

Impaired Theta-Gamma Coupling as Biomarker for a Mouse Model of Dravet Syndrome

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Preface

This thesis titled 'Impaired Theta-Gamma Coupling as Biomarker for a Mouse Model of Dravet Syndrome' is submitted to obtain the Degree of Master of Science in Biomedical Engineering at the Delft University of Technology, the Netherlands. The research described was conducted under the supervision of Dr. Ir. A.C. Schouten in the Department of Biomechanical Engineering at the Faculty of Mechanical, Maritime and Materials Engineering at the Delft University of Technology and N.A. Jansen MSc in the Department of Human Genetics at the Leiden University Medical Center. This work is to best of my knowledge original, except where acknowledgments and references are made to previous work.

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List of abbreviations

AC	- Alternating current
AEDs	- Anti-epileptic drugs
aWk	- Active wakefulness
EEG	- Electroencephalograph
ECoG	- Electrocorticogram
KL	- Kullback-Leibler
MI	- Modulation-index
PAC	- Phase-amplitude coupling
REM	- Rapid-eye movement
SEM	- Standard error of the mean
SMEI	- Severe myoclonic epilepsy in infancy
SUDEP	- Sudden unexpected death in epilepsy
TG	- Theta-gamma
THG	- Theta-high gamma
V1rAC	- Alternative current of primary right visual cortex
WT	- Wild type

Impaired Theta-Gamma Coupling as Biomarker for a Mouse Model of Dravet Syndrome

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Abstract - Dravet Syndrome is a rare epileptic disorder, which is caused in more than 70% of all patients by a loss-of-function mutation in the SCN1A gene encoding sodium channel Na_v1.1. One of the main problems for treatment of patients with Dravet Syndrome is drug resistance and there are no biomarkers to monitor the efficacy of a particular treatment. The Dravet Syndrome phenotype can be recapitulated in mice by knockout of the Scn1a gene. Previous studies revealed a reduced sodium current in inhibitory GABAergic interneurons in a mouse model of Dravet Syndrome which leads to reduced excitability of inhibitory GABAergic inter-neurons. Inhibition of inhibitory GABAergic interneurons can lead to decreased phase-amplitude coupling between theta (5-10 Hz) and gamma (50-70 Hz) or theta and high gamma (100-140 Hz) oscillations in the brain. To investigate whether impaired phase-amplitude coupling could be a potential biomarker to monitor the efficacy of treatment for Dravet Syndrome, we recorded electrocorticogram signals from the primary visual cortex in two mice models of Dravet Syndrome and wild type mice. We found a decrease in theta-high gamma coupling for mice with Dravet Syndrome in the early epileptic stage and a decrease in theta-gamma and theta-high gamma coupling in mice with Dravet Syndrome in which seizure onset had already started, during rapid-eye-movement sleep. Reduced excitability of inhibitory GABAergic interneurons can also cause hyper-excitability that leads to seizures. These results suggest that phase amplitude coupling could be a potential biomarker to monitor the efficacy of treatment for Dravet Syndrome. Efficient treatment decreases the number of seizures and therefore we expect to see an increase in theta-gamma and theta-high gamma coupling.

I. INTRODUCTION

Dravet Syndrome, also known as severe myoclonic epilepsy in infancy (SMEI), is a rare severe epileptic disorder [1,2]. The first manifestations of the disorder are febrile seizures within the first year of life. These febrile seizures evolve quickly into spontaneous seizures that are often pharmacoresistant [1,3]. After seizure onset multiple comorbidities develop, including sleep disturbance, ataxia and cognitive and social impairments [4,5]. In most

patients, these symptoms stabilize after five years with a decrease in number of seizures and improvement of mental development. Nevertheless, there are exceptions in which the symptoms worsen. Approximately 15% of the patients with Dravet Syndrome die early due to accidents, drowning, severe status, infections or sudden unexpected death (SUDEP) [3].

Dravet Syndrome is only clinically diagnosed with a screening test when the first symptoms are already present [1]. In more than 70% of all patients with Dravet Syndrome a loss-of-function mutation is found in the SCN1A gene, which encodes the pore-forming α -subunit of sodium channel Na_v1.1 [6,7]. Therefore, the most important symptoms for diagnosis are the repeated occurrence of prolonged febrile seizures in infants younger than 1 year, other types of seizures, photosensitivity, delay in development and the presence of a SCN1A gene mutation. The screening test consists in total of eight items which are examined on presence or absence in the patient. To each item in the screening test a fixed score of one, two or three points is granted. When an item is present in the patient, the patient gets the full score for this item and when a patient has a total clinical score of 6 points or above out of a maximum of 16 points, this indicates high risk of Dravet Syndrome [1,3].

There are three principles essential in the treatment of patients with Dravet Syndrome [3]. The first is to avoid classic anti-epileptic drugs (AEDs) that target the Na_v1.1 sodium channel, because of the risk of aggravating the condition of the patient. One of the main features of Dravet Syndrome is drug resistance. However, due to pharmacological progress, there are some new AEDs that are promising for the treatment of Dravet Syndrome [1]. The second is to apply drugs against infections and temperature variations and the third is to stop convulsive seizures before they evolve into status epilepticus [3]. A seizure is classified as status epilepticus when there is more than 30 minutes of continues seizure activity [8]. Being able to monitor the efficacy of a particular treatment in a patient would be helpful to start with appropriate treatment at young age.

The Dravet Syndrome phenotype can be recapitulated in mice. Just as in humans, the Scn1a gene encodes the pore-forming α -subunit of sodium channel Na_v1.1 in mice [9]. A mouse model of Dravet Syndrome can be generated by global heterozygous knockout of the Scn1a gene. This mouse model develops several features of Dravet Syndrome. The mice have epilepsy with early onset, are susceptible to heat-induced seizures, get spontaneous

seizures with age-dependent severity, have premature deaths and have the same comorbidities [10,11]. Another method to generate a mouse model is by local knockout of the *Scn1a* gene. Local knockout can be used to investigate the role of specific brain areas [12]. Mice models are used because mice allow for specific measurement which cannot be obtained technically and ethically in patients and which will give valuable insight into Dravet Syndrome [13].

There are already some studies that have investigated the network dynamics of the brain of mice with Dravet Syndrome. There are a couple of frequency bands distinguishable in neuronal brain oscillations, including delta (1-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-30 Hz), gamma (30-80 Hz) and high gamma (> 80 Hz) [14]. The most important findings were that most studies did not find changes in the power spectra of brain rhythms over a large frequency range between wild type mice and mice with Dravet Syndrome, independent of the depth of the recordings [15-17]. However one study found an increase of theta power and a decrease of gamma power during active wakefulness [18] and another study found a decrease in theta power during rapid-eye movement (REM) sleep [19]. Nevertheless, all studies reported an unaltered sodium current in the excitatory pyramidal neurons and a reduced sodium current in the inhibitory GABAergic interneurons, which leads to reduced excitability of the inhibitory GABAergic interneurons [17,19,20].

Different types of neurons are mainly responsible for the generation of different oscillatory rhythms and the interaction between oscillatory rhythms [14]. Brain oscillations of different frequencies can interact with each other. In particular, the phase of a low-frequency rhythm can modulate the amplitude of a high-frequency rhythm, which is called phase-amplitude cross-frequency coupling (PAC) [14]. PAC gives additional information about the network dynamics of the brain up to the power spectrum [21]. The network of hippocampal inhibitory GABAergic interneurons is important for the interaction between theta and gamma oscillations. Inhibition of inhibitory GABAergic interneurons leads to altered theta-gamma PAC [22]. Furthermore, vigilance state plays an important role in the interaction between theta and gamma oscillations. Especially during REM sleep and active wakefulness there is a strong interaction between theta and gamma oscillations [15,23,24]. Therefore, there might be an altered theta-gamma PAC during REM sleep and active wakefulness for Dravet Syndrome.

In this study we investigated if theta-gamma PAC is a potential biomarker to monitor the efficacy of treatment for Dravet Syndrome. We explored PAC, using the Modulation Index (MI) metric [25], from EEG signals in two mouse models of Dravet Syndrome and wild type mice during seizure free periods for different vigilance states. We found a decrease in theta-gamma and theta-high gamma PAC during REM sleep in mice with Dravet Syndrome compared to wild type mice.

II. METHODS

Animals

Data was collected from two different mouse models of Dravet Syndrome and wild type mice (no mutation). In both mouse models the gene encoding the sodium channel type 1 α -subunit ($Na_v1.1$) was removed. The first model consisted of heterozygous *Scn1a*^{+/-} mice. This model was a global knock-out, in which there was a loss-of-function mutation in the *Scn1a* gene from birth onwards. The second model consisted of homozygous *Scn1a*^{fl/fl} mice and heterozygous *Scn1a*^{fl/+} mice. In this model a localized knock-out of *Scn1a* was induced by a viral infection, limited to a specific brain area. The virus was injected into the hippocampus after birth. For the first model wild type mice were used as control and for the second model wild type mice which got a placebo injection in the hippocampus were used as control. Both males and females were used in the age between 1 and 3 months old (see appendix A for a more detailed overview of all mice).

