitry disruptions relate to neuropsychiatric symptoms, and how NAS and BDZs differentially modulate these circuits to produce distinct profiles of anxiolytic, sedative, and antidepressant effects, is still needed. Such insights could ultimately inform personalized GABAergic treatment strategies tailored to individual symptom profiles and their underlying neurocircuitry dysfunction.

Limitations

This small, proof-of-mechanism, crossover study excluded females to avoid potential menstrual-cycle-related variability in cortical excitability,⁴⁹ limiting the translatability of our findings to women. No direct concentration-effect relationships were established for TMS-EMG endpoints. Intrinsic variability determined from TMS recordings performed in the off-drug conditions demonstrated moderate to high variability, which could have contributed to the model not converging. Given that the mean peak plasma concentration of lorazepam was comparable between the current study and the study by Ruijs et al.,¹⁷ the failure to replicate the significant effect could be attributed to intra-subject variability or differences in sampling time preventing us from capturing lorazepam's peak TMS response (+4H in current study vs. +7H in study by Ruijs et al.). Prior studies have suggested that the P180 may reflect residual auditory-evoked activity.^{50,51} Given our cross-over design and the use of continuous masking noise,⁵² we believe auditory confounds were sufficiently controlled, and that the observed lorazepam effects on the late TEP components represent genuine neurophysiological effects. TMS-EEG was recorded using a 32-channel system, potentially reducing spatial resolution compared to 64- or 128-channel caps used in previous TMS-EEG studies. There are different artefact-rejection methods to reduce artefacts caused by the magnetic

pulse and scalp muscle activation,⁵³ among which principal component analysis (PCA) has been shown to be effective.⁵⁴ As there is currently no consensus or gold-standard approach,⁵⁵ we applied PCA to keep analysis uniform and to facilitate comparisons across this and our prior studies. We also used a placebo-controlled baseline-adjusted analysis, preferred for this design, in contrast to prior studies comparing pre- and post-dose TEPs. Finally, due to the exploratory nature of the study, no multiple testing corrections were applied. Therefore, replication in larger, diverse cohorts is essential to clarify the translational relevance of our findings.

CONCLUSIONS

Our study demonstrates neurocircuitry modulation by the NAS brexanolone is distinct from the BDZ lorazepam, reflecting its unique mechanism of action through combined synaptic+extrasynaptic GABA_AR engagement. Frontal theta-power and LIC100, and possibly specific TEP modulation, emerged as sensitive and mechanism-specific biomarkers, while beta-power remains a robust biomarker for more general GABAergic activity. Together, these findings underscore the potential of a multimodal neurophysiological biomarker approach to guide the development of novel therapies with more selective targeting of the GABAergic neurocircuitry in psychiatric disorders.

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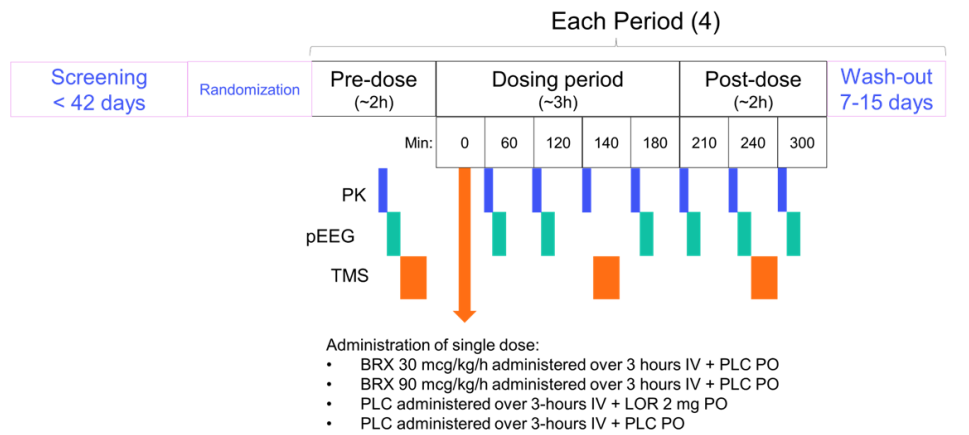
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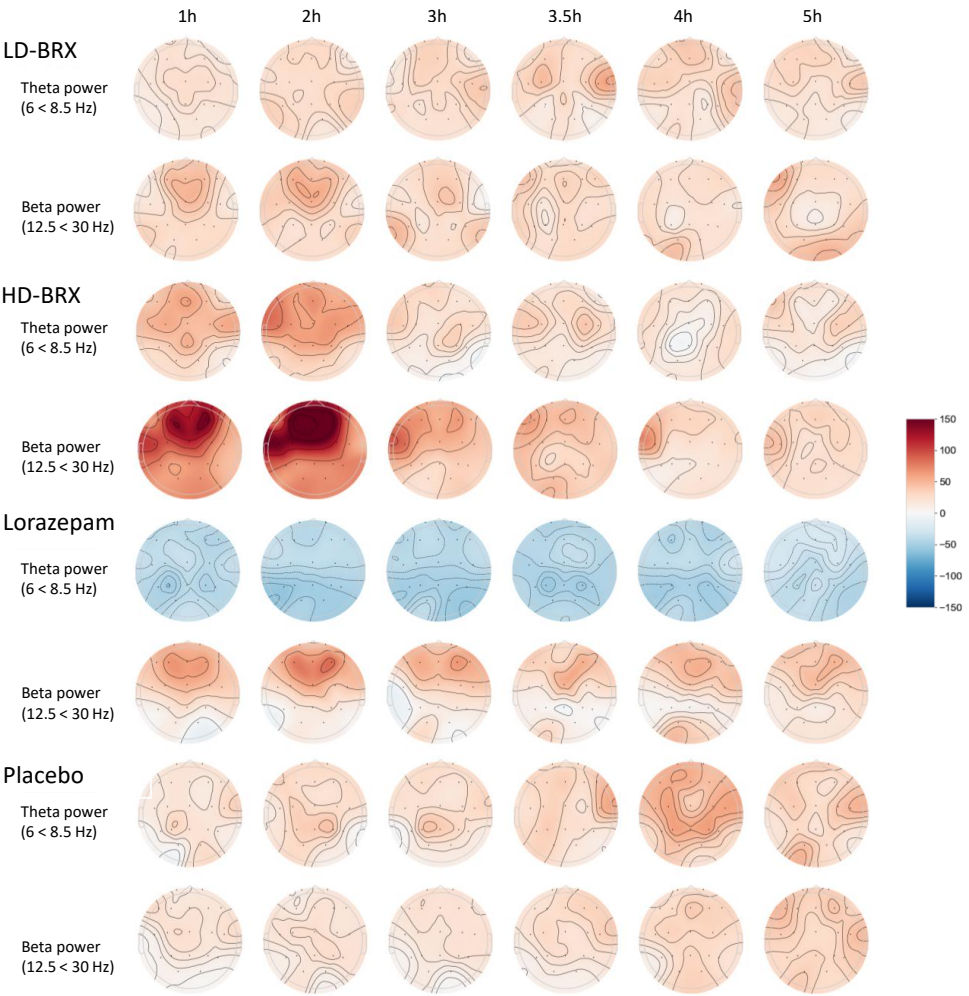
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FIGURE 1 Study design.
Schematic overview of study design.



Abbreviations: H = hour; HD-BRX = high-dose brexanolone (90 mcg/kg/h); IV = intravenous infusion; kg = kilogram; LD-BRX = low-dose brexanolone (30 mcg/kg/h); LOR = lorazepam; mcg = microgram; mg = milligram; min = minutes; PK = pharmacokinetic assessment; PLC = placebo; PO = per os; pEEG = pharmaco-electroencephalogram; TMS = transcranial magnetic stimulation

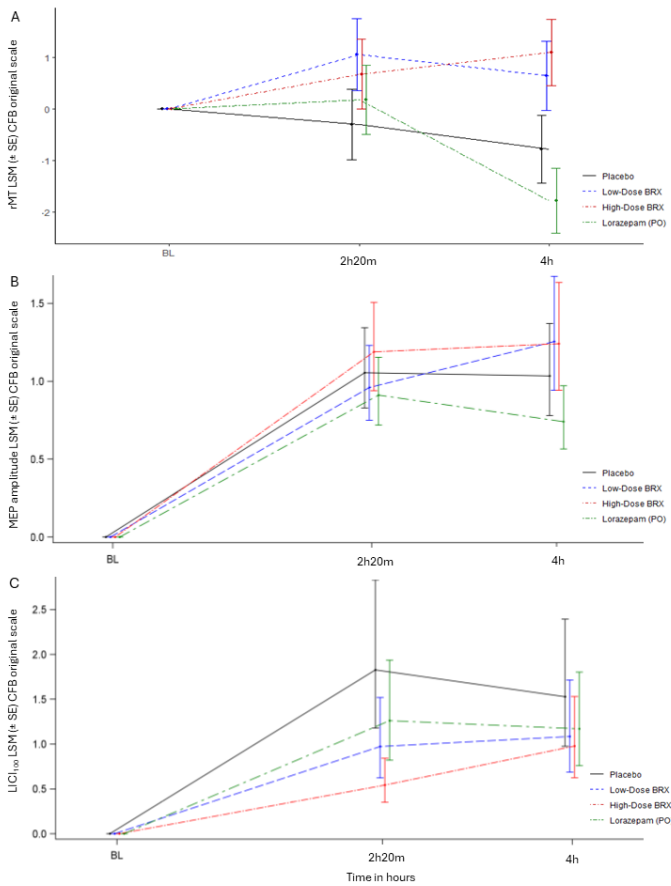
FIGURE 2 pEEG spectral power topoplots for beta and theta frequency in Fz-Cz with eyes closed. Topoplots of pEEG spectral power measured at Fz-Cz in the eyes closed condition for the theta (θ): 6.0 < 8.5 Hz and beta (β): 12.5 < 30.0 Hz frequency bands, for all treatments. Results are presented per frequency band (rows) and post-dose timepoint (column). On the right side, the change from baseline median amplitude of the power (μ V²) in the topographical distribution is presented.



Abbreviations: FZ-CZ = frontocentral leads; H = hours; HD-BRX = high-dose brexanolone (90 mcg/kg/h); IV = intravenous; kg = kilogram; LD-BRX = low-dose brexanolone (30 mcg/kg/h); mcg = microgram; pEEG = pharmaco-electroencephalography; PO = per os.

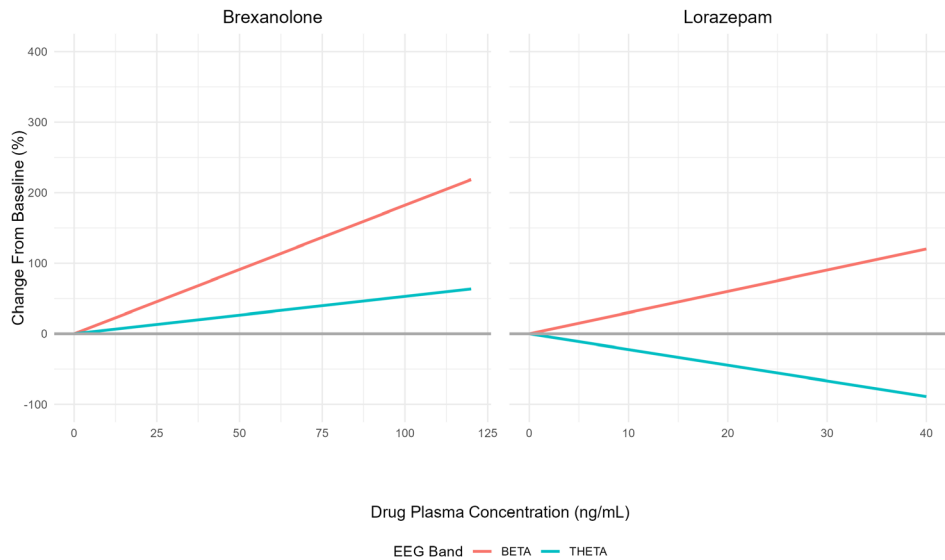
FIGURE 3 Summary graphs for TMS-EMG parameters RMT (A), MEP amplitude (B) and LIC1₁₀₀ (C) – Change from baseline.

Summary graphs of the change from baseline LSM with the SE as error bars for the RMT (3A), and the LSM ratio with the SE as error bars for MEP amplitude (3B) and LIC1₁₀₀ (3C) for all treatments. RMT was impacted by brexanolone in a dose-dependent manner relative to placebo reaching significance for high-dose brexanolone at +4H timepoint and differentiated from lorazepam overall and at the +4H timepoint (3A). Lorazepam demonstrated a trend towards a significant difference overall and at the +4H timepoint (3B). MEP amplitude also differentiated between low-dose brexanolone (30 mcg/kg/h) and high-dose brexanolone (90 mcg/kg/h) relative to lorazepam at the +4H timepoint. LIC1₁₀₀ was significantly impacted by brexanolone, in a dose-dependent manner, but not by lorazepam (3C). LIC1₁₀₀ was significantly enhanced by both low-dose (30 mcg/kg/h) and high-dose brexanolone (90 mcg/kg/h) overall and at the +2H20M timepoint and also differentiated between high-dose brexanolone (90 mcg/kg/h) and lorazepam overall and at the +2H20M timepoint.



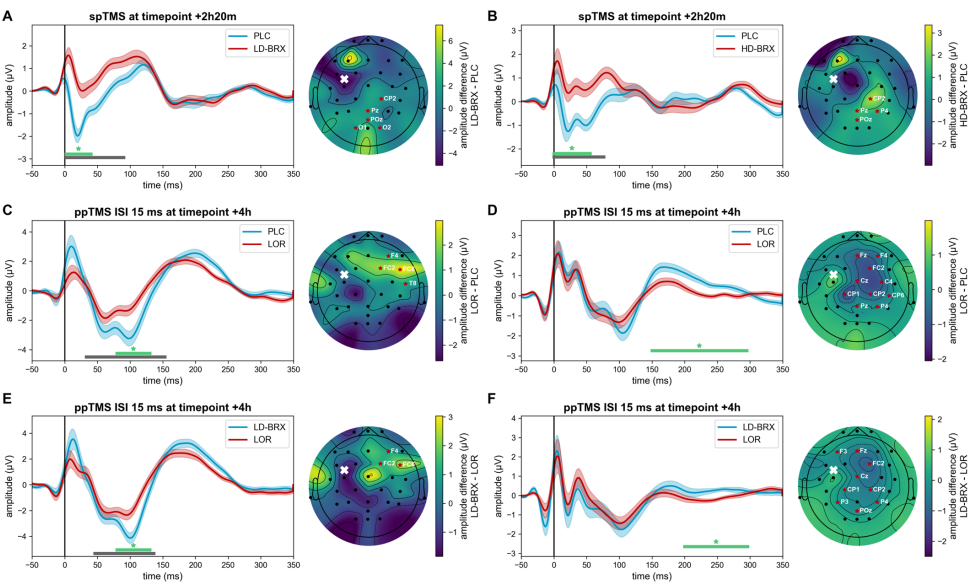
Abbreviations: BL = baseline; BRX = brexanolone; CFB = change from baseline; H = hours; kg = kilogram; LIC1₁₀₀ = long-interval intracortical inhibition at interstimulus interval of 100 milliseconds; LSM = least square means; M = minutes; mcg = microgram; MEP = motor evoked potential; mL = milliliters; ng = nanograms; PO = oral; RMT = resting motor threshold; SE = standard error; TMS-EMG = transcranial magnetic stimulation combined with electromyography.

FIGURE 4 Concentration-Response Comparison of PEEG Endpoints in FZ-CZ with Eyes Closed per Treatment. Concentration-response graphs for PEEG endpoints for brexanolone on the left and lorazepam on the right. Solid lines: Change from baseline in spectral power of the beta (β): 12.5 < 30.0 HZ and theta frequency bands (θ): 6.0 < 8.5 HZ measured in FZ-CZ with eyes closed.



