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

PhD Thesis

Gábor Kozák

Supervisor:
Dr. Antal Berényi

Department of Physiology
Faculty of Medicine, University of Szeged

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LIST OF PUBLICATIONS

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.
Scientific reports 7:(1) Paper 6300. 10 p. (2017)
DOI: 10.1038/s41598-017-06684-0II.

II. Kozák G, Földi T, Berényi A (2018) Chronic Transcranial Electrical Stimulation and Intracortical Recording in Rats
Journal of Visualized Experiments. 2018 May 11;(135).
DOI: 10.3791/56669

III. Kozák G. (2019) Insights on the Role of Thalamocortical HCN Channels in Absence Epilepsy
Journal of Neuroscience 23 January 2019, 39 (4) 578-580
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IV. Kozák G (2019) Absence epilepsy might build its own nest
Journal of Physiology. 2019 Mar;597(6):1437-1438
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V. Kozák G, Földi T, Berényi A (2019) Spike and Wave Discharges Are Not Pathological Sleep Spindles - Network-level Aspects of Age Dependent Absence Seizure Development in Rats (under review)

OTHER PUBLICATIONS

VI. Szabó T, Bencsik G, Magyar M, Visy C, Gingl Z, Nagy K, Váró G, Hajdu K, Kozák G, Nagy L Photosynthetic reaction centers/ITO hybrid nanostructure
Materials Science and Engineering: C 33 (2), 769-773
DOI: 10.1016/j.msec.2012.10.031

VII. Bere Z, Obrenovitch TP, Kozák G, Bari F, Farkas E (2014) Imaging reveals the focal area of spreading depolarizations and a variety of hemodynamic responses in a rat microembolic stroke model
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DOI: 10.1038/jcbfm.2014.136

VIII. Clark D, Institoris A, Kozák G, Bere Z, Tuor U, Farkas E, Bari F (2014) Impact of aging on spreading depolarizations induced by focal brain ischemia in rats
Neurobiology of aging 35 (12), 2803-2811
DOI: 10.1016/j.neurobiolaging.2014.06.013

IX. Vöröslakos M, Takeuchi Y, Brinyiczki K, Zombori T, Oliva A, Fernández-Ruiz A, Kozák G, Kincses ZT, Iványi B, Buzsáki G, Berényi A (2018) Direct effects of transcranial electric stimulation on brain circuits in rats and humans
Nature Communications 9 (1), 483
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2 LIST OF ABBREVIATIONS

AP: anteroposterior

CA: Cornu Ammonis

CSD: current source density

DAPI: 4′,6-diamidino-2-phenylindole

EEG: electroencephalography

ETX: ethosuximide

GABA: gamma-aminobutyric acid

GAERS: Genetically Absence Epilepsy Rat from Strasbourg

HCN: hyperpolarization-activated cyclic nucleotide-gated

LFP: local field potential

LTS: low-threshold spike

ML: mediolateral

NREM: non-rapid eye movement sleep

NRT: nucleus reticularis thalami

REM: rapid eye movement sleep

SWD: spike-and-wave discharge

SWS: slow-wave sleep

TC: thalamocortical

TES: transcranial electrical stimulation

WAG/Rij: Wistar Albino Glaxo rats from Rijswijk

3 INTRODUCTION

Absence epilepsy – the most common type of epilepsy of the childhood (Glauser et al., 2010) – is an idiopathic, primary generalized, non-convulsive epilepsy with a polygenic background. It is characterized by sudden, transient loss of consciousness, behavioral arrest, and an EEG pattern dominated by spike-and-wave discharges (SWDs) of thalamocortical origin (Crunelli and Leresche, 2002). Although absence epilepsy is considered a relatively benign form of epilepsy, as in most cases seizures vanish during late infancy, this condition is often accompanied by comorbidities, such as attentional deficit, depression, learning difficulties (Caplan et al., 2008), which might even persist when the patients become seizure-free. Furthermore, antiepileptic drugs fail to adequately treat a substantial portion of the patient and in addition, sufficient seizure control often fails to improve comorbid disorders (Glauser et al., 2013).

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, since the introduction of ethosuximide and valproic acid in the 1960s, there was no significant improvement of the pharmaceutical possibilities (Vrielynck, 2013). Furthermore, as by the time of the diagnosis most patients were already exposed to frequent seizures for a substantial amount of time, we still have scarce knowledge on the epileptogenesis of the seizure-susceptible brain. The pre-existing epileptogenic conditions and the seizure induced alterations of the brain have yet to be separated.

Thus a better understanding of the seizure-related changes of brain function and new therapeutic approaches are needed to overcome these shortages. For these and other reasons, my present work is focusing on the cortical aspects of seizure development and possible treatment alternatives of the absence epilepsy.

ANATOMY AND CONNECTIVITY

The thalamo-cortico-thalamic system consists of highly interconnected loops of neurons of the thalamic relay nuclei located in the dorsal thalamus, the nucleus reticularis thalami (NRT) of the ventral thalamus and the cerebral cortex (Jones, 2007). The cells of the thalamic relay

nuclei (thalamocortical (TC) or relay cells) receive their inputs either from subcortical sources such as sensory or motor systems (first order relay nuclei) or from cortical domains (higher order nuclei) and send their axons to their target cortical area together with collaterals to the NRT (Sherman, 2005). They receive feedback excitation from cortical pyramidal cells and inhibition from their target NRT cells. Axons of the NRT remain exclusively intrathalamic and serve as a powerful modulatory and inhibitory system of the thalamus. GABAergic inhibitory cells within the NRT are highly interconnected via both axon collaterals and gap junctions, making it possible to both powerfully synchronize and desynchronize thalamic activity depending on the precise timing of their firing patterns (Fuentealba and Steriade, 2005).

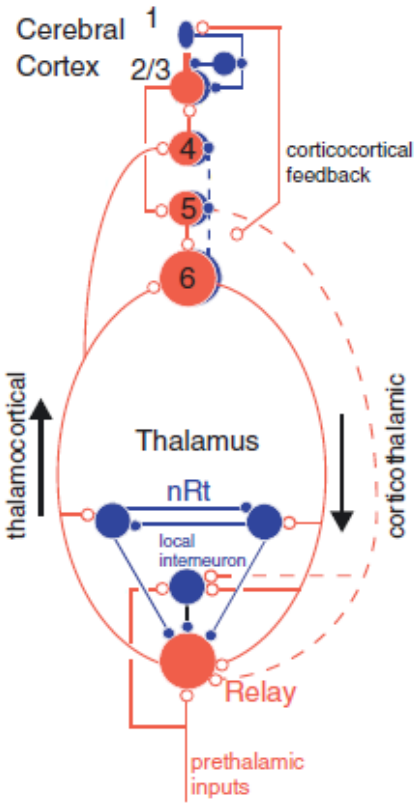

Figure 1. The anatomy of the thalamocortical loop. *Modified from McCormick et al. (2015)*

Cortical pyramidal cells provide feedback and feed-forward excitation to their peers in the thalamic relay nuclei, innervate NRT via collaterals and also project to other cortical regions. Thus, the convergence and divergence inside the thalamocortical loop combined with the extensive reciprocal connections between the thalamus and the cortex allow a highly

confined topographic cortico-thalamic communication among subsets of cells (Sherman and Guillery, 2002), but also provides opportunity for a widespread, bidirectional, generalized activation which may entrain large domains of the cerebral cortex simultaneously (Steriade et al., 1993; Lewis et al., 2015). Furthermore, the thalamocortical circuitry is greatly influenced by neuromodulatory systems of the brain, which shape the thalamocortical activity in a brain-state dependent manner (Steriade et al., 1986; Steriade et al., 1993; McCormick and Bal, 1997; McCormick et al., 2015).

Altogether, the structure of the dorsal thalamus not only allows it to relay sensory information to the cortex, but with its recurrent connectivity it also participates in sensory processing. Importantly, governed by its neuromodulatory inputs, it can gate sensory flow to the cortex. Thus it has a critical role in governing attention and orchestrating sleep, when cortical computation is greatly isolated from the external environment. Preventing cortical processing from being contaminated with online sensory information is a key element of memory consolidation during sleep.

SINGLE CELL AND NETWORK PHYSIOLOGY OF THE THALAMOCORTICAL CIRCUITRY

The electrophysiological properties of both the thalamocortical relay cells and NRT cells enable them to respond to incoming excitation with two different firing patterns: burst and tonic modes. Especially the high density of low threshold T-type calcium channels and the hyperpolarization-activated cyclic nucleotide–gated (HCN) channels (responsible for I_h currents) attributes them to work in these strikingly different manners, depending on their actual membrane potential and on their past activity (Figure 2).

In burst mode, cells are relatively hyperpolarized, that de-inactivates T-type calcium channels and so called low-threshold calcium spikes (LTS) might occur, which are relatively long-lasting, high amplitude depolarization events of the individual cells. These are frequently accompanied by bursts of ‘regular’ sodium action potentials. Hence, cells are able to respond to inhibition with postinhibitory rebound burst.

In tonic mode, relatively depolarized cells respond to activation with single action potentials as the T-type channels are inactivated, providing a linear input-output characteristics

of the cells. Thus, hyperpolarization of thalamic cells facilitate the occurrence of LTS and burst firing, capacitating cells to respond to inhibition with postinhibitory rebound burst, while depolarization might switch cells to tonic firing mode. As the actual membrane potential of thalamic cells is greatly influenced by subcortical neuromodulatory inputs, firing mode of thalamic cells is largely brain-state dependent.

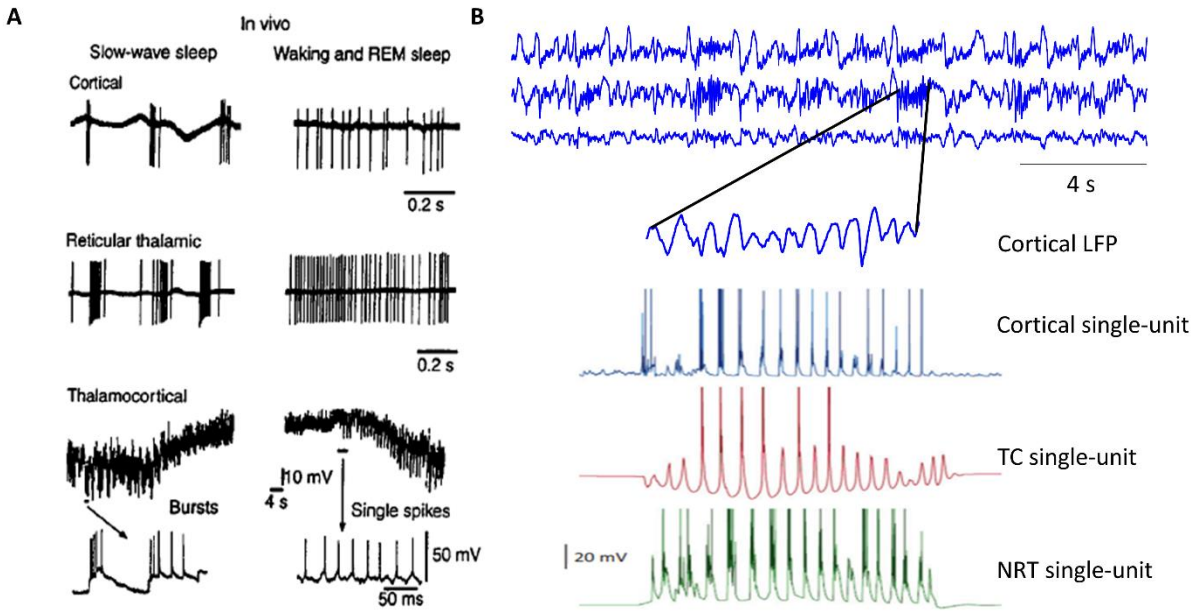

Figure 2. Brain-state dependent neuronal firing patterns within the thalamocortical circuitry. **(A)** *Single-cell recordings of neurons of the cortex, NRT and thalamic relay nuclei during periods of slow-wave sleep and waking in behaving animals showing the different firing modes within the thalamocortical loop.* **(B)** *Slow-wave sleep activity in multiple depth of the cortex. Inlet highlights a sleep spindle with representative intracellular traces. Note the interweaving activity patterns of the NRT and TC cells. (A) is reproduced from Steriade et al. (1993), single cell traces on (B) are modified from Bonjean et al. (2011) and Astori et al. (2013)*

These firing modes (and particularly burst firing) supported with the high density intrathalamic and cortical interconnectivity is ideal for the emergence of oscillatory patterns. Indeed, the thalamocortical loop gives rise to sleep related oscillations (when the overall neuromodulatory influence on the thalamus results in hyperpolarization favoring the burst firing mode), such as sleep spindles, delta and slow waves. During sleep spindles, activity of NRT cells impose bursts of inhibition on TC cells, which in turn greatly hyperpolarize, responding with postinhibitory rebound bursts, activating NRT cells to fire again. These periodic inhibitory-excitatory bursts via thalamocortical projections relayed to the cortex shaping its

activity rhythmically. Furthermore, rhythmic alteration of firing modes within the thalamus are thought to be one of the main contributors of cortical UP and DOWN states.

PATHOPHYSIOLOGY OF THE THALAMOCORTICAL CIRCUITRY

Beside the diverse physiological functions, the high degree of anatomical interconnectivity and the cellular features of the thalamocortical loop is in favor of easily spreading pathologic (e.g. seizure) activity. In principle, hyperexcitation at any point of the thalamocortical loop can result in absence-like spike-and-wave seizures (Meeren et al., 2002; Sorokin et al., 2016b; Makinson et al., 2017) and selective interventions at various points of the thalamocortical loop are able to reduce ongoing seizure activity (Berenyi et al., 2012; Paz et al., 2013; Sorokin et al., 2016b). Indeed, absence epilepsy is thought to be disorder of the thalamocortical loop. However, no gross anatomical alteration can be identified as a source of seizure activity, many different channel deficiencies were identified as possible causes or contributors of the absence epilepsy both in human and in animal models (van Luijtelaar et al., 2000; Crunelli and Leresche, 2002; Reid et al., 2012).

Although both thalamus and cortex are required to have fully developed seizures, a large body of evidence suggests that a hyperexcitable initiating zone located in the fronto-parietal region of the cortex drives the thalamocortical loop into seizure activity (Meeren et al., 2002; Holmes et al., 2004; Polack et al., 2007; Studer et al., 2018). To our current understanding, seizures emerge as local paroxysmal activity of hyperactive deep layer pyramidal cells of the cortical focus (Polack and Charpier, 2006) that excite cells of the relay nuclei and the NRT; this excitation drives NRT cells to periodically impose bursts of inhibition on TC cells, which in turn send rebound excitatory feedback to cortex and NRT (Steriade et al., 1993; McCormick and Contreras, 2001). There are ample experimental demonstrations on the spiking dynamics of the major components of the thalamocortical loop during absence seizures. However, the pathophysiological mechanism of seizure initiation is still under debate and the relative contribution of the different structures to seizure initiation and ictal activity still remains elusive (Blumenfeld, 2005).