All mice were kept under standard housing conditions in a shielded Faraday cage with a 12-hour light/dark cycle (lights on from 6 a.m. to 6 p.m.). Mice were allowed unlimited access to food and water. The protocol for the experiments was approved by the 'Leiden University Medical Centre Animal Experiments Committee' (DEC 2014-13193), in line with the directives of the European Communities Council Directive (2010/63/EU) and the ARRIVE guidelines. Animal harm and discomfort was avoided at all costs.

Surgery for data recording

All surgical procedures were performed under initial 4% isoflurane anesthesia, followed by 1.5% isoflurane to maintain anesthesia in oxygen-enriched air. During surgery mice were placed in a stereotactic device. The skin of the skull got removed and a small hole was drilled into the skull using a dentist's bore (Fine Science Tools Inc; Foster City, USA). For electrocorticogram (ECoG) recordings custom made silver ball-tip electrodes (Ag) were implanted in the right primary visual cortex (V1rAC) (mm to bregma: -3.5 anterior/-2.0 lateral/-0.5 ventral) and left primary visual cortex (mm to bregma: -3.5 anterior/+2.0 lateral/0.0 ventral) and local field potential electrodes (single 75 μ m platinum/iridium, PT6718; Advent Research Materials, Oxford, UK) were bilaterally implanted in the hippocampus (mm to bregma: -3.0 anterior/-3.0 lateral/-0.0 ventral). Furthermore, two ball-tip electrodes were bilaterally inserted above the cerebellum as reference (Ag) and ground (Ag/AgCl). For electrocardiogram recordings a local field potential electrode was placed subcutaneously over the left latissimus dorsi muscle and tunneled to the skull. A seven-channel pedestal connected all electrodes (E363/0 socket contacts and MS373 pedestal; Plastic One, Roanoke, USA) and was glued to the skull using dental cement (DiaDent Europe, Almere, The Netherlands). Carprofen (5 mg/kg, s.c.) and buprenorphine (0.1 mg/kg, s.c.) were administered to the mice for postoperative pain relief.

Data acquisition

Data acquisition was performed as described previously by Houben et al. [26] and Loonen et al. [27]. Summarizing, directly after surgery, animals were connected to custom-built recording hardware. ECoG signals were pre-amplified 3× and fed into an amplifier (0.05-500 Hz bandpass filter; 800× gain). Thereafter, ECoG signals were digitized (Power 1401 and Spike2 software; CED, Cambridge, UK) at a sampling rate of 5000 Hz. Locomotor activity was recorded using a custom-built passive infrared motion detection sensor, and drinking activity was recorded with a custom-built beam-break sensor in front of the drinking bottle. Video recordings were made using a CCD camera (acA 1300-60gmNIR, 30 frames/s, Basler; Luebeck, Germany). Global Dravet mice were recorded for 7 consecutive days after surgery and local Dravet mice for 14 consecutive days after surgery.

Histology

After completion of all recordings, animals were euthanized by CO₂ and transcardially perfused [0.1 M phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer]. After transcardial perfusion, brains were post-fixed in 4% paraformaldehyde for 2.5 hours at room temperature and sucrose-processed. Coronal brain sections (40 µm) were made using a sliding microtome (Leica; Wetzlar, Germany) and Nissl-stained to confirm electrode positions.

Data analysis

Data analysis was performed in three steps. Spike2 (Spike2 8.0; CED, Cambridge, UK) was used for determination of vigilance state. Scored Spike2 files were imported to MATLAB (Matlab 2019a, The Mathworks Inc.; Natick, USA). MATLAB was used for pre-processing of the data, for spectral analysis, for the calculation of the MI and all other calculations. In all calculations 5-10 Hz was taken as theta frequency range, 50-70 Hz as gamma frequency range and 100-140 Hz as high gamma frequency range. SPSS (SPSS 25.0, IBM SPSS; Armonk, USA) was used for all statistical analysis.

Vigilance state determination

All raw data recordings were converted to Spike2 for determination of vigilance state. In general, the alternating current (AC) potential of the V1rAC electrode, the reference signal and the passive infrared sensor were used for determination of vigilance state. Three virtual channels were deducted from the V1rAC. The first consisted of the ratio of theta power over delta power, the second consisted of only the power of theta and the third consisted of only the power of gamma. High level of gamma power was used to indicate wakefulness. When wakefulness was established, high level of theta power and activation of the passive infrared sensor were used to determine active wakefulness. Like in active wakefulness, a high level of theta power is present during REM sleep. No activation of the passive infrared sensor and a ratio over 2.5 for theta

power over delta power were used to separate REM sleep from active wakefulness. When there was doubt about the vigilance state, video footage was used as control mechanism.

For the global Dravet mice and corresponding wild type mice a data recording of 6 hours on day 7 after surgery was scored and for the local Dravet mice and corresponding wild type mice a data recording on day 2, 7, and 14 after surgery was scored. Vigilance state was scored manually in epochs of 5 seconds. At least 120 clear epochs (10 minutes) of active wakefulness and REM sleep were scored in each recording and exactly 120 random chosen epochs were used for all calculations.

Pre-processing

Scored Spike2 files were imported to MATLAB. In MATLAB an anti-aliasing filter was applied to all ECoG signals and then signals were down-sampled from 5000 Hz to 1000 Hz. Subsequently, the down-sampled signals were high-pass filtered with a 4th order Butterworth filter with a cut-off frequency of 1 Hz. Epochs with artefacts got removed from the data by using an iterative implementation of Grubbs Test ([28], see appendix B) with a significance level of $\alpha=0.05$.

Spectral analysis

Power spectra were computed using Welch's averaging method, using epochs of 5 seconds with 50% overlap. A Hamming window was applied on each epoch. The fast Fourier transform of the epochs was calculated and then the resulting power spectra were averaged. To normalize the power spectra, the power in each frequency was divided by the total power in the range of 1-200 Hz. Band power was defined as the average power in the specified frequency band.

Modulation Index

Phase-amplitude coupling was measured using the MI metric. The MI was calculated similar as previously described by Tort et al. [25]. In short, The ECoG signals were bandpass-filtered at the frequency ranges of interest. The Hilbert transform was applied to extract phase and amplitude information. Afterwards, phase of the low frequency was divided in 18 bins of 20° and mean amplitude of the high frequency was calculated inside each phase bin. A phase-amplitude distribution was achieved by normalization of mean amplitude in each phase bin by the sum over the bins. There is no coupling when the mean amplitude is uniformly distributed over the phase bins. The further away the phase-amplitude distribution is from the uniform distribution, the higher the coupling. The Kullback-Leibler (KL) distance was used to compare the phase-amplitude distribution with the uniform distribution [29]. The MI was obtained by normalization of the KL distance by dividing with a constant factor ($\log(18)$, the logarithm of the number of phase bins), to achieve values between 0 and 1 (see appendix C for more details).

The comodulogram maps were obtained by expressing the MI in a pseudocolor code scheme for multiple frequency pairs. For the phase 12 frequency bands were

used in the range of 3-14 Hz (1 Hz step, 2 Hz bandwidth) and for amplitude 81 frequency bands were used in the range of 40-200 Hz (2 Hz step, 4 Hz bandwidth).

We first generated typical examples of the comodulogram maps from recordings of the hippocampus for global Dravet mice and wild type mice, since the hippocampus plays a key factor in the pathophysiology of Dravet Syndrome [12,22,30]. Local knockout of *Scn1a* in the hippocampus can presumably induce the global Dravet Syndrome phenotype [12]. Therefore, we did expect the biggest effect of impaired PAC in the hippocampus. We did not perform a group comparison for the hippocampal recordings, because electrodes could not be placed with enough precision. Electrode placement comes very precise within the hippocampus because the hippocampus consist of multiple layers with different characteristics for PAC [22]. Furthermore, due to the location of the hippocampus deep in the brain, it is methodologically challenging to record non-invasive EEG signals from the hippocampus of good quality without influences of other brain areas [31]. A potential biomarker to monitor treatment efficacy of Dravet Syndrome would be far more valuable when it could be measured non-invasive. Taken together, we decided not to go deeper into the PAC of the hippocampus and only show if there was an effect visible in the hippocampus.

Second, we generated the comodulogram maps from recordings of the primary visual cortex for global Dravet mice and wild type mice to investigate whether Dravet Syndrome influences the primary visual cortex. The primary visual cortex is located more superficial in the brain which makes it easier to record non-invasive EEG

signals from the primary visual cortex [32]. Last, we generated the comodulogram maps from recordings of day 2, 7 and 14 after surgery of the primary visual cortex for local Dravet mice and wild type mice to investigate the effect of Dravet Syndrome on PAC over time.

