Investigation of the absence epilepsy in freely moving Long-Evans rats

Summary of the Ph.D. thesis

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Introduction

Despite the great advances of the recent decades in understanding the pathomechanism of absence epilepsy, still there are fundamental caveats regarding epilepsy research and clinical practice. From the clinical perspective, there was no significant improvement of the pharmaceutical possibilities since the introduction of ethosuximide and valproic acid in the 1960s. Furthermore, as most patients already suffer from frequent seizures when diagnosed, we still only have scarce knowledge on the epileptogenesis of the seizure-susceptible brain. For these and other reasons, my present work is focusing on the cortical aspects of seizure development and possible treatment alternatives of the thalamocortical absence epilepsy.

Absence epilepsy is characterized by sudden, transient loss of consciousness, behavioral arrest, and an EEG pattern dominated by spike-and-wave discharges. Studies performed on brain slices and anesthetized animals, as well as recent investigations of local circuits in awake animals, elucidated many aspects of the basic mechanisms of absence epilepsy. This type of seizure emerges as a result of promptly generalizing, highly synchronous interaction among neurons within the thalamocortical loop. In these loops, cortical pyramidal cells excite thalamocortical (TC) cells of the relay nuclei and the nucleus reticularis thalami (NRT); this excitation drives NRT cells to periodically impose bursts of inhibition on TC cells, which in turn send synchronous excitatory feedback to cortex and NRT. In principle, hyperexcitation at any point of the thalamocortical loop can result in absence-like spike-and-wave seizures and selective

interventions at various points of the thalamocortical loop are able to reduce ongoing seizure activity. No gross anatomical alteration, but many different channel deficiencies were identified as possible causes or contributors of the absence epilepsy both in human and in animal models.

Importantly, the interplay between the cortex and the thalamus also gives rise to major physiologic oscillations such as spindles and delta waves during sleep. Although these physiological and pathological oscillations can co-exist in the same circuitry, very little is known about how the emergence of seizures influence the physiological sleep oscillations. To our knowledge, no study has investigated yet the evolution of thalamocortical oscillations in relation to the emerging seizure activity. Altogether, despite the genotypic and phenotypic variations of absence epilepsy are relatively well studied, we still have scarce knowledge on the epileptogenesis in the seizure-susceptible brain. Beyond giving a mechanistic insight into seizure generation, understanding epileptogenesis may help to identify possible nodes of the epileptic circuitry as potential targets for new treatments.

Despite the intensive efforts to develop new pharmacotherapies, antiepileptic drugs fail to adequately treat approximately one-third of the patients, and even responsive subjects often suffer from side effects. Electrical stimulation techniques might provide an alternative, but require extensive investigation of the effects of time-targeted electrical perturbation of epileptic seizures in animal experiments. Importantly, most studies have not expanded

beyond the acute effects of the treatment. Given the chronic nature of the epilepsy in the majority of patients, understanding the long-term effects of a stimulation paradigm is critical. TES has already been proven to effectively reduce the duration of spike-and-wave discharges in rats with absence epilepsy. However, the clinical applicability of TES has not yet been realized, and the successful treatment seen in animal experiments was suspected to become ineffective over longer timescales due to habituation.

Long-Evans rats can express spontaneous spike-and-wave discharges and the behavioral correlates of absence seizures, and they also show similar pharmacological responses to anti-epileptic treatment as human patients. In general, absence epilepsy possesses an early age-related onset with persistent ictal activity during the adulthood in rodent models. The evolution of absence seizures spans months in Long-Evans rats that capacitates this strain to be an ideal model to investigate how seizure emergence impacts other oscillations of the thalamocortical circuitry parallel to the progression of the epileptic condition. Furthermore, its pharmacological face validity allows it to use as a model to test alternative treatments for absence epilepsy.

Aims of the study

The aims of the present study were to examine the development of absence seizures in the thalamocortical circuitry of freely moving Long Evans rats and to investigate the long-term outcome of on-demand transcranial electrical seizure interruption. The concrete goals of my work were the following:

- To design and build a closed-loop intervention system, which continuously supervises brain activity for months and provides on demand seizure interruption for the early termination of seizures.
- To determine whether the effective on-demand TES treatment of epileptic seizures over an extended period of time leads to a long-term therapeutic effect.
- To describe the evolution of spontaneous seizures and the related co-occurring alterations of sleep architecture.
- To causally investigate the cortical mechanisms of SWD generalization and to understand how maturation influences seizure susceptibility.