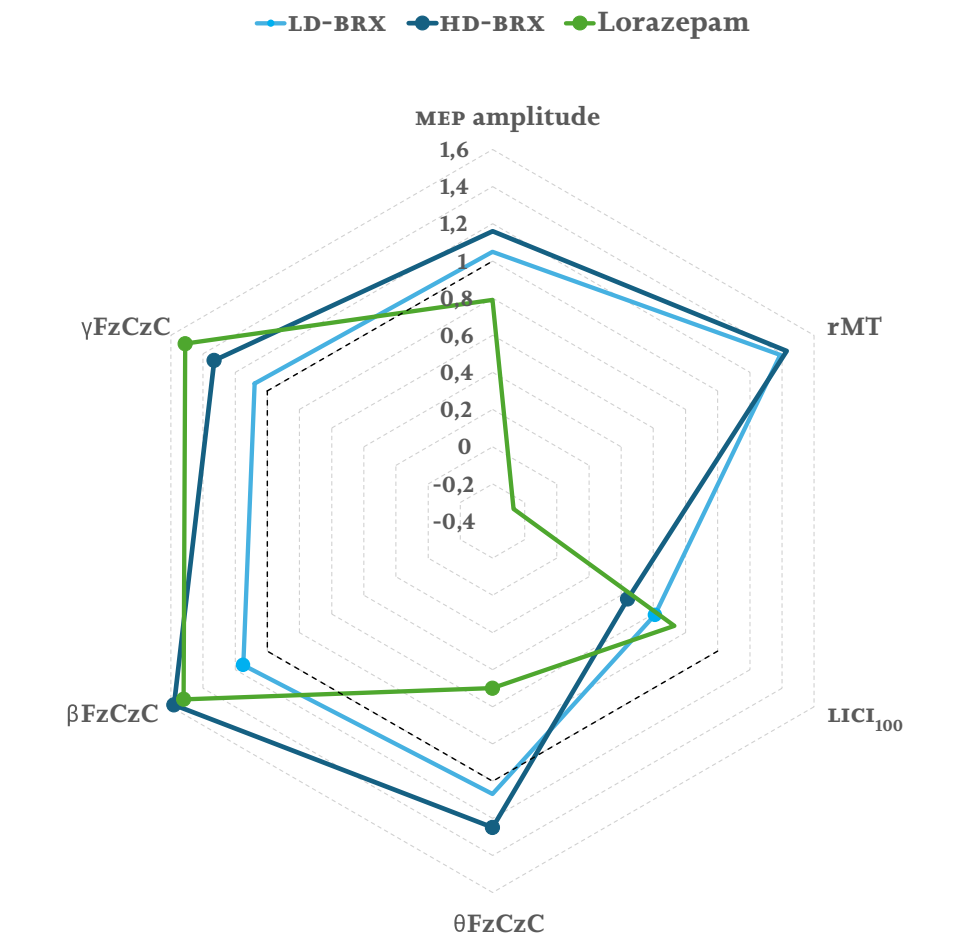
Abbreviations: FZ-CZ = frontocentral leads; mL = milliliter; ng = nanogram; PEEG = pharmaco-electroencephalography

FIGURE 5 Exploratory TMS-EEG analysis identifies distinct cortical activation patterns between brexanolone (A-B) and lorazepam (C-D) relative to placebo, and brexanolone relative to lorazepam (E-F). Overview of the significant clusters found when comparing TEPs of low- and high-dose brexanolone (in red) compared to the placebo (in blue) for SPTMS-EEG at the timepoint +2H20M (A-B), and for lorazepam (in red) compared to placebo (in blue) for PPTMS-EEG at the timepoint +4H (C-D), and for low-dose brexanolone (in blue) compared to lorazepam (in red) for PPTMS-EEG at the timepoint +4H (E-F). On the left side, the grand average (mean $\pm$ SEM) over all significant electrodes is presented. On the right side, the amplitude difference in topographical distribution (brexanolone-placebo, lorazepam-placebo or brexanolone-lorazepam) at the time of the cluster is presented. The thick green bar represents the time window of significant differences, the thick grey bar the time window of the entire cluster (including non-significant parts), the white cross the stimulation site, and the black dots the electrode positions with the significant electrodes as red stars. Modulation of specific TEP components and topographical location of clusters differentiate between brexanolone and lorazepam's effects.



Abbreviations: H = hours; HD-BRX = high-dose brexanolone; ISI = interstimulus interval; kg = kilogram; LD-BRX = low-dose brexanolone; LOR = lorazepam; mcg = microgram; ms = millisecond; PLC = placebo; PPTMS = paired pulse TMS; SEM = standard error of the mean; SPTMS = single pulse TMS; TEP = transcranial magnetic stimulation evoked potential; TMS-EEG = transcranial magnetic stimulation combined with electroencephalography.

FIGURE 6 Pharmacodynamic profile of brexanolone and lorazepam. Effect sizes of low-dose brexanolone (light blue), high-dose brexanolone (dark blue) and lorazepam (green) on TMS-EMG and PEEG biomarkers, visualized using the change from baseline LSM ratio for all treatments relative to placebo, with exception of the RMT for which we report the LMS difference. Negative values thus favor placebo. The round marker indicates the significant effects for the overall treatment effects ($p < 0.05$).



Abbreviations: θ FzCzC = theta power measured in frontocentral leads with eyes closed; β FzCzC = beta power measured in frontocentral leads with eyes closed; γ FzCzC; gamma power measured in frontocentral leads with eyes closed; HD-BRX = high-dose brexanolone (90 mcg/kg/h); LD-BRX = low-dose brexanolone (30 90 mcg/kg/h); LICI₁₀₀ = long-interval intracortical inhibition at interstimulus-interval of 100 ms; LSM = least square means; MEP = motor evoked; PEEG = pharmaco-electroencephalogram; RMT = resting motor threshold; TMS-EMG = transcranial magnetic stimulation combined with electromyography.

CHAPTER V

SUPPLEMENTARY MATERIALS

Full supplementary materials available at the publisher’s website:
<https://www.authorea.com/doi/full/10.22541/authorea.15007748/v1>
 Selected supplementary text, figures and tables are provided next.

TABLE S1 Summary of results for PEEG in eyes closed condition for all treatments – change from baseline. Summary table of PEEG results change from baseline for all treatments relative to placebo, and for both doses of brexanolone relative to lorazepam, in the eyes closed condition. The changes in PEEG spectral power are reported per frequency band (delta (δ): 1.5 < 6.0 Hz; theta (θ) 6.0 < 8.5 Hz; alpha (α): 8.5 < 12.5 Hz; beta (β): 12.5 < 30.0 Hz; low-beta (β1): 12.5 < 18.5 Hz; and gamma (γ): 30.0 < 40.0 Hz), and electrode of interest FZ-CZ, PZ-O1, and PZ-O2, and are abbreviated to e.g. δFZCZ for delta power in FZ-CZ. P-values are derived from LMS comparisons between treatments using a mixed model repeated measures analysis. Results are reported in LMS ratio, with 95%-CI and p-value. Significant p-values are indicated in bold.

Parameter	Timepoint/ Statistic	Overall*	1H	2H	3H	3.5H	4H	5H
LD-BRX VERSUS PLC								
δFzCZ	LSM ratio	0.95	0.98	0.99	0.93	0.95	0.93	0.94
	95%-CI	0.85, 1.07	0.78, 1.25	0.83, 1.19	0.82, 1.06	0.83, 1.09	0.77, 1.13	0.83, 1.07
	p-value	0.44	0.89	0.93	0.3	0.45	0.45	0.34
δPzO1	LSM ratio	1.17	1.4	1.42	1.17	1.2	0.96	0.97
	95%-CI	0.95, 1.44	1.01, 1.95	1.06, 1.89	0.84, 1.63	0.90, 1.60	0.77, 1.20	0.81, 1.16
	p-value	0.13	0.05	0.02	0.33	0.22	0.74	0.73
δPzO2	LSM ratio	1.13	1.16	1.24	1.15	1.2	1.04	1.02
	95%-CI	0.92, 1.39	0.93, 1.43	0.99, 1.56	0.82, 1.60	0.86, 1.66	0.79, 1.37	0.78, 1.33
	p-value	0.24	0.19	0.07	0.41	0.27	0.79	0.9
θFzCz	LSM ratio	1.07	1.15	1.06	1.15	1.1	1.01	0.98
	95%-CI	0.91, 1.26	0.90, 1.47	0.85, 1.31	0.96, 1.39	0.90, 1.36	0.80, 1.27	0.82, 1.18
	p-value	0.38	0.27	0.6	0.13	0.35	0.95	0.86
θPzO1	LSM ratio	1.17	1.44	1.42	1.26	1.13	0.93	0.96
	95%-CI	0.94, 1.47	1.07, 1.95	1.07, 1.89	0.94, 1.70	0.85, 1.49	0.71, 1.22	0.75, 1.23
	p-value	0.16	0.02	0.02	0.12	0.4	0.6	0.74
θPzO2	LSM ratio	1.13	1.24	1.23	1.24	1.1	0.95	1.04
	95%-CI	0.89, 1.42	0.99, 1.55	0.98, 1.53	0.90, 1.71	0.80, 1.52	0.70, 1.30	0.76, 1.42
	p-value	0.3	0.07	0.07	0.19	0.54	0.77	0.81
αFzCz	LSM ratio	1.01	1.04	1.03	1.07	1.01	0.99	0.93
	95%-CI	0.89, 1.15	0.86, 1.25	0.85, 1.24	0.91, 1.28	0.86, 1.19	0.85, 1.16	0.81, 1.07
	p-value	0.88	0.69	0.79	0.41	0.86	0.89	0.28
αPzO1	LSM ratio	1.09	1.25	1.21	1.15	1.05	0.99	0.91
	95%-CI	0.88, 1.34	0.89, 1.75	0.86, 1.69	0.87, 1.53	0.81, 1.36	0.79, 1.24	0.73, 1.13
	p-value	0.42	0.19	0.27	0.31	0.7	0.93	0.39
αPzO2	LSM ratio	1.06	1.05	1.04	1.15	1.07	1.04	1
	95%-CI	0.87, 1.29	0.81, 1.37	0.79, 1.35	0.86, 1.54	0.82, 1.40	0.81, 1.33	0.76, 1.33
	p-value	0.57	0.7	0.8	0.33	0.63	0.78	0.98
βFzCz	LSM ratio	1.15	1.32	1.4	1.15	1.11	1	1
	95%-CI	1.03, 1.29	1.14, 1.54	1.19, 1.64	1.00, 1.32	0.98, 1.25	0.89, 1.14	0.87, 1.15
	p-value	0.01	<0.01	<0.01	0.04	0.11	0.95	0.98
βPzO1	LSM ratio	1.15	1.35	1.37	1.21	1.08	0.98	0.97
	95%-CI	0.96, 1.37	1.04, 1.75	1.06, 1.77	0.97, 1.51	0.88, 1.33	0.81, 1.18	0.80, 1.17
	p-value	0.13	0.03	0.02	0.09	0.46	0.82	0.71
βPzO2	LSM ratio	1.04	1.07	1.11	1.06	1.08	0.96	0.97
	95%-CI	0.87, 1.24	0.89, 1.29	0.92, 1.34	0.83, 1.34	0.85, 1.37	0.76, 1.20	0.75, 1.26
	p-value	0.67	0.45	0.27	0.66	0.54	0.69	0.83

Parameter	Timepoint/ Statistic	Overall*	1H	2H	3H	3.5H	4H	5H
β1FzCz	LSM ratio	1.13	1.33	1.36	1.11	1.08	0.98	0.98
	95%-CI	1.00, 1.28	1.12, 1.58	1.13, 1.64	0.95, 1.29	0.94, 1.23	0.85, 1.13	0.84, 1.14
	p-value	0.05	<0.01	<0.01	0.19	0.28	0.77	0.8
β1PzO1	LSM ratio	1.15	1.35	1.38	1.22	1.09	1.01	0.94
	95%-CI	0.97, 1.37	1.04, 1.76	1.07, 1.77	0.98, 1.52	0.89, 1.33	0.85, 1.19	0.78, 1.13
	p-value	0.1	0.2	0.01	0.07	0.39	0.94	0.5
β1PzO2	LSM ratio	1.06	1.12	1.12	1.09	1.07	0.99	0.95
	95%-CI	0.89, 1.25	0.95, 1.33	0.95, 1.32	0.87, 1.38	0.85, 1.35	0.80, 1.21	0.74, 1.22
	p-value	0.52	0.18	0.17	0.43	0.58	0.9	0.68
γFzCz	LSM ratio	1.08	1.18	1.27	1.12	1.05	0.94	0.98
	95%-CI	0.98, 1.19	1.04, 1.34	1.12, 1.45	0.98, 1.28	0.94, 1.17	0.83, 1.06	0.88, 1.10
	p-value	0.1	<0.01	<0.01	0.1	0.41	0.31	0.78
γPzO1	LSM ratio	1.06	1.18	1.22	1.16	0.98	0.84	1.05
	95%-CI	0.81, 1.39	0.85, 1.64	0.88, 1.71	0.86, 1.57	0.70, 1.36	0.58, 1.23	0.74, 1.48
	p-value	0.65	0.32	0.23	0.32	0.9	0.37	0.79
γPzO2	LSM ratio	0.93	0.89	0.99	0.9	1.01	0.84	0.96
	95%-CI	0.70, 1.23	0.64, 1.23	0.72, 1.35	0.66, 1.22	0.73, 1.40	0.58, 1.23	0.65, 1.40
	p-value	0.61	0.47	0.95	0.5	0.94	0.37	0.81
HD-BRX VERSUS PLC								
δFzCZ	LSM ratio	1.1	1.32	1.34	0.99	0.97	1.02	1.02
	95%-CI	0.98, 1.24	1.05, 1.68	1.12, 1.61	0.87, 1.13	0.85, 1.11	0.84, 1.23	0.90, 1.16
	p-value	0.11	0.02	<0.01	0.9	0.66	0.86	0.72
δPzO1	LSM ratio	1.19	1.72	1.53	1.08	1.04	1.05	0.94
	95%-CI	0.97, 1.47	1.23, 2.39	1.14, 2.04	0.78, 1.50	0.78, 1.39	0.84, 1.31	0.78, 1.13
	p-value	0.09	<0.01	<0.01	0.65	0.79	0.67	0.5
δPzO2	LSM ratio	1.21	1.48	1.47	1.11	1.08	1.16	1.05
	95%-CI	0.99, 1.49	1.19, 1.83	1.17, 1.84	0.80, 1.56	0.78, 1.49	0.88, 1.52	0.81, 1.36
	p-value	0.07	<0.01	<0.01	0.52	0.65	0.29	0.73
θFzCz	LSM ratio	1.25	1.62	1.59	1.24	1.15	1.03	1.04
	95%-CI	1.07, 1.47	1.27, 2.07	1.29, 1.97	1.03, 1.49	0.93, 1.41	0.82, 1.29	0.87, 1.24
	p-value	<0.01	<0.01	<0.01	0.03	0.19	0.82	0.68
θPzO1	LSM ratio	1.14	1.63	1.61	1.15	1	0.84	0.88
	95%-CI	0.91, 1.43	1.21, 2.21	1.21, 2.14	0.85, 1.54	0.75, 1.31	0.64, 1.10	0.69, 1.12
	p-value	0.24	<0.01	<0.01	0.36	0.98	0.2	0.29
θPzO2	LSM ratio	1.19	1.5	1.46	1.24	1.06	0.96	1.06
	95%-CI	0.95, 1.50	1.19, 1.88	1.17, 1.82	0.89, 1.71	0.77, 1.46	0.70, 1.30	0.77, 1.44
	p-value	0.13	<0.01	<0.01	0.2	0.7	0.77	0.73
αFzCz	LSM ratio	1.1	1.28	1.39	1.05	1.06	0.91	0.97
	95%-CI	0.96, 1.25	1.07, 1.54	1.15, 1.68	0.89, 1.25	0.90, 1.25	0.78, 1.06	0.84, 1.11
	p-value	0.16	<0.01	<0.01	0.57	0.49	0.22	0.61
αPzO1	LSM ratio	0.96	1.12	1.12	0.94	0.96	0.81	0.84
	95%-CI	0.78, 1.18	0.80, 1.58	0.80, 1.58	0.71, 1.25	0.75, 1.24	0.65, 1.02	0.68, 1.04
	p-value	0.7	0.49	0.49	0.68	0.78	0.07	0.11