Gamma bursts and theta phase of maximal gamma amplitude

The calculation of PAC seems very straightforward. However, it is not necessary that all signatures of PAC are caused by physiological processes [33]. For assessment of PAC it is important that random fluctuations in gamma amplitude are averaged out. This can be done by using long enough epochs. It is not possible to assess PAC in a single theta cycle [25]. A decrease in PAC could be due to lower occurrence of theta cycles with nested gamma or due to lower variations of gamma amplitude within theta cycles. We investigated the amount of ‘gamma bursts’ for wild type mice and global Dravet mice during REM sleep and active wakefulness in the primary visual cortex to answer this question. A gamma burst was specified as: a crossing of the instantaneous gamma amplitude of a threshold 2 standard deviations above the mean gamma amplitude [34]. Furthermore we calculated for which theta phase the amplitude of gamma was maximal in wild type mice and global Dravet mice.

Statistical analysis

Unless indicated otherwise, data were reported as mean $\pm$ standard error of the mean (SEM). Group comparisons were performed with the unpaired t-test for normally distributed data and the Mann-Whitney U-test

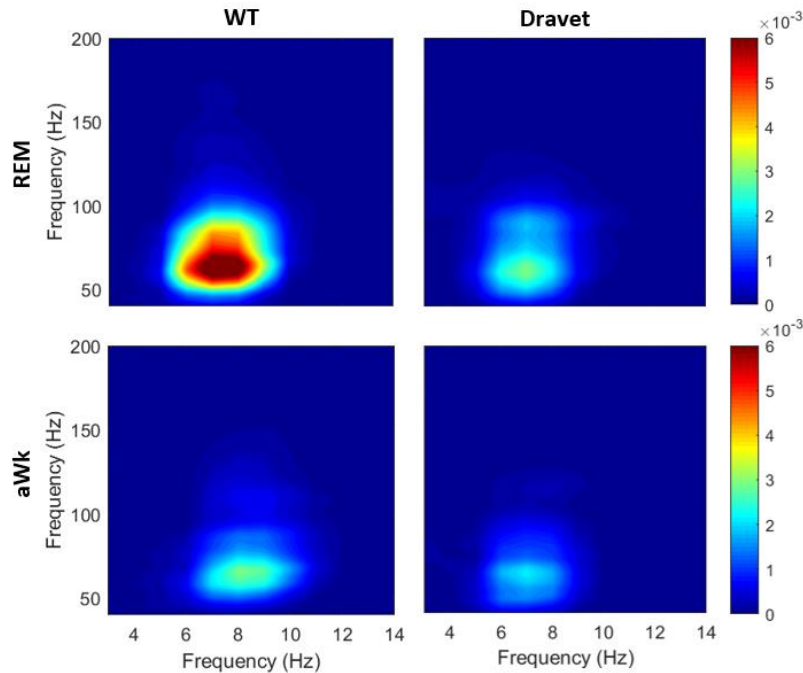


Figure 1: Theta-gamma coupling from recordings of the hippocampus. Typical example of a comodulation map of a wild type mouse (WT, left plots) and global Dravet mouse (right plots) during REM sleep (top plots) and active wakefulness (aWk, bottom plots). Pseudocolor scale indicates the MI. Based on visual inspection there is a decrease in MI for the global Dravet mouse compared to the wild type mouse.

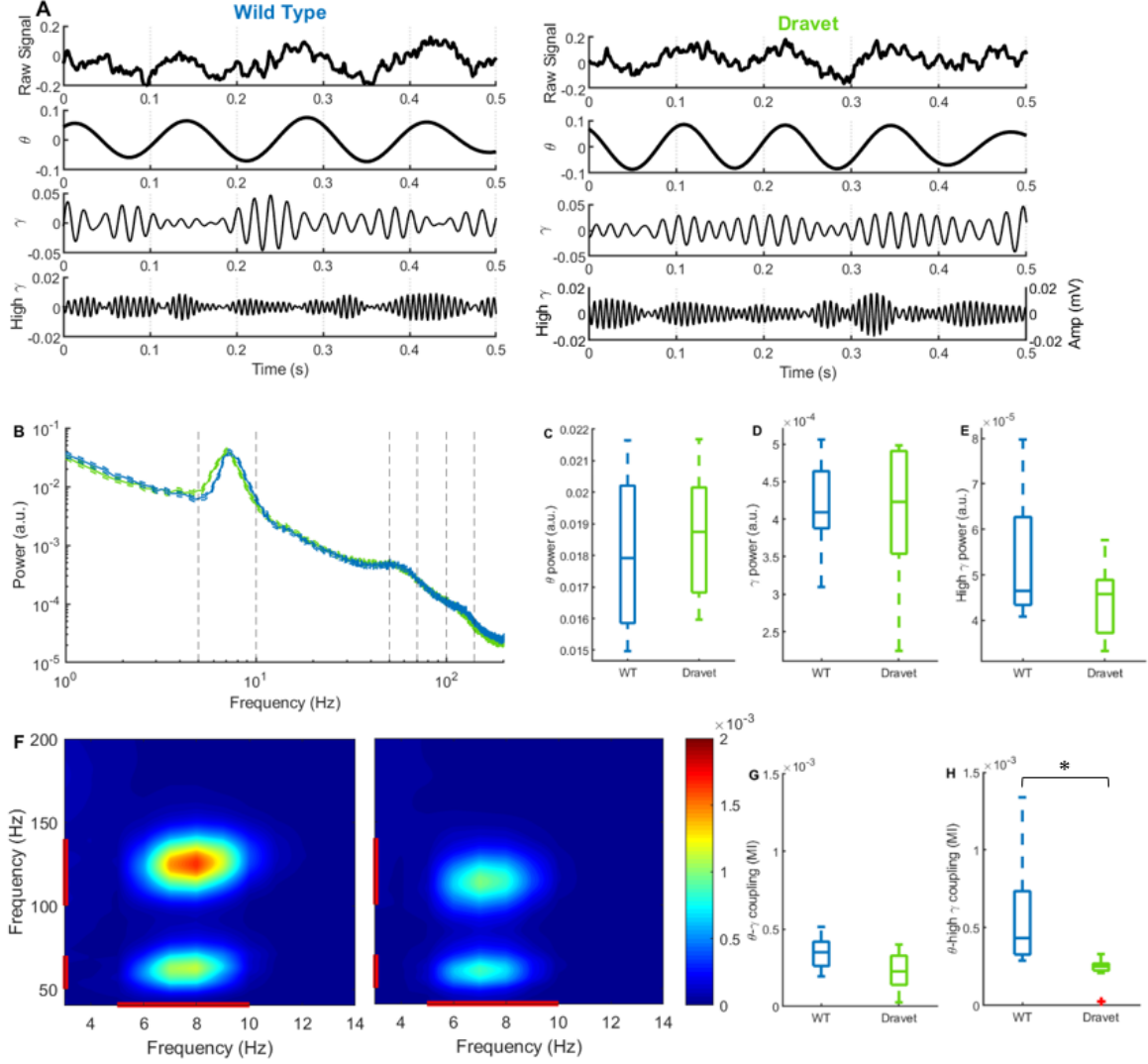


Figure 2: Decreased theta-high gamma coupling in global Dravet mice during REM sleep in recordings from the primary visual cortex. (A) Representative example of raw and filtered (theta, gamma and high gamma bandpass) recordings from the right primary visual cortex during REM sleep of a wild type mouse (n=1) and a global Dravet mouse (n=1). (B) Mean ($\pm$ SEM) power spectra from 1 to 200 Hz of wild type (blue, n=8) and global Dravet mice (green, n=9). The vertical dashed lines indicate the theta, gamma and high gamma frequency range. (C-E) Boxplot distribution of mean theta (C), gamma (D), and high gamma (E) band power. There is no significant difference between wild type mice and global Dravet mice. (F) Mean comodulation map of wild type (left) and global Dravet mice (right). The horizontal red line indicates the theta frequency range and the vertical red lines indicate the gamma and high gamma frequency ranges. Pseudocolor scale indicates the MI. (G) Boxplot distribution of mean theta-gamma coupling in wild type and global Dravet mice. Coupling strength is not significantly decreased in global Dravet mice ($p=0.06$). (H) Boxplot distribution of mean theta-high gamma coupling in wild type and global Dravet mice. The red dot indicates an outlier. Coupling strength is significantly decreased in global Dravet mice ($*p<0.05$).

for non-normally distributed data. Within group comparisons were performed with a related-samples Wilcoxon Signed Rank Test. All tests were performed two-tailed and significance levels were indicated by one or two asterisks ($*p<0.05$, $**p<0.01$). Significant coupling between theta and (high)-gamma has previously been demonstrated [25,34,35], therefore we focused on comparing coupling levels between Dravet Syndrome and wildtype mice. We took 4.3×10^{-5} as significance threshold because this was previously shown as reliable threshold value based on surrogate control analyses [25].

