Seizure: European Journal of Epilepsy 81 (2020) 1–7

Contents lists available at ScienceDirect

Seizure: European Journal of Epilepsy

journal homepage: www.elsevier.com/locate/seizure

Epileptogenesis-induced changes of hippocampal-piriform connectivity

Mark D. Skopin[a], Arezou Bayat[a], Lalitha Kurada[a], Mithilesh Siddu[a], Sweta Joshi[a], Christina M. Zelano[b], Mohamad Z. Koubeissi[a,*]

[a] Department of Neurology, George Washington University, Washington, DC, 20037, USA
[b] Department of Neurology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

ARTICLE INFO

Keywords:
Cerebro-cerebral evoked potentials (CCEPs)
Low-frequency stimulation (LFS)
Biomarker
Anterior piriform cortex (aPC)
Spontaneous recurrent seizures (SRS)

ABSTRACT

Objective: Tissue remodeling has been described in brain circuits that are involved in the generation and propagation of epileptic seizures. Human and animal studies suggest that the anterior piriform cortex (aPC) is crucial for seizure expression in focal epilepsies. Here, we investigate the effect of kainic-acid (KA)-induced seizures on the effective connectivity of the aPC with bilateral hippocampal CA3 regions using cerebro-cerebral evoked potentials (CCEPs).

Methods: Adult male Sprague-Dawley rats were implanted with a tripolar electrode in the left aPC for stimulation and recording, and with unipolar recording electrodes in bilateral CA3 regions. Single pulse stimulations were given to the aPC and CCEPs were averaged before KA injections and after the emergence of spontaneous recurrent seizures (SRS). Similar recordings at equivalent time intervals were obtained from animals that received saline injections instead of KA (controls).

Results: In the experimental group, the percentage change of increased amplitude of the contralateral (but not ipsilateral) CA3 CCEPs between pre-KA injection and after the emergence of SRS was significantly greater than in controls. No significant single-pulse-induced spectral change responses were observed in either epileptic or control rats when comparing pre- and post-stimulus time intervals. Also, we found no correlation between seizure frequency and the extent of amplitude changes in the CCEPs.

Conclusions: In the KA model, epileptogenesis results in plastic changes that manifest as an amplification of evoked potential amplitudes recorded in the contralateral hippocampus in response to single-pulse stimulation of the aPC. These results suggest epileptogenesis-induced facilitation of interhemispheric connectivity between the aPC and the hippocampus. Since the amplitude increase of the contralateral CCEP is a possible in vivo biomarker of epilepsy, any intervention (e.g. neuromodulatory) that can reverse this phenomenon may hold a potential antiepileptic efficacy.

1. Introduction

Human experience [1,2] and many animal models [3,4] of epilepsy follow a classic scenario of an inciting epileptogenic event followed by a latency phase before emergence of spontaneous recurrent seizures (SRS). There is evidence that tissue remodeling occurs during the latency period, which facilitates the initiation and propagation of epileptic seizures [5–7]. Biomarkers of such remodeling can be of value for diagnosis and assessment of response to therapeutic interventions. Cerebro-cerebral evoked potentials (CCEPs) recorded in the seizure network in response to low frequency stimulation (LFS) are measurable signals that may serve as such biomarkers.

Specific brain circuits are known to contribute to the spread and maintenance of seizure discharges [8–10]. In focal epilepsies, SRS consistently propagate along specific anatomic pathways [11,12]. Although there is a considerable individual variability, some areas are common to most seizure networks [13]. In humans, the piriform cortex appears to be a common area of activation during interictal epileptiform discharges in focal epilepsy regardless of the localization of the seizure focus [14,15]. The extensive connections of the piriform cortex with limbic, cortical, and subcortical regions [16,17] facilitate and intensify seizure activity. The anterior piriform cortex (aPC), in particular, has been shown to be involved in the propagation of limbic seizures into generalized motor seizures [18–20]. Thus, the aPC is a critical target for further study and potential therapeutic interventions.