Parameter	Timepoint/ Statistic	Overall*	1H	2H	3H	3.5H	4H	5H
αPzO2	LSM ratio	0.96	0.96	0.98	0.98	1	0.88	0.98
	95%-CI	0.79, 1.17	0.73, 1.24	0.75, 1.27	0.73, 1.31	0.76, 1.31	0.68, 1.13	0.74, 1.29
	p-value	0.68	0.73	0.86	0.88	1	0.3	0.87
βFzCz	LSM ratio	1.58	2.43	2.78	1.53	1.25	1.12	1.07
	95%-CI	1.42, 1.76	2.09, 2.82	2.38, 3.25	1.34, 1.76	1.10, 1.41	0.99, 1.27	0.93, 1.23
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	0.06	0.34
βPzO1	LSM ratio	1.24	1.74	1.72	1.16	1.2	0.92	0.93
	95%-CI	1.04, 1.47	1.34, 2.26	1.33, 2.22	0.93, 1.45	0.97, 1.48	0.76, 1.11	0.77, 1.12
	p-value	0.02	<0.01	<0.01	0.19	0.08	0.37	0.43
βPzO2	LSM ratio	1.17	1.42	1.45	1.14	1.15	0.95	1
	95%-CI	0.98, 1.40	1.18, 1.71	1.21, 1.75	0.90, 1.44	0.90, 1.45	0.76, 1.19	0.78, 1.29
	p-value	0.08	<0.01	<0.01	0.29	0.25	0.66	0.98
β1FzCz	LSM ratio	1.62	2.7	3.14	1.53	1.23	1.09	1.06
	95%-CI	1.44, 1.83	2.27, 3.20	2.61, 3.78	1.32, 1.78	1.08, 1.40	0.94, 1.25	0.91, 1.23
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	0.25	0.47
β1PzO1	LSM ratio	1.25	1.8	1.85	1.21	1.17	0.91	0.9
	95%-CI	1.06, 1.49	1.38, 2.34	1.44, 2.37	0.98, 1.50	0.96, 1.43	0.77, 1.08	0.75, 1.08
	p-value	<0.01	<0.01	<0.01	0.08	0.11	0.27	0.26
β1PzO2	LSM ratio	1.2	1.52	1.58	1.18	1.13	0.95	0.98
	95%-CI	1.02, 1.42	1.28, 1.81	1.34, 1.86	0.94, 1.49	0.90, 1.42	0.78, 1.17	0.77, 1.26
	p-value	0.03	<0.01	<0.01	0.14	0.29	0.65	0.89
γFzCz	LSM ratio	1.33	1.7	1.85	1.34	1.15	1.11	1.03
	95%-CI	1.21, 1.47	1.50, 1.93	1.62, 2.11	1.17, 1.54	1.03, 1.29	0.98, 1.26	0.92, 1.16
	p-value	<0.01	<0.01	<0.01	<0.01	0.01	0.1	0.58
γPzO1	LSM ratio	1.2	1.46	1.41	1.12	1.29	1	0.99
	95%-CI	0.92, 1.56	1.05, 2.03	1.01, 1.96	0.83, 1.52	0.93, 1.79	0.69, 1.45	0.70, 1.40
	p-value	0.18	0.03	0.04	0.45	0.12	0.99	0.96
γPzO2	LSM ratio	1.03	1.16	1.1	0.97	1.11	0.92	0.95
	95%-CI	0.78, 1.36	0.84, 1.60	0.81, 1.51	0.71, 1.31	0.80, 1.54	0.64, 1.34	0.65, 1.38
	p-value	0.83	0.37	0.53	0.82	0.52	0.67	0.77
LD-BRX VERSUS LOR								
δFzCZ	LSM ratio	1.05	1.08	1.05	0.99	1.08	1.08	1.04
	95%-CI	0.94, 1.18	0.86, 1.36	0.88, 1.26	0.87, 1.13	0.95, 1.24	0.89, 1.30	0.91, 1.18
	p-value	0.38	0.5	0.56	0.89	0.25	0.44	0.58
δPzO1	LSM ratio	1.31	1.3	1.41	1.13	1.43	1.31	1.32
	95%-CI	1.07, 1.61	0.94, 1.79	1.06, 1.87	0.82, 1.56	1.08, 1.90	1.05, 1.64	1.10, 1.59
	p-value	<0.01	0.11	0.02	0.45	0.01	0.02	<0.01
δPzO2	LSM ratio	1.32	1.29	1.34	1.18	1.44	1.35	1.35
	95%-CI	1.08, 1.62	1.04, 1.59	1.07, 1.68	0.85, 1.65	1.05, 1.98	1.03, 1.77	1.04, 1.76
	p-value	<0.01	0.02	0.01	0.32	0.03	0.03	0.03
θFzCz	LSM ratio	2.17	2.18	2.31	2.23	2.3	2.21	1.82
	95%-CI	1.85, 2.54	1.72, 2.77	1.87, 2.85	1.85, 2.68	1.87, 2.81	1.76, 2.78	1.52, 2.19
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Parameter	Timepoint/ Statistic	Overall*	1H	2H	3H	3.5H	4H	5H
θPzO1	LSM ratio	2.28	2.35	2.64	2.42	2.44	2	1.93
	95%-CI	1.83, 2.84	1.76, 3.15	1.99, 3.49	1.81, 3.24	1.86, 3.21	1.53, 2.62	1.51, 2.46
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
θPzO2	LSM ratio	2.34	2.36	2.63	2.51	2.64	2	2
	95%-CI	1.87, 2.94	1.89, 2.94	2.12, 3.26	1.83, 3.46	1.93, 3.61	1.48, 2.71	1.47, 2.73
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
αFzCz	LSM ratio	1.92	1.88	2.09	2.13	1.95	1.88	1.63
	95%-CI	1.69, 2.18	1.57, 2.24	1.73, 2.53	1.80, 2.52	1.66, 2.29	1.61, 2.20	1.42, 1.87
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
αPzO1	LSM ratio	2.53	2.38	2.85	3.11	2.57	2.39	2.05
	95%-CI	2.07, 3.11	1.72, 3.29	2.05, 3.96	2.36, 4.09	2.00, 3.30	1.92, 2.98	1.65, 2.54
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
αPzO2	LSM ratio	2.74	2.51	2.95	3.43	3	2.54	2.2
	95%-CI	2.26, 3.32	1.94, 3.23	2.27, 3.82	2.57, 4.57	2.30, 3.91	1.98, 3.26	1.67, 2.89
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
βFzCz	LSM ratio	0.76	0.84	0.85	0.74	0.73	0.71	0.72
	95%-CI	0.68, 0.85	0.72, 0.97	0.73, 0.99	0.65, 0.85	0.64, 0.82	0.63, 0.80	0.63, 0.82
	p-value	<0.01	0.02	0.04	<0.01	<0.01	<0.01	<0.01
βPzO1	LSM ratio	1.16	1.11	1.12	1.27	1.1	1.18	1.16
	95%-CI	0.97, 1.37	0.86, 1.43	0.87, 1.44	1.02, 1.58	0.90, 1.35	0.98, 1.43	0.96, 1.40
	p-value	0.1	0.41	0.37	0.03	0.36	0.08	0.11
βPzO2	LSM ratio	1.25	1.15	1.15	1.36	1.3	1.28	1.29
	95%-CI	1.05, 1.49	0.96, 1.37	0.96, 1.38	1.08, 1.72	1.03, 1.65	1.02, 1.59	1.00, 1.65
	p-value	0.01	0.13	0.13	0.01	0.03	0.03	0.05
β1FzCz	LSM ratio	0.85	0.95	0.91	0.83	0.81	0.79	0.83
	95%-CI	0.76, 0.96	0.81, 1.13	0.76, 1.09	0.71, 0.96	0.71, 0.92	0.68, 0.91	0.72, 0.97
	p-value	<0.01	0.57	0.31	0.01	<0.01	<0.01	0.02
β1PzO1	LSM ratio	1.25	1.22	1.22	1.39	1.23	1.27	1.21
	95%-CI	1.06, 1.48	0.95, 1.58	0.96, 1.55	1.13, 1.72	1.02, 1.50	1.07, 1.50	1.01, 1.45
	p-value	<0.01	0.12	0.11	<0.01	0.03	<0.01	0.04
β1PzO2	LSM ratio	1.33	1.26	1.24	1.48	1.44	1.32	1.27
	95%-CI	1.13, 1.57	1.07, 1.49	1.05, 1.45	1.18, 1.86	1.14, 1.81	1.08, 1.62	0.99, 1.61
	p-value	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	0.06
γFzCz	LSM ratio	0.72	0.74	0.78	0.7	0.73	0.65	0.71
	95%-CI	0.65, 0.79	0.66, 0.84	0.69, 0.89	0.61, 0.80	0.65, 0.81	0.58, 0.74	0.63, 0.79
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
γPzO1	LSM ratio	0.87	0.85	0.85	0.95	0.7	0.8	1.13
	95%-CI	0.67, 1.13	0.62, 1.18	0.61, 1.17	0.71, 1.28	0.50, 0.96	0.55, 1.16	0.80, 1.60
	p-value	0.29	0.33	0.31	0.75	0.03	0.24	0.47
γPzO2	LSM ratio	1.05	0.97	0.95	1.07	0.96	1	1.42
	95%-CI	0.80, 1.38	0.71, 1.32	0.70, 1.30	0.79, 1.44	0.70, 1.32	0.69, 1.44	0.98, 2.06
	p-value	0.72	0.82	0.76	0.66	0.8	0.98	0.07

Parameter	Timepoint/ Statistic	Overall*	1H	2H	3H	3.5H	4H	5H
HD-BRX VERSUS LOR								
δFzCZ	LSM ratio	1.21	1.45	1.42	1.05	1.11	1.18	1.13
	95%-CI	1.08, 1.36	1.16, 1.83	1.19, 1.70	0.93, 1.20	0.97, 1.26	0.98, 1.42	0.99, 1.28
	p-value	<0.01	<0.01	<0.01	0.43	0.14	0.09	0.06
δPzO1	LSM ratio	1.34	1.59	1.52	1.04	1.24	1.43	1.28
	95%-CI	1.09, 1.64	1.16, 2.19	1.14, 2.01	0.75, 1.44	0.94, 1.64	1.15, 1.78	1.07, 1.54
	p-value	<0.01	<0.01	<0.01	0.8	0.13	<0.01	<0.01
δPzO2	LSM ratio	1.42	1.65	1.59	1.15	1.29	1.5	1.39
	95%-CI	1.16, 1.73	1.34, 2.03	1.27, 1.98	0.82, 1.60	0.94, 1.77	1.15, 1.97	1.07, 1.80
	p-value	<0.01	<0.01	<0.01	0.41	0.11	<0.01	0.01
θFzCz	LSM ratio	2.53	3.08	3.47	2.39	2.38	2.25	1.92
	95%-CI	2.16, 2.96	2.43, 3.90	2.82, 4.27	1.99, 2.87	1.95, 2.92	1.80, 2.82	1.61, 2.30
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
θPzO1	LSM ratio	2.22	2.67	2.99	2.2	2.16	1.81	1.76
	95%-CI	1.79, 2.76	1.99, 3.56	2.26, 3.94	1.65, 2.94	1.65, 2.84	1.39, 2.36	1.38, 2.24
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
θPzO2	LSM ratio	2.48	2.85	3.13	2.51	2.54	2	2.04
	95%-CI	1.98, 3.10	2.29, 3.55	2.53, 3.88	1.83, 3.44	1.86, 3.47	1.48, 2.71	1.50, 2.77
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
αFzCz	LSM ratio	2.08	2.32	2.83	2.08	2.03	1.73	1.69
	95%-CI	1.83, 2.36	1.95, 2.77	2.35, 3.42	1.76, 2.47	1.73, 2.39	1.48, 2.01	1.48, 1.94
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
αPzO1	LSM ratio	2.24	2.14	2.65	2.54	2.35	1.97	1.89
	95%-CI	1.83, 2.74	1.55, 2.96	1.91, 3.69	1.93, 3.34	1.83, 3.02	1.58, 2.44	1.53, 2.34
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
αPzO2	LSM ratio	2.49	2.27	2.78	2.91	2.81	2.15	2.14
	95%-CI	2.06, 3.01	1.76, 2.93	2.15, 3.60	2.18, 3.87	2.16, 3.65	1.68, 2.75	1.63, 2.81
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
βFzCz	LSM ratio	1.04	1.53	1.69	0.99	0.82	0.79	0.77
	95%-CI	0.94, 1.16	1.33, 1.77	1.45, 1.97	0.86, 1.13	0.73, 0.93	0.70, 0.89	0.67, 0.88
	p-value	0.44	<0.01	<0.01	0.85	<0.01	<0.01	<0.01
βPzO1	LSM ratio	1.25	1.43	1.41	1.22	1.22	1.11	1.12
	95%-CI	1.05, 1.48	1.12, 1.84	1.10, 1.81	0.98, 1.51	0.99, 1.49	0.92, 1.34	0.93, 1.35
	p-value	0.01	<0.01	<0.01	0.07	0.06	0.27	0.23
βPzO2	LSM ratio	1.41	1.52	1.5	1.47	1.39	1.27	1.33
	95%-CI	1.19, 1.68	1.28, 1.82	1.26, 1.80	1.16, 1.86	1.10, 1.75	1.02, 1.58	1.03, 1.70
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	0.03	0.03
β1FzCz	LSM ratio	1.23	1.93	2.1	1.15	0.93	0.88	0.9
	95%-CI	1.09, 1.38	1.64, 2.28	1.75, 2.52	0.99, 1.33	0.82, 1.06	0.76, 1.01	0.78, 1.04
	p-value	<0.01	<0.01	<0.01	0.07	0.26	0.06	0.15
β1PzO1	LSM ratio	1.37	1.62	1.64	1.38	1.33	1.15	1.16
	95%-CI	1.16, 1.61	1.26, 2.10	1.29, 2.09	1.12, 1.70	1.09, 1.61	0.97, 1.35	0.97, 1.38
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	0.1	0.11