Materials and Methods

Long-Evans rats were operated under isoflurane anesthesia and all rats were chronically implanted with tungsten triplet electrodes, which targeted the frontal and parietal cortical areas of both hemispheres and over the right hippocampus. Rats for seizure interruption experiments were additionally equipped with transcranial stimulating electrodes. Rats for unit recordings and seizure induction experiments were equipped with an additional silicone probe (right

prefrontal or motor cortex) and a bipolar tungsten stimulation electrode in the motor cortex of the left hemisphere. In all surgeries miniature stainless steel screws (serving as reference and ground) were implanted bilaterally above the cerebellum. A copper mesh (serving as a Faraday cage) was built around the probes and electrodes and enforced with dental cement. All recording sessions took place in the same room in 12h light-dark cycles. The recorded signals were preamplified, amplified 400×, multiplexed on head and stored after digitalization.

In seizure interruption experiments, the preamplified signals were analyzed on-line by a digital signal processor using a custom made seizure detection algorithm. The LFP of pre-selected tripolar electrodes (with the earliest seizure appearance) were demultiplexed (one triplet for each rat) in real time, and the current source density (CSD) of those triplets was bandpass-filtered, rectified and integrated in a time window of 20 ms. Threshold crossing for both the raw CSD and the integrated signal were monitored. Synchronous multiple threshold crossing (minimum twice, separated by 40–50 ms interval) triggered a charge neutral, triphasic single-pulse (100 ms) stimulation. The control animals were not stimulated at all, the open-loop and closed-loop stimulated animals received treatment in an alternating fashion: each non-stimulated day was followed by a stimulated day, the stimulation timing of every open-loop treated animal was driven by the stimulation timing of a randomly chosen closed-loop treated animal. To investigate the effect of antiepileptic treatment on sleep spindles, animals received an intraperitoneal injection of saline

(control day) then on the following day an intraperitoneal injection of ethosuximide (treatment day, 100 mg/kg body weight). Injections were given at 8 a.m. and animals were monitored for 12 hours in daylight. In intracortical stimulation experiments monophasic single-pulses (0.1 ms, 7-15 V, sufficient to induce delta waves during NREM sleep) were delivered to the deep layers of the motor cortex. Pulses were delivered every 10 seconds, with no respect to the behavioral states.

For histological verification of the recording locations and possible pathologic changes, i.e. stimulus induced gliosis, animals were transcardially perfused with saline followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer saline. 50-μm thick coronal sections were prepared with vibratome and either immunostained for GFAP with DAPI counterstaining or subjected to cresyl violet staining with standard histological techniques. Sections from every 1 mm throughout the entire anterior–posterior extent of the cerebrum were investigated.

All off-line analyses were performed in MATLAB. To detect SWD episodes, band-pass filtered LFP was thresholded, seizures were detected if both the high [30-200 Hz] and the low [8-12 Hz] passed Z-scored signal conjunctively exceeded 5. Consecutive SWD episodes separated less than 1 second were merged. For delta wave and sleep spindle detections, we used only non-theta epochs. Non-theta epochs were detected automatically using the ratio of the power in theta band (5-11 Hz) to the power of nearby bands (1-4 Hz, 12-14 Hz) of hippocampal LFP. To detect global delta waves, LFP was filtered (0–

6 Hz) and Z-scored. Consecutive upward-downward-upward zero-crossings of the first temporal derivative within a temporal window of 150 and 500 ms were considered as putative delta events. Delta waves corresponded to epochs where Z-score exceeded 2 at the peak, or exceeded 1 and fell below −1.5 at the end of the event bilaterally. For spindle detection, LFP was filtered (10-20 Hz) and Z-scored. Spindles corresponded to epochs where Z-score exceeded 2 for more than 0.2 s and peaked at >4. Events separated by less than 0.4 s were merged, events lasting more than 3 s were discarded. Brain state of the animals was estimated based on delta wave density. Segments with high delta wave occurrence rate (>10/min) were considered as slow wave sleep (SWS). SWS epochs separated by less than 1 min, were merged. The first and last minutes of SWS with the neighboring 1 minute long non-SWS epochs were labeled as 'Transition to SWS' and 'Transition from SWS', respectively. Remaining epochs were labeled as 'Awake/REM'. The spikes were automatically clustered using Kilosort and manually curated in Phy.