In the kainic acid (KA) model of multifocal epilepsy, seizures originate in (or invariably propagate to) the hippocampi [21]. Furthermore, following KA injections, CA3 regions show damage [22] and thus

* Corresponding author at: 2150 Pennsylvania Ave. NW., Washington, DC 20037, USA.
E-mail address: mkoubeissi@mfa.gwu.edu (M.Z. Koubeissi).

https://doi.org/10.1016/j.seizure.2020.07.008
Received 11 November 2019; Received in revised form 6 July 2020; Accepted 9 July 2020

are suitable to study seizure-induced changes. The objective of this study was to investigate whether epileptogenesis induces plastic changes that are measurable as the effective connectivity between the aPC and bilateral hippocampi. We used hippocampal evoked potentials in response to aPC stimulation and studied the changes in waveform responses in the CA3 regions before KA injection and after the emergence of SRS.

2. Materials and methods

2.1. Animals

Fourteen (10 experimental and 4 control) adult male Sprague-Dawley rats weighing 250 − 390 g were used in this study (Hilltop; Scottdale, PA). All animal procedures were conducted in accordance with the NIH guidelines (NIH Publications No. 8023, for the care and use of Laboratory animals), reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the George Washington University. Upon arrival, rats were pair-housed in conventional plastic animal cages and allowed to acclimate for a week before surgery or experimentation. The cages and experimental rooms were climate-controlled and illuminated on a standard light-dark cycle (7 a.m. on/7 p.m. off). Animal chow (Harlan Laboratories, Madison, WI) and water were provided ad libitum throughout the study.

2.2. Electrode implantation

Prior to electrode implantation, induction of anesthesia was performed using a 5% isoflurane in a gas mixture of 70 % compressed air and 30 % oxygen. After inducing anesthesia, the surgical site was shaved, animals were transferred to the stereotaxic frame (Kopf Instruments, Tujunga, California), and 2–2.5 % isoflurane was used for maintenance. Ophthalmic lubricant was applied to both eyes to prevent drying during the surgery. Before the surgery, carprofen was injected subcutaneously along the nape (Pfizer, New York, NY; 5 mg/kg; 1:5 dilution from 50 mg/mL stock). Using a homeothermic blanket system (Harvard Apparatus, Kent, UK), body temperature was maintained at 37 °C and was monitored with a rectal thermometer throughout the surgery. The dorsal surface of the skull was exposed by a midline incision along the head of the animal. Using a 1.397-mm-diameter drill bit (Plastics One, Roanoke, VA) burr holes were drilled for implantation of electrodes, screw-electrodes and anchor screws. Intraperitoneal injections of cefazolin sodium (Chem-Impex International Inc., Wood Dale, IL; 22 mg/kg) were done before the surgery and weekly thereafter for the duration of experiment.

Three depth electrodes were placed using stereotactic coordinates according to Paxinos and Watson [23]. A tripolar stimulating and recording and two unipolar recording electrodes (0.010″ polyamide-insulated stainless steel; Plastics One) were implanted into the aPC (2.5 mm anterior, 3.0 mm lateral on the left, and 7.0 mm ventral to bregma) and bilateral CA3 regions (3.0 mm posterior, 3.0 mm lateral on either side, and 3.4 mm ventral to bregma) respectively (Fig. 2). Two stainless steel screw- electrodes were placed as a ground over the cerebellum (10 mm posterior and 3 mm lateral to bregma) and as a reference over the frontal lobe (3 mm anterior and 2 mm lateral to bregma), along with two anchor screws. All electrodes were inserted into their respective positions of a 5-pin connector and then fixed to the skull using dental cement. Animals were monitored after surgery before they were moved, when ambulatory, to cages provided with wet food and hydrogel.

2.3. Acquisition of CCEPs

One week postoperatively, single-pulse stimulations were given to all animals before KA or saline injections. This was repeated after the emergence of SRS in epileptic animals and after a comparable duration in controls. Single-pulse (1 ms; biphasic) was given to the aPC at 0.1 Hz starting at 200 μA and increasing by 200 μA increments up to a maximum of 1 mA. A total of 100 pulses were delivered at each current intensity. Animals were monitored throughout the stimulation and kept awake by manual agitation. EEGs were then averaged time-locked to these stimuli using an in-house software (MATLAB, Natwick, MA).