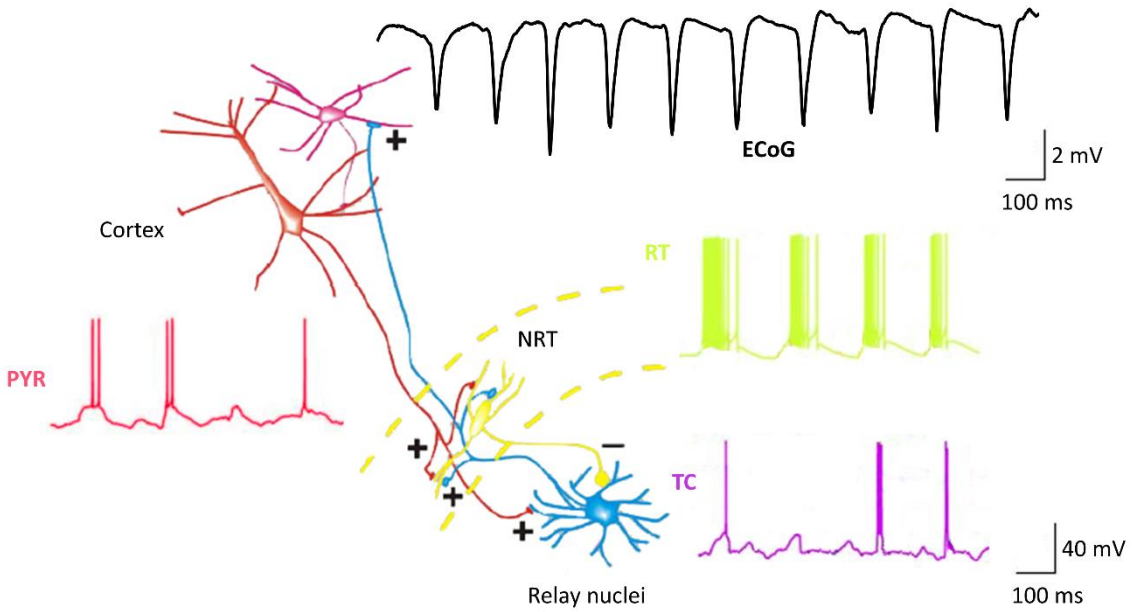

Figure 3. Ictal firing within the thalamocortical loop. *A schematic depiction shows single-unit traces of a cortical pyramidal cell (PY, red), a reticular cell (RT, yellow) and a thalamocortical cell (TC, blue) together with cortical LFP activity of the seizure onset zone. Modified from Huguenard (2019) and McCafferty et al. (2018)*

One main criticism of the current model is that it oversimplifies the thalamocortical circuitry. It treats the thalamic nuclei as a homogeneous mass of identical cells and omits all but the pyramidal cells of the hyperexcitable core in the cortex. Very little to nothing is known about the way how cortical microcircuitry participates in seizure activity, particularly the role of cortical interneurons remains to be elucidated. Furthermore, fully developed absence seizures are generalized, thus the contribution of non-focus areas should not be neglected.

Channel and receptor deficiencies (Crunelli and Leresche, 2002) – which are thought to be the underlying cause of the seizures – might not explain all the features of absence epilepsy and the co-occurring morbidities. It is plausible to assume, that the cortical microcircuitry might be altered as a consequence of frequent seizing, which affects cortical processing (Studer et al., 2018; Kozak, 2019). 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. Therefore the pre-existing epileptogenic conditions and the seizure induced alterations of the thalamocortical circuitry have yet to be separated, if possible. Beyond giving a mechanistic insight into seizure generation, these investigations can help to

identify possible nodes of the epileptic circuitry as potential pharmacological targets for new treatments.

POSSIBLE TREATMENTS FOR ABSENCE EPILEPSY

Being the most common type of epilepsy of the childhood, absence epilepsy imposes a huge societal burden worldwide. To date, there are some drugs, such as ethosuximide, valproic acid and lamotrigine (Glauser et al., 2010), which can effectively make ~70% of the patients seizure free either in monotherapy or in combination, however, regardless of the intensive efforts to develop new pharmacotherapies, antiepileptic drugs fail to adequately treat approximately one-third of the patients (Kwan et al., 2010; Glauser et al., 2013), and even responsive subjects often suffer from side effects (Walia et al., 2004). Surgical removal of epileptic foci is not an option for patients with pharmaceutically-resistant absence seizures. Various studies have developed methods to implement different intervention strategies in the recent years: i) optical (Krook-Magnuson et al., 2013; Paz et al., 2013; Krook-Magnuson et al., 2014; Krook-Magnuson et al., 2015), ii) intracranial electrical stimulation (Fountas and Smith, 2007), and iii) transcranial electrical stimulation (TES) (Berenyi et al., 2012).

Since optically driven seizure disruption requires the expression of genetically engineered foreign proteins by the targeted cells, there are substantial barriers to its use in the short term for human medical applications. Electrical stimulation techniques are more accessible, 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. Those few studies focusing on longer time scales involve exclusively intracranial electrical stimulation (Fountas and Smith, 2007; Salam et al., 2015; Salam et al., 2016; van Heukelum et al., 2016). This method has the disadvantage of being more invasive compared to the TES and more importantly, the specific targeting of intracranial electrodes requires the clear initial identification of a small number of key seizure choke points (Vercueil et al., 1998; Feddersen et al., 2007; Nelson et al., 2011; Blik, 2015; Paz and Huguenard, 2015; Sorokin et al., 2016b).

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, as this treatment effect was reported only on short timescales so far and it has been suggested that electrical treatments might be ineffective over longer timescales due to habituation (Vercueil et al., 1998; Feddersen et al., 2007; Blik, 2015).

RAT MODELS OF SPONTANEOUS ABSENCE EPILEPSY

Genetic models of absence epilepsy (Genetic absence epilepsy rats of Strasbourg, GAERS; Wistar Albino Glaxo rats from Rijswijk, Wag/Rij) (Marescaux et al., 1992; Coenen and Van Luijtelaar, 2003), which are inbred strains from the Wistar rats, can express spontaneous spike-and-wave discharges and these animals also show the behavioral correlates of absence seizures like sudden behavioral arrest, vibrissal twitches and facial myoclonus. Importantly, some study confirmed that genetic strains also show similar pharmacological responses to anti-epileptic treatment as human patients (Coenen et al., 1992; Danober et al., 1998), making them useful models of epilepsy research, with an emphasis on testing new pharmaceutics for absence epilepsy.

Beside the inbred genetic models, it is known that Long-Evans rats can express spike-and-wave discharges with similarity to absence seizures on the cellular (Polack and Charpier, 2006), behavioral (Semba et al., 1980) and pharmacological (Shaw, 2004) levels. Thus Long-Evans rats meet all the criteria for being an absence model like GAERS and Wag/Rij.

Interestingly, the WAG/Rij, GAERS and Long-Evans strains show a different age characteristics regarding seizure development (Coenen and Van Luijtelaar, 1987; Shaw, 2007; Jarre et al., 2017). In genetic rodent models, absence epilepsy possess an early age-related quick onset with persistent ictal activity during the adulthood (Stafstrom, 2014; Jarre et al., 2017). 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. Although the persisting incidence of seizures in rat models are in contrast with those observed in human, where thalamocortical epilepsy disappears in the majority of the cases by the adulthood, understanding what makes the rodents progressively more susceptible to seizures may be a very valuable tool to understand how seizures develop in human.

4 AIMS OF THE STUDY

The aims of the present study were to examine and evaluate the development of absence seizures within the thalamocortical circuitry in 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.

5 MATERIALS AND METHODS

All experiments were performed in accordance with European Union guidelines (2003/65/CE) and the National Institutes of Health Guidelines for the Care and Use of Animals for Experimental Procedures. The experimental protocols were approved by the Ethical Committee for Animal Research at the Albert Szent-Györgyi Medical and Pharmaceutical Center of the University of Szeged (XIV/218/2016 and XIV/471/2012).

FABRICATION OF THE STIMULATION ELECTRODES

Double stranded, miniature hook-up wires (Phoenix Wire Inc., South Hero, VT, USA), peeled at both ends were introduced through the holes of a strip of the tape packaging of an integrated circuit with SOT-353 case (74HC1G00GW, Nijmegen, Netherlands). Dental cement (Duracryl™ Plus, Spofa Dental, Jičín, Czech Republic) was put on the holes to fix the wires and a thin layer of cyano-acrylic glue (Loctite 401, Henkel, Düsseldorf, Germany) was dropped into the hole to form a watertight sealing towards the porous cement (Kozák and Berényi, 2017; Kozak et al., 2018).

FABRICATION OF THE INTRACORTICAL ELECTRODES

Tripolar electrodes were prepared to record neocortical and hippocampal local field potentials (LFP). Three 50-µm diameter polyimide-insulated tungsten wires (Tungsten 99.95%, California Fine Wire, Grover Beach, CA, USA) were inserted into a 180-µm inner diameter stainless steel tube (Vita Needle Company, Needham, MA, USA), and their tips were spaced 400 µm vertically from each other (Figure 4C-D). Impedances of the wire electrodes varied between 30–90 kΩ at 1 kHz. Bipolar stimulation electrodes for intracortical stimulation experiments consisting of two insulated tungsten wires were inserted into a 180-µm inner diameter stainless steel tube, each peeled at the tip for 200 µm (inter-wire spacing, 0.5 mm). Impedances of the wire electrodes varied between 10–30 kΩ at 1 kHz.

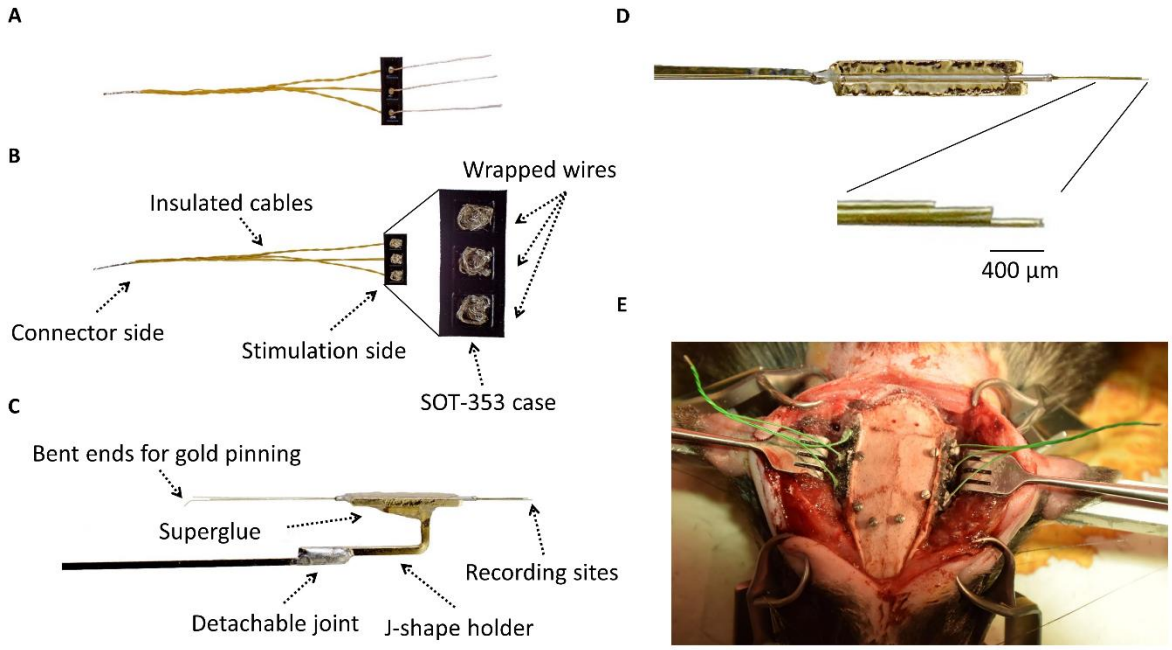

Figure 4. Steps of stimulation and recording electrode fabrication. (A) *Twisted wires stitched through the holes and fixed to the packaging.* **(B)** *Final form of the stimulation electrodes. Inset: wrapped wires inside the packaging.* **(C)** *Side view of the recording electrodes* **(D)** *Top view of the recording electrodes. Inset: tip of the recording sites, 400 µm spacing.* **(E)** *Intraoperative picture of the transcranial stimulation electrode placement. The stimulation electrodes are already in position, together with some of the anchoring screws (Kozak et al., 2018).*

SURGERY

All animals were operated under isoflurane anesthesia and chronically implanted with intracortical recording and intracortical/transcranial stimulating electrodes according to the following procedure: For long term experiments and for pharmacological experiments rats were implanted with multiple tripolar electrodes (Figure 4C-D), which targeted the frontal and parietal cortical areas of both hemispheres and over the right hippocampus. The electrodes were vertically advanced through individual holes in the skull, until the most superficial wire of the electrode reached the surface of the cortex. The holes around the electrodes were filled in with non-conductive silicone (Dow Corning Corporation, Auburn, MI, USA) and the electrodes

were fixed carefully with dental cement (Duracryl™ Plus, Spofa Dental, Jičín, Czech Republic). Rats for seizure interruption experiments were additionally equipped with transcranial stimulating electrodes. Transcranial stimulation electrodes were glued onto the temporal bone bilaterally, using cyano-acrylic glue (Loctite, Henkel, Germany). Pockets were filled with conductive paste (Super Visc, Brain Products, Gilching, Germany) and were electrically isolated with cyanoacrylic glue and dental cement from the tissues (Figure 4E). Unilateral pockets were wired together serving as a single pole (Figure 4B).

Unit recordings and intracortical stimulation experiments were performed in rats divided into two age groups: young (~3 months, n = 5) and adult (~6 months, n=4) animals. Rats were implanted with tripolar electrodes, which targeted the frontal and parietal cortical areas of the left hemisphere and unilaterally the CA1 subfield of the hippocampus and a silicone probe (Neuronexus, Poly2, 32 channels) with a custom-built microdrive (Vandecasteele et al., 2012) allowing for the vertical adjustment during targeting the prefrontal cortex (AP: +2.7 mm from bregma; ML: +1.5 mm, angled at 10° from the sagittal plane) or motor cortex (AP: -2 mm from bregma, ML: +1.5 mm in the sagittal plane) of the right hemisphere. A custom-built bipolar electrode was implanted in the left neocortex (AP: +2 mm; ML: −2 mm; DV: −1.5 mm from the dura (motor area)(Maingret et al., 2016). 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.