Parameter	Timepoint/ Statistic	Overall*	1H	2H	3H	3.5H	4H	5H
β1PzO2	LSM ratio	1.52	1.71	1.74	1.61	1.52	1.28	1.31
	95%-CI	1.29, 1.78	1.45, 2.01	1.48, 2.04	1.28, 2.01	1.22, 1.91	1.05, 1.57	1.03, 1.67
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	0.02	0.03
γFzCz	LSM ratio	0.88	1.07	1.14	0.84	0.8	0.77	0.74
	95%-CI	0.80, 0.97	0.95, 1.21	1.00, 1.29	0.73, 0.96	0.72, 0.89	0.68, 0.87	0.66, 0.83
	p-value	<0.01	0.28	0.05	0.01	<0.01	<0.01	<0.01
γPzO1	LSM ratio	0.98	1.06	0.97	0.92	0.92	0.95	1.07
	95%-CI	0.76, 1.27	0.77, 1.45	0.70, 1.35	0.68, 1.24	0.67, 1.27	0.66, 1.38	0.76, 1.51
	p-value	0.88	0.74	0.86	0.58	0.6	0.78	0.68
γPzO2	LSM ratio	1.16	1.26	1.06	1.15	1.05	1.09	1.4
	95%-CI	0.89, 1.52	0.92, 1.71	0.79, 1.44	0.85, 1.55	0.77, 1.45	0.76, 1.57	0.97, 2.02
	p-value	0.27	0.15	0.69	0.36	0.74	0.64	0.07
LOR VERSUS PLC								
δFzCZ	LSM ratio	0.91	0.91	0.94	0.94	0.88	0.86	0.91
	95%-CI	0.81, 1.02	0.72, 1.15	0.79, 1.13	0.83, 1.07	0.77, 1.00	0.71, 1.04	0.80, 1.03
	p-value	0.1	0.43	0.5	0.36	0.06	0.13	0.13
δPzO1	LSM ratio	0.89	1.08	1.01	1.04	0.84	0.73	0.73
	95%-CI	0.73, 1.09	0.78, 1.49	0.76, 1.34	0.75, 1.44	0.63, 1.11	0.59, 0.92	0.61, 0.88
	p-value	0.27	0.65	0.96	0.83	0.21	<0.01	<0.01
δPzO2	LSM ratio	0.85	0.9	0.92	0.97	0.83	0.77	0.75
	95%-CI	0.70, 1.05	0.72, 1.11	0.74, 1.16	0.70, 1.36	0.61, 1.15	0.58, 1.01	0.58, 0.98
	p-value	0.13	0.31	0.49	0.86	0.26	0.06	0.03
θFzCz	LSM ratio	0.5	0.53	0.46	0.52	0.48	0.46	0.54
	95%-CI	0.42, 0.58	0.41, 0.67	0.37, 0.57	0.43, 0.62	0.39, 0.59	0.36, 0.57	0.45, 0.65
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
θPzO1	LSM ratio	0.51	0.61	0.54	0.52	0.46	0.46	0.5
	95%-CI	0.41, 0.64	0.46, 0.82	0.41, 0.71	0.39, 0.70	0.35, 0.61	0.36, 0.61	0.39, 0.64
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
θPzO2	LSM ratio	0.48	0.53	0.47	0.49	0.42	0.48	0.52
	95%-CI	0.38, 0.60	0.42, 0.66	0.38, 0.58	0.36, 0.68	0.31, 0.57	0.35, 0.65	0.38, 0.71
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
αFzCz	LSM ratio	0.53	0.55	0.49	0.5	0.52	0.53	0.57
	95%-CI	0.46, 0.60	0.46, 0.66	0.40, 0.59	0.43, 0.60	0.44, 0.61	0.45, 0.61	0.50, 0.65
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
αPzO1	LSM ratio	0.43	0.53	0.42	0.37	0.41	0.41	0.44
	95%-CI	0.35, 0.53	0.38, 0.73	0.30, 0.59	0.28, 0.49	0.32, 0.53	0.33, 0.52	0.36, 0.55
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
αPzO2	LSM ratio	0.39	0.42	0.35	0.34	0.36	0.41	0.46
	95%-CI	0.32, 0.47	0.32, 0.54	0.27, 0.46	0.25, 0.45	0.27, 0.46	0.32, 0.52	0.35, 0.60
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
βFzCz	LSM ratio	1.52	1.58	1.64	1.55	1.52	1.42	1.39
	95%-CI	1.36, 1.69	1.37, 1.84	1.41, 1.92	1.36, 1.78	1.35, 1.71	1.26, 1.60	1.21, 1.59
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Parameter	Timepoint/ Statistic	Overall*	1H	2H	3H	3.5H	4H	5H
βPzO1	LSM ratio	0.99	1.21	1.22	0.95	0.98	0.83	0.83
	95%-CI	0.83, 1.18	0.94, 1.56	0.95, 1.57	0.76, 1.18	0.80, 1.21	0.69, 1.00	0.69, 1.00
	p-value	0.93	0.13	0.12	0.65	0.88	0.05	0.05
βPzO2	LSM ratio	0.83	0.93	0.97	0.77	0.83	0.75	0.76
	95%-CI	0.70, 0.99	0.78, 1.12	0.80, 1.16	0.61, 0.98	0.65, 1.04	0.60, 0.93	0.59, 0.97
	p-value	0.04	0.46	0.7	0.03	0.11	0.01	0.03
β1FzCz	LSM ratio	1.32	1.4	1.49	1.34	1.33	1.24	1.17
	95%-CI	1.17, 1.49	1.18, 1.65	1.24, 1.79	1.15, 1.55	1.16, 1.51	1.08, 1.43	1.01, 1.36
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.03
β1PzO1	LSM ratio	0.92	1.11	1.13	0.88	0.88	0.79	0.78
	95%-CI	0.78, 1.09	0.85, 1.43	0.88, 1.44	0.71, 1.09	0.73, 1.07	0.67, 0.94	0.65, 0.93
	p-value	0.32	0.44	0.33	0.23	0.21	<0.01	<0.01
β1PzO2	LSM ratio	0.79	0.89	0.91	0.74	0.74	0.75	0.75
	95%-CI	0.67, 0.93	0.75, 1.06	0.77, 1.07	0.59, 0.92	0.59, 0.93	0.61, 0.92	0.59, 0.96
	p-value	<0.01	0.18	0.23	<0.01	0.01	<0.01	0.02
γFzCz	LSM ratio	1.51	1.59	1.63	1.61	1.44	1.44	1.39
	95%-CI	1.38, 1.66	1.41, 1.80	1.43, 1.85	1.40, 1.84	1.29, 1.61	1.27, 1.63	1.24, 1.55
	p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
γPzO1	LSM ratio	1.22	1.38	1.45	1.22	1.41	1.05	0.92
	95%-CI	0.94, 1.59	1.00, 1.91	1.04, 2.01	0.90, 1.65	1.02, 1.94	0.72, 1.53	0.65, 1.30
	p-value	0.13	0.05	0.03	0.19	0.04	0.79	0.65
γPzO2	LSM ratio	0.89	0.92	1.04	0.84	1.05	0.85	0.68
	95%-CI	0.67, 1.17	0.67, 1.27	0.76, 1.41	0.62, 1.14	0.76, 1.45	0.58, 1.23	0.46, 0.98
	p-value	0.38	0.61	0.82	0.26	0.74	0.38	0.04

Abbreviations: 95%-CI = 95% confidence interval; H = hours; HD-BRX = high-dose brexanolone (90 mcg/kg/h); LD-BRX = low-dose brexanolone (30 mcg/kg/h); LOR = lorazepam; LSM = least square means; PEEG = pharmaco-electroencephalogram; PLC = placebo
* Overall includes all post-dose timepoints.

TABLE S2 Summary of results for single- and paired-pulse TMS-EMG – change from baseline.
Summary table of TMS-EMG results change from baseline for all treatments relative to placebo, and for both doses of brexanolone relative to lorazepam. P-values are derived from LMS comparisons between treatments using a mixed model repeated measures analysis. Results are reported in LMS ratio, with exception of the RMT for which we report the LMS difference ± standard error, with 95%-CI and p-values. Significant p-values are indicated in bold.

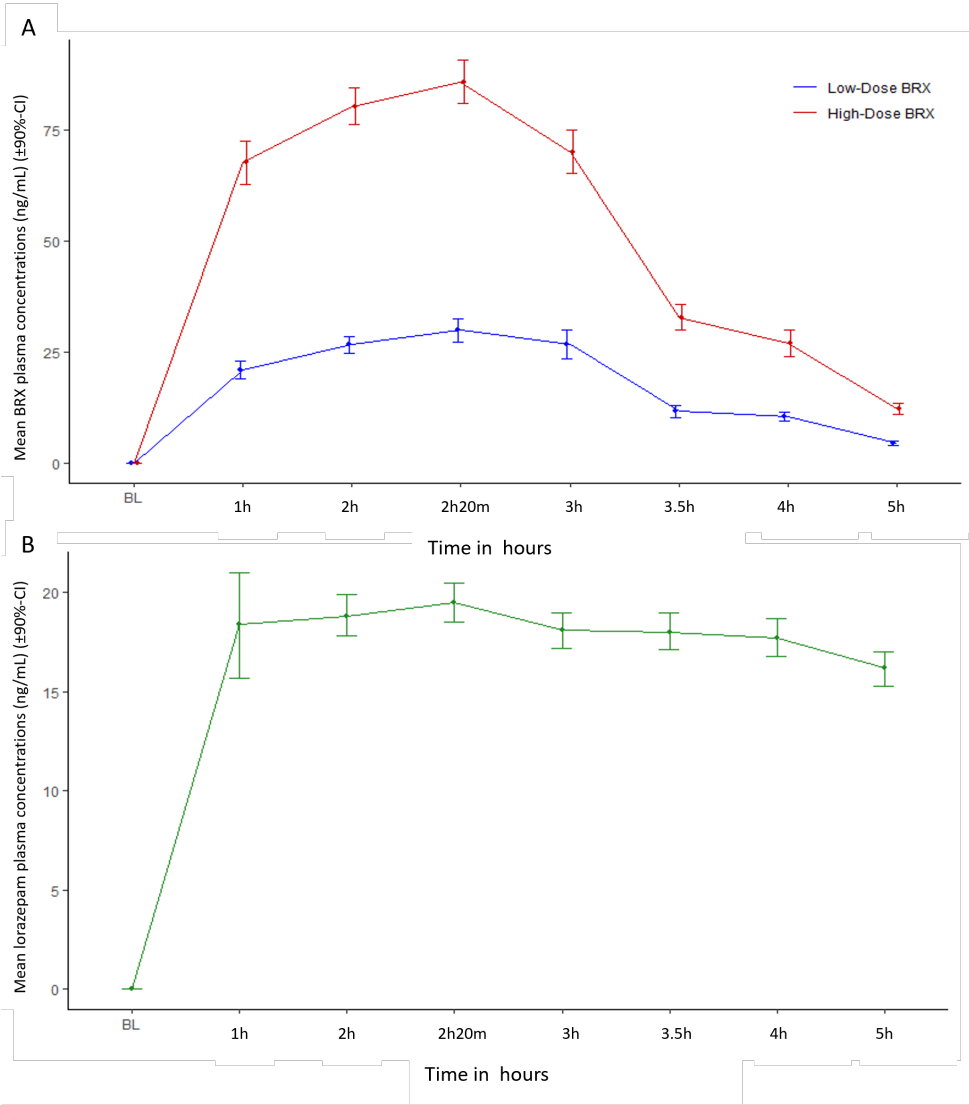
Treatment/ Timepoint	Statistic	Single pulse		Paired pulse		
		MEP (μV)	RMT† (%)	SICI ₁₂ (%)	ICF ₁₅ (%)	LICI ₁₀₀ (%)
LD-BRX VERSUS PLC						
Overall*	LSM ratio	1.05	1.39 (0.83)	0.99	1.1	0.61
	95%-CI	0.79, 1.40	-0.27, 3.04	0.75, 1.32	0.87, 1.39	0.39, 0.96
	p-value	0.72	0.10	0.96	0.40	0.03
2H20M	LSM ratio	0.91	1.35 (0.98)	1.11	1.16	0.53
	95%-CI	0.67, 1.24	-0.61, 3.31	0.79, 1.54	0.83, 1.61	0.31, 0.92
	p-value	0.55	0.17	0.55	0.38	0.02
4H	LSM ratio	1.21	1.42 (0.94)	0.89	1.05	0.71
	95%-CI	0.84, 1.76	-0.44, 3.28	0.62, 1.27	0.75, 1.47	0.40, 1.25
	p-value	0.30	0.13	0.52	0.76	0.23
HD-BRX VERSUS PLC						
Overall*	LSM ratio	1.16	1.43 (0.82)	0.91	0.93	0.44
	95%-CI	0.88, 1.53	-0.19, 3.05	0.70, 1.19	0.75, 1.16	0.28, 0.68
	p-value	0.28	0.08	0.50	0.49	< 0.01
2H20M	LSM ratio	1.13	0.98 (0.97)	1.01	0.92	0.30
	95%-CI	0.83, 1.52	-0.94, 2.90	0.74, 1.39	0.67, 1.25	0.17, 0.51
	p-value	0.43	0.31	0.94	0.58	< 0.01
4H	LSM ratio	1.2	1.88 (0.92)	0.83	0.94	0.64
	95%-CI	0.84, 1.73	0.05, 3.70	0.59, 1.16	0.68, 1.29	0.36, 1.13
	p-value	0.32	0.04	0.26	0.69	0.12
LD-BRX VERSUS LOR						
Overall*	LSM ratio	1.34	1.66 (0.82)	0.91	1.21	0.85
	95%-CI	1.01, 1.77	0.03, 3.29	0.69, 1.22	0.95, 1.54	0.54, 1.31
	p-value	0.04	0.05	0.53	0.13	0.45
2H20M	LSM ratio	1.05	0.87 (0.97)	0.98	1.18	0.77
	95%-CI	0.78, 1.43	-1.06, 2.81	0.70, 1.38	0.83, 1.66	0.45, 1.33
	p-value	0.73	0.37	0.93	0.35	0.35
4H	LSM ratio	1.69	2.44 (0.92)	0.85	1.24	0.93
	95%-CI	1.18, 2.44	0.61, 4.27	0.60, 1.21	0.89, 1.73	0.53, 1.62
	p-value	< 0.01	< 0.01	0.36	0.2	0.79
HD-BRX VERSUS LOR						
Overall*	LSM ratio	1.48	1.70 (0.80)	0.84	1.02	0.60
	95%-CI	1.13, 1.94	0.11, 3.29	0.64, 1.11	0.81, 1.27	0.39, 0.92
	p-value	< 0.01	0.04	0.21	0.89	< 0.01
2H20M	LSM ratio	1.31	0.50 (0.95)	0.9	0.93	0.43
	95%-CI	0.97, 1.76	-1.39, 2.40	0.65, 1.25	0.68, 1.28	0.25, 0.74
	p-value	0.08	0.60	0.53	0.66	< 0.01

Treatment/ Timepoint	Statistic	Single pulse		Paired pulse		
		MEP (μV)	RMT† (%)	SI _{CI} ₂ (%)	ICF ₁₅ (%)	LICI ₁₀₀ (%)
4H	LSM ratio	1.67	2.90 (0.90)	0.79	1.11	0.84
	95%-CI	1.17, 2.39	1.11, 4.68	0.56, 1.11	0.81, 1.52	0.48, 1.45
	p-value	< 0.01	< 0.01	0.17	0.53	0.52
LOR VERSUS PLC						
Overall*	LSM ratio	0.79	-0.27 (0.81)	1.09	0.91	0.73
	95%-CI	0.60, 1.04	-1.88, 1.34	0.83, 1.42	0.73, 1.15	0.47, 1.12
	p-value	0.09	0.74	0.55	0.43	0.15
2H20M	LSM ratio	0.86	0.48 (0.96)	1.12	0.98	0.69
	95%-CI	0.64, 1.17	-1.44, 2.39	0.82, 1.54	0.71, 1.36	0.40, 1.18
	p-value	0.34	0.62	0.47	0.92	0.17
4H	LSM ratio	0.72	-1.02 (0.91)	1.05	0.85	0.77
	95%-CI	0.50, 1.03	-2.83, 0.79	0.75, 1.47	0.62, 1.17	0.44, 1.33
	p-value	0.07	0.26	0.78	0.31	0.34

Abbreviations: 95%-CI = 95% confidence interval; H = hours; HD-BRX = high-dose brexanolone (90 mcg/kg/h); ICF₁₅ = intracortical facilitation at interstimulus interval of 15 ms; LD-BRX = low-dose brexanolone (30 mcg/kg/h); LICI₁₀₀ = long-interval intracortical inhibition at interstimulus interval of 100 ms; LOR = lorazepam; LSM = least square means; MEP = motor evoked; PLC = placebo; RMT = resting motor threshold; SI_{CI}₂ = short-interval intracortical inhibition at interstimulus interval of 2 ms; TMS-EMG = transcranial magnetic stimulation combined with electromyography.

*Overall includes all post-dose timepoints. † For RMT, LSM difference ± standard error is reported instead of the LSM ratio.