III. RESULTS

PAC in the hippocampus

We generated comodulation maps from hippocampal ECoG signals during REM sleep and active wakefulness for global Dravet mice and wild type mice to investigate the effect of Dravet Syndrome on hippocampal PAC. Figure 1 shows a typical example of the comodulation map for a wild type mouse (n=1) and a global Dravet mouse (n=1) for REM sleep and active wakefulness. The levels of theta-gamma coupling are above the established

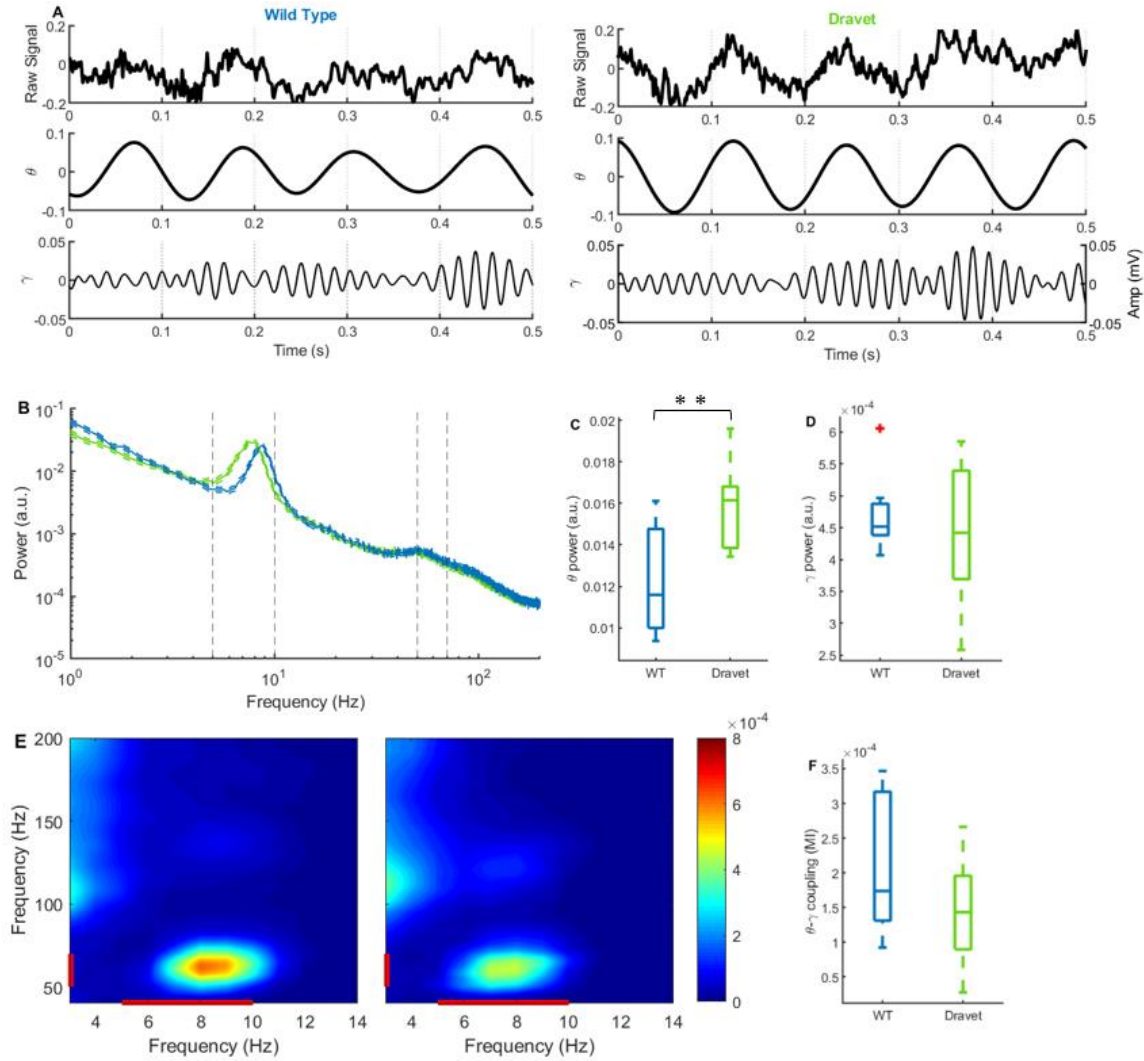


Figure 3: Theta-gamma coupling during active wakefulness in recordings from the primary visual cortex. (A) Representative example of raw and filtered (theta and gamma bandpass) recordings from the right primary visual cortex during active wakefulness of a wild type mouse (n=1) and global Dravet mouse (n=1). (B) Mean ($\pm$ SEM) power spectra from 1 to 200 Hz of wild type (blue, n=8) and global Dravet mice (green, n=9). The vertical dashed lines indicate the theta and gamma frequency range. (C) Boxplot distribution of mean theta band power. Global Dravet mice have significant increased theta power (**<p<0.01). (D) Boxplot distribution of mean gamma band power. The red dot indicates an outlier. There is no significant difference between wild type mice and global Dravet mice. (E) Mean comodulation map of wild type mice (left) and global Dravet mice (right). The horizontal red line indicates the theta frequency range and the vertical red line indicates the gamma frequency range. Pseudocolor scale indicates the MI. (F) Boxplot distribution of mean theta-gamma coupling in wild type and global Dravet mice. Coupling strength is not significantly decreased in global Dravet mice (p=0.08).

significance threshold of 4.3×10^{-5} in both mice for both states. For REM sleep the level of theta-gamma coupling is higher than for active wakefulness, which is in accordance with previous studies [15,34] and there is a decrease in the level of theta-gamma coupling for global Dravet mice compared to wild type mice based on visual inspection.

Impaired PAC in recordings from the primary visual cortex of global Dravet mice

To investigate whether Dravet Syndrome influences the primary visual cortex we calculated the absolute (see appendix D) and normalized power spectra and generated the comodulation maps from ECoG signals of the right

primary visual cortex in wild type mice (n=8) and global Dravet mice (n=9) during REM sleep and active wakefulness.

During REM sleep there were clear theta, gamma and high gamma oscillations observed for both groups (fig. 2a). The power levels were similar for both groups and theta, gamma and high gamma peak frequency were unaltered (fig. 2b-e). Wild type mice had high levels of theta-gamma and theta-high gamma coupling. The level of PAC in the primary visual cortex was lower than in the hippocampus based on visual inspection, but still abundantly above the significance threshold. Despite the normal power levels, the levels of coupling were decreased for global Dravet mice (fig. 2f-h). An unpaired t-test