ELECTROPHYSIOLOGICAL RECORDINGS AND STIMULATION

All recording sessions took place in the same room in 12h light-dark cycles. The rats were housed individually in plastic cages (42x38 cm, 18 cm tall), the walls were made of clear plexiglas and food and water were given ad libitum. After recovery from the surgery (minimum 3 days), the rats were connected to the recording system. To avoid the twisting and overtension of the cables, a very thin, lightweight recording cable (40 AWG Nylon Kerrigan-Lewis Litz wire, Alpha Wire, Elizabeth, NJ, USA) and a suspended commutator (Adafruit, New York, NY, USA) sliding vertically on guide rails with the help of a counterweighted trolley system were

used. The recorded signals were preamplified, amplified 400×, multiplexed on head and stored after digitalization (KJE-1001, Amplipex, Szeged, Hungary).

SEIZURE INTERRUPTION EXPERIMENTS

The preamplified signals of all parallel recorded rats (up to 8) were analyzed on-line by a programmable digital signal processor (RX-8, Tucker-Davis Technologies, Alachua, FL, USA) using a custom made seizure detection algorithm, as follows. The LFP of pre-selected tripolar electrodes were demultiplexed (one triplet for each rat) in real time, and the current source density (CSD) of those triplets were calculated [(CSD= 2 x intermediate - (deep + superficial electrode))]. The manual selection of the triplet was based on the consideration of the anatomical location of the electrodes, earliest seizure appearance and the signal-to-noise ratio of the triplets. The derived signal was bandpass-filtered, rectified and integrated in a time window of 20 ms (reflecting the monotonicity of the signal within the temporal window). Threshold crossing for both the raw CSD and the integrated signal (coincidence) were monitored. Synchronous multiple threshold crossing (minimum 2, separated by 40–50 ms, regularity) triggered a charge neutral, triphasic single-pulse (100 ms) stimulation (STG4008; Multi Channel Systems, Reutlingen, Germany). The thresholds of the detection algorithm were set for each rat separately and were periodically fine-tuned based on the variance of the signal, when it was necessary. The stimulation was performed either in voltage or current controlled mode, the stimulus intensity was titrated until a reliable seizure-stopping effect was observed (approx. 1.5–2 mA in all animals). The generated intracranial electrical gradient was approximated as the stimulus induced voltage difference measured between multiple recording triplets divided by the intertriplet distance. During the long-term observations, the stimulus intensity was regularly re-adjusted to maintain the same intracranial gradient.

PHARMACOLOGICAL EXPERIMENTS

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. These sessions were repeated three times, with leaving 2 days rest between the consecutive control-treatment sessions to ensure the elimination of ethosuximide between measurements (Bachmann et al., 1988).

SEIZURE INDUCTION

In intracortical stimulation experiments the optimal stimulation voltage was determined in advance for each animal as the minimum voltage (7-15 V) necessary to reliably induce delta waves during non-REM (NREM) sleep. During stimulation a monophasic single-pulse (0.1 ms) was delivered by an isolated stimulator generator (STG4008; Multi Channel Systems, Reutlingen, Germany) to the deep layers of the motor cortex (anode: −1.5 mm from the dura, cathode: -1.0 mm from the dura).During stimulation sessions intracortical pulses were delivered every 10 seconds, with no respect to the behavioral states.

HISTOLOGY

For histological verification of the recording locations and possible pathologic changes, i.e. stimulus induced gliosis, animals were deeply anaesthetized with 1.5 g/kg urethane (i.p.) and transcardially perfused with saline followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer saline. After overnight postfixation, 50-µm thick coronal sections were prepared with vibratome. The sections were then either immunostained for GFAP with DAPI counterstaining (Millipore Cat# MAB360, RRID:AB_2109815), or subjected to cresyl violet staining with standard histological techniques (Takeuchi et al., 2014). GFAP signaling were examined with a Zeiss laser scanning microscopy (LSM880) from sections every 1 mm throughout the entire anterior–posterior extent of the cerebrum.

OFF-LINE ANALYSIS

Off-line detection of the epileptic activity was performed in a similar way to the online method. SWD episodes were detected from the off-line calculated CSD signals. The LFP was band-pass filtered with a 4th order zero phase lag Butterworth filter between 8 and 12 Hz (low BW) and 30 and 200 Hz (high BW), and the peaks of the spike components were detected if

the high BW signal and the low BW signal conjunctively exceeded +5 standard deviation of their corresponding baseline activity. The results of the automated algorithm were manually revised and the threshold of the spike detection was manually re-adjusted in cases when the automatic detection generated false positives or missed events. Consecutive SWD episodes were merged if they followed each other within 1 second. For aging experiment, one randomly chosen day's recording of each week was included. For delta wave and sleep spindle detections, we used only non-theta epochs. Non-theta epochs were detected automatically using the ratio of the FFT power in theta band (5-11 Hz) to the power of nearby bands (1-4 Hz, 12-14 Hz) of hippocampal LFP. Threshold was manually adjusted for all animals. To detect global delta waves, the LFPs recorded bilaterally in the somatosensory were filtered (0–6 Hz) and Z-scored. Zero-crossings of the first temporal derivatives were calculated. Consecutive upward-downward-upward zero-crossings 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 (Maingret et al., 2016). Segmentation of the recording sessions into slow-wave sleep (SWS), transition to SWS, transition from SWS and awake/REM was performed as follows. Recordings were segmented into 1 min long epochs. Those epochs, in which delta wave occurrence rate exceeded 10/min, were considered as SWS. SWS epochs separated by less than 1 min, were merged. The first minutes of SWS with the previous non-SWS epochs were labeled as 'Transition to SWS'. Similarly we identified the last minutes of SWS sessions and the first minutes of non-SWS as 'Transition from SWS'. Remaining epochs were labeled as 'Awake/REM'. Spindle detection was performed as follows. The LFP recorded in the somatosensory cortex was band-pass 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, and combined events lasting more than 3 s were discarded (Maingret et al., 2016). The events induced by intracortical stimulation were classified manually. Continuous wavelet spectra and power spectra were calculated in Matlab using Wavelet Toolbox and Chronux Toolbox (http://chronux.org/), respectively.

The spike sorting was performed using Kilosort with its default settings and were manually curated in Phy (Rossant et al., 2016). Putative interneurons and pyramidal cells were discriminated based on their spike widths and autocorrelograms. For all individual units,

spiking activity in a [-88, +88] ms window centered around the peak of spike-and-wave discharges were collected and firing rate histograms (0.8 ms time bin) were constructed. The firing rate histograms were Z-scored for each unit individually and smoothed with a Gaussian filter. Raster plots were constructed with all the smoothed Z-scored histograms of individual units.

STATISTICAL ANALYSIS

All statistical analyses were performed in MATLAB (Mathworks, Natwick, MA). No statistical methods were used to pre-determine sample sizes, but the number of animal and recorded cells were similar to those employed in previous works. All tests were two-tailed. Non-parametric Wilcoxon's signed rank test, Kruskal-Wallis one-way analysis of variance, repeated measures analysis of variance, Kolmogorov-Smirnov-test, Rayleigh's tests were used. To investigate the spindle characteristics' change over time, from each analyzed recording sessions we randomly sampled 500 events of each animal and applied repeated measures ANOVA. To perform similar analysis on seizures, we randomly sampled 200 seizure events in each animal. Box-plots represent median and 25^{th}, 75^{th} percentiles and their whiskers the data range. Outlier values are not displayed on the figures, but they were always included in the statistical analysis. Linear regressions were performed using robust-linear regression and p-values for linear regressions tests the hypothesis that true correlation exists against a null-correlation. Modulation strength was calculated using mean resultant length of the phases (peaks of spike component of SWDs are at 180°) and units were considered to have phase-locking to SWDs when $\alpha < 0.05$ and $\kappa > 0.1$ on Rayleigh's test of non-uniformity using the circular statistics toolbox provided by P. Berens (Berens, 2009).

6 RESULTS

REAL-TIME DETECTION AND INTERRUPTION OF EPILEPTIC SEIZURES

We developed an unsupervised closed-loop intervention system, which is capable of monitoring brain activity and automatically terminates epileptic seizures for durations comparable to the life expectancy of the animals (Figure 5A, Methods). In addition, we designed and built a cage system that allowed us to record electrophysiological signals 0-24 h for months without disconnecting the animals during the replacement of bedding and cages. 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 (Figure 5B, Methods).

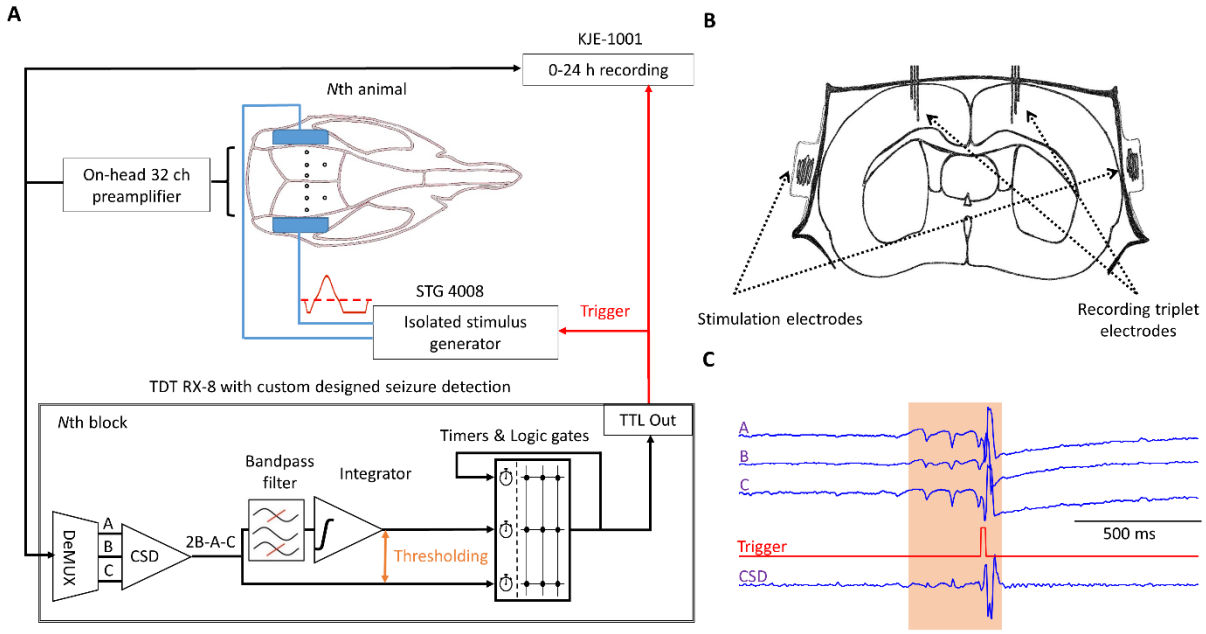

Figure 5. Overview of the closed loop system. (A) *Cortical LFP from every rat (up to 8 simultaneously, max. 32 channel each) is amplified and multiplexed on-head, and fed to the digital signal processor (TDT RX-8) running a custom designed seizure detection algorithm. The signals were first digitally demultiplexed and CSD of one preselected tripolar electrode was calculated in each rat. Pre-set threshold crossings of the raw CSD and the bandpass filtered, integrated CSD was detected and in case of fulfilling the predetermined seizure criteria (coincidence, regularity) a single isolated, triphasic, charge neutral stimulus pulse was*

delivered. All LFP signals and the on-line seizure triggers were continuously recorded for every rat. **(B)** *Horizontal schematics shows triplet recording electrodes implanted in the parietal cortex and stimulation electrodes placed bitemporally on the skull.* **(C)** *Representative LFP traces of a preselected tripolar electrode and the corresponding CSD during a spontaneously emerging interrupted spike-and-wave seizure.*

Intracortical recording electrodes and transcranial stimulating electrodes were implanted in Long-Evans rats (n = 13). All animals were showing the electrophysiological and behavioral symptoms of absence epilepsy. Seizures were detected based on specific ECoG parameters (Methods) and after each single detected SWD-event a charge-balanced, triphasic stimulus pulse was applied transcranially to interrupt the ictal activity (Figure 5C).

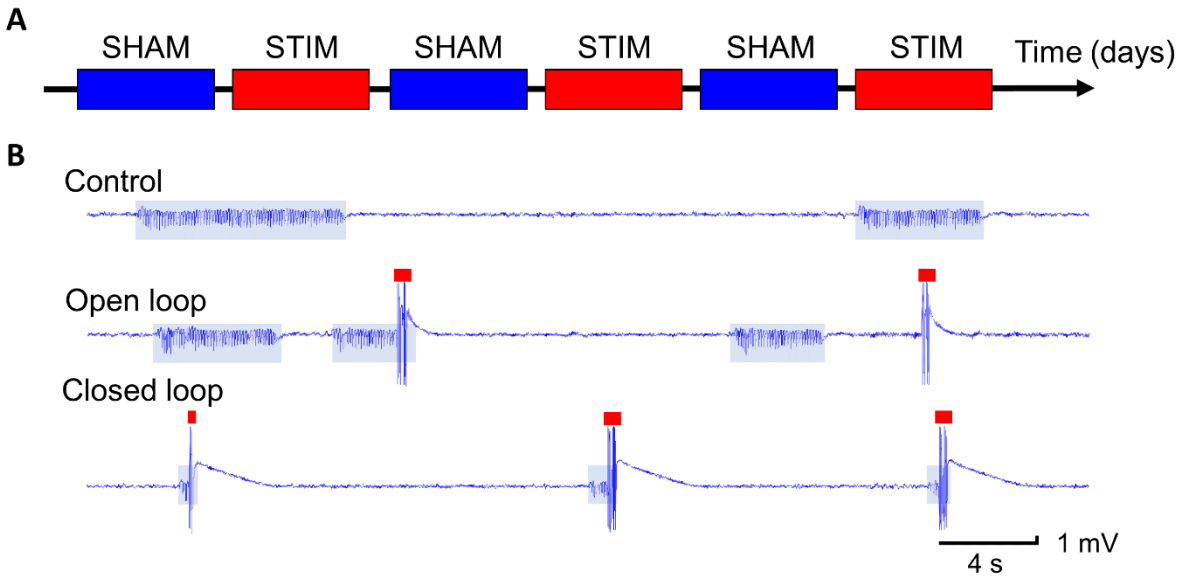

Figure 6. Experimental protocol for short-term seizure intervention. (A) *Experimental timeline shows a day-by-day alternation of sham and stimulated sessions. This pattern was repeated for 6 days.* **(B)** *Representative examples of LFP traces for the sham, pseudorandom and closed-loop seizure interruption protocols. Shaded bars and red ticks indicate the identified seizures and stimuli, respectively.*

First, for validation purposes we demonstrated the short-term (day-long) effects of our seizure intervention system. Animals were divided randomly into three groups: control (n = 3), open-loop stimulated (n = 5) and closed-loop stimulated (n = 5) rats. 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 (SHAM) was followed by a stimulated day (STIM) (Figure 6A-B). The stimulation timing of every open-loop treated animal was driven by the stimulation timing of a randomly chosen closed-loop treated animal (known as "yoked" stimulation).