Results

Real-time detection and interruption of epileptic seizures

We developed an unsupervised closed-loop intervention system, which is capable to monitor brain activity and automatically terminates epileptic seizures for durations comparable to the life expectancy of the animals. Furthermore, we established a fabrication and surgical protocol of stimulation and recording electrodes that provides sufficient mechanical stability and signal-to-noise ratio to perform long-term stimulation experiments. For validation purposes we demonstrated the short-term (day-long) effects of our seizure intervention system. The overall time spent in seizure per day and the duration of the seizures only changed significantly in the closed loop stimulated group, confirming the effectiveness of the on-demand treatment on the short timescale. There was a marked, but not significant increase in the time spent in seizures for some open loop animals without changing the individual seizure duration. These results raise the possibility that the randomly delivered stimulation may induce seizures, but it does not prolong them. These findings emphasize the crucial importance of the timing of the stimulation.

Long-term closed loop seizure control

Closed-loop stimulation was found to be effective in longer time scales as well, as we could successfully disrupt seizure activity for 6 weeks continuously. We found that during the treatment both the total time in seizures and the duration of the seizures significantly

decreased. These effects were immediate and did not deteriorate over the time course of treatment, and returned to the pre-treatment baseline level as the treatment was suspended. The seizure rate increased significantly during the treatment and returned to the baseline level after suspending the treatment. Importantly, we did not observe traces of glial remodeling due to the intervention. In one animal, we continued the treatment for an additional three months. The prolonged closed-loop treatment (altogether 4 months) also resulted in qualitatively similar results. Since the efficacy of the seizure suppression was maintained, this further supports that long-term closed loop treatment is possible in terms of the efficacy of the early termination of the seizures.

Absence seizure development and sleep spindles

In another set of experiments, we performed a longitudinal study in Long-Evans male rats to determine the evolution of spontaneous seizures and the related co-occurring alterations of sleep architecture. Altogether, we found that the total time spent in seizures and individual seizure length progressively increased during the observation period and saturated around 5 months with no change in the seizure occurrence rate. In parallel to the progressively emerging seizure activity, we observed a substantial fall of spindle occurrence rate that raises the possibility of a causal relationship. Furthermore, a single high dose peritoneal injection of the antiepileptic drug, ethosuximide (ETX) resulted in a prompt seizure suppression (in minutes), which was accompanied with a further reduction in sleep spindle occurrence, too. Hours later, as the drug's plasma level

decreased, ETX still had the potential to suppress seizure activity, but simultaneously sleep spindle occurrence was higher than the time-matched baseline activity of vehicle injection. In contrast, treatment with transcranial electrical stimulation was only effective in quickly terminating the seizures, but it did not increase sleep spindle occurrence rate.

The incidence of spontaneous sleep spindles and SWDs with respect to the depth of the slow-wave sleep (SWS) was very diverse. Unlike sleep spindles, seizures emerged at the transitions between wakefulness and sleep and their occurrence rate progressively fell with deepening of the SWS. Furthermore, we found that the seizure occurrence showed a substantial asymmetry regarding SWS. Seizures were very likely to occur when the animals entered SWS, but very few seizures were observed during SWS and during transitions from SWS. Although the majority of the seizures occurred in awake/REM state, taking into account the time spent in different brain states, the transition to SWS found to be the most seizure susceptible state.

Seizure activity outside the seizure initiation zone

We observed topographic differences in seizure activity. In juvenile animals (~14 weeks old), the LFP in the somatosensory cortex already expressed 8 Hz spike and wave discharges during ictal activity, but the off-focus motor cortex displayed a slower frequency oscillation ictally, that with aging, gradually increased its frequency to reach the main SWD-frequency. It became highly coherent with the activity of the seizure onset zone in mature animals (>20 weeks old).