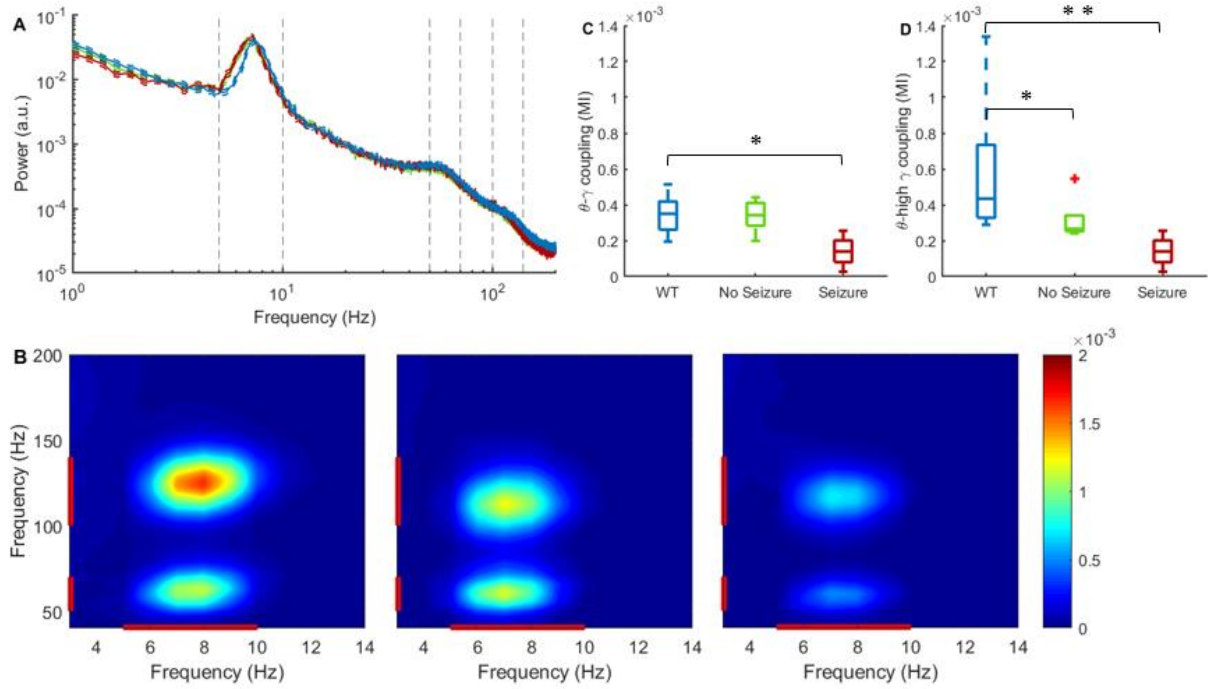


Figure 4: Theta-gamma coupling in the primary visual cortex during REM sleep for global Dravet mice which have and have not perceived seizures yet. (A) Mean ($\pm$ SEM) power spectra from 1 to 200 Hz of wild type mice (blue, $n=8$), global Dravet mice without seizures (green, $n=5$) and global Dravet mice with seizures (red, $n=4$). The vertical dashed lines indicate the theta, gamma and high gamma frequency ranges. There is no significant difference in power for all frequency ranges between wild type mice and global Dravet mice with and without seizures (boxplot not shown). (B) Mean comodulation map of wild type mice (left), global Dravet mice without seizures (middle) and global Dravet mice with seizures. The horizontal red line indicates the theta frequency range and the vertical red lines indicate the gamma and high gamma frequency ranges. Pseudocolor scale indicates the MI. (C) Boxplot distribution of mean theta-gamma coupling in wild type mice and global Dravet mice with and without seizures. There is a significant decrease in coupling strength for global Dravet mice with seizures compared to wild type mice ($*p<0.05$). (D) Boxplot distribution of mean theta-high gamma coupling in wild type mice and global Dravet mice with and without seizures. The red dot indicates an outlier. There is a significant decrease in coupling strength for global Dravet mice with seizures compared to wild type mice ($**p<0.01$) and a significant decrease in coupling strength for global Dravet mice without seizures compared to wild type mice ($*p<0.05$).

showed a significant decrease for theta-high gamma coupling ($p<0.05$) and almost significant decrease for theta-gamma coupling ($p=0.06$).

During active wakefulness there were also clear theta and gamma oscillations observed for both groups (fig. 3a). For global Dravet mice the mean band power of theta was significant increased compared to wild type mice ($p<0.01$, fig. 3b,c). This might be due to the slightly altered peak frequency of theta for global Dravet mice (fig. 3b). The mean band power of gamma was similar for both groups (fig. 3d). Just as in the hippocampus, the level of PAC was lower during active wakefulness than during REM sleep in the primary visual cortex. Nevertheless, the levels of theta-gamma coupling were above the established significance threshold for both wild type mice and global Dravet mice. The level of theta-gamma coupling in global Dravet mice looked decreased compared to wild type mice by visual inspection (fig. 3e). However, the level of theta-gamma coupling was not significant decreased in global Dravet mice compared to wild type mice ($p=0.08$). We did not find theta-high gamma coupling during active wakefulness, which is in accordance with previous studies [15,34].

Influence of seizures on PAC in the primary visual cortex

Until now we have taken all global Dravet mice as one group. We can separate the global Dravet mice in a group in which seizure onset has already started and a group in which seizure onset has not started yet. To investigate whether seizures have influence on the level of PAC we calculated the absolute (see appendix D) and normalized power spectra and generated the comodulation maps for wild type mice ($n=8$), global Dravet mice in the early epileptic stage (no seizures, $n=5$) and global dravet mice with seizures ($n=4$) during REM sleep and active wakefulness for recordings of the primary visual cortex.

The separation between mice with seizures and without seizures did not have any effect on the power spectra for REM sleep. There was no significant difference in mean band power or peak frequency for theta, gamma and high gamma (fig. 4a). As showed before, wild type mice showed high levels of theta-gamma and theta-high gamma coupling. Global Dravet mice without seizures showed only a decrease in theta-high gamma coupling and global Dravet mice with seizures showed a decrease in both theta-gamma and theta-high gamma coupling (fig. 4b). The

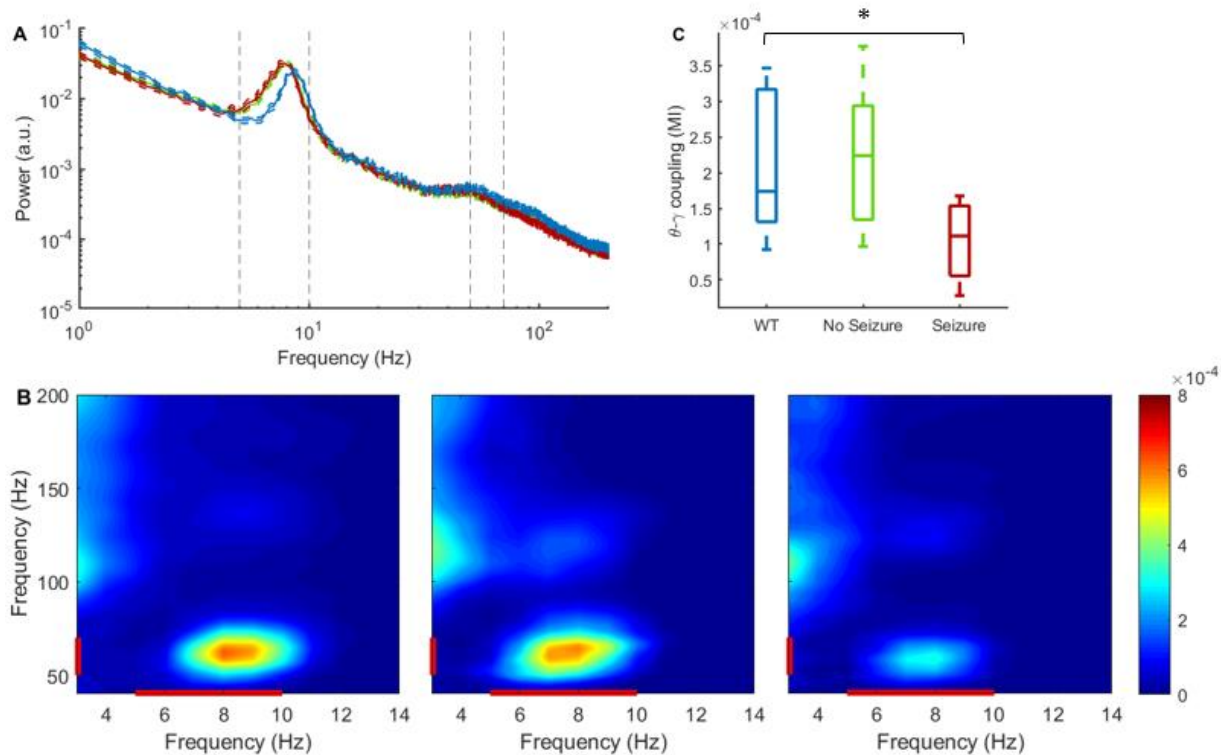


Figure 5: Theta-gamma coupling in the primary visual cortex during active wakefulness for global Dravet mice which have and have not perceived seizures yet. (A) Mean ($\pm$ SEM) power spectra from 1 to 200 Hz of wild type mice (blue, $n=8$), global Dravet mice without seizures (green, $n=5$) and global Dravet mice with seizures (red, $n=4$). The vertical dashed lines indicate the theta and gamma frequency ranges. There is a significant increase in mean theta band power for both global Dravet mice without seizures and global Dravet mice with seizures compared to wild type mice ($*p<0.05$). There are no differences in mean gamma band power (boxplot not shown, see fig. 3c). (B) Mean comodulation map of wild type mice (left), global Dravet mice without seizures (middle) and global Dravet mice with seizures. The horizontal red line indicates the theta frequency range and the vertical red line indicates the gamma frequency range. Pseudocolor scale indicates the MI. (C) Boxplot distribution of mean theta-gamma coupling in wild type mice and global Dravet mice with and without seizures. There is a significant decrease in coupling strength for global Dravet mice with seizures compared to wild type mice ($*p<0.05$).

decrease in theta-high gamma coupling was significant for global Dravet mice without seizures ($p<0.05$) and global Dravet mice with seizures ($p<0.01$) compared to wild type mice following a Mann-Whitney U test (fig. 4c). The decrease in theta-gamma coupling was also significant for global Dravet mice with seizures ($p<0.05$) compared to wild type mice following a Mann-Whitney U test (fig. 4d).