The overall time spent in seizure per day (Time in Seizure - TiS) only changed significantly in the closed loop stimulated group (5 of 5 animals showed a significant decrease, $p < 0.05$, paired two sample t test, STIM TiS was 33.0 ± 13.2% of SHAM TiS on the group level, $p < 0.01$, paired two sample t-test), confirming the effectiveness of the on-demand treatment on the short timescale (Figure 7A). Furthermore, the stimulation significantly shortened the duration of the seizures in the closed-loop group (5 of 5 animals showed a significant decrease, $p < 0.05$, Wilcoxon rank sum test, STIM seizure durations were 32.1 ± 16.1% of SHAM seizure durations, $p < 0.01$, Wilcoxon rank sum test), but had no effect in the other group (Figure 7B). These observations suggest that the timing of the TES is a critical factor in achieving an effective seizure control.

Stimulation, either open- or closed-loop protocols, had a variable effect on the occurrence rate of seizures, but resulted no significant change on the group level (significant decrease in one of the open-loop treated animals (n = 5 rats) and significant increase in two of the open-loop and two of the closed-loop treated animals (n = 5 rats), $p < 0.05$, paired two sample t-test, no change on the group level, $p = 0.2494$ for open loop and $p = 0.5418$ for closed loop animals, Figure 7C), pointing out the likeliness of a rebound effect after stimulation.

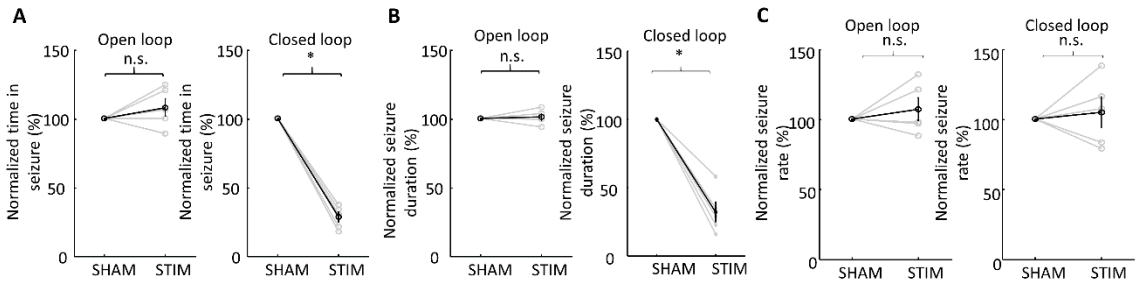

Figure 7. Results of the short-term intervention protocol. (A) *Time in seizure,* **(B)** *Average seizure duration and* **(C)** *Seizure rates during the SHAM and STIM days (normalized to the average seizure rate during the SHAM days for each animal). Grey lines denote individual animals, while black lines represent the population data. Error bars represent SEM.*

There was a marked, but not significant increase in the TiS for some open loop animals, but the mean seizure duration did not change in this group (Figure 7B). These results raise the possibility that the randomly delivered stimulation may induce seizures, but it does not prolong them. Furthermore, considering the higher seizure rate that can be observed in some animals with the open-loop and closed-loop stimulation (Figure 8), we tested if the TES exerts a pro-convulsive effect.

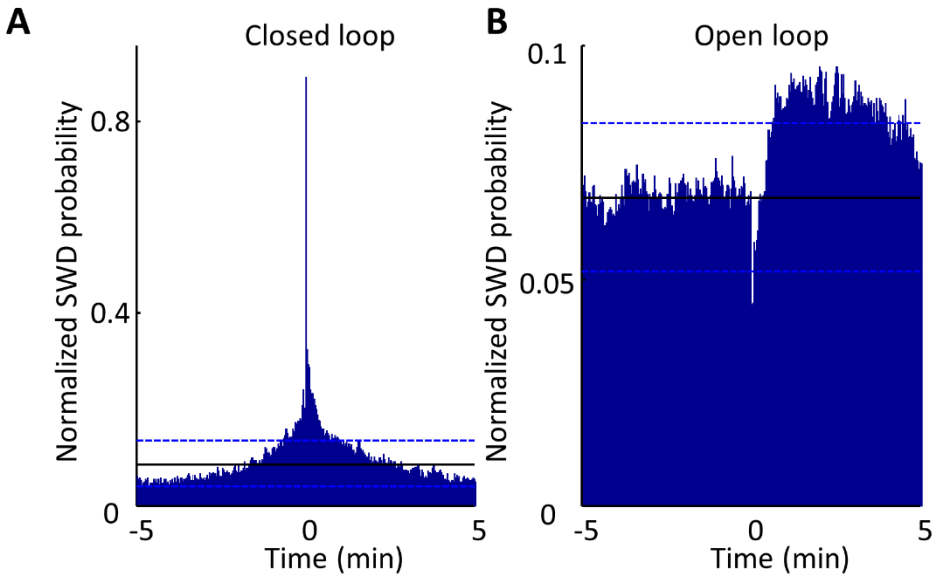

Figure 8. Possible pro-convulsive effect of stimulation. *Peri-stimulus time histogram of the SWDs of a* **(A)** *closed-loop and an* **(B)** *open-loop stimulated rat across all the stimulated days. Black lines denote the average SW occurrence rate along the whole recording period and dashed blue lines refer to the confidence intervals (95%).*

The peri-stimulus time-histogram of the SWs in the open loop animals revealed that following the random transcranial stimuli, the probability of SW occurrence is substantially lower than the baseline for approximately 30 seconds, but later the probability raises above the mean occurrence probability and can be even become significantly higher. In contrast to this, in case of the closed loop stimulation, the SW occurrence rate remains significantly higher following the first stimulus approximately for a minute, reflecting that the first stimulus cannot always stop the seizure activity or seizures may quickly recur in cases of incomplete termination and importantly, closed loop seizure intervention found to have no proconvulsive effect. These

findings suggest that there is no large direct seizure inducing effect of the stimulation and emphasize the crucial importance of the timing of the stimulation.

LONG-TERM CLOSED LOOP SEIZURE CONTROL

Next, we investigated whether long-term application of the closed-loop TES modifies the occurrence of the spontaneous seizures. As the open-loop stimulation did not have the capacity to improve seizure activity during the short stimulation sessions, we only investigated further the effects of closed-loop stimulations. Closed-loop stimulated (n = 5) and control (n = 4) rats were monitored for at least an additional 8 weeks continuously. The control rats did not receive any treatment during the observations. In case of the closed-loop stimulated rats the first week of the observation served as a control period (Pre-Treatment), then for 6 weeks the animals were stimulated in a closed-loop fashion (Treatment). After finishing the treatment, the rats were observed at least for one more week (Post-Treatment, Figure 9A). Additionally, in one animal we continued the closed-loop treatment after the 8 week long experimental session for three more months with a week-long control session at the end.

The control group (n = 4) did not show any significant change in terms of the TiS (one-way ANOVA, $F(7,209) = 0.95$, $p = 0.4665$, not shown), in accordance with previous findings (Shaw, 2007). We found that during the treatment all the animals (n = 5) spent significantly less time in seizure (38.8 ± 20.2%, mean across treatment weeks, one-way ANOVA, $F(7,264) = 72.47$, $p < 0.001$, Figure 9B), and the duration of the seizures significantly decreased (33.8 ± 17.8%, mean across treatment weeks, Kruskal-Wallis test, group $\chi 2 = 97.86$, $p < 0.001$, Figure 9C). These effects were immediate and did not deteriorate over the time course of treatment (TiS = 41.8 ± 19.3% vs 41.9 ± 15.3%, $p = 1.000$; Normalized seizure duration = 33.4 ± 16.8% vs 38.6 ± 15.8%, $p = 0.9932$, for Week 1 vs Week 6, respectively, *post-hoc* Tukey HSD), and returned back to the Pre-Treatment level as the treatment was suspended (Post-Treatment TiS = 99.1 ± 20.4% of the Pre-Treatment TiS, $p = 1.000$; normalized Post-Treatment seizure duration = 111.3 ± 24.3% of the Pre-Treatment seizure duration, $p = 0.5814$, *post-hoc* Tukey HSD). The seizure rate was significantly increased in all animals during the treatment (133 ± 16.8% of the Pre-Treatment seizure rate, one-way ANOVA, $F(2,9) = 12.07$, $p < 0.05$, Figure 9E, see Discussion on page 37-38) and returned to the original level during the Post-Treatment period in 4 of 5

animals. One animal maintained a significantly lower seizure rate even after the treatment (75.86 ± 32.93% of the Pre-Treatment, $p < 0.05$, post-hoc Tukey HSD). Importantly, we did not observe any overt, major behavioral change (alteration in nest building, rearing, sleep-awake cycle) or traces of glial remodeling due to the treatment (Figure 9F), which is in accordance with the similarity of Pre- and Post-Treatment seizure parameters.

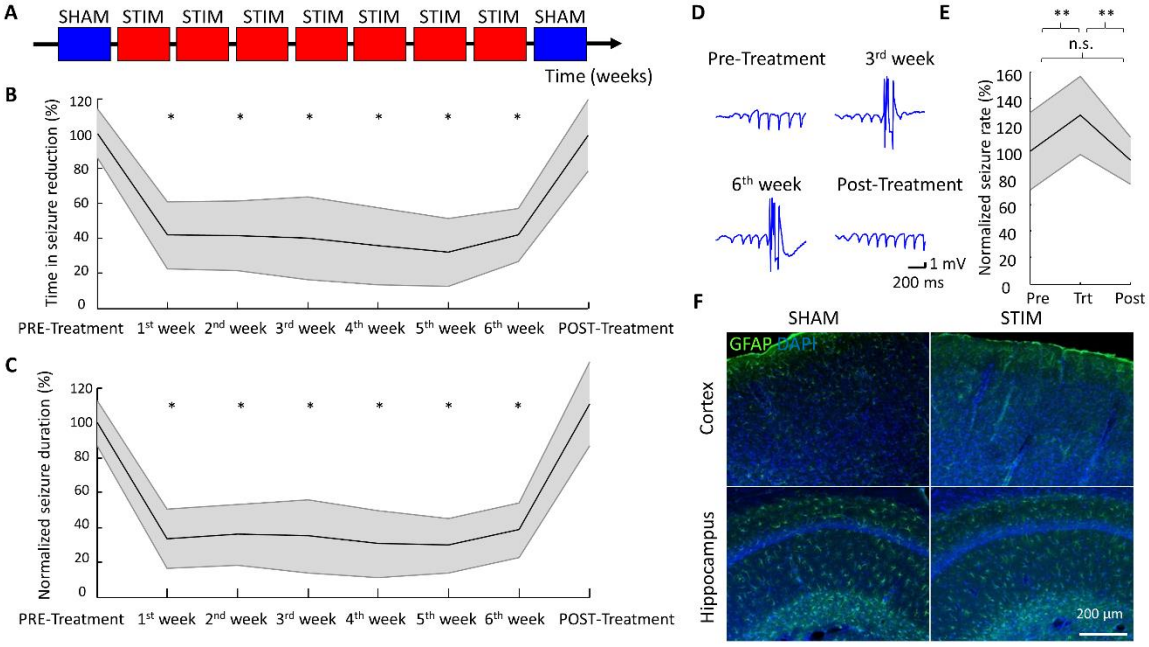

Figure 9. Results of the seizure suppression during the long-term stimulation protocol. **(A)** *Experimental timeline. Group data show the stable and similar decrease of* **(B)** *Time in Seizure and* **(C)** *average seizure duration as long as the closed-loop seizure suppression is on (each measure is normalized to its corresponding mean Pre-Treatment value for each animal).* **(D)** *Example LFP traces of one representative rat demonstrating the recording quality over the weeks. Note that the amplitudes and signal to noise ratios are qualitatively similar, suggesting a negligible change of the electrode conductance.* **(E)** *Group data show seizure rates before (Pre), during (Trt) and after (Post) the treatment (normalized to the average seizure rates during the Pre days for each animal). Shaded area represents ±SEM.* **(F)** *Representative histological examples of cortical and hippocampal regions of control (SHAM) and long term treated (STIM) animals, stained for GFAP (green) and DAPI (blue). Note that stimulation did not induce overt gross histological changes (i.e. gliosis) despite the long-term application. *p < 0.001, **p < 0.05 vs Pre-Treatment, one-way ANOVA (B,E), Kruskal-Wallis test (C), both followed by post hoc HSD.*

The prolonged closed-loop treatment (4 months) also resulted in qualitatively similar results, suggesting that lifelong TES treatment of epilepsy is possible, since the efficacy of the seizure suppression was maintained (Figure 10A-B). After the termination of this four month long treatment, we observed that both TiS (133.1% ± 22.2% and 137.3% ± 21.2% of the Pre-Treatment TiS, for the control and the treated animal, respectively) and normalized seizure duration were substantially increased (138.8% ± 10.7% and 209.6% ± 23.5% of the Pre-Treatment seizure duration, for the control and the treated animal, respectively) in accordance with the trends observed in the control animal. This observation further supports that long-term closed loop treatment is possible in terms of the efficacy of the early termination of the seizures, but it may have no effect on the natural progression of the disease.