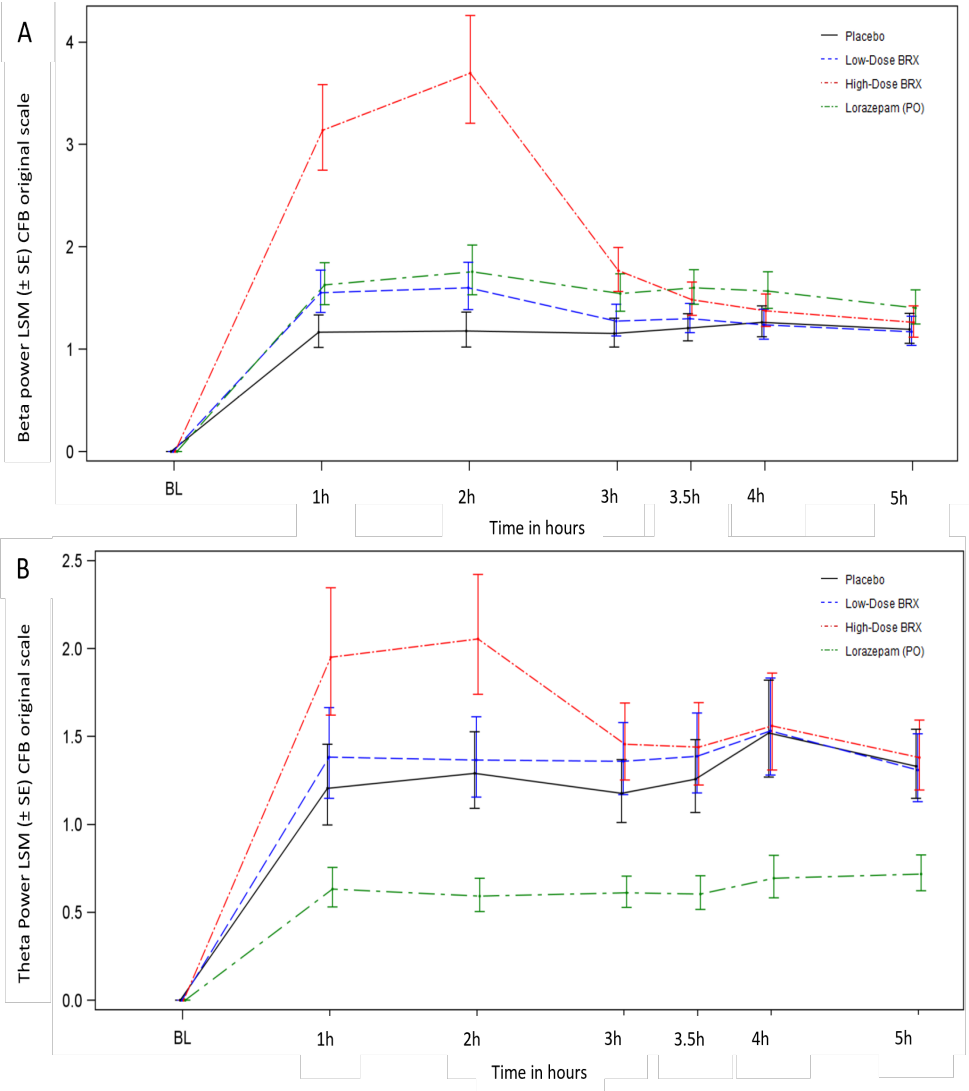
FIGURE S1 Pharmacokinetic time course of (A) low- and high-dose brexanolone and (B) lorazepam. Summary graphs of mean (±90%-CI) brexanolone (S1A) and lorazepam (S1B) measured drug plasma concentrations (ng/mL) - linear scales. C_{max} was (S1A) 28.3 ng/mL and 85.8 ng/mL for low-dose BRX (30 mcg/kg/h) and high-dose BRX (90 mcg/kg/h), respectively, and (S1B) 19.3 ng/mL for lorazepam. T_{max} median was (S1A) roughly the same for both doses BRX at 3H and (S1B) at 1.6H for lorazepam.



Abbreviations: BL = baseline; BRX = brexanolone; CI = confidence interval; H = hours; mL = milliliters; ng = nanograms; PO = oral; SD = standard deviation.

FIGURE S2 Summary graphs for PEEG beta power (A) and theta power (B) at FZ-CZ for the eyes closed condition – Change from baseline.

Summary graphs of the change from baseline of the LSM ratio with the SE as error bars for PEEG spectral power measured at FZ-CZ in the eyes closed condition for the beta (β): 12.5 < 30.0 HZ (S2A) and theta frequency band (θ): 6.0 < 8.5 HZ (S2B), for all treatments. Beta power at the FZ-CZ electrodes with eyes closed (S2A) was significantly increased for all treatments relative to placebo. Theta power at the FZ-CZ electrodes with eyes closed (S2B) was significantly increased for brexanolone relative to placebo and lorazepam and decreased for lorazepam relative to placebo.



Abbreviations: BL = baseline; BRX = brexanolone; FZ-CZ = frontocentral leads; LSM = least square means; PEEG = pharmaco-electro-encephalography; PO = oral; SE = standard error.

CHAPTER VII

GENERAL DISCUSSION

AND CONCLUSIONS

Cortical excitability reflects the dynamic balance between excitatory glutamatergic and inhibitory gamma-aminobutyric acid (GABA) signaling, which together form the core mechanisms governing neuronal activation and inhibition.¹ This balance is further shaped by neuromodulatory systems, including monoaminergic and cholinergic pathways, as well as intrinsic membrane properties, ion channel dynamics, and network-level connectivity.^{2,3} Disruptions in this balance are implicated across a wide range of neurological and psychiatric disorders,⁴⁻⁷ making abnormal cortical excitability a compelling target for drug development. However, progress in central nervous system (CNS) drug development continues to be hindered by the lack of reliable and translatable biomarkers capable of capturing mechanistic drug effects in humans during early-phase trials.^{8,9} In this context, cortical excitability has emerged as a promising mechanism-based biomarker for early-phase CNS drug development.¹⁰ This thesis aims to address critical gaps in early-phase CNS drug development by examining whether cortical excitability, measurable through transcranial magnetic stimulation (TMS), can serve as a mechanistic and translational biomarker. Central to this objective is the question whether TMS can capture drug-induced cortical modulation fingerprints across distinct mechanisms of action, moving from *signal* to *signature*, and thereby support a biomarker-driven framework for early CNS drug development that ultimately bridges the translational gap from animal to human and from healthy participants to patient outcomes and advances tools for precision pharmacology.

To achieve this objective, the thesis was structured around a series of aims: building a benchmark portfolio of drug-induced cortical signatures in humans; assessing the translatability of these drug-specific cortical excitability signatures from healthy participants to relevant patient populations; applying TMS as a mechanistic biomarker for drugs with novel mechanism of action (MOA); determining whether TMS can distinguish between benchmark drugs and drugs with novel MOA; and exploring the value of TMS-derived measures as mechanistic biomarkers relative to conventional pharmaco-electroencephalography (PEEG). These aims were operationalized through a sequence of proof-of-mechanism studies using standardized TMS-electromyography (EMG), TMS-EEG, and PEEG protocols, evaluating market-approved CNS-active compounds (S-ketamine, lorazepam, and levetiracetam) alongside investigational agents (TRV045 and brexanolone). Together, these studies enabled systematic characterization of pharmacodynamic responses and refinement based on their putative

mechanisms of action. Extending these investigations to patients with generalized epilepsy further enabled examination of the translational value of cortical excitability signatures from healthy participants to a clinically relevant patient population.

Benchmark portfolio of drug-induced cortical signatures

Building upon our previous work characterizing acute glutamatergic modulation using an investigational compound (Osavampator, also known as TAK-653 or NBI-1065845) with pro-glutamatergic effects on cortical excitability,¹¹ **Chapter II** investigated both the acute and sustained pharmacodynamic effects of intravenously and orally administered S-ketamine in healthy participants with the goal of benchmarking a rapid antiglutamatergic antidepressant (RAAD) for the future development of novel RAAD. To determine whether TMS and PEEG can detect S-ketamine's delayed and sustained effects on cortical excitability beyond the acute concentration-driven response,¹² we assessed these measures over several days following a single administration. This chapter provides the first evidence of S-ketamine's delayed pharmacodynamic (PD) effects, extending at least 7 days post-dose, well after plasma concentrations of the parent compound and its metabolites have dissipated. Importantly, these sustained effects differed in directionality from the acute phase: whereas S-ketamine caused acute CNS depression,¹³ the delayed and sustained phases were characterized by increased cortical excitability and resting-state brain activity. The emergence of these increases approximately 24 hours post-dose aligns with the known onset of ketamine's antidepressant effects,¹⁴ suggesting potential clinical relevance of these biomarkers. Together, these findings highlight the potential of S-ketamine as a benchmark compound for RAAD development and suggest that TMS and PEEG may provide insight into neurophysiological processes relevant to RAAD mechanisms.

Translatability of these drug-specific cortical excitability signatures from healthy participants to relevant clinical populations

After validating TMS-derived biomarkers to profile antiseizure medication (ASM) in HVs, **Chapter III** evaluated the translatability of cortical excitability signatures from healthy participants to clinical populations by replicating a previously conducted healthy volunteer study of single-dose ASM¹⁵ in patients with generalized epilepsy. Levetiracetam was selected

as the benchmark compound based on its robust and well-characterized effects in healthy participants. In patients with well-controlled generalized epilepsy, a single 2000 mg dose of levetiracetam decreased cortical excitability, reflected by reductions in motor evoked potential (MEP) amplitude and enhancements in long-interval intracortical inhibition (LICI) at interstimulus interval (ISI) of 50 ms (LICI₅₀), replicating the findings observed in healthy participants. Beyond TMS measures, PEEG also demonstrated increased frontal gamma and beta power, suggesting their potential as sensitive biomarkers of levetiracetam's pharmacodynamic activity. Exploratory pharmacokinetic/pharmacodynamic (PK/PD) analyses revealed a linear concentration–response relationship across these biomarkers, reinforcing their validity and potential value for guiding dose selection. Together, these findings demonstrate that drug-induced cortical excitability signatures observed in healthy participants translate to patients with generalized epilepsy, underscoring the value of TMS as both a proof-of-mechanism and proof-of-concept biomarker for early-phase ASM development. TMS-based measures may help fill the current gap in reliable biomarkers capable of confirming target engagement in healthy participants while remaining applicable to patient populations and potentially serving as surrogate indices of antiepileptic efficacy.

TMS as mechanistic biomarker for drugs with novel MoA

Chapter IV extended our earlier work on established ASM¹⁵ by evaluating the pharmacodynamic effects of the investigational ASM TRV045, a Selective Sphingosine-1-Phosphate Subtype 1 Receptor (S1PR1) agonist with a novel MOA, in healthy participants.¹⁶ This multiple-dose study examined whether TRV045 influences cortical excitability, and whether these effects persist with repeated dosing, to provide early clinical evidence of its antiepileptic potential following promising preclinical findings.¹⁷ This is the first study to characterize the neurophysiological effects of S1PR1 modulation in humans using combined TMS-EMG, TMS-EEG and PEEG. TRV045 produced a marked shift in PEEG spectral power, with increases in alpha, beta, and gamma activity and decreases in delta power, which became more pronounced over four days of dosing. TMS-EEG also demonstrated cortical modulation after four days of dosing, consistent with the PEEG findings. TMS-EMG showed a non-significant decrease in MEP amplitude after the first dose, of comparable effect size to acute responses reported for other ASM,¹⁵ suggesting a possible early reduction in corticospinal excitability that did not persist on Day 4. Higher exposures may have been required

to detect significant TMS-EMG effects, as exploratory PK/PD analyses did reveal a linear concentration–response relationship for MEP amplitude. Overall, the PEEG and TMS-EEG pharmacodynamic profile hints at indirect glutamatergic modulation via S1PR1,^{16,18,19} although limited exposure and day-to-day variability may have constrained the detection of robust cortical excitability changes. Further evaluation across a broader dosing range and in an epilepsy patient population is warranted to determine whether these effects translate into clinically meaningful antiepileptic efficacy.

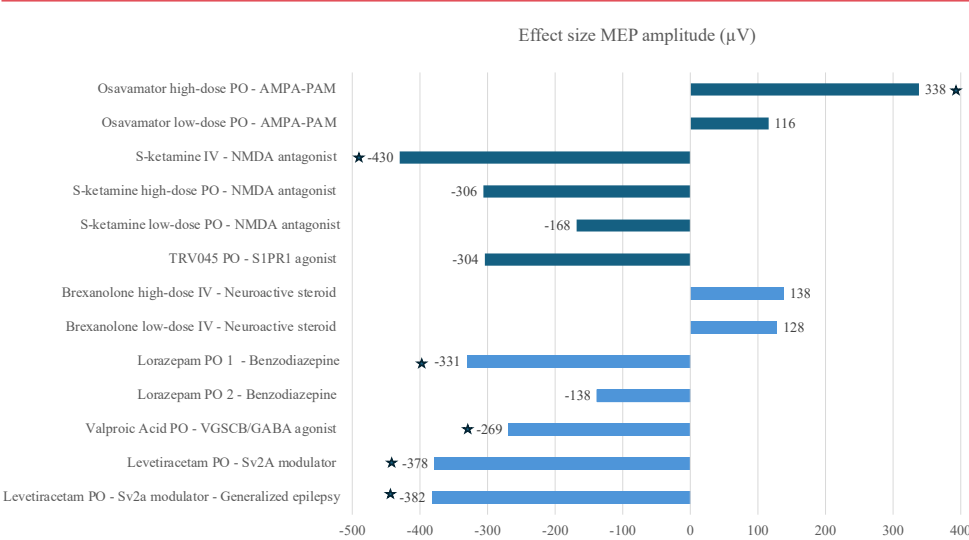
TMS as mechanistic biomarker to distinguish between benchmark drugs and drugs with novel MoA

Chapter V presents the first head-to-head comparison of cortical excitability signatures of two mechanistically distinct GABA_AR-modulating drugs, the benzodiazepine lorazepam, used as a benchmark comparator, and the investigational neurosteroid brexanolone, to evaluate whether TMS can differentiate mechanism-specific neurocircuit modulation in healthy participants. These compounds were selected for their distinct mechanisms of action. Benzodiazepines enhance phasic inhibition by targeting synaptic GABA_AR at the classical benzodiazepine-binding site, whereas neurosteroids such as brexanolone act at a distinct neurosteroid-binding domain on both synaptic and extrasynaptic GABA_AR, allowing combined modulation of phasic and tonic inhibition.^{20–22} Brexanolone produced distinct pharmacodynamic effects on both PEEG and TMS-derived measures, clearly differentiating it from both lorazepam. Notably, brexanolone induced dose-dependent increases in theta power and enhancement of LICI₁₀₀, a pattern that resembles modulation of tonic inhibition through GABA_BR,^{23–25} but through extrasynaptic GABA_AR. These findings demonstrate that TMS-EMG, TMS-EEG, and pEEG are sensitive to the differential engagement of synaptic versus combined synaptic and extrasynaptic GABAergic circuits, supporting their utility for mechanism-based differentiation. Importantly, they further indicate that TMS-based measures can contribute to defining neurophysiological fingerprints of drug effects, even for compounds affecting the same receptor. Together, these results underscore the potential of a multimodal neurophysiological biomarker strategy, leveraging both PEEG and TMS, to inform early-phase compound differentiation and to support the development of next-generation GABAergic therapies through mechanism-specific characterization of neurocircuitry modulation relevant to neuropsychiatric disorders.