Investigation of the neuronal spiking of motor and prefrontal cortices in relation to the spontaneously emerging seizure activity revealed that although the firing rate of pyramidal cells did not change, interneurons were less active compared to their pre-seizure baseline activity, causing the inhibitory/excitatory balance to shift towards excitation. As this was only observed in mature animals, it raises the possibility of an active cortical inhibitory veto of seizure spread that disappears with maturation. In addition, most cells were only firing in ~25% of the total SWD cycles, suggesting that cortical cells are not entrained to seizure activity on a cycle-by-cycle basis. Thus, the LFP pattern of spike-and-wave discharges seems more likely an emergent network property with loosely coupled unit activity, than a hypersynchronous firing of all the units at each discharge.

Brain-state dependent seizure susceptib-ility

Given that a single pulse intracortical stimulation has the potential to elicit global delta waves, we asked if it is also sufficient to switch the brain into seizure activity. We found that both juvenile and mature animals' somatosensory cortices could respond with spike-and-wave activity to the stimulation. However, juvenile animals' ictal-like activity often remained local, mature animals developed generalized seizures. Importantly, the susceptibility of the cortex for seizures was highly brain state dependent. Investigations of the pre-stimulation power of characteristic oscillations (hippocampal theta, cortical beta and delta) revealed that both hippocampal theta and cortical delta power negatively correlated with seizure induction

probability. Interestingly, cortical beta band in juveniles negatively correlated with seizure induction probability, but showed positive correlation in matured animals.

Discussion

Here we report that the unsupervised, time-targeted transcranial electrical seizure suppression in rats remains steadily effective over months of treatment (tested up to four months), and it has no deteriorating post-stimulation effect on the internal brain dynamics. These findings establish the safe transition of this approach to human clinical trials. Importantly, although our disease model was absence epilepsy, the long-term efficacy and safety points far beyond the absence epilepsy. It holds a great promise, that transcranial electrical stimulation might be a new effective tool in disrupting seizure activity in various forms of epilepsy with low risk, when applied in a temporally and spatially constrained manner.

As absence epilepsy is very rarely recognized in the early stages in children, we have scarce knowledge on the mechanism of epileptogenesis. Long Evans rats develop thalamocortical spike and wave discharges and the characteristic seizure related behavioral symptoms over months, providing an opportunity to track the network level changes of the thalamocortical loop that lead to fully-developed generalized seizures. We observed the relationship of seizure related patterns to sleep-related physiologic oscillations. We found that although there is a strong inverse relationship between the occurrence of spindles and SWDs, they appear at different stages of non-REM

sleep. In young, spindles are already global patterns, but SWDs are focal and become generalized only during maturation. Based on our observations we suggest that maturation gradually shifts the focal ictal events into generalized seizure activity as a secondary step, in a network that was already capable to generate global patterns. Along the same lines, the decreasing spindle incidence and the emergence of SWDs are two confound, but distinct consequences of the underlying changes of the thalamocortical network.

A clinically important key finding of our work is that although high doses of ethosuximide, the first-choice drug in absence epilepsy, suppresses both SWDs and the remaining spindles, carefully chosen smaller doses are still capable to eliminate SWDs, while spindles become more frequent, recovering to a similar occurrence rate as in healthy animals. These findings put emphasis on the importance of using a proper dosing of antiepileptic substances that matches a narrow therapeutic window, where the seizures are already suppressed, but the sleep quality might improve. As spindles are essential for memory consolidation, bearing this in mind may help to properly treat the absence related sleep and learning disturbances.

Altogether the precisely timed closed-loop electrical seizure suppression in combination with carefully exploiting the narrow therapeutic window of spindle-sparing drug dosing offer yet unutilized therapeutic potential for patients with pharmaceutically yet intractable epileptic seizures.

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Publications related to the subject of the thesis

I. Kozák G, Berényi A (2017) Sustained efficacy of closed loop electrical stimulation for long-term treatment of absence epilepsy in rats.
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