The separation between mice with seizures and without seizures did also not have effect on the power spectra for active wakefulness. There was a significant increase in mean theta power for both global Dravet mice with and without seizures compared to wild type mice ($p<0.05$). There was no difference in mean theta power between global Dravet mice with and without seizures and there was also no difference in mean gamma power between all groups (fig 5a, boxplot not shown, see fig. 3c). Global Dravet mice without seizures did not show a decrease in theta-gamma coupling compared to wild type mice. However, global Dravet mice with seizures did show a significant decrease theta-gamma coupling compared to wild type mice following a Mann-Whitney U test ($p<0.05$, fig. 5b,c).

Dravet Syndrome does not affect the occurrence of gamma burst nor the theta phase of maximal gamma amplitude in the primary visual cortex

The decrease in theta-(high) gamma coupling for global Dravet mice could be due to lower occurrence of theta cycles with nested gamma or due to lower variations of gamma amplitude within theta cycles. We investigated the amount of gamma bursts for wild type mice ($n=8$) and global Dravet mice ($n=9$) during REM sleep and active wakefulness in the primary visual cortex to answer this question. There was no difference in the mean amount of gamma bursts during REM sleep (wild type mice: 3.11 ± 0.10 Hz; global Dravet mice: 3.15 ± 0.09 Hz) and active wakefulness (wild type mice: 3.21 ± 0.09 Hz; global Dravet mice: 3.16 ± 0.11 Hz) for wild type mice and global Dravet mice. There was also no difference in the theta phase of maximal (high) gamma amplitude between wild type mice and global Dravet mice during REM sleep and active wakefulness in the primary visual cortex (fig. 6). This indicates that Dravet Syndrome does not influence the amount of gamma bursts nor the theta phase where these gamma bursts occur.

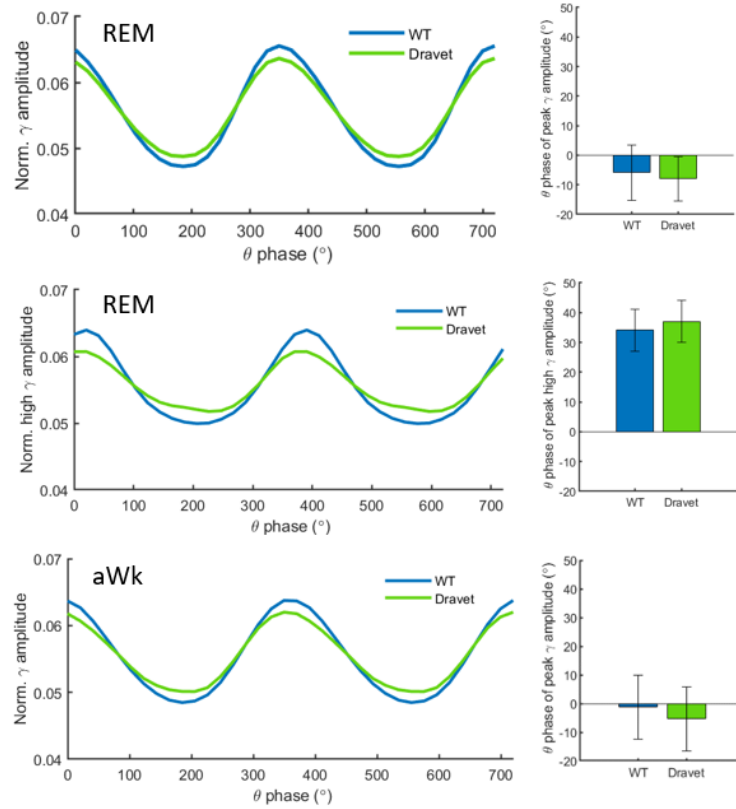


Figure 6: Dravet Syndrome does not affect the theta phase of maximal (high) gamma amplitude. The line plots show the normalized mean (high) gamma amplitude as a function of theta phase for wild type mice (blue, $n=8$) and global Dravet mice (green, $n=9$) from recordings of the primary visual cortex during REM sleep and active wakefulness. The bar graphs show the mean theta phase of maximal (high) gamma amplitude. The error bars denote $\pm$ standard deviation. Notice the reduced variations in normalized (high) gamma amplitude within theta cycles for global Dravet mice. The theta phase of maximal (high) gamma amplitude is not different for wild type mice and global Dravet mice.

Table 1: Mean ($\pm$ SEM) PAC in the primary visual cortex during REM sleep for wild type mice and local Dravet mice on day 2, 7 and 14 after surgery.

	TG* 2 ($\times 10^{-3}$)	THG** 2 ($\times 10^{-3}$)	TG 7 ($\times 10^{-3}$)	THG 7 ($\times 10^{-3}$)	TG 14 ($\times 10^{-3}$)	THG 14 ($\times 10^{-3}$)
Wild type	0.38 $\pm$ 0.13	0.48 $\pm$ 0.15	0.31 $\pm$ 0.11	0.49 $\pm$ 0.17	0.26 $\pm$ 0.05	0.51 $\pm$ 0.08
Local Dravet	0.46 $\pm$ 0.08	0.90 $\pm$ 0.24	0.12 $\pm$ 0.02	0.43 $\pm$ 0.13	0.06 $\pm$ 0.01	0.30 $\pm$ 0.06

*TG = theta-gamma, **THG = theta-high gamma

Changes in PAC over time for local Dravet mice in the primary visual cortex

To investigate whether PAC changes over time we generated the comodulation maps of wild type mice ($n=4$) and local Dravet mice ($n=4$) from ECoG signals of the primary visual cortex on day 2, 7 and 14 after surgery during REM sleep (fig. 7). Table 1 gives the mean PAC for theta-gamma coupling and theta-high gamma coupling. A Wilcoxon signed rank test showed no difference in level of coupling for both theta-gamma and theta-high gamma coupling in wild type mice between day 2 and day 14 after surgery. For the local Dravet mice a Wilcoxon signed rank test showed a significant decrease in theta-high gamma coupling ($p<0.05$) between day 2 and day 14 after surgery and an almost significant decrease in theta-gamma coupling ($p=0.06$) between day 2 and day 14 after surgery.

IV. DISCUSSION

In this study we investigated if theta-gamma PAC could be a potential biomarker to monitor the efficacy of treatment for Dravet Syndrome. Despite the power spectra did not contain differences for wild type mice and global Dravet mice for recordings of the primary visual cortex during REM sleep, we found that theta-high gamma coupling was decreased during REM sleep in global Dravet mice during the early epileptic stage compared to wild type mice. Furthermore, we found that both theta-gamma and theta-high gamma coupling were strongly decreased during REM sleep in global Dravet mice in which seizure onset has already started, compared to wild type mice. In global Dravet mice with seizures theta-

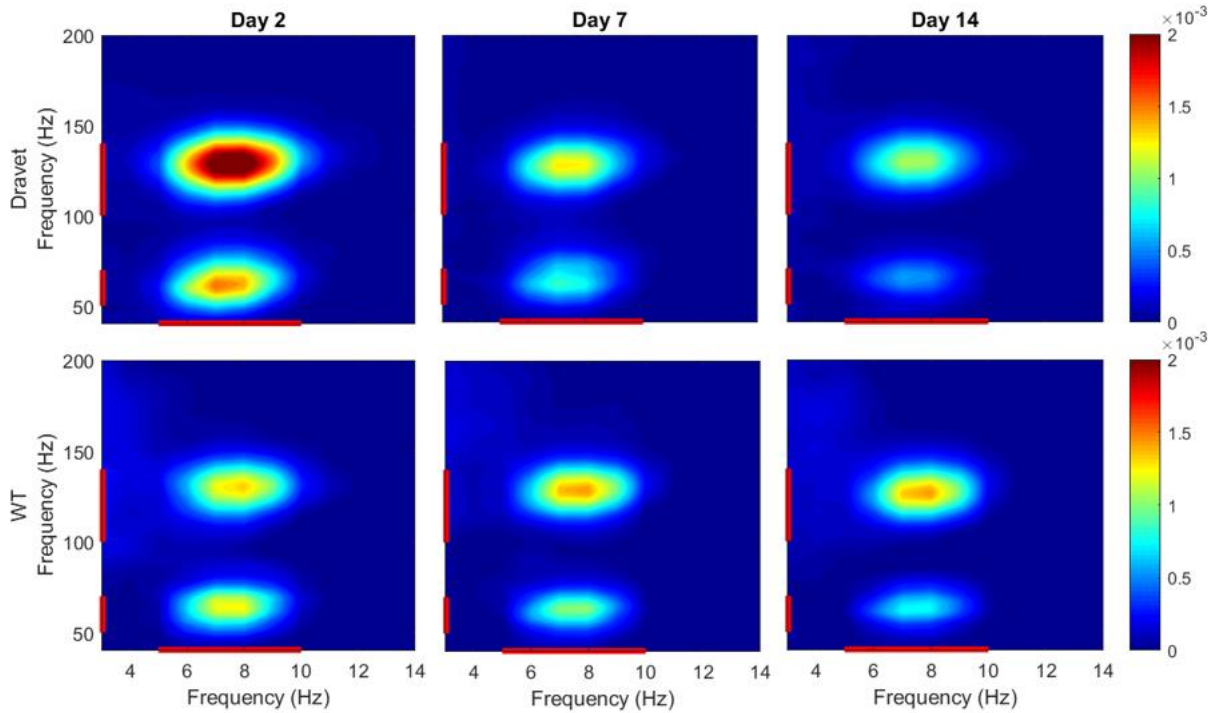


Figure 7: PAC over time from recordings of the primary visual cortex. Mean comodulation maps of local Dravet mice (top plots) and wild type mice (bottom plots) during REM sleep on day 2 (left), 7 (middle) and 14 (right) after surgery. The horizontal red line indicates the theta frequency range and the vertical red lines indicate the gamma and high gamma frequency ranges. Pseudocolor scale indicates the MI. There is a significant decrease in the level of theta-high gamma coupling for local Dravet mice between day 2 and day 14 ($p < 0.05$). For wild type mice there are no significant changes in PAC over time.