Considerations for using TMS as a biomarker-driven framework for early CNS drug development

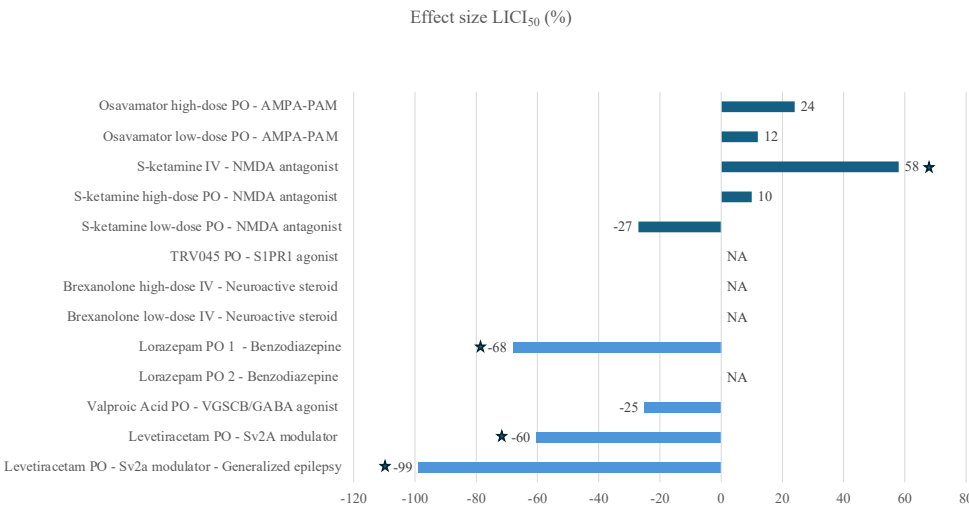
Collectively, these studies support the development of a coherent TMS-based biomarker platform capable of identifying pharmacodynamic response, elucidating mechanism of action, and providing early signals of potential efficacy, thereby strengthening decision-making in early CNS drug development. Importantly, the sensitivity and applicability of TMS-derived measures were demonstrated not only in healthy participants but also in patients with generalized epilepsy. Across studies, we benchmarked several compounds acting on GABAergic and glutamatergic systems and showed that TMS is sensitive to both inhibitory and excitatory pharmacological effects (Figure 1, 2 and 3). Moreover, TMS was able to detect effects of compounds that indirectly modulate cortical excitability (TRV045) and, moreover, was able to differentiate between GABA_AR modulators with distinct MOA. The MEP amplitude emerged as the most sensitive TMS-EMG biomarker (Figure 1), followed by LIC1 (Figure 2-3), whereas no consistent effects were observed for SIC1 or ICF for the compounds tested. Taken together, the combined use of TMS-EMG and TMS-EEG measures enabled the characterization of distinct neurophysiological fingerprints across compounds, underscoring the value of a multimodal TMS-based approach for mechanism-based differentiation in early-phase drug development.

FIGURE 1 Summary of drug effects on MEP amplitude. Effect sizes of different CNS active drugs on TMS-EMG MEP amplitude as expressed by the estimated difference of the least square mean of the active treatment relative to placebo in μ V. Compounds that modulate glutamatergic neurotransmission are indicated in dark blue. Compounds that modulate GABAergic inhibition are indicated in light blue. Significant effects ($p < 0.05$) are indicated with a star symbol (★). Lorazepam was administered in two different trials using the same dose and are therefore indicated with 1) Ruijs et al. 2022, and 2) de Cuba et al. 2026.



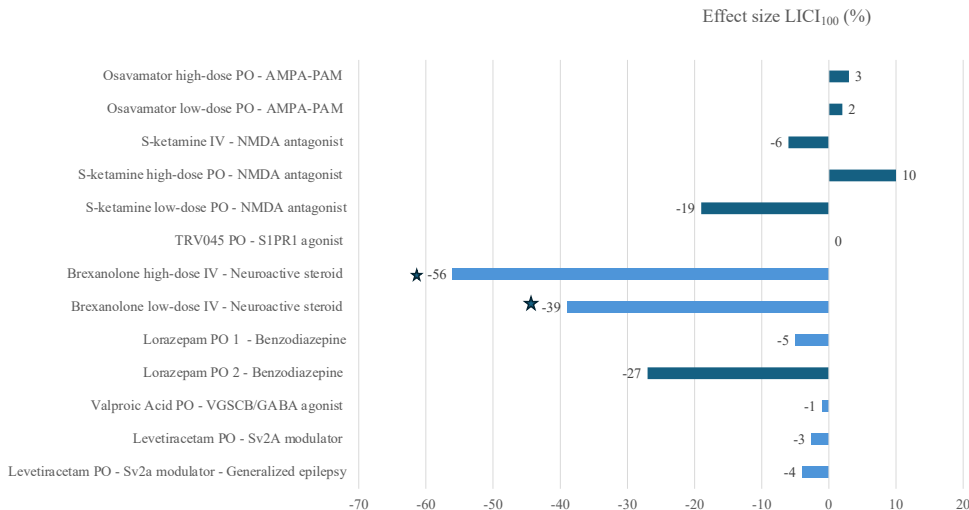
Abbreviations: μ V = microvolts; AMPA-PAM = α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor – Positive Allosteric Modulator; GABA = Gamma-Aminobutyric Acid receptor; IV = intravenous; NMDA = N-Methyl-D-Aspartate receptor; MEP = motor evoked potential; PO = oral; S1PR1 = Sphingosine-1-Phosphate Subtype 1 Receptor; SV2A = Synaptic Vesicle Glycoprotein 2A; VGSCB = voltage-gated sodium channel blocker.

FIGURE 2 Summary of drug effects on LICl₅₀.
Effect sizes of different CNS active drugs on TMS-EMG LICl₅₀ as expressed by the estimated difference of the least square mean of the active treatment relative to placebo in percentages. Compounds that modulate glutamatergic neurotransmission are indicated in dark blue. Compounds that modulate GABAergic inhibition are indicated in light blue. Significant effects (p < 0.05) are indicated with a star symbol (★). Lorazepam was administered in two different trials using the same dose and are therefore indicated with 1) Ruijs et al. 2022, and 2) de Cuba et al. 2026.



Abbreviations: AMPA-PAM = α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor – Positive Allosteric Modulator; GABA = Gamma-Aminobutyric Acid receptor; IV = intravenous; LICl₅₀ = long-interval intracortical inhibition at interstimulus interval of 50 ms; NMDA = N-Methyl-D-Aspartate receptor; PO = oral; S1PR1 = Sphingosine-1-Phosphate Subtype 1 Receptor; Sv2A = Synaptic Vesicle Glycoprotein 2A; VGSCB = voltage-gated sodium channel blocker.

FIGURE 3 Summary of drug effects on LICl₁₀₀.
Effect sizes of different CNS active drugs on TMS-EMG LICl₁₀₀ as expressed by the estimated difference of the least square mean of the active treatment relative to placebo in percentages. Compounds that modulate glutamatergic neurotransmission are indicated in dark blue. Compounds that modulate GABAergic inhibition are indicated in light blue. Significant effects (p < 0.05) are indicated with a star symbol (★). Lorazepam was administered in two different trials using the same dose and are therefore indicated with 1) Ruijs et al. 2022, and 2) de Cuba et al. 2026.



Abbreviations: AMPA-PAM = α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor – Positive Allosteric Modulator; GABA = Gamma-Aminobutyric Acid receptor; IV = intravenous; LICl₁₀₀ = long-interval intracortical inhibition at interstimulus interval of 100 ms; NMDA = N-Methyl-D-Aspartate receptor; PO = oral; S1PR1 = Sphingosine-1-Phosphate Subtype 1 Receptor; Sv2A = Synaptic Vesicle Glycoprotein 2A; VGSCB = voltage-gated sodium channel blocker.

The findings discussed in this thesis emphasize the value of combining TMS-EMG and TMS-EEG with resting-state pEEG within a multimodal biomarker framework, as these approaches probe distinct but complementary aspects of drug-induced neurophysiology. TMS-EMG and TMS-EEG provide mechanistic insight by directly interrogating cortical excitability and excitation–inhibition (E/I) balance under controlled perturbation and, for several of the compounds tested (e.g. levetiracetam, TRV045), also revealed clear PK/PD relationships. In contrast, pEEG primarily captures spontaneous, resting-state brain dynamics and has shown high sensitivity for detecting concentration–effect relationships, likely due to lower intrinsic variability and the ability to sample more frequently. However, pEEG lacks spatial specificity, which could be enhanced by higher-density EEG and advanced analytical approaches, such as cluster-based permutation testing²⁶ and source-localization approaches (e.g., LORETA),²⁷ but at the cost of increased logistical and technical burden and additional challenges related to standardization, potentially limiting feasibility in early-phase clinical trials.

The key differences between TMS and resting-state pEEG reflect fundamental distinctions in the neurophysiological processes they probe and, consequently, the types of pharmacological mechanisms to which they are most sensitive. Because pEEG relies on resting-state activity, it is primarily sensitive to pharmacological effects that alter ongoing oscillatory dynamics or global network activity, such as compounds that broadly dampen or enhance spontaneous cortical rhythms. In contrast, pEEG may be less sensitive to drug effects that are expressed predominantly during specific excitability states or pathological conditions, or that require sufficient displacement of the system from baseline to become detectable. This includes mechanisms that normalize aberrant excitation–inhibition balance without substantially affecting baseline activity. An example of such a mechanism is the pharmacological restoration of potassium-chloride cotransporter 2 (KCC2) hypofunction, which is expected to normalize chloride homeostasis and strengthen inhibitory efficacy in hyperexcitable cortical circuits.²⁸ Such state-dependent drug effects are more likely to become apparent when cortical circuits are driven away from equilibrium and may remain largely latent and undetectable during resting-state recordings in the absence of overt hyperexcitability. In this context, TMS offers a powerful complementary approach to resting-state pEEG by enabling controlled and localized cortical perturbation, for example at the motor hotspot, allowing direct interrogation of

excitation–inhibition circuits. This perturbational capability makes TMS particularly well suited for characterizing neurophysiological fingerprints of drug effects, including compounds that modulate excitability indirectly through homeostatic or higher-order regulatory mechanisms (e.g. via KCC2, sodium-potassium-chloride cotransporter 1 (NKCC1) or SIPR1), which are increasingly explored as pharmacological targets in part due to their potentially favorable tolerability profiles or disease-modifying potential.^{28–31}

The biggest challenge in developing TMS-based biomarkers, and in advancing methodological standardization more broadly, is the absence of established gold standards across the entire TMS pipeline, including endpoint selection, procedural elements, stimulation paradigms, data processing, and analytical approaches.³² This lack of consensus contributes to substantial heterogeneity in the literature and limits the comparability of findings across research groups.

Effective implementation of TMS-based biomarkers requires careful consideration of how high intra- and inter-subject variability of TMS signals³³ influences study design and endpoint selection. This variability often necessitates crossover designs in which participants serve as their own control, typically requiring sample sizes of approximately 16 to 24 participants. Such designs are feasible in early proof-of-mechanism studies but are more difficult to implement in first-in-human settings, especially within standard SAD and MAD study designs where the number of participants per dose level is limited. Alternative approaches that may balance the need for within-subject control with practical and ethical constraints on participant numbers include partial cross-over designs, which can mitigate some of these constraints but typically reduce statistical power.

With respect to endpoint selection, intrinsic variability has limited the routine use and reporting of MEP amplitude,³³ despite its demonstrated value as a measure of corticospinal excitability when collected under carefully standardized conditions. Alternative TMS-EMG endpoints, such as input/output (I/O) recruitment curves and the stimulator intensity required to evoke ~1 μ V MEPs (SI1MV), are frequently reported in the literature.^{34–38} However, these paradigms introduce practical trade-offs: I/O curves cannot be collected concurrently with TMS-EEG, and SI1MV procedures require substantial setup time, both of which increase operational burden and reduce feasibility in early-phase clinical drug studies. Although TMS-EMG–derived biomarkers are sensitive to CNS drugs with diverse mechanisms of action,³⁹ they reflect excitability across the entire

corticospinal pathway rather than cortical responses alone, making them more susceptible to physiological variability. In light of these limitations, there has been growing interest in TMS-EEG-derived endpoints as a complementary approach that may offer a more direct assessment of cortical excitability.

In addition, several procedural strategies can help reduce variability, including increasing the number of stimulation trials,^{33,40} optimizing coil placement,⁴¹⁻⁴³ and standardizing procedural elements. Although neuronavigation systems are often recommended to ensure accurate and stable coil positioning,⁴⁴ manual placement remains widely used in clinical trials due to its speed and practicality. To date, available studies have not demonstrated superior reliability of neuronavigation compared with manual positioning for either TMS-EMG,^{45,46} or TMS-EEG endpoints,⁴⁷ and no significant differences in intra- or inter-subject variability of MEP amplitude between navigated and manual positioning have been observed.^{48,49} At the same time, direct head-to-head comparisons assessing superiority or non-inferiority remain scarce, and conclusions regarding the relative performance of these approaches should therefore be interpreted with caution. Importantly, implementing neuronavigation entails substantial cost and logistical complexity, for example due to the requirement for individual MRI scans. Taken together, these considerations help explain why manual coil placement continues to be favored, particularly in early-phase studies.

Beyond these differences in endpoint selection and procedural elements, physiological sources of variability are inherently more difficult to control. A key contributor to such physiological variability is the dependence of TMS responses on the ongoing oscillatory state of the cortex. Prior work has shown that MEP amplitude varies systematically with the phase of ongoing EEG rhythms;⁵⁰⁻⁵⁴ for example, TMS pulses delivered at high-excitability phases of the μ -rhythm elicit larger responses than pulses delivered at low-excitability phases.⁵¹ This state dependence of the TMS signal underscores the potential of real-time EEG-triggered or closed-loop TMS to enhance the reliability of TMS-derived biomarkers by aligning stimulation with specific excitability states. Although closed-loop paradigms could be used as methodological refinement to reduce physiological variability, they introduce considerable technical complexity, require additional setup time, and increase analytical burden, which may limit their practicality in early-phase clinical trials. What should also be considered is that substantial variability also exists in data processing

and analysis frameworks, primarily for TMS-EEG.⁵⁵ Broadly, two ways of quantifying the neural response to a TMS pulse are used: the phase-locked EEG response, designated the TMS-evoked EEG potential (TEP), and the oscillatory, non-phase-locked TMS-EEG response labelled as TMS-induced oscillations (TIO).³² In addition, there are different artefact-rejection methods to reduce artefacts caused by the magnetic pulse and scalp muscle activation,⁵⁶ among which principal component analysis (PCA) has been shown to be effective.⁵⁷ Moreover, we used a placebo-controlled, baseline-adjusted design to analyze the data. We believe this approach represents a superior method for capturing true treatment effects relative to placebo, although it differs from the pre-/post-dose comparisons commonly reported in the literature,⁵⁸⁻⁶¹ thereby limiting direct comparability with historical findings. Thus, we have applied a standardized framework across all studies to ensure internal comparability and to build a coherent historical dataset. Importantly, this framework reflects what we consider the most feasible and clinically appropriate approach for implementing TMS as a biomarker in early-phase trials, balancing scientific rigor with operational practicality.