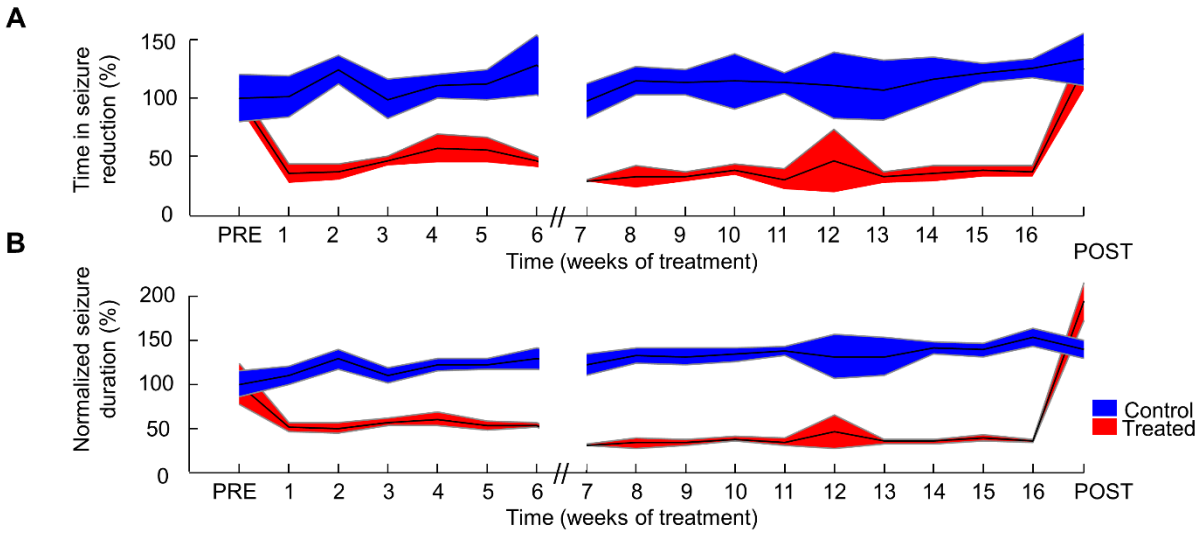

Figure 10. Sustained efficacy of the seizure suppression during the four-month long stimulation. *Results of an extended 4 months long treatment are similar to those obtained during the 6 weeks protocol, both for the* **(A)** *Time in Seizure and* **(B)** *average seizure duration. Shaded area represents ±SD. (Control period at the end of the 6 week treatment is removed)*

ABSENCE SEIZURE DEVELOPMENT

In an other set of experiments, we performed a longitudinal study in Long-Evans male rats using multisite recording electrodes in multiple cortical areas to determine the evolution of spontaneous seizures and the related co-occurring alterations of sleep architecture. First, we

determined how seizure incidence is influenced by maturation. We found that the total time spent in seizures progressively increased during the observation period and saturated around 5 months (n= 5 animals; ANOVA F(14,56)=13.60; $p < 0.05$; *post hoc* LSD vs 14th week, Figure 11B), which is in accordance with previous observations of Long-Evans rats reporting constant seizure parameters during late adulthood (Shaw, 2007). Seizures were significantly longer in older animals (n = 5 animals; ANOVA F(14,56) = 18.51; $p < 0.05$; *post hoc* LSD vs 14th week, Figure 11C) and mean seizure length showed the same saturation tendency as the total time spent in seizure. Seizure occurrence rate showed an early modest increase that remained steady (n = 5 animals; F(14,56) = 2.67; $p = 0.1773$, Figure 11D). The temporal distribution of the seizures also changed with age. In mature animals the median inter-event interval was shorter (Kolmogorov-Smirnov, Inter-event interval: $p < 0.001$, Figure 11E). This result suggests that in well-developed absence epilepsy bursts of long seizures can be observed, which accounts for most of the time spent in seizures (Kolmogorov-Smirnov, Distribution of seizures, $p < 0.001$, Figure 11F). We refer to ~14 weeks old animals as 'juvenile', while ages above 20 weeks were considered as ‘mature’ from here on.

Investigating the relationship between sleep spindles and SWDs, we found that sleep spindles and spike-and-wave discharges’ duration and amplitude distributions were overlapping in juvenile animals, but they became distinct in older ones (Figure 12A-B). The peak spindle frequency did not change with age (data not shown, n = 5 animals; ANOVA $F(14,34930) = 2.37$; $p = 0.12$), however, a substantial fall of spindle occurrence rate (n = 5 animals; ANOVA F(14,56) = 7.10; $p = 0.0562$, *post hoc* LSD vs 14th week, Figure 12C) and spindle duration (n = 5 animals; ANOVA F(14,34930) = 14.94; $p < 0.001$, *post hoc* Tukey vs 14th week, Figure 12D) was observed. The increase of total time spent in seizures, indicative of the progression of the disease, showed strong inverse correlation with the decline of spindle incidence rate that raises the possibility of a causal relationship ($R^2 = 0.73$; $p < 0.001$; t = -13.65, Figure 12E). In order to clarify this, we investigated the effect of ethosuximide (ETX), the drug of first choice in absence epilepsy, on sleep spindle occurrence (Figure 12F). A single high dose injection of ETX resulted in a prompt seizure suppression (0-120 min), which was accompanied with a reduction in sleep spindle occurrence, too. 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. To further investigate,

whether this increased spindle occurrence rate was only the mechanistic consequence of the increased seizure-free time of the animals, we analysed our short term on-demand transcranial electrical stimulation experiments. We found, that although transcranial electrical stimulation was effective in quickly terminating the seizures, it did not decrease their incidence, and did not influence positively sleep quality by leaving sleep spindle occurrence rate unchanged (Figure 12G).

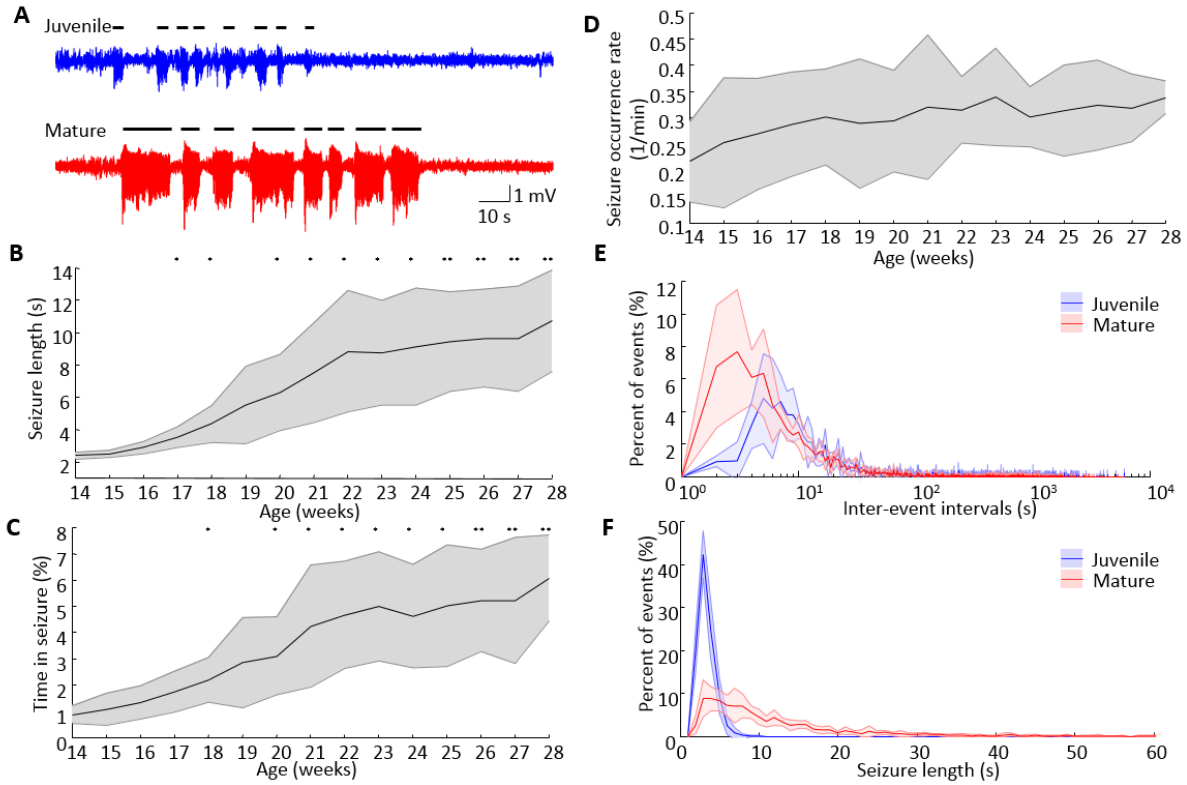

Figure 11. Seizure frequency and duration increase with maturation. (A) *Representative LFP traces of seizure activity at ages of 3 months (blue) and 6 months (red). Black ticks label individual seizures. Group data shows an increasing average seizure duration* **(B)**, *an increasing time spent in seizure with age* **(C)**, *expressed as the percentage of the total observation time and an increasing seizure rate* **(D)** *over the observation period. Shaded area represent ±SD. Population data shows distribution of inter-event intervals* **(E)** *and seizure length* **(F)** *at 3 months (blue) and at 6 months (red), shaded area represents ±SD. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$*

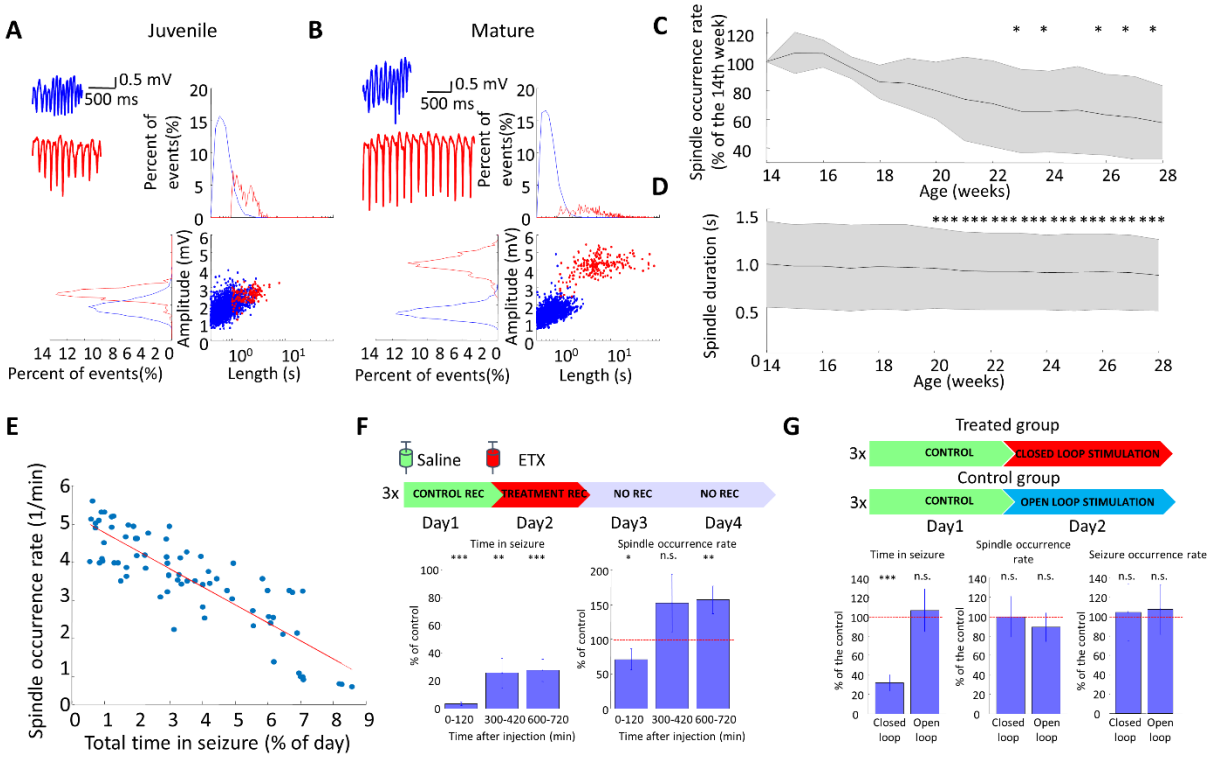

Figure 12. Relationship between sleep spindles and SWDs. (A and B) *Sleep spindle (blue) and seizure (red) characteristics in a representative animal at 3 months and at 6 months, with example sleep spindles and spike-and-wave episodes (upper left panel).* **(C and D)** *Population data shows the decrease of sleep spindle duration and sleep spindle incidence normalized to the first week of observation.* **(E)** *Regression of the total time spent in seizure and sleep spindle occurrence rate, red line corresponds to best linear fit.* **(F)** *The effect of ethosuximide treatment on time spent in seizures (left) and on sleep spindle occurrence rate (right), expressed as the percentage of the control days' values (Wilcoxon's signed rank test, control vs treatment).* **(G)** *The effect of closed loop and open loop transcranial electrical stimulation on time spent in seizures, on sleep spindle occurrence rate and on the seizure occurrence rate expressed an the percentage of the control days' values (Wilcoxon's signed rank test, control vs treatment). $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$*

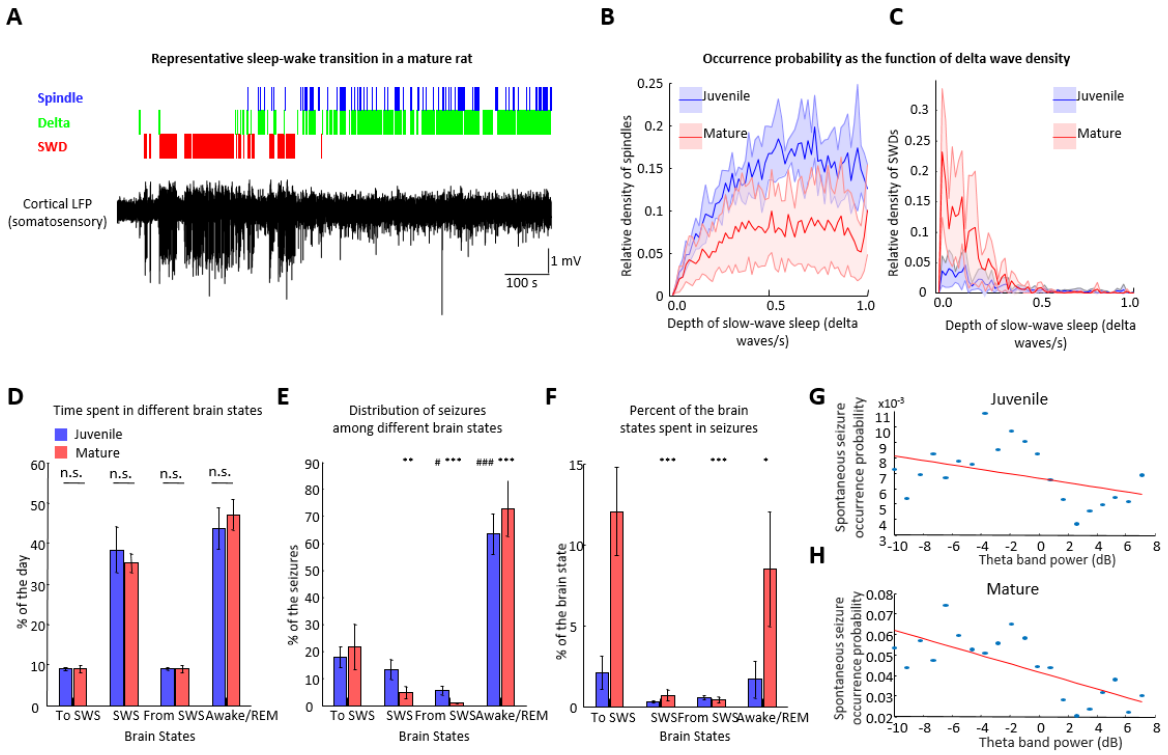

Figure 13. Brain state dependence of seizure susceptibility. (A) *Representative wake-sleep transition of a mature rat. Black trace is the LFP of the somatosensory cortex, colored ticks highlight individual SWDs (red), delta waves (blue), and sleep spindles (green).* **(B and C)** *Relative occurrence of sleep spindles and spike-and-waves as the function of delta wave density at 3 months (blue) and 6 months (red).* **(D)** *Population data shows the time spent in different brain states in juvenile (blue) and mature (red) animals.* **(E)** *Population data shows the distribution of seizures among different brain states (post hoc Tukey-Kramer; vs juvenile To SWS: #$p \leq 0.05$; ###$p \leq 0.001$; vs mature To SWS: **$p \leq 0.01$, ***$p \leq 0.001$).* **(F)** *Population data shows the percent of time spent in seizures in different brain states (post hoc Tukey-Kramer, vs mature To SWS: *$p \leq 0.05$, ***$p \leq 0.001$).* **(G and H)** *Probability of seizure occurrence as the function of pre-seizure hippocampal theta-band activity in juvenile and mature rats. Red lines: best linear fit.*