2.4. Kainic acid injections

After acquisition of baseline CCEPs, KA (Milestone PharmTech USA Inc., New Brunswick, NJ) was injected intraperitoneally at a dose of 5 mg/kg of body weight dissolved in 2 mg/mL of normal saline [24] at one-hour intervals until animals developed at least 10 stage-4 or -5 seizures (modified Racine Scale) or began exhibiting excessive inactivity or hyperactivity. However, after the occurrence of 5–9 stage-4 or -5 seizures, KA dosage was reduced to 2.5 mg/kg/hour [24]. The control group was injected with similar volumes of normal saline intraperitoneally each hour up to 3 times. To reduce mortality, intraperitoneal diazepam (Hospira, Inc., Lake Forest, IL; 5 mg/kg) was given to all animals after KA injections were completed. Additionally, 1 mL of lactated Ringer's solution was injected intraperitoneally to all the animals to prevent dehydration.

2.5. Video-EEG recordings

One week after KA injections, animals were transferred to cylindrical cages that allowed free movement. A tethered video-EEG recording system (M20; Triangle Biosystems International, Durham, NC) with a video camera (Logitech C270 HD Webcam, Newark, CA) and in-house video acquisition system (MATLAB, Natwick, MA) were used for seizure quantification. EEGs were sampled at 1000 Hz using Neuroware (Triangle Biosystems International, Durham, NC). Both video and EEG recordings were obtained in units of 24 h each and analyzed entirely by a blinded EEG reader – No automated spike- or seizure-detection programs were used. Seizure duration, severity (according to the Racine scale) and frequency were assessed throughout all the recordings (Fig. 1).

2.6. Analysis of CCEPs

The amplitudes of stimulation artifacts invariably exceeded 2000 μV, which was higher than the recorded brain activity, and were thus chosen as stimulus onsets by an in-house software for averaging the CCEPs. We then verified the automatically detected stimuli and manually excluded ones that did not correspond to single-pulse stimulation keeping a mean (± SD) of 83.6 ± 1.7 out of the 100 stimuli delivered. Mean responses were calculated by averaging 1 s prior to the stimulus (baseline) and 3 s after the stimulus. Spectral analysis was done for various frequency bands using Fast-Fourier Transform as follows: Low Gamma (30 − 55 Hz; 0.5 s epoch; 50 % overlap); Mid-frequency Gamma (55 − 80 Hz; 0.2 s epoch;50 % overlap); High Gamma (80 − 100 Hz; 0.1 s epoch; 50 % overlap); Ripple (100 − 250 Hz; 0.05 s epoch; 50 % overlap); and Fast Ripple (250 − 500 Hz; 0.05 s epoch; 50 % overlap). The focus on high frequency activity was due to its relevance to epileptogenesis as described by other authors [25]. The spectral response was determined by the percentage change from the average power from the baseline. GraphPad Prism 6 software (San Diego, CA) was used to test the statistical significance between control and epileptic animals.

2.7. Histology

To verify electrode locations, rats were injected with Euthasol intraperitoneally (75 mg/kg; 50 mg/mL; Virbac AH, Inc., Fort Worth, TX) and then transcardially perfused using 0.9 % NaCl with heparin, followed by 10 % cold buffered formalin phosphate (Fisher Scientific, New Jersey, USA). Brains were extracted and fixed in 10 % formalin