Future perspectives

The work presented in this thesis highlights the potential of TMS-EEG and TMS-EMG as mechanistic and translational biomarkers for early-phase CNS drug development. A central theme underlying this work is the progression from neurophysiological signal to compound-specific signature. In this context, a signature is not intended to represent a single biomarker or unidimensional readout, but rather a reproducible pattern across complementary neurophysiological measures that together capture the pharmacodynamic and mechanistic footprint of a compound. For TMS-EMG, such signatures are relatively intuitive, as changes in measures such as MEP amplitude or long-interval intracortical inhibition can be directly interpreted in terms of altered corticospinal excitability or inhibitory tone. For TMS-EEG, signatures take a different form: the amplitude and polarity of TMS-evoked EEG components cannot be mapped one-to-one onto excitation or inhibition, but instead reflect the engagement of distinct neuronal populations and synaptic processes with different temporal dynamics. As such, TMS-EEG contributes primarily at a mechanistic level, providing insight into how specific cortical circuits respond to perturbation rather than yielding a scalar index of excitability. Within this framework, neurophysiological signatures emerge most clearly through

the combined interpretation of TMS-EMG and TMS-EEG, rather than within either modality alone. This multimodal perspective is exemplified by the differentiation between lorazepam and brexanolone, where both TMS-EMG and TMS-EEG showed distinguishable profiles, and their combined interpretation yielded a more informative compound-specific signature than either modality alone. At the same time, fully interpreting and generalizing such signatures will require substantially broader benchmarking across compounds with well-characterized and diverse MOA, to establish how specific neurophysiological patterns map onto pharmacological classes and targets. Building on these findings, several avenues for future research and methodological refinement should be considered. First, broader validation across clinical populations is essential. Our patient study focused on individuals with well-controlled generalized epilepsy, selected for safety reasons and because they exhibit prominent bilateral excitability abnormalities, facilitating standardized stimulation. While this approach enabled a strong proof-of-concept, it limits extrapolation to more heterogeneous and treatment-resistant populations, including focal epilepsy. Future studies should extend this framework of pharmacological translatability from healthy participants to other disorders characterized by altered cortical excitability, such as major depressive disorder, amyotrophic lateral sclerosis, and chronic neuropathic pain, to evaluate the generalizability and disease-specific signatures of TMS-derived biomarkers. Third, implementation of TMS in first-in-human (FIH) studies represents an important next step. TMS has not yet been widely incorporated into SAD or MAD designs, largely due to high signal variability. Partial crossover schemes, where each participant receives a subset of dose conditions, may provide a practical way to embed TMS into early development while preserving internal controls, albeit at the cost of reduced statistical power. Accordingly, strategies aimed at limiting signal variability are likely critical for the successful integration of TMS into FIH trials. Fourth, methodological refinement of TMS-EEG endpoints will be critical. To allow for further methodological refinement, it is important to understand the physiological underpinnings of paired-pulse TMS-EEG. Currently, it remains poorly understood how in paired-pulse TMS-EEG the conditioning pulse affects the morphology of the TEP elicited by the test pulse. Emerging evidence indicates that ISI-dependent effects in TMS-EEG do not fully correspond to those observed in TMS-EMG,⁶² likely reflecting fundamental differences between cortico-cortical and corticospinal pathways. Our systematically acquired TMS-EEG dataset provides a unique

opportunity to investigate these dynamics further, and follow-up work will specifically aim to address these remaining questions. Moreover, cross-validation with computational or spectral proxies of the excitation/inhibition balance, including r/f slope and mean-field modelling,⁶³⁻⁶⁶ may strengthen mechanistic interpretation and anchor TMS-derived measures within a broader neurophysiological framework. Finally, standardization remains an urgent priority. Harmonized stimulation protocols, acquisition settings, analysis pipelines, and reporting standards are necessary to reduce methodological heterogeneity and enable cumulative science. As the field advances, coordinated efforts -through consortia, regulatory science collaborations, or methodological working groups- will be essential for establishing recommended practices for incorporating TMS-EMG and TMS-EEG into early-phase drug development. Together, these directions outline a pathway toward a validated, scalable, and mechanistically informative TMS-based biomarker platform capable of supporting precision pharmacology across CNS indications.

Conclusions

This thesis sets out to address critical gaps in early-phase CNS drug development by examining whether cortical excitability, measurable with TMS, can serve as a mechanistic and translational biomarker. Central to this objective was the question of whether TMS can capture drug-induced cortical modulation fingerprints across distinct mechanisms of action, moving from signal to signature, and thereby support a biomarker-driven framework that ultimately bridges the translational gap from animals to humans and from healthy participants to patient outcomes. Across a series of proof-of-mechanism studies, we systematically evaluated key characteristics required for cortical excitability to function as a pharmacodynamic biomarker in early-phase CNS drug development. First, in **Chapter II**, pharmacodynamic sensitivity and temporal relevance were demonstrated by showing that TMS and PEEG can detect delayed and sustained drug effects following administration of S-ketamine in healthy participants, supporting their ability to capture CNS PD effects beyond acute exposure windows. Second, in **Chapter III**, translational validity across populations was established by demonstrating that cortical excitability signatures induced by levetiracetam in healthy participants are preserved in patients with generalized epilepsy, supporting the use of TMS-derived measures as translatable biomarkers bridging healthy-volunteer studies and patient populations. Third, in **Chapter IV**, mechanism-of-action characterization,

including sensitivity to indirect effects on cortical excitability, was supported by the evaluation of TRVo45, where combined TMS-EEG and PEEG revealed emerging pharmacodynamic profiles consistent with indirect glutamatergic modulation, even under exposure-limited conditions. Finally, mechanism-based differentiation at the circuit level was demonstrated by showing that multimodal TMS-EMG, TMS-EEG, and PEEG can distinguish between a benchmark GABA_A positive allosteric modulator and a novel GABA_AR modulator (lorazepam versus brexanolone), even for compounds with subtle differences in pharmacological targets. Taken together, these findings demonstrate that cortical excitability measures fulfill multiple key criteria of a pharmacodynamic/response biomarker as defined in the United States Food and Drug Administration and National Institutes of Health Biomarkers, EndpointS, and other Tools (FDA–NIH BEST) framework. Specifically, they enable assessment of CNS target engagement, capture pharmacodynamic sensitivity and temporal relevance, support mechanism-of-action characterization at the circuit level, and provide translatable readouts of drug-induced modulation across populations (healthy versus patient). While limitations remain, particularly regarding high signal variability, standardization, and validation in broader, more heterogeneous patient populations, the overall body of work supports TMS-derived biomarkers of cortical excitability as a promising mechanistic and translational biomarker platform that can be applied in early proof-of-mechanism and proof-of-concept studies. As such, this thesis lays important groundwork for a TMS-based, biomarker-driven approach to early-phase CNS drug development in support of precision pharmacology.

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APPENDICES

NEDERLANDSE SAMENVATTING

Onze hersenen werken dankzij een nauwkeurig evenwicht tussen prikkelende en remmende signalen. Dit evenwicht zorgt ervoor dat hersencellen informatie kunnen verwerken zonder overactief of juist te geremd te raken. Bij veel neurologische en psychiatrische aandoeningen, zoals epilepsie en depressie, is dit evenwicht verstoord. Veel geneesmiddelen voor deze aandoeningen zijn erop gericht dit evenwicht te herstellen. Toch is het in de vroege fase van geneesmiddelenontwikkeling vaak moeilijk om bij mensen vast te stellen of en hoe een nieuw middel daadwerkelijk effect heeft op de hersenen. Dit gebrek aan goede meetinstrumenten vertraagt de ontwikkeling van nieuwe behandelingen. In dit proefschrift is onderzocht of een techniek genaamd transcraniële magnetische stimulatie (TMS) kan helpen om effecten van medicijnen op de hersenen vroegtijdig en betrouwbaar in kaart te brengen. TMS gebruikt korte magnetische pulsen om specifieke hersengebieden tijdelijk te activeren. Door te meten hoe de hersenen (EEG) en spieren (EMG) hierop reageren, kan informatie worden verkregen over hoe prikkelbaar de hersenschors is. Deze prikkelbaarheid wordt gezien als een belangrijke indicator van de balans tussen activerende en remmende processen in de hersenen. In een reeks zorgvuldig uitgevoerde studies is gekeken of veranderingen in hersenactiviteit na toediening van verschillende geneesmiddelen kan worden gemeten met behulp van TMS. Hierbij zijn zowel bestaande medicijnen (zoals middelen tegen epilepsie en een antidepressivum) als nieuwe, experimentele middelen onderzocht. De metingen werden gedaan bij gezonde vrijwilligers en, in een deel van het onderzoek, ook bij patiënten met epilepsie. De resultaten laten zien dat TMS subtiele maar consistente veranderingen in hersenactiviteit kan vastleggen die optreden na gebruik van geneesmiddelen. Zo bleek dat het antidepressivum S-ketamine niet alleen direct na toediening effect heeft, maar ook dagen later nog meetbare veranderingen veroorzaakt in hersenactiviteit. Deze latere veranderingen vielen samen met het moment waarop het antidepressieve effect in de klinische praktijk wordt verwacht. Dit suggereert dat TMS kan helpen om niet alleen of een middel werkt, maar ook *wanneer* het effect heeft.

Daarnaast bleek dat de veranderingen die we bij gezonde vrijwilligers meten, ook zichtbaar zijn bij epilepsiepatiënten die een bekend anti-epilepticum kregen. Dat is belangrijk, omdat het laat zien dat TMS-metingen kunnen helpen om resultaten uit onderzoek bij gezonde personen te vertalen naar patiënten. Ook bleek dat TMS onderscheid kan maken tussen

geneesmiddelen die op verschillende manieren op de hersenen werken, zelfs wanneer die verschillen klein zijn. Dit maakt de techniek waardevol om nieuwe medicijnen beter te begrijpen en met elkaar te vergelijken in een vroege onderzoeksfase. Tegelijkertijd laat dit proefschrift zien dat er nog uitdagingen zijn. Het TMS-signaal, en hersenactiviteit in zijn algemeen, kunnen per persoon sterk variëren en vereisen daarom een zorgvuldige en gestandaardiseerde aanpak. Verdere methodologische verfijning en samenwerking tussen onderzoeksgroepen is nodig voordat TMS op grote schaal kan worden ingezet in geneesmiddelenontwikkeling. Samengevat laat dit proefschrift zien dat TMS een veelbelovend hulpmiddel is om in een vroege fase effecten van nieuwe medicijnen op de hersenen te meten. Door beter te begrijpen hoe geneesmiddelen het evenwicht in de hersenen beïnvloeden, kan de ontwikkeling van effectievere en beter gerichte behandelingen voor aandoeningen van het centrale zenuwstelsel worden versneld.

RESUMEN NA PAPIAMENTO

Nos celebroy ta funciona danki na un balans meticuloso entre señalnan cu ta stimula y frena celula cerebral. E balans aki ta percura cu e celula cerebral por procesa informacion sin bira di mas of di otro banda di menos activo. Den hopi caso di trastorno neurológico of psiquiátrico, manera epilepsia of depresion, e balans aki ta interumpi. Hopi remedi pa trata e trastornonan aki ta enfoca riba restauracion di e balans aki. Den e fase inicial di desaroyo di remedi, hopi biaha ta difícil pa determina si of con un remedi nobo tin efecto riba e cerebro di e ser humano. Desaroyo di tratamiento nobo ta wordo stroba pa falta di instrumento confiabel pa midi e efectonan di remedi. Den e tesis aki a investiga si e tecnica di estimulacion magnetico transcranial, tambe yama TMS, por yuda pa visualisa e efecto di remedi riba e cerebro den un forma sensitivo y confiabel. TMS ta usa impulso magnetico cortico pa temporalmente activa parti específico di e cerebro. Door di midi con e cerebro (EEG) y musculo (EMG) ta reacciona riba e impulso aki, por obtene informacion con sensitivo e cortex cerebral ta. E sensibilidad aki ta wordo mira como un indicacion importante di e balans entre e procesonan cu ta activa y frena e cerebro. Den un serie di estudio menuciosamente realisa a investiga si cu ayudo di TMS, despues di a suministra diferente sorto di remedi, tin cambio den e actividad cerebral. Pa cu esaki nos a investiga remedi existente (por ehempel remedi contra epilepsia y antidepresivo) y remedi experimental. Nos a investiga e efectonan aki den voluntario saludabel y den un parti di e investigacion, tambe den paciente cu epilepsia. Resultado ta muestra cu TMS por midi cambio chiquito pero consistente den e actividad cerebral cu ta presenta despues di suministro di remedi. Asina a sali na cla cu e antidepresivo esketamine no solamente tin efecto inmediato despues di suministro di e remedi, pero tambe efectonan retarda, cu ta visibel ainda diana despues. E cambionan retarda ta cai hunto cu e momento unda e efecto antidepresivo ta wordo spera den practica. Esaki ta sugeri cu TMS por yuda pa no solamente demonstra si e remedi ta funciona, pero tambe ki ora e tin su efecto. Ademá ta muestra cu e cambionan cerebral midi cerca voluntarionan saludabel despues di suministra un anti-epileptico conoci, tambe ta wordo mira den e paciente cu epilepsia. Esaki ta importante, pasobra esaki ta muestra cu TMS por yuda pa interpreta e resultadonan di e estudio cerca pacientenan cu epilepsia.

Tambe a resulta cu TMS por distingui entre remedi cu ta traha a traves di diferente mecanismo riba e cerebro, hasta si e diferencianan ta chiquito.

Esaki ta hasi e tecnica valioso pa por investiga remedi cu mecanismo nobo y pa compara nan efecto cu remedi conosi den e fase inicial di desaroyo di remedi. Simultaneamente e tesis aki ta muestra cu ainda tin reto. E señal di TMS, y señal di cerebro en general, por varia hopi di persona pa persona, y ta rekeri un metodo meticuloso y standarisa. Mas refinamento metodológico y colaboracion entre gruponan di investigacion científico ta necesario pa TMS por wordo usa na gran escala den e desaroyo di remedi nobo. En resumen, e tesis aki ta muestra cu TMS ta un instrumento cu hopi potencial pa midi efecto di remedi nobo den e cerebro den e fase inicial di desaroyo di remedi. A traves di mihor comprendemento di e influencia di remedi riba e balans den e cerebro, por accelera e desaroyo di tratamentonan di un manera mas eficiente y específico pa trastornonan di e sistema nervioso central.

PHD PORTFOLIO

This portfolio provides a factual overview of the academic development, training activities, and scholarly output during the PhD trajectory. The PhD project focused on the development and translational application of neurophysiological biomarkers, primarily transcranial magnetic stimulation (TMS) and electroencephalography (EEG), to characterize pharmacodynamic effects of central nervous system (CNS), active compounds in early-phase drug development. The work was conducted within a clinical pharmacology framework, with an emphasis on mechanistic insight, biomarker validation, and translatability from healthy volunteers to patient populations.

The PhD trajectory commenced with foundational training in clinical drug development, neurophysiology, pharmacokinetics/pharmacodynamics (PK/PD), and research governance. Early in the project, emphasis was placed on acquiring broad methodological expertise, including not only TMS-EMG, TMS-EEG, and pharmaco-EEG, but also nerve and muscle stimulation methods (nerve excitability threshold tracking and muscle velocity recovery cycle) to assess drug effects on the peripheral nervous system. In addition, training was undertaken to develop a thorough understanding of the regulatory and ethical frameworks governing clinical research, including Good Clinical Practice (GCP). Introductory courses in population PK and PK/PD modeling and clinical development provided a basis for interpreting pharmacological exposure–response relationships and situating biomarker findings within the broader context of drug development.

Between 2021 and 2022, the candidate focused on establishing methodological competence and contributing to early proof-of-mechanism studies by conducting multiple clinical drug trials and implementing neurophysiological pharmacodynamic biomarkers. This period also included formal coursework in ‘medicamenteuze therapie’ (Boerhaave/LUMC) and training in clinical drug development, including pathways toward market approval (Paul Janssen Futurelab Leiden). Furthermore, the PhD candidate participated in international scientific meetings such as the International Congress of Clinical Neurophysiology (ICCN) and British Pharmacological Society (BPS), where preliminary findings were presented in poster format. These activities supported the development of scientific communication skills and facilitated engagement with the international neurophysiology and clinical pharmacology communities.

From 2022 onwards, the PhD project progressed toward hypothesis-driven experimental studies focusing on the evaluation of drug-induced modulation of cortical excitability using TMS. This phase resulted in multiple PhD project chapters addressing distinct pharmacological mechanisms and the characterization of both novel and established CNS-active medications developed for neurological and psychiatric disorders using the TMS biomarker framework. During this period, the candidate increasingly assumed responsibility for study design, data analysis, interpretation, and manuscript preparation, reflecting growing scientific independence.

In parallel, the candidate contributed to peer-review activities, including multiple review articles for the *British Journal of Clinical Pharmacology*, strengthening critical appraisal skills and familiarity with publication standards. Attendance and presentations at major international conferences (ECCN, Tremor Congress, MDS, EEC, EACPT, ACNP) supported dissemination of findings to both neuroscience and clinical pharmacology audiences and facilitated interdisciplinary exchange.