gamma coupling was also decreased during active wakefulness. Besides, in local Dravet mice, in which Dravet Syndrome starts developing through a virus injection in the hippocampus, we found a decrease in theta-high gamma coupling over time during REM sleep for recordings of the primary visual cortex.

The only difference in the power spectra between global Dravet mice and wild type mice was an increase in theta power during active wakefulness in recordings from the primary visual cortex. This finding is in accordance with the study of Salgueiro-Pereira et al. [18]. Salgueiro-Pereira et al. proposed that the increase in theta power was a direct effect of seizures. However, we also found an increase in theta power in global Dravet mice during the early epileptic stage. Therefore, there need to be other factors influencing theta power than only seizures. Another possible explanation for the increase in theta power is that active wakefulness consisted of various types of locomotor activities for which theta power might not be exactly the same. Due to some of the comorbidities going with Dravet Syndrome the most performed type of locomotor activities could be slightly different for mice with Dravet Syndrome than for wild type mice, which could influence the level of theta power.

There is a discussions whether the level of theta power influences the level of theta-gamma coupling [16]. The studies of Canolty et al. [36] and Tort et al. [37] showed that the level of theta-gamma coupling increased when there was an increase in theta power if other confounding variables were controlled. A later study of Tort et al. [16]

found that the increase in theta-gamma coupling could not be explained by changes in theta power. Our results also suggest that an increase in theta-gamma coupling cannot be explained by changes in theta power. For global Dravet mice with seizures we found an increase in theta power together with a decrease in theta-gamma coupling during active wakefulness. Since it is not clear what causes the increase in theta power during active wakefulness for global Dravet mice and because it is not sure whether the level of theta power influences the level of theta-gamma coupling, it might be better to use another vigilance state for PAC calculations.

The presence of PAC in a specific brain area is not direct evidence that the found PAC has physiological significance. The interpretation of PAC in terms of modulating or causally interacting frequencies can be misleading [33]. It is possible to find high levels of PAC when there are abrupt changes in a signal or when a signal contains non-stationarities in the absence of interactions between different oscillatory rhythms [14,33]. However, we did not find a difference in the amount of gamma bursts nor in the theta phase where these gamma bursts occur between wild type mice and global Dravet mice for all vigilance states. This makes it more likely that the PAC we found here is physiologically meaningful.

Inhibition of inhibitory GABAergic interneurons can lead to decreased theta-gamma and theta-high gamma PAC [15,22]. Therefore, we expected to find a decrease in theta-gamma and theta-high gamma coupling for mice with Dravet syndrome. Indeed, we found a decrease in

theta-gamma and theta-high gamma coupling for global Dravet mice in recordings from the primary visual cortex. However, we found a stronger decrease in coupling levels for mice in which seizures onset had already started than for mice which were in the early epileptic period. Besides, leads the decreased sodium current in inhibitory GABAergic interneurons in mice with Dravet Syndrome to a decreased ability for sustained action potential firing of GABAergic interneurons. The reduced GABAergic output enhances the excitability of neighboring neurons, which can cause hyperexcitability that leads to seizures [20]. These two could possibly go together. When the reduction of excitability is larger, the chance on seizures could be higher and theta-gamma PAC could be more decreased.

Here, we calculated PAC from ECoG signals in mice. If we want to use PAC as biomarker in practice it would be desirable to calculate PAC from non-invasive EEG signals in humans. There are already some studies that have shown it is possible to calculate PAC from scalp EEG in humans [38,39]. Since the main effects of impaired PAC in mice with Dravet Syndrome are within the high gamma range, at least a sampling frequency of 512 Hz, or preferably even higher, is needed in human recordings. Furthermore, the location of the electrodes used for recordings is of importance. We need to be able to calculate the PAC from a specific brain area, since there are large differences in PAC between brain areas [34,38]. More research is needed to investigate the patterns and levels of theta-gamma PAC for specific brain areas during different vigilance states in humans.

In conclusion, these results suggest that PAC could be a potential biomarker to monitor the efficacy of treatment for Dravet Syndrome. When treatment is efficient in a patient the number of seizures decreases [3]. Therefore we expect to see an increase in theta-gamma and theta-high gamma coupling when a patient responds positive to a particular treatment. The changes in level of PAC within a patient could be used to check the efficacy of a particular treatment. The biomarker would be most reliable when it is measured during REM sleep.

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Appendices

APPENDIX A

Table 2: Detailed overview of all mice

Name	Genotype	Gender	Date of birth	Date of surgery
SCN-AU	Scn1a ^{fl/fl}	Female	12/23/2018	02/25/2019
SCN-AV	Scn1a ^{fl/fl}	Female	12/23/2018	02/26/2019
SCN-CB	WT*	Female	04/23/2019	06/03/2019
SCN-CP	WT*	Male	05/20/2019	06/20/2019
SCN-CT	WT*	Female	05/20/2019	06/27/2019
SCN-CU	WT	Male	05/27/2019	06/19/2019
SCN-CV	Scn1a ^{+/-}	Male	05/27/2019	06/18/2019
SCN-CX	WT	Male	05/27/2019	06/19/2019
SCN-CZ	Scn1a ^{+/-}	female	06/14/2019	07/04/2019
SCN-DA	Scn1a ^{+/-}	Female	06/14/2019	07/04/2019
SCN-DB	Scn1a ^{+/-}	Female	06/14/2019	07/04/2019
SCN-DC	Scn1a ^{+/-}	Male	06/14/2019	07/04/2019
SCN-DD	Scn1a ^{+/-}	Female	06/24/2019	07/15/2019
SCN-DG	WT	Female	06/24/2019	07/15/2019
SCN-DI	Scn1a ^{fl/+}	Male	06/10/2019	07/16/2019
SCN-DJ	Scn1a ^{fl/+}	Male	06/10/2019	07/16/2019
SCN-DK	WT*	Female	06/10/2019	07/17/2019
SCN-DO	WT	Male	07/19/2019	08/12/2019
SCN-DQ	WT	Male	07/19/2019	08/12/2019
SCN-DR	WT	Female	07/19/2019	08/12/2019
SCN-DS	Scn1a ^{+/-}	Male	07/21/2019	08/12/2019
SCN-DT	WT	Male	07/21/2019	08/13/2019
SCN-DV	WT	Female	07/21/2019	08/13/2019
SCN-DW	Scn1a ^{+/-}	Female	07/21/2019	08/13/2019
SCN-DX	Scn1a ^{+/-}	Female	07/21/2019	08/13/2019

(* = wild type with placebo injection)

APPENDIX B

Grubbs test

Before rejecting observations in a dataset, there need to be evidence that an observation contains outliers. This can be done with a statistical test. Grubbs test [28] is a test that is used to detect outliers. To apply Grubbs test a dataset needs to be univariate and the dataset is assumed to come from a normal or Gaussian population. Grubbs test can only detect one outlier at a time. The detected outlier is removed from the dataset and the test is iterated until no outliers are detected. With each iterations the mean and standard deviation of the dataset change and therefore the probabilities of detection change. Grubbs test should not be used for a sample size smaller than seven since it frequently tags most of the points as outliers in that case.

Grubbs test is defined for the hypothesis:

- H_0 : There are no outliers in the dataset
- H_a : There is exactly one outlier in the dataset

A significance level for when to reject H_0 is chosen and the Grubbs test statistic is calculated as:

$$G = \frac{\max_{i=1,\dots,N} |Y_i - \bar{Y}|}{s}$$

The test statistic is compared with the critical T-value. In the case $G > T$, H_0 is rejected.