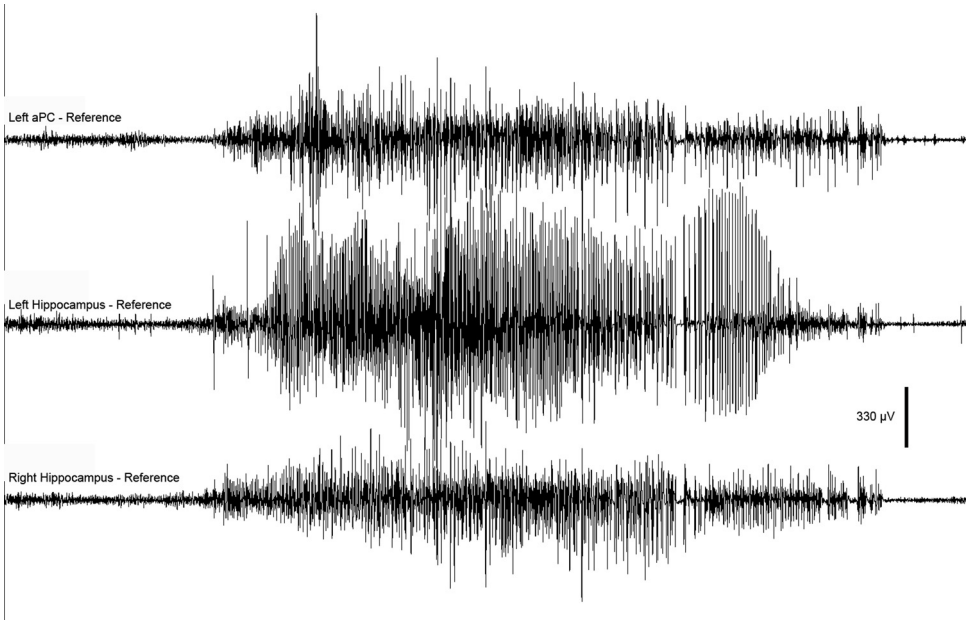

Fig. 1. EEG trace of seizure. The channels are in the anterior piriform cortex, left and right hippocampi, respectively. The whole duration of the shown epoch is one minute, and the corresponding seizure is stage 5 on the Racine scale.

followed by 30 % sucrose 24 h later for histological analysis. They were then embedded with Tissue-Tek O.C.T. (Sakura Finetek USA, Torrance, CA) and frozen in 2-methylbutane with liquid nitrogen, stored at − 20 °C and coronally sectioned at 30 μm using a cryostat. Sections were stained with hematoxylin and eosin and imaged using a Leica Microsystems DM 6000B microscope and software (LAS AF Version: 2.6.0.7266.2). Electrode locations were histologically verified by a blinded reviewer. All animals had electrodes in the intended brain regions, and none were excluded from the analysis (Fig. 2).

3. Results

Histological assessment in all rats (10 experimental and 4 control rats) confirmed electrode locations in the aPC and bilateral hippocampi. The typical morphology of the CCEP responses consisted of an initial prominent negative deflection (N1), as shown in Fig. 3. N1 amplitude was measured from the pre-stimulus baseline to the peak of the N1 potential for each electrode, at each stimulus intensity. Since artifacts from electrical stimulation can interfere with interpretation of CCEP profiles and network analysis, we used a ground electrode made of the same material (stainless steel) to minimize direct current artifacts.

The latency, amplitude, and slope of the CCEPs were measured before and after kainate-induced seizures in the ipsi- and contralateral CA3 as well as the ipsi- and contralateral CA3 referenced to each other. The statistical significance between control and epileptic animals was analyzed by two-way ANOVA using post hoc Tukey with a $p < 0.05$ threshold. Standard error of mean was used to represent the percentage change from baseline to epileptic CCEPs.

The percentage change in CCEPs amplitudes (i.e., from pre- to post-injection stimulations) of animals receiving KA were significantly greater than those of control animals in the contralateral (Fig. 4C; $p = 0.023$) as well as the contralateral-minus-ipsilateral CA3 amplitude (Fig. 4B; $p = 0.045$). No significant change in percentage change of the ipsilateral hippocampal CCEP response was observed ($p = 0.553$; Supplementary Materials). Furthermore, there was a significant decrease in the contralateral-minus-ipsilateral CA3 latency ($p < 0.001$)