The incidence of spontaneous sleep spindles and SWDs with respect to the depth of the 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 (Kolmogorov-Smirnov, sleep spindles: $p < 0.001$, seizures: $p < 0.05$, Figure 13A-C). To further

quantify the relationship of absence episodes to the slow-wave sleep, we analysed the brain state dependence of seizure occurrence. Segmenting the recordings into SWS, transition to SWS, transition from SWS (two minutes epochs with one minute SWS and one minute awake/REM period) and the rest as awake/REM sleep (Methods, Figure 13D-F). According to our results, animals' sleeping time and their active period did not change with maturation (two-way ANOVA, interaction between age and brain state, $F(3,32) = 1.9$; $p = 0.1492$; Figure 13D). We found that the seizure occurrence showed a substantial asymmetry regarding SWS (two-way ANOVA, interaction between age and brain state, $F(3,32) = 4.78$, $p < 0.01$; Figure 13E). 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 (two-way ANOVA, interaction between age and brain state, $F(3,32) = 21.78$, $p < 0.001$; Figure 13F). We additionally investigated the seizure occurrence probability as the function of hippocampal theta power (Juvenile: $R = 0.1746$; $p = 0.0944$; $t = -1.7656$; Mature: $R = 0.5189$; $p < 0.001$; $t = -4.0085$; Figure 13G-H), and found that even those seizures which occurred during awake episodes, were more likely to occur when the animal was not active, in accordance with previous findings (Marescaux et al., 1992; Steriade et al., 1993; Shaw, 2004).

Traditionally, absence epilepsy was considered as an instantaneously generalizing, global seizure type, but to date there is an increasing body of evidence suggesting that these seizures emerge locally from a cortical focus located in the somatosensory cortex (Meeren et al., 2002; Polack et al., 2007; Crunelli et al., 2011). So far most studies have investigated only the putative initiation zone of the seizures (Polack et al., 2007; Williams et al., 2016; Jarre et al., 2017), but have not clarified how other cortical areas are invaded or being surpassed by the ictal activity. Therefore, we systematically investigated the effect of seizures emerging in the somatosensory cortex, on the neuronal activity of out-of-focus cortical areas. First, we compared the local field potentials (LFPs) of the motor and the somatosensory cortices of the maturing rats during seizure activity (Figure 14A). The ictal LFP spectra of the different areas revealed that in juvenile animals the somatosensory cortex was already expressing the ~8Hz oscillations at 3 months (Figure 14B-C), characteristic of spike-and-wave discharges. On the other hand, the motor cortex displayed a slower frequency oscillation, gradually increasing its

frequency with maturation to reach the main SWD-frequency (n = 5 animals; ANOVA F(14,41902) = 27.27; $p < 0.001$, post hoc Tukey vs 14th week; Figure 14B-C). Importantly, the bilateral somatosensory cortices showed higher coherence in juvenile animals than the unilateral adjacent structures, but in mature animals seizures became widely coherent with LFP activity locked to the SWD-frequency (n = 5 animals; ANOVA F(14,41902) = 700.46; $p < 0.001$, post hoc Tukey vs 14th week; Figure 14D). An interesting observation is that in juvenile animals the characteristic frequency of the off-focus motor cortex often falls in the range of slow-wave sleep during seizures in the somatosensory cortex and even delta waves are present, which suggests some sort of independence of the out-of-focus areas (Figure 14E).

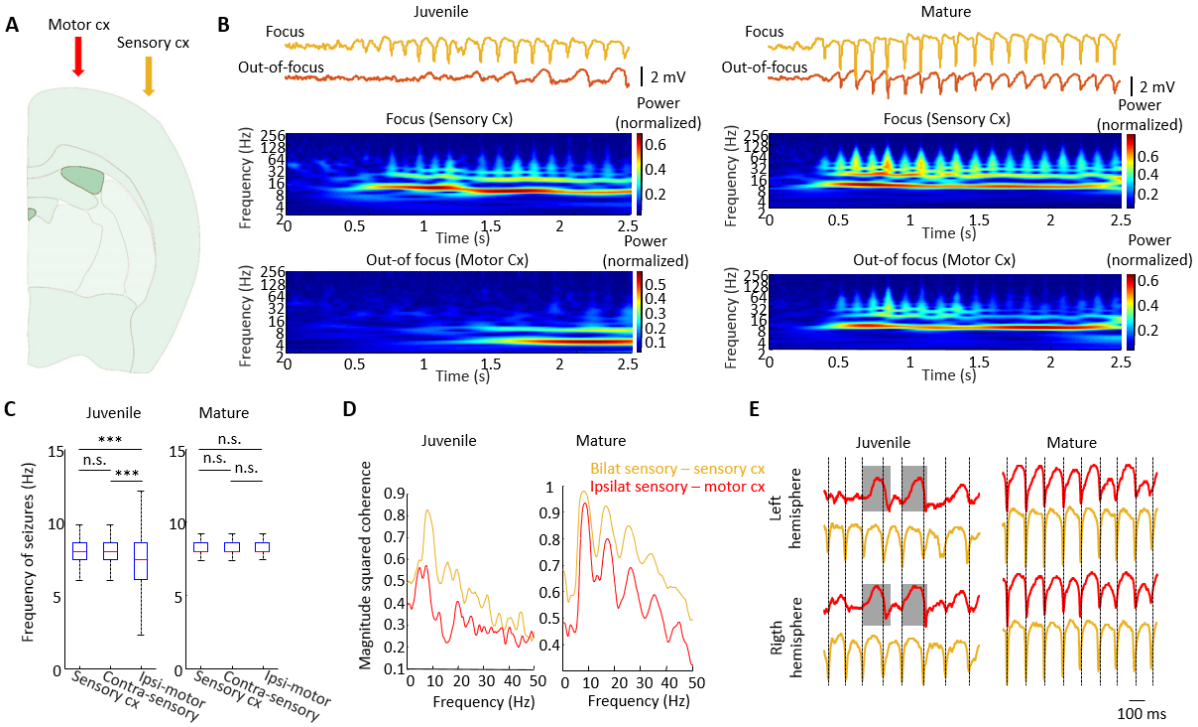

Figure 14. Topographic differences and characteristics of seizures. (A) *Typical arrangement of unilateral recording electrodes in the putative 'initiation zone' (orange) and in the adjacent motor cortex (red).* **(B)** *Representative LFP traces and corresponding wavelet spectrum of typical intraseizure activity of these cortical areas.* **(C)** *Population data shows mean frequency of spike and wave episodes in different cortical recording sites (ipsi- and contralateral somatosensory cortices and ipsilateral motor cortex) at 3 months and 6 months.* **(D)** *Increasing coherence of intraseizure LFP signals in young and adult animals between different cortical recording sites (bilateral somatosensory cortices (orange), ipsilateral somatosensory and motor cortices (red)).* **(E)** *Representative LFP traces (orange –*

somatosensory cortex, red – motor cortex) showing intraseizure dynamics at 3 months and at 6 months in the same animals. Dashed lines refer to the peak of spike-and-wave discharges, delta-wave like activity of motor cortices are highlighted. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$

Next, we investigated how neuronal spiking of motor and prefrontal cortices were related to the spontaneously emerging seizure activity. We compared the firing patterns during seizure emergence by looking at the firing rate of neurons (all neurons, putative excitatory pyramidal cells, putative inhibitory interneurons and inhibitory/excitatory ratio) in a 2 s windows prior and following the seizure onset (Figure 15A). During the preictal periods, the firing rate relationship of the putative excitatory cells (i.e. pyramidal cells) and the putative inhibitory cells (i.e. interneurons) did not change with maturation (firing rates of 118 vs 318 pyramidal cells, $Z = 1.05$, $p = 0.30$; 77 vs 113 interneurons' firing rate, $Z = 1.69$, $p = 0.09$, from young and old animals, respectively, Wilcoxon's signed rank test), suggesting no gross change in the overall inhibitory/excitatory balance under non-seizure conditions. During the seizures though, the firing rate of pyramidal cells did not change, while interneurons in mature animals were less active compared to the pre-seizure baseline activity, causing the inhibitory/excitatory balance to shift towards excitation (Wilcoxon signed rank test, preSeiz vs Seiz, young: 3 animals, 126 events, N = 118 pyramidal cell, N = 77 interneuron; total p = 0.3136; Z = 1.01, excitatory p = 0.4542; Z = 0.75, inhibitory p = 0.6986; Z = 0.39, inhibitory/excitatory p = 0.82485; Z = -0.22; old: 4 animals, 215 events; N = 318 pyramidal cells, N = 113 interneuron, total $p < 0.05$; Z = -2.26, excitatory p = 0.9832; Z = 0.02, inhibitory $p < 0.001$; Z = -3.98, inhibitory/excitatory $p < 0.001$; Z = -5.93; Figure 15B). Furthermore, we found that 53.3% (42.7 % of interneurons and 59.4 % of pyramidal cells) of units in juvenile and 88 % (89.6 % of interneurons and 87.3 % of pyramidal cells) of mature animals' units show phase locking to ongoing seizure activity at different phases of SWDs (Kruskal Wallis one-way analysis of variance, *post hoc* Wilcoxon signed rank test, young: n = 3 animals, N = 115/225 (82/143 pyramidal cell, 33/82 interneuron), old: n = 4 animals, N = 351/408 (252/293 pyramidal cell, 99/115 interneuron; phase preference: $F(3,462) = 50.64$ $p < 0.001$; Figure 15C-D). Interestingly, the preferred phase showed no cell-specific difference, but the pyramidal cells had significantly higher coupling strength during SWDs (mean resultant length: $F(3,462) = 72.57$ $p < 0.001$**;** Figure 15D). We next asked whether cells were entrained to seizure activity on a cycle-by-cycle basis or the LFP pattern of spike-

and-wave discharges was more likely an emergent network property with loosely coupled unit activity, as it was reported in the thalamus (Buzsaki, 1991; Steriade and Contreras, 1995; McCafferty et al., 2018). Indeed, most cells were only firing in ~25% of the total SWD cycles (Wilcoxon signed rank test, young (N = 225 units) vs old (N = 408 units) Z = 8.30; $p < 0.001$**;** Figure 15E), suggesting that once an absence seizure emerged from the cortical focus it can spread and generalize via thalamic and cortico-cortical connections. However, since thalamocortical cells are not entrained in every cycle of the ictal activity (McCafferty et al., 2018), they do not provide thalamic feedback to their cortical counterparts on a cycle-to-cycle basis, thus most of the cortical cells are not driven at each discharge.

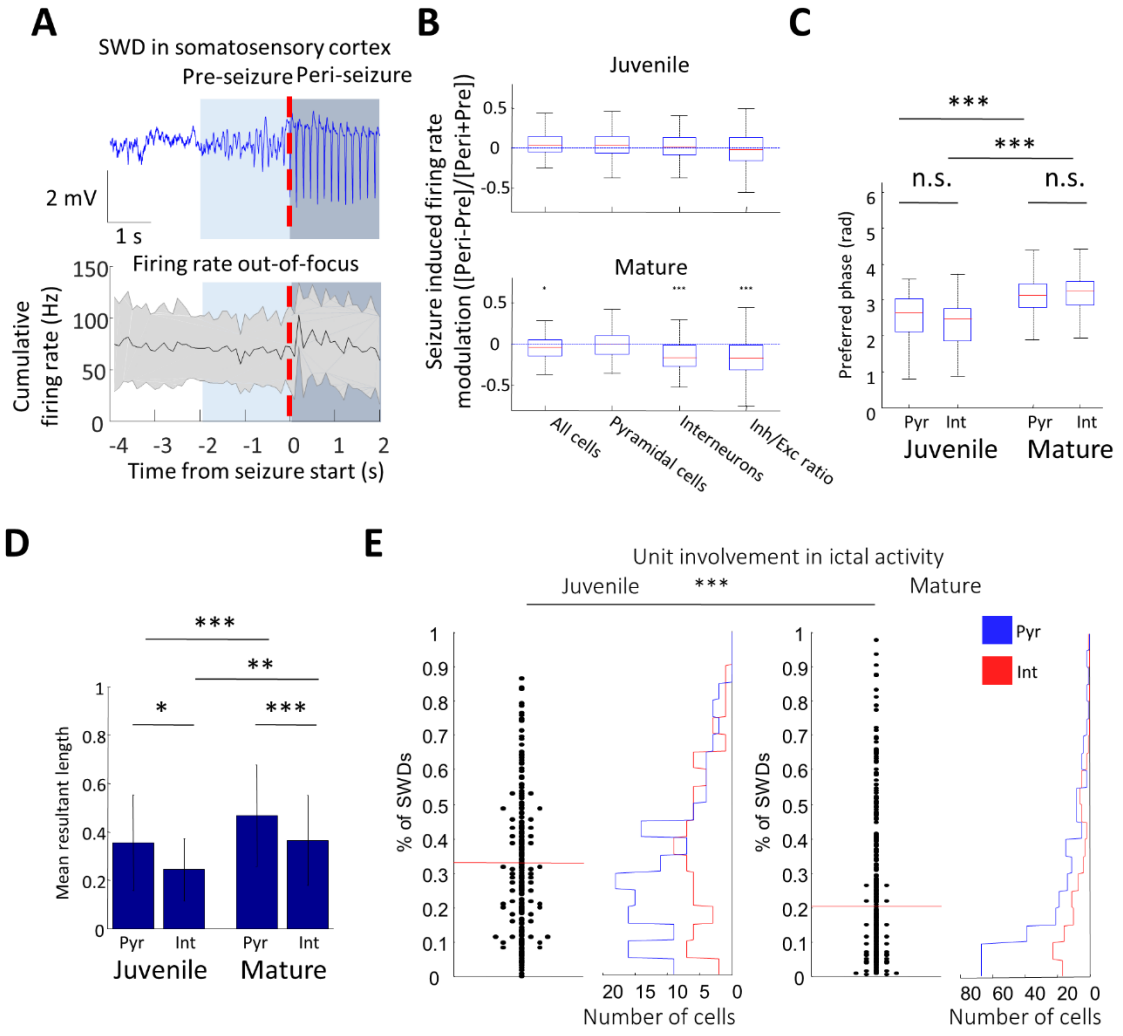

Figure 15. Firing dynamics of single units around seizures. (A) *LFP trace of somatosensory cortex and total firing rate of motor cortex around seizure initiation.* **(B)** *Boxplot of firing rate ratios of units (all cells, pyramidal cells, interneurons and inhibitory/excitatory ratio) prior to*

and following the seizure onset ([Peri-Pre] / [Peri+Pre]). **(C and D)** *Phase preference and coupling strength of unit firing around SWDs for modulated interneurons and pyramidal cells,* **(E)** *Unit involvement in ictal activity at each SWDs are plotted as percentage of SWDs in which they fire for each unit (left) and as the distribution of cell type specific contribution (right, blue – pyramidal, red - interneuron). Red line represents mean.* $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$