APPENDIX C

Modulation Index

There is no gold standard for assessing phase-amplitude coupling. Several methods exist with different advantages and limitations [16,25,33]. The measure used in this study to detect phase-amplitude coupling between two frequency ranges of interest is called the Modulation Index (MI) [25]. The first frequency range is the ‘phase-modulating’ denoted as the phase (f_p) which is consisting of low frequencies and the second frequency range is the ‘amplitude-modulated’ denoted as amplitude (f_A) which is consisting of high frequencies. First we will explain how the MI is calculated and then we will give some advantages of using the MI for our purposes compared to other methods.

To calculate the MI a phase-amplitude distribution like plot is generated:

- 1) The same raw signal is bandpass-filtered two times at the two frequency ranges under analysis (f_p and f_A). The filtered signals are denoted as $x_{f_p}(t)$ and $x_{f_A}(t)$.
- 2) The standard Hilbert transform is applied to $x_{f_p}(t)$ to extract the time series of the phases [denoted as $\phi_{f_p}(t)$] and also to $x_{f_A}(t)$ to extract the time series of the amplitude envelope [denoted as $A_{f_A}(t)$]. The composite time series [$\phi_{f_p}(t), A_{f_A}(t)$] is then constructed, which gives the amplitude of the f_A oscillation at each phase of the f_p rhythm.
- 3) Next, the phases $\phi_{f_p}(t)$ are binned ($N = 18$ bins of 20°) and the mean of A_{f_A} over each phase bin j is calculated. The mean A_{f_A} value at the phase bin j is denoted as $\langle A_{f_A} \rangle_{\phi_{f_p}(j)}$.
- 4) As last step to obtain the phase-amplitude distribution plot, the mean amplitude $\langle A_{f_A} \rangle_{\phi_{f_p}(j)}$ is normalized by dividing each bin value by the sum over the bins:

$$P(j) = \frac{\langle A_{f_A} \rangle_{\phi_{f_p}(j)}}{\sum_{k=1}^N \langle A_{f_A} \rangle_{\phi_{f_p}(k)}}$$

where N is the number of used phase bins. The normalized amplitude P has the same characteristics as a discrete probability density function: $P(j) \geq 0 \forall j$ and $\sum_{j=1}^N P(j) = 1$. By plotting P as a function of the phase bins the phase-amplitude plot is obtained. Visual inspection of the phase-amplitude plot can already give an indication of the level of phase-amplitude coupling. Figure 8 gives examples of phase-amplitude plots with the associated MI values. A value for the MI is calculated from the phase-amplitude plot.

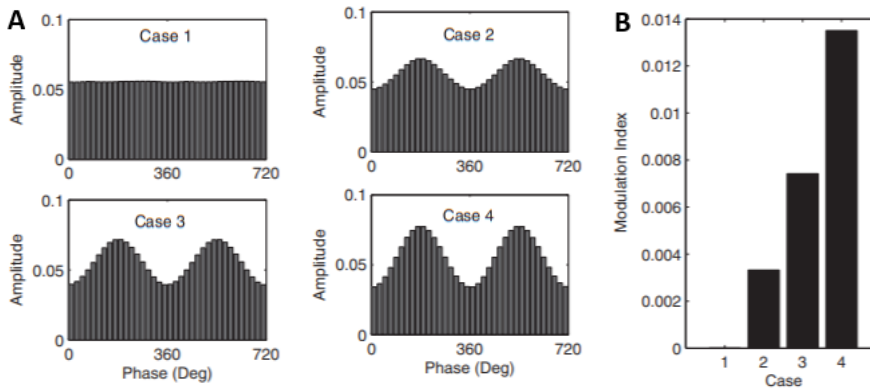


Figure 8: Example of phase-amplitude plots with associated MI values. (A) Mean amplitude is plotted as function of the phase for 4 cases with a different level of coupling. Two cycles of theta phase are plotted for clarity. **(B)** MI values for the 4 cases of A .Figure obtained from Tort et al. [25].

The amplitude distribution P over the phase bins is uniform (the mean amplitude of f_A is the same for all phases of f_p), when there is no phase-amplitude coupling between the pair of frequencies (f_p, f_A) under study. The MI is calculated from the deviation of P from the uniform distribution, which is achieved by an adaptation of the Kullback-Leibler (KL) distance. The adaptation was made to achieve values between 0 and 1 for the MI. The KL distance is a premetric that is widely used to infer the amount of difference between two distributions [29]. The KL distance of a discrete distribution P from a distribution Q is defined as:

$$D_{KL}(P, Q) = \sum_{j=1}^N P(j) \log \left[\frac{P(j)}{Q(j)} \right]$$

The MI is calculated by dividing the KL distance by $\log(N)$:

$$MI = \frac{D_{KL}(P, Q)}{\log(N)}$$

in which $\log(N)$ represents maximal entropy. Therefore the MI has always a value between 0 and 1. The value of the MI is 0 when $P = Q$. The MI increases the further away P gets from Q and the MI has a value of 1 when P has a Dirac-like distribution, that is $P(k) = 1$ for a given bin k and $P(j) = 0$ for all bins j different from k .

Table 3 gives a summary of the quality of performance of the MI compared to other methods. The MI is the only method that has a positive score for all factors.

Table 3: Performance of PAC measures. Obtained from Tort et al. [25]

Measure	Tolerance to noise	Amplitude independent	Sensitivity to multimodality	Sensitivity to modulation width
Modulation Index	Good	Yes	Good	Good
Heights ratio	Good	Yes	No discrimination	No
Mean vector length	Good	No	Restricted	Reasonable
Amplitude PSD	Low	No	Restricted	Good
Phase-locking value	Low	No	Restricted	Low
Correlation measure	Low	No	Restricted	Low
GLM measure	Low	No	Restricted	Low
Coherence value	Low	No	Restricted	Low

APPENDIX D

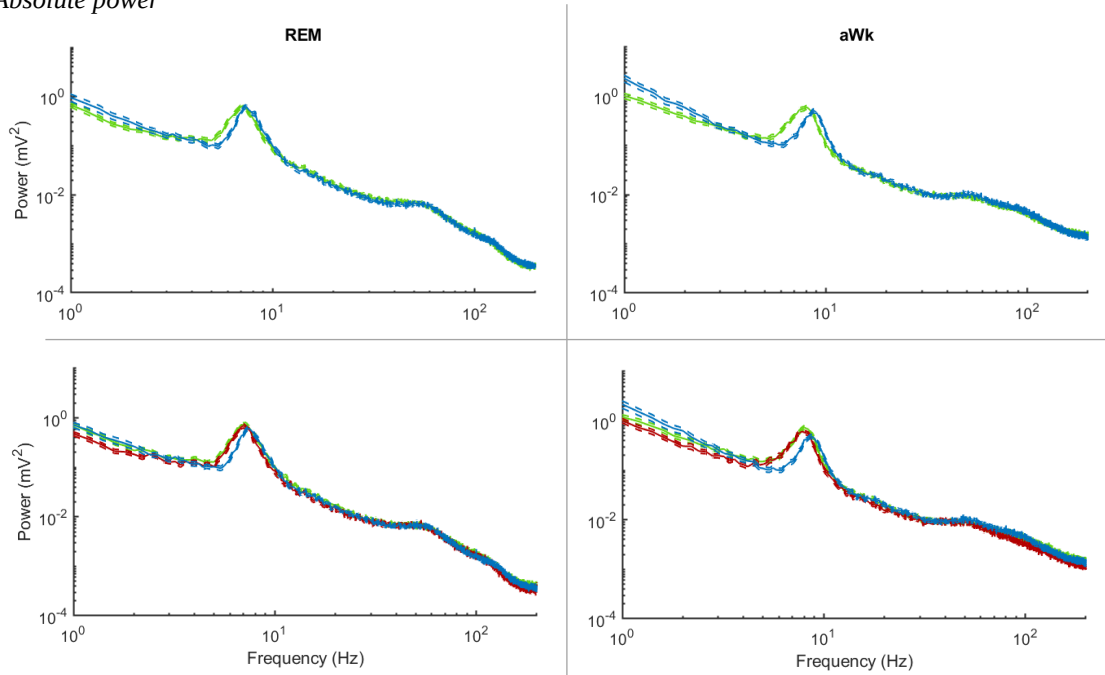
Absolute power

Figure 9: Absolute power spectra from recordings of the primary visual cortex. Top plots: Mean ($\pm$ SEM) power spectra from 1 to 200 Hz of wild type mice (blue, $n=8$) and global Dravet mice (green, $n=9$) during REM sleep (left) and active wakefulness (right). Bottom plots: Mean ($\pm$ SEM) power spectra from 1 to 200 Hz of wild type mice (blue, $n=8$), global Dravet mice without seizures (green, $n=5$) and global Dravet mice with seizures (red, $n=4$) during REM sleep (left) and active wakefulness (right).