In the later phase of the PhD trajectory (2024–2025), the focus shifted toward integration and synthesis of findings across studies. This culminated in a general discussion chapter contextualizing cortical excitability biomarkers within early-phase CNS drug development. Additional training in supervision and modern drug development complemented the candidate’s role in mentoring and collaborative research settings. The candidate also contributed to further methodological development by implementing new protocols for nerve excitability threshold tracking and TMS-EMG input–output curves, which were subsequently implemented in new clinical trials following completion of the PhD.

Throughout the trajectory, the candidate maintained compliance with Good Clinical Practice through repeated WMO-GCP training and completed formal biostatistics training to support robust data analysis. Transferable skills training further supported professional development, including responsible research conduct and research integrity. In addition, the candidate participated in yearly training activities focused on neuronal excitability measures from 2022 onwards to remain up to date with methodological and conceptual developments in the field.

TABLE 1 CRediT table.
Schematic overview of the scientific contributions per thesis chapter.

CRediT table for the thesis of Catherine Pieters												
Ch.	Type*	Short Title	Conceptualization	Data Curation	Formal Analysis	Funding Acquisition	Investigation	Methodology	Project Administration	Resources	Software	Supervision
1	Introduction	Introduction TMS biomarkers										
2	PhD project chapter	Delayed effects esketamine										
3	PhD project chapter	Levetiracetam generalized epilepsy										
4	PhD project chapter	TRV045 novel ASM										
5	PhD project chapter	Differentiating GABAAR-PAMs										
6	Discussion	General discussion TMS biomarkers										

*PhD project chapters are the direct result of the PhD project of the PhD candidate.
Some theses also include Collaboration Chapters, to which the PhD candidate has contributed but fall outside the PhD project.

Research and biomedical training activities

2025	Aarhus Excitability course
2024	ACNP Conference: attendance and poster presentation
2024	EEC Conference: attendance and poster presentation, including Excitability course
2024	EACPT Conference: attendance and poster presentation
2024	Supervision Educational Course: Ontwikkelingstraject Moderne Geneesmiddelen
2024	Review article for British Journal of Clinical Pharmacology
2023	ECCN Conference: attendance and oral presentation, including Excitability course
2023	MDS Conference: attendance and oral presentation
2023	Review article for British Journal of Clinical Pharmacology
2023	International Tremor Congress: attendance, oral and poster presentation
2022	BPS Conference: attendance and poster presentation
2022	ICCN Conference: attendance and poster presentation, including Excitability course
2022	Aarhus Excitability workshop
2022	Medicamenteuze therapie (Boerhaave/LUMC)
2022	Paul Janssen Future Lab: Market Approval (online)
2021	Paul Janssen Future Lab: Clinical Development (online)
2021	Introduction to Population PK and PK/PD Modeling

Transferable skills training activities

2026	Resume and motivation letter training (Career Café)
2025	Introductory course
2025	Reasonable Research
2023	Basic Methods and Reasoning in Biostatistics
2022	Scientific Integrity
2020-'26	CHDR WMO-GCP Basic Training (retraining every 3 years)

CRediT authorship contributions

A detailed CRediT table is included to transparently describe the candidate’s contributions to each chapter in the scientific part of the thesis (Table 1). PhD project chapters represent work directly resulting from the PhD project, while introductory and discussion chapters reflect integrative and conceptual contributions.

CURRICULUM VITAE

Catherine M.E. de Cuba was born on 11 November 1990 in Oranjestad, Aruba. In 2009 she completed her pre-university education at Colegio Arubano in Oranjestad before moving to the Netherlands to pursue her lifelong ambition of studying Medicine at the University of Amsterdam. She obtained her Bachelor's degree in 2013 and her Master's degree in Medicine in 2016. Following her graduation, Catherine pursued clinical training in neurosurgery and neurology at Amsterdam University Medical Centers, Slotervaart Ziekenhuis in Amsterdam, and Noordwest Ziekenhuisgroep in Alkmaar. During this period, she developed a strong interest in neuroscience and neurophysiology and was involved in research on deep brain stimulation alongside her clinical training.

In 2020, she joined the Centre for Human Drug Research (CHDR) in Leiden as a Research Physician and Clinical Scientist and simultaneously started her training as a clinical pharmacologist at Leiden University. At CHDR, she became involved in early-phase clinical drug development, with a focus on neurophysiological biomarkers to assess pharmacodynamic effects of central nervous system-active compounds. This work formed the basis of her PhD research, conducted under the supervision of prof. dr. G.J. Groeneveld and dr. J.A.A.C. Heuberger.

During her PhD trajectory, Catherine developed expertise in a broad range of neurophysiological biomarkers, including transcranial magnetic stimulation (TMS), electroencephalography, and peripheral nerve and muscle excitability assessments. After the initial years of her PhD, her research increasingly focused on the further development of TMS-derived biomarkers to assess drug-induced modulation across compounds for neurological and psychiatric indications. This phase of the project was further supported by the appointment of dr. A.A. de Goede as co-thesis supervisor.

In 2024, Catherine was appointed Experienced Clinical Scientist, and in 2025 she was promoted to Senior Clinical Scientist. In this role, she contributes to the design and execution of early-phase clinical trials, supports methodological development, and mentors PhD students during their own doctoral trajectories.

Catherine has been together with her partner, Damiano Pieters, since 2014, and they welcomed their son, Matteo Pieters, in 2022 during her PhD trajectory. Outside of work, she enjoys traveling with her family, particularly their annual visits to the sunny island of Aruba.

LIST OF PUBLICATIONS

de Cuba, C. M. K. E., Albanese, A., Antonini, A., Cossu, G., Deuschl, G., Eleopra, R., Galati, A., Hoffmann, C. F. E., Knudsen, K., Landi, A., Lanotte, M. M. R., Marcante, A., Mosch, A., Pilleri, M., Reich, M. M., Ricchi, V., Rinaldo, S., Romito, L. M., Saba, F. S., Contarino, M. F. (2016). Idiopathic delayed-onset edema surrounding deep brain stimulation leads: Insights from a case series and systematic literature review. *Parkinsonism and Related Disorders*, 32. <https://doi.org/10.1016/j.parkreldis.2016.09.007>

de Cuba, C. M. K. E., de Goede, A. A., Klaassen, E. S., Otto, M. E., Doll, R. J., Kim, J., Demitrack, M. A., Chen, R., Groeneveld, G. J., & Heuberger, J. A. A. C. (2024). TMS and EEG pharmacodynamic effects of a selective sphingosine-1-phosphate subtype 1 receptor agonist on cortical excitability in healthy subjects. *Clinical Pharmacology and Therapeutics*, 117(3), 787–797. <https://doi.org/10.1002/cpt.3521>

Gorbenko, A. A., **de Cuba, C. M. K. E.**, de Goede, A. A., Post, T. E., Bohoslavsky, R., Strugala, P. K., Heuberger, J. A. A. C., & Groeneveld, G. J. (2025). Cannabidiol lacks direct effect on cortical excitability: a randomized, double blind, placebo controlled, 3-way crossover trial. *Clinical Pharmacology and Therapeutics*. <https://doi.org/10.1002/cpt.70038>

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Skov, M., Ruijs, T. Q., Grønnebak, T. S., Skals, M., Riisager, A., Winther, J. B., Dybdahl, K. L. T., Findsen, A., Morgen, J. J., Huus, N., Broch-Lips, M., Nielsen, O. B., **de Cuba, C. M. K. E.**, Heuberger, J. A. A. C., de Kam, M. L., Tannemaat, M., Verschuuren, J. J. G. M., Knutsen, L. J. S., Kelly, N. M., Pedersen, T. H. (2024). The CLC-1 chloride channel inhibitor NMD670 improves skeletal muscle function in rat models and patients with myasthenia gravis. *Science Translational Medicine*, 16(739). <https://doi.org/10.1126/scitranslmed.adk9109>

de Cuba, C. M. E., Ruijs, T. Q., de Goede, A. A., Doll, R. J., Juachon, M., van Putten, M. J. A. M., Groeneveld, G. J., & Heuberger, J. A. A. C. (2026). Transcranial magnetic stimulation as a translational biomarker in early-phase anti-seizure medication development: a randomized, double-blind, placebo-controlled study in generalized epilepsy. *Clinical Pharmacology and Therapeutics*. <https://doi.org/10.1002/cpt.70327>

de Cuba, C. M. K. E., de Goede, A. A., van Mechelen, J. C., Borghans, L. G. J. M., van der Aa, L., Olofsen, E., Juachon, M. J., Doll, R. J., Gillespie, A., Harmer, C. J., Jacobs, G. E., & Heuberger, J. A. A. C. (2026). Sustained pharmacodynamic effects of S-ketamine on cortical excitability and resting-state brain activity: A randomized, placebo-controlled trial. *British Journal of Clinical Pharmacology*. <https://doi.org/10.1002/bcp.70649>

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To my promotor, Prof. Dr. Geert Jan Groeneveld, thank you for your scientific vision and confidence in the potential value of excitability biomarkers, which have been invaluable throughout my PhD journey. I am truly grateful for the opportunities you provided and for your continued trust and support in shaping both this work and the future application of these biomarkers in early-phase drug development.

To my co-promotor, Dr. Jules Heuberger, thank you for your expertise, critical feedback, and guidance, which have greatly strengthened this thesis. I particularly appreciate the time you invested in my development and the open and intellectually stimulating scientific discussions that characterized our collaboration; your ability to combine scientific rigor with genuine personal engagement is something I will carry forward throughout my career.

To my co-promotor, Dr. Annika de Goede, thank you for your critical eye and commitment to scientific rigor, which have challenged me throughout this journey and undoubtedly made me a better scientist. After years of collaboration, I am thankful for your dedication to the TMS team and look forward to continuing our work together in the years ahead.

To my Aruban village, thank you for your warmth, love, and unwavering support. A special word of gratitude goes to my mother, Ria, whose sacrifices created opportunities for me that would otherwise not have been possible. To Super Oma, Lisette, Ben, Vera, Lynne, and Doayenne, despite the distance, you have always encouraged me to pursue my ambitions and accepted my life choices without hesitation.

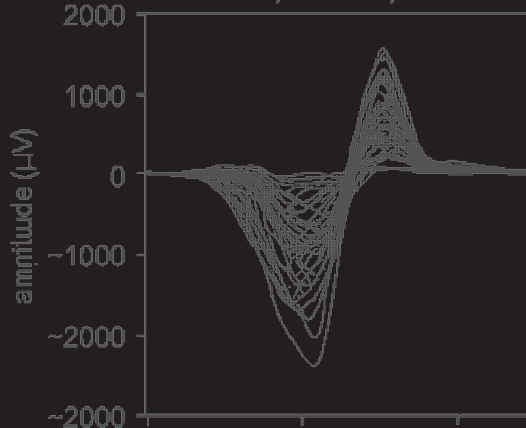
To my Dutch village, especially Erienne, my eldest sister, thank you for always being a role model to me. Although being the eldest sometimes might feel like a burden, you have always carried it with grace and style. Thank you for giving me my extended family: Jan, Benjamin, and Phillippe. To my Aruban friends who crossed the ocean with me in pursuit of our dreams, thank you for sticking together throughout the years. The strength

of our community has contributed to many of our accomplishments and successes. Together, we created a home away from home.

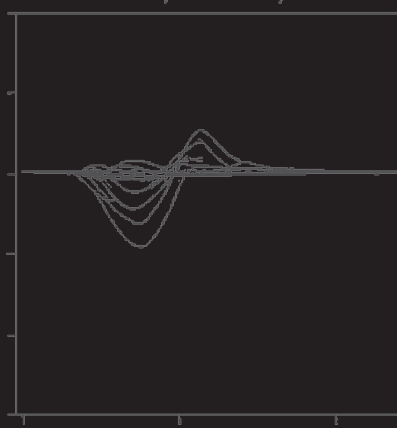
To my CHDR village, thank you for making this thesis possible through the combined efforts of many individuals across different departments. I am particularly grateful to the members of the TMS team for their expertise, dedication, and hard work. The collaborative environment within the team made it possible to execute technically challenging studies and continuously improve our methodologies. To Ingrid Koopmans, my confidant, who is always ready to help and provide a listening ear when needed, thank you for your friendship and support throughout the years. To Karen Broekhuizen, thank you for support that extended far beyond medical writing. To Marieke de Kam, thank you for always indulging my statistically driven questions and helping me whenever my statistical knowledge reached its limits. To all Neuroscience research directors, senior researchers, and PhD researchers, thank you for welcoming me as a senior researcher within the team. I am thankful for the trust you placed in me and look forward to continuing our collaboration and conducting many more exciting studies together. To my teammates, thank you for your enthusiasm, dedication, and for constantly keeping me on my toes. Your shared ambition to push the boundaries of translational neuroscience has made this journey both inspiring and rewarding. It has been a privilege to work alongside such a talented and driven group as we help pave the way for the development of new biomarkers that we hope will improve drug development and, ultimately, the lives of patients. To the clinical, recruitment, data management, statistics, and operational teams, thank you for your professionalism, flexibility, and commitment. Your contributions were essential to the successful execution of the studies presented in this thesis. To all study participants and patients, thank you. Your willingness to volunteer your time and effort made this research possible and contributed to advancing our understanding of cortical excitability and neurophysiological biomarkers.

This thesis marks the completion of an important chapter, and for each and every contribution, I am sincerely and forever grateful.

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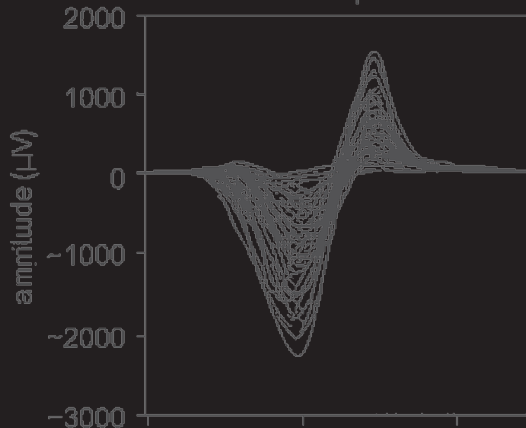
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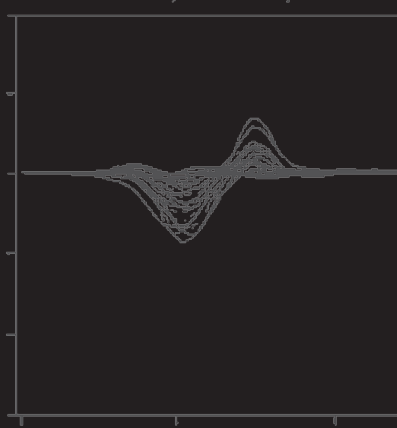
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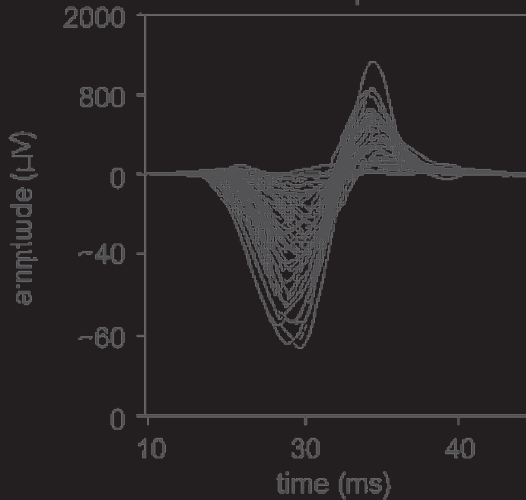
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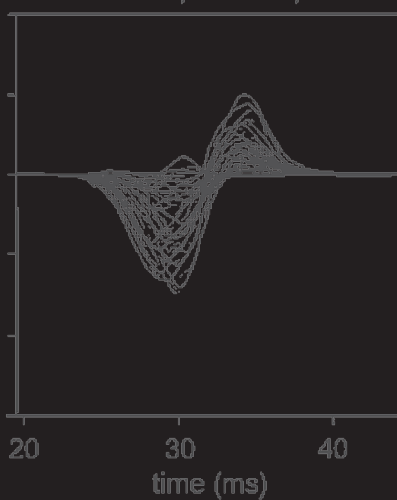
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