Furthermore, to investigate seizure susceptibility, we tested how single pulse intracortical electrical stimulation can influence brain activity. It is known that short single pulse stimulation during NREM sleep can induce global delta waves, which are identical to the spontaneously emerging ones (Vyazovskiy et al., 2009; Maingret et al., 2016). Mechanistically, such electrical stimulation causes the simultaneous depolarization of some pyramidal cells, which in turn can entrain distant cortical regions into sleep oscillation via cortico-cortical connections (Vyazovskiy et al., 2009). Given that single pulse stimulation has the potential to elicit global sleep patterns, we asked if it is also sufficient to switch the brain into seizure activity. After determining the minimal intensity required to induce delta waves during slow wave sleep (Methods), we applied the same intensity stimulation during each brain state. Indeed, we were able to induce delta waves successfully as reflected by their characteristic LFP and unit-firing patterns (Figure 16A). Interestingly, both juvenile and mature animals' somatosensory cortices could respond with spike-and-wave activity to the stimulation, although juvenile animals' ictal-like activity often remained local, but mature animals developed generalized seizures and the susceptibility of the cortex for seizures was highly brain state dependent (Figure 16B).

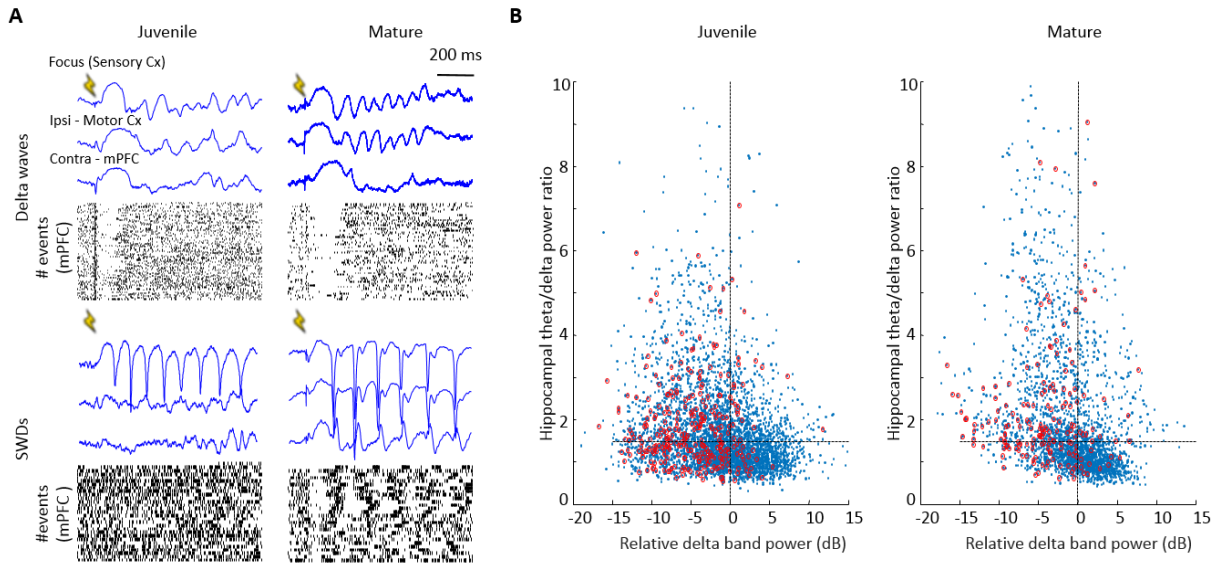

Figure 16. Events induced by intracortical single pulse stimulation. (A) *Representative LFP traces (single event) of different cortical areas and raster plot of cortical units (mPFC) during a single stimulation, which successfully induced delta waves or spike and waves in a young and an old animal.* **(B)** *Brain state dependence of single pulse stimulations. Each blue dot represents a single trial as the function of cortical delta band power and hippocampal theta/delta ratio. Dashed lines divide trials into four panels. Lower right panel corresponds to slow wave sleep. Red circles denote successful seizure inductions. (Young: 5 animals, 10 sessions, Old: 4 animals, 7 sessions).*

We compared spiking activity around electrical stimuli in order to see how promptly the cortex can react to stimulation. In order to do that, we compared the firing rate of units (total, excitatory, inhibitory and inhibitory/excitatory) in 1 s windows prior and following stimuli. In juvenile animals, no change was observed in the investigated parameters, but in mature ones total and excitatory firing rate increased after stimulation, therefore the inhibitory/excitatory balance shifted similarly to the spontaneously emerging seizures (Wilcoxon signed rank test, preSeiz vs Seiz, young: 4 animals, N = 107 pyramidal cell, N = 60 interneuron; total $p = 0.8319$; $Z = -0.21$, excitatory $p = 0.564$; $Z = -0.58$, inhibitory $p = 0.5366$; $Z = -0.62$, inhibitory/excitatory $p = 0.74743$; $Z = -0.32$; old: 4 animals, N = 317 pyramidal cells, N = 117 interneuron, total $p < 0.05$; $Z = 2.28$, excitatory $p < 0.001$; $Z = 3.48$, inhibitory $p = 0.4969$; $Z = -0.68$, inhibitory/excitatory $p < 0.01$; $Z = -3.01$; Figure 17A).

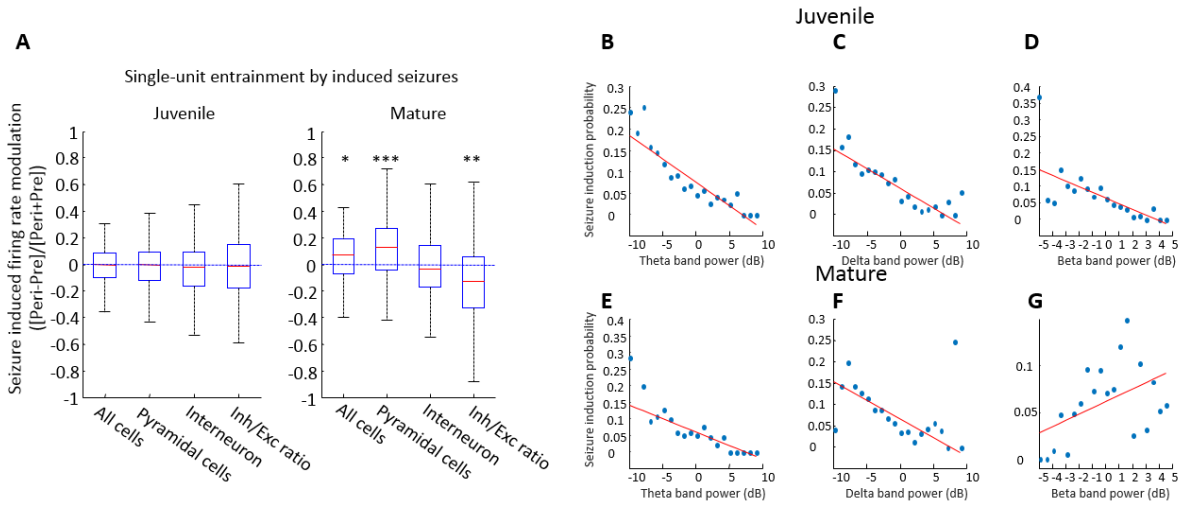

Figure 17. State-dependent seizure susceptibility (A) *Boxplot of firing rate ratios of units (all cells, pyramidal cells, interneurons and inhibitory/excitatory ratio) around successful seizure induction ([Peri-Pre]/[Peri+Pre]).* **(B and E)** *Probability of seizure induction as the function of pre-seizure hippocampal theta-band activity,* **(C and F)** *pre-seizure cortical delta band activity and* **(D and G)** *pre-seizure cortical beta-band activity. Red lines: best linear fit.* $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$

In accordance with the spontaneously emerging seizures, stimulation during slow wave sleep were not able to induce seizure activity neither in juvenile nor in mature animals, but other brain states were susceptible for seizure induction. In order to determine what makes the cortex susceptible for seizure induction, we tested the correlation of seizure induction efficacy and the pre-stimulation power of characteristic oscillations (hippocampal theta, cortical beta and delta). We found that both hippocampal theta power and cortical delta power negatively correlated with seizure induction probability (Young, theta: $R = 0.8425$; $p < 0.001$; $t = -9.6547$; delta: $R = 0.7205$; $p < 0.001$; $t = -8.8036$; $t = -6.3559$; Adult, theta: $R = 0.6645$; $p < 0.001$; $t = -6.4961$; delta: $R = 0.1416$; $p < 0.001$; $t = -6.3944$**;** Figure 17B, C, E, D). The latter relationship was expected from the rare occurrence of spontaneous seizures and low yield of electrical triggering during NREM sleep. Interestingly, we found a different seizure susceptibility regarding cortical beta band. In juveniles, beta power negatively correlated with seizure induction probability, but in contrast, it showed positive correlation in mature animals (Young, beta: $R = 0.4724$; $p < 0.001$; $t = -6.3559$; Adult, beta: $R = 0.2363$; $p < 0.05$; $t = 2.2121$; Figure 17D, G).

7 DISCUSSION

Our results provide evidence that TES can effectively control absence seizures in a closed loop fashion from days to months, the treatment effect is instantaneous and it does not deteriorate over time (Kozák and Berényi, 2017). Many attempts to prove the potential long term efficacy of electrical treatment of epilepsy have failed, possibly due to the lack of temporal selectivity (Vercueil et al., 1998; Blik, 2015) or due to selective intracranial targeting that misses the choke point locations of the epileptic network (Paz and Huguenard, 2015). The diffuse effect of the TES (Ozen et al., 2010; Ali et al., 2013) may be remarkably beneficial in generalized seizures, where the pattern generation is the result of a distributed network even if the initiation is triggered from a specific location (Meeren et al., 2002).

In this study we investigated spontaneously emerging absence seizures, which is a widely used epilepsy model to test different non-pharmaceutical treatment approaches in rodents (van Luijtelaar et al., 2016). Although this is a considerably mild form of epilepsy, owing to the high spontaneous seizure rate it is an ideal model to investigate the seizure – stimulus interactions (Pitkanen, 2017). It is not possible to directly draw conclusions how other generalized seizure types would respond to TES treatment, nevertheless it is promising that in a particular type of generalized epilepsy it is feasible to control seizure activity on the long term. Clearly, further studies are necessary to test TES on other seizure models to reveal the full potential of this treatment, such as temporal lobe epilepsy, which is the most common type of seizures in the adulthood (Tellez-Zenteno and Hernandez-Ronquillo, 2012).

Previous papers describing an increased seizure frequency during electrical suppression of absence seizures suggest (Feddersen et al., 2007; Luttjohann and van Luijtelaar, 2013) that this phenomenon may be related to the very high spontaneous seizure rate of this epilepsy model. Nonetheless, this side effect of the closed loop stimulation has to be taken into account when considering the feasibility of clinical application, the possible seizure rate increase in response to the treatment may be a potential limiting factor determining the tolerance of the patients, which may be balanced by the quick seizure termination.

Spike-and-wave discharges are most likely to occur during periods of slow-wave sleep and drowsiness (Timofeev and Steriade, 2004). Previous studies reported that antiepileptic stimuli neither change the behavioral state of the animal (Feddersen et al., 2007) nor induce

arousal (Berenyi et al., 2012), therefore the animal – when the seizures are stopped – remains in the seizure susceptible behavioral state. Consequently, the higher seizure rate may be coming from the fact that the due to the early termination, there is more room for further seizures within the given period when the brain is more prone to develop seizures.

Furthermore it cannot be excluded, that the recurrence of suppressed seizures reflected by the increased seizure rate may be the result of incomplete switching between the firing modes of thalamic cells (Sorokin et al., 2016b), leading to a rebound phenomenon (Vercueil et al., 1998; Blik, 2015). Importantly, these rebound or recurrent seizures did not increase the total time spent in seizure, as the shortening of the average seizure duration more than compensated the increase in the seizure rate, thus altogether it decreased significantly.

It is long known that the cells of the thalamus can express various response patterns depending on their firing modes (Steriade et al., 1993). Consequently, the strikingly different effect of the random and closed-loop TES can be explained by this state-dependent response of the thalamocortical loop. Stimulation during a random initial state, the external stimulus may weakly synchronize cells making them susceptible to randomly reach a level of synchrony and initiating a seizure, which leads to an indirect increase of seizure rate. Importantly, as we showed that even a single intracortical stimulus is able to induce epileptic seizures in a seizure susceptible brain state, therefore it might be possible, that the increased seizure rate seen in our experiments might be related to the hyperexcitable core of the cortex. Altogether, these results underlie the importance of timing (Kozák and Berényi, 2017).

An important conclusion of our experiments is that the seizure-related synchronous electrical activity is not required for the maintenance of seizure susceptibility, as we could not achieve a curative effect when the seizures were quickly suppressed, but not prevented for months (Kozák and Berényi, 2017). In our study, we investigated the seizure disruption efficacy on mature animals, whose seizure parameters were already constant at the beginning of the treatment. Some recent findings suggest, that the frequent seizures themselves might play a role in the pathophysiological alterations related to absence epilepsy (Studer et al., 2018). Therefore, further investigations should address the question, whether early intervention with transcranial electrical stimulation has a seizure-modifying effect. To date, only pharmacological treatment with ethosuximide has been shown to have a seizure-modifying effect, as administration of

ETX seems to be capable to improve long-term outcome of the epileptic condition (Blumenfeld et al., 2008).

Similarly to the deep brain stimulation (DBS) for Parkinson's disease (PD) (Temperli et al., 2003; Lang and Obeso, 2004), the investigated seizure parameters returned to the baseline as we stopped the treatment in most of the animals, suggesting that the effect does not cumulate, and the seizure generating networks do not habituate, which parallels the lack of histological alteration (Figure 4f). Furthermore, the closed loop TES was not able to stop the progression of the disease in the prolonged closed-loop treatment either, despite the effective seizure control during this period (Figure 5a, 5b). Note that similar observations were reported regarding the lack of lasting DBS effect in PD (Hilker et al., 2005), as it does not stop the loss of dopaminergic neurons, but rather treats the symptoms of the disease (Dauer and Przedborski, 2003; Hilker et al., 2005). In contrast to this, we cannot yet exclude that the reason for the lack of the curative effect in our epilepsy related TES application is due to the fact, that the closed loop detection requires a seizure to be generated, thus the seizures were not prevented but only reversed. There are efforts to predict the seizure emergence (Ramgopal et al., 2014; van Luijtelaar et al., 2016) and if reliable predictors can be identified, it may ultimately clarify the therapeutic effects of TES when used for preventing and not only terminating the seizures.

Very often the general condition of patients with epilepsy deteriorates over time due to the overexcitation of the microcircuitry and cell death, leading to memory impairment, decline in intellectual performance and a higher risk of sudden unexpected death in epilepsy (SUDEP) (Dodrill, 2002; Vingerhoets, 2006; Surges et al., 2009). The main effect of TES according to the dataset presented here — namely the sustained lower time spent in seizure and shorter seizures during treatment — might have the potential to attenuate or prevent the secondary consequences of epilepsy, but it requires further investigations. Our results suggest, that TES might have a favorable impact on the long term outcome of the disease, even if it cannot achieve a cumulative effect regarding the seizure activity.

Importantly, effective seizure control of epileptic patients can improve their quality of life (Jones, 1998), even if the treatment is not able to cure the underlying mechanism of epilepsy. There are available comparisons about the happiness, self-esteem and other psychosocial parameters with and without successful treatment (Poochikian-Sarkissian et al., 2008), showing a clear correlation between the effectivity of treatment and general well-being

of the patients, but the importance of terminating seizures as soon as possible and reducing the non-responsive periods to avoid accidents (e.g. driving vehicles, handling machinery, etc.) is probably even larger. Therefore, many secondary positive effects might be expected from closed-loop TES treatment besides those which can be tested in animal models.

These findings demonstrate the safety and effectiveness of TES treatment, and suggest that it could be used as a minimally invasive, lifelong palliative treatment of certain types of epilepsy, alone or as a complement to pharmaceuticals. To date, short-delay, system-on-a-chip solutions are already available for real-time epileptic seizure detection tested in animal models (van Luijtelaar et al., 2016), which can be combined with reliable detection algorithms optimized for human data (Ihle et al., 2012; Furbass et al., 2015). In addition, brain-machine-brain interfacing is feasible through medically approved non-penetrating electrodes. Therefore every aspects are given to translate our previous and current results on the proof of the concept and safety of a temporally targeted on-demand transcranial seizure suppression approach into a potentially effective, nonpharmaceutical antiepileptic therapy for human patients.

To further optimize the efficacy of these treatments, our efforts lead - to our knowledge - to the first study, which follows the evolution of thalamocortical rhythms in the same animals over the time course of 3 months in multiple cortical locations. We showed that maturation gradually shifts local ictal activity of the somatosensory cortex into global seizures of the thalamocortical circuitry, which parallels the progressive disappearance of sleep spindles. Importantly, the sleep related changes seem to be reversible by pharmacological therapy. Furthermore, we showed that even a single depolarization burst is sufficient to kick a susceptible brain into seizure activity.

It is known, that absence epilepsy is very often accompanied with sleep disturbances in human (Myatchin and Lagae, 2007) and the progressive disappearance of sleep spindles might account for an impairment of learning abilities (Radek et al., 1994; Nolan et al., 2004), but the exact mechanisms are still elusive. We found that sleep architecture has changed during maturation. The NREM sleep became less spindle-rich, as the spindle occurrence markedly decreased, but the peak frequency of the individual spindles did not change over the observation period. Altogether these findings raise the possibility of an impaired spindle initiation. Here we showed that the parallel disappearance of sleep spindles and the emergence of spike-and-waves might be two joint consequences of the same underlying mechanism even if they affect different

phases of sleep, as the treatment of absence epilepsy with ETX can increase the occurrence rate of sleep spindles in a dose-dependent manner. Similar results were found in human (Kellaway et al., 1990), although no systematic clinical study on antiepileptic drugs confirmed this effect of ETX treatment yet. The initial suppression of spindles is understandable as high dose of ETX might massively decrease the availability of T-type Ca2+ channels necessary for spindle initiation, but smaller plasma concentrations of ETX resulted in reduced seizure activity and high spindle occurrence. ETX exerts its pharmacological effect mainly via blocking T-type $Ca2^{+}$-channels, although it has other targets as well and its seizure suppression effect is not confined to the thalamus (Manning et al., 2004). Thus, it has to be further investigated whether the frequent seizing is impairing the thalamocortical circuitry (Kozak, 2019) or a maturation-related cellular alteration may be in favor of seizure spread and causing spindle initiation difficulties in an already epileptic brain. Nevertheless, the restoration of sleep spindles by the antiepileptic drug of ETX suggests a network effect of channel deficiencies which causes spindle initiation difficulties in the epileptic brain, especially since the on-demand seizure control did not have similar positive effect on sleep spindles.

An interesting aspect of the results is that most cortical units, similarly to their thalamic counterparts (Buzsaki, 1991; McCafferty et al., 2018), are not firing during most of the spike-and-waves and altogether a distributed firing pattern of always-changing set of spike-and-wave entrained ensembles was observed during ictal activity with a decreased inhibitory/excitatory balance. Furthermore, it is possible that the symptoms of absence epilepsy (i.e. subjects being non-responsive for environmental stimuli during the seizures) are connected not only to the generalized synchronous activity of the minority of the units, but to the silence of the majority at the same time. Some studies showed intact cortical processing and partial consciousness during absence seizures in animals (Drinkenburg et al., 2003) and in human as well (Chipaux et al., 2013; Guo et al., 2016). The underlying reason for this might be that in each cycle, most cortical and thalamic units are not entrained to seizure activity, therefore the unaffected loops can still process incoming information from the external world and can explain why epileptic activity can be interrupted by unexpected sensory stimuli.

The overall decrease of firing of the inhibitory cells suggests that the silence of the units is not due to the powerful inhibition on the pyramidal cells but rather due to their weaker thalamocortical drive. On the other hand, our results show a higher inhibitory/excitatory

balance in juvenile animals during ictal activity compared to mature ones, what raises the possibility of an active cortical inhibitory veto of seizure spread that disappears with age. Indeed, there is evidence that epileptic animals have an impairment of intracortical GABAergic inhibition (Luhmann et al., 1995; Crunelli et al., 2011). Importantly, our observations are in line with the results of Meyer et al. (2018), who showed using calcium imaging in the visual cortex of stargazer mice, that most inhibitory cells decrease their activity during absence seizures and most cells are not entrained to all of the SWD cycles.

An alternative mechanistic explanation of the decreased inhibitory activity is that while the bursts of the otherwise sparsely firing pyramidal cells during the spikes can counterbalance their decreased activity during the waves resulting in no gross change in their average firing rate, the already fast-firing interneurons cannot further increase their activity, and thus cannot compensate for their relative suppression during the wave phases. This speculation suggests that the decreased interneuron activity is a mere consequence of the altered rhythmicity, and thus, it may help to maintain the SWD patterns but may not encounter for the increased susceptibility for seizure invasion of the given region. Further investigations are needed to clarify this question of mutual causality.

It is noteworthy, that while spindles are mainly global patterns already in the juvenile animals, the majority of both the spontaneous or induced ictal activity are local without generalization. This underscores that the hyperexcitable focus *per se* is not sufficient to drive generalized seizures, even though the thalamocortical circuitry is already capable to generate generalized patterns. However, during maturation some yet undetermined changes happen which turn the whole brain to be susceptible for epileptic seizures. The hyperexcitability of the cortical focus develops earlier than the propensity of the adjacent areas to participate in the ictal activity, which suggests that seizure development is a multiple-step process.

One possible candidate for maturation-related alterations that might contribute to the differential processing of emerging seizure activity of juvenile and adult animals is the changing TRN-connectivity with aging (Halassa and Acsady, 2016). Many evidence suggests that intra-TRN chemical synapses – that can potentially prevent widespread synchronization and confine emerging seizure activity – disappear during maturation in rodents (Pinault and Deschenes, 1998; Hou et al., 2016), but gap junctions remain present allowing intranuclear fast synchronization (Deleuze and Huguenard, 2006). This alteration might help spike-and-wave

discharges emerging in the somatosensory cortex to entrain large portions of the TRN and this increased thalamic contribution might be necessary to have full-blown seizures with self-sustaining oscillations.

A study showing that the unilateral inactivation of thalamus can disrupt epileptic seizures ipsilaterally presented similar cortical traces during contralateral seizure activity than we report here in juvenile animals (Buzsaki et al., 1988). As the cortical spread of ictal activity is hypothesized to happen through cortico-cortical connections, this highlights the importance of thalamocortical feedback on cortical units which frames them to fire synchronously to the seizure drive. Furthermore, the observed delta wave like activity of the motor cortex in response to the ongoing seizure activity of the somatosensory cortex in juvenile animals taken together with the very strong state-dependence of seizure susceptibility raises the possibility that in juvenile animals the different cortical areas might be concomitantly in different microstates. Indeed, global sleep is thought to be an emergent property of the synchronization of local sleeping networks (Krueger and Obal, 1993; Krueger et al., 2008; Krueger and Roy, 2016), and importantly cortical areas also can selectively go 'offline' independently from other cortical areas, a phenomenon known as local sleep (Vyazovskiy et al., 2011). Therefore, it is possible that the differential upstream determinants of cortical states locally can result in different response to incoming seizure drive, as one column can develop a spike-and-wave while the other skips a cycle of the seizure while generating a delta wave. The existence of microstates reflects the independence of local thalamocortical loops to some extent and it may be possible that maturation increases the intrathalamic synchrony resulting in a more uniform response to cortical stimulation and generalization. A previous study already showed that ion channel loss in the thalamic reticular nucleus (TRN) cause the hypersynchrony of TRN neurons, leading to absence seizures (Makinson et al., 2017). Although it is important to mention that in such a multiple-step process a slight imbalanced shift to higher synchrony together with the already hyperexcitable cortex might probably be sufficient to initiate and maintain generalized seizures.

We showed here that spontaneous seizure occurrence correlates with changes in wakefulness. While the seizure probability is very high around transitions from wakefulness to light sleep, as sleep deepens, seizure patterns are overturned by slow waves and even rhythmic cortical activation fails to induce seizures in this state. It is known that arousal related brain states are controlled locally via the locus coeruleus noradrenergic system (Constantinople and

Bruno, 2011). Furthermore, during transition to slow wave sleep the synchrony among TRN neurons is increased which is physiologically responsible for gating the sensory information to the cortex (Halassa et al., 2014). Alternatively, this also can be in favor of spreading highly synchronous activity of epileptic seizures (Makinson et al., 2017). It is possible that during wake-sleep transitions in maturing animals the thalamic synchrony is imbalanced in a way that the continuously decreasing arousal together with increasing TRN synchrony provides a seizure-permissive time-window. The cortical excitability is still high enough to initiate a putative seizure and the TRN is already sufficiently synchronous to provide a sufficiently broad feedback to the cortex via the thalamocortical cells to frame seizure activity for the next cycle. We found that seizure susceptibility oppositely correlates with cortical beta oscillations in juvenile and in older animals. The opposite correlation raises the possibility that the observed beta oscillations have different origin depending on the age of the animals. In juvenile animals it might represent physiological beta oscillation, which is associated with attention and wakefulness, therefore negative correlation with seizure susceptibility is understandable. On the contrary, in mature animals, pathological beta oscillation (Grandi et al., 2018) may emerge, which can contribute to seizure susceptibility. A recent study using optogenetic thalamic stimulation to induce seizures (Sorokin et al., 2016a) described similar relationship between beta power and seizure induction probability as we found in mature rats. As they hypothesized, this cortical beta oscillation might synchronize and frame TRN firing to be pro-epileptic and making generalization of seizures possible.

We propose here that the development of absence epilepsy in polygenic models requires multiple steps, which very likely includes the development of a hyperexcitable cortical initiation zone and the occasional imbalance between thalamocortical synchrony and cortical excitability, which might be present in the form of pathological beta oscillation. Altogether these multiple minor alterations might turn the thalamocortical dynamics to favor seizure spread in polygenic animal models and human subjects.

8 SUMMARY

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. Since TES requires only a minor surgery and the device is cosmetically compatible for ambulatory and chronic use, the closed-loop feedback stimulation can become a clinically effective solution of seizure control. 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.

Although transcranial direct current stimulation is already medically approved for the treatment of major depressive disorder and many studies focus on showing the cognitive effects of transcranial stimulation in humans, there are still many open questions regarding the detailed mechanism of action, potential disease specific side-effects and the long-term outcome of applying this method. Given that many disorders (e.g. depression, epilepsy and schizophrenia) can only be extensively investigated in awake animal models and the nature of these medical conditions necessitate usually long-term treatment, the protocol provided here for chronical implantation of transcranial electrodes in rats might be a powerful tool in testing hypotheses regarding the use of transcranial electrical stimulation.

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, thus using our long-term recording methods, they provide an opportunity to track the network level changes of the thalamocortical loop that lead to fully-developed generalized seizures. We investigated two important perspectives of seizure development in this strain. By performing large-scale extracellular neuronal recordings continuously for months from childhood to adolescence and recording single unit activity in

young and adult rats, we could analyze population activity and firing of individual cells during and between seizures.

Our observations suggest that absence seizures are not accompanied by widespread phase-locking of neuronal activities. Interestingly, most cortical interneurons are tonically suppressed during absence seizures and pyramidal cells do not increase their mean firing rate either. As we could not provide mechanistic explanation of these alterations, further investigations are required in order to understand how these interneurons are suppressed in parallel with the emerging seizure activity. This might help to identify new seizure choke points, serving as a potential new pharmaceutical target to disrupt seizure activity.

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. This finding puts 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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