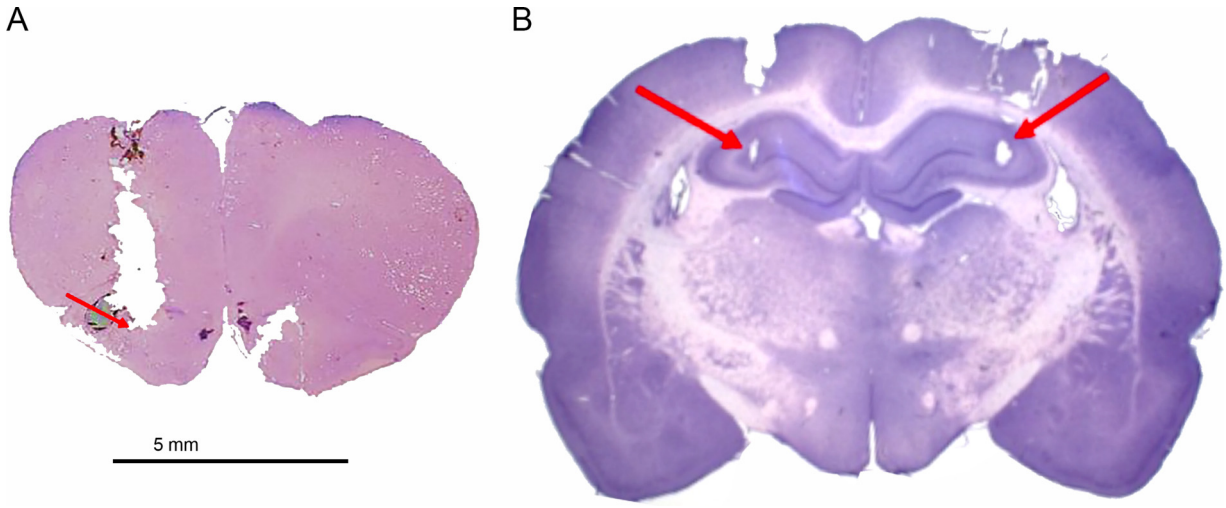

Fig. 2. Stereotactic electrode placement. Hematoxylin and eosin staining of the sectioned brain confirm electrode locations (arrowheads) in the aPC (A), and bilateral CA3 region of the hippocampi (B).

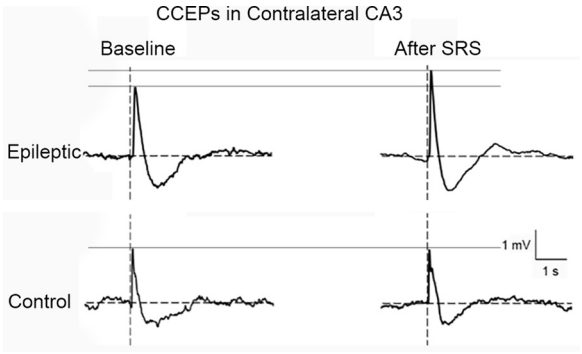

Fig. 3. Example of contralateral CCEP amplitude change. CCEPs recorded in the right CA3 to single-pulse stimulation of the left aPC at the same current intensity (200-400 μA) showing increased amplitude after the emergence of spontaneous recurrent seizures (SRS) in an epileptic rat (upper), but not in a control rat (lower) after an equivalent period of time. Such amplitude changes were not observed over ipsilateral CA3.

and a significant increase in slope in both the contralateral CA3 as well as the contralateral-minus-ipsilateral CA3 ($p = 0.004$ and $p < 0.001$, respectively) following SRS in epileptic group compared to control group (Supplementary Materials). There was no significant difference between pre- and post-injection normal saline data in control group. Additionally, there was no significant change in the spectral responses, including the gamma power and high frequency oscillations-, pre- and post-single pulse stimuli (data not shown). Overall, there were increases in contralateral amplitude of CCEPs in the hippocampus, which were not observed in the control group.

The correlation between number of seizures per day and percentage change of amplitude at different current intensities was analyzed by linear regression, and no significant correlation was found ($p > 0.05$). Additionally, the relation between latency of the emergence of SRS after KA injection and contralateral CCEP amplitude increases was analyzed by linear regression. No significant correlation ($p > 0.05$) was found at all current intensities, except at 800 μA where higher contralateral CCEP amplitude increases correlated with longer durations between KA injections and emergence of SRS ($p = 0.026$).

4. Discussion

In a previous study, we have demonstrated that LFS of the aPC abolishes severe motor seizures with a prolonged carry-over effect in the KA model, suggesting a network role of LFS, which is in agreement with the findings of other authors that LFS of the aPC can delay seizure development in amygdala kindling model [26,27]. These findings suggest the involvement of a network of cortical and subcortical structures modulating epileptiform activity. The purpose of the current study was to investigate the epileptogenesis-related connectivity changes by evoked potentials recorded in ipsilateral and contralateral hippocampi upon stimulation of the aPC in epileptic animals. Such changes over time possibly reflect plastic changes within the seizure network. The principal finding of this study is that emergence of SRS in the KA model was associated with a significant increase of the CCEP amplitude recorded in the contralateral hippocampus upon stimulation of the aPC, whereas no such change occurred in the controls. Additionally, as a possible indicator of enhanced connectivity, the latency of the initial component of the CCEP became shorter after emergence of seizures and its slope increased. Although seizure frequency per se did not correlate with CCEP changes, it is possible that these results reflect enhancement of interhemispheric connectivity in epileptic animals, which, one may speculate, may underlie increased propensity for secondary generalization of seizures in the KA model. We cannot comment on the role of subcortical structures in CCEP amplification since we did not sample them with electrodes. A CCEP study in humans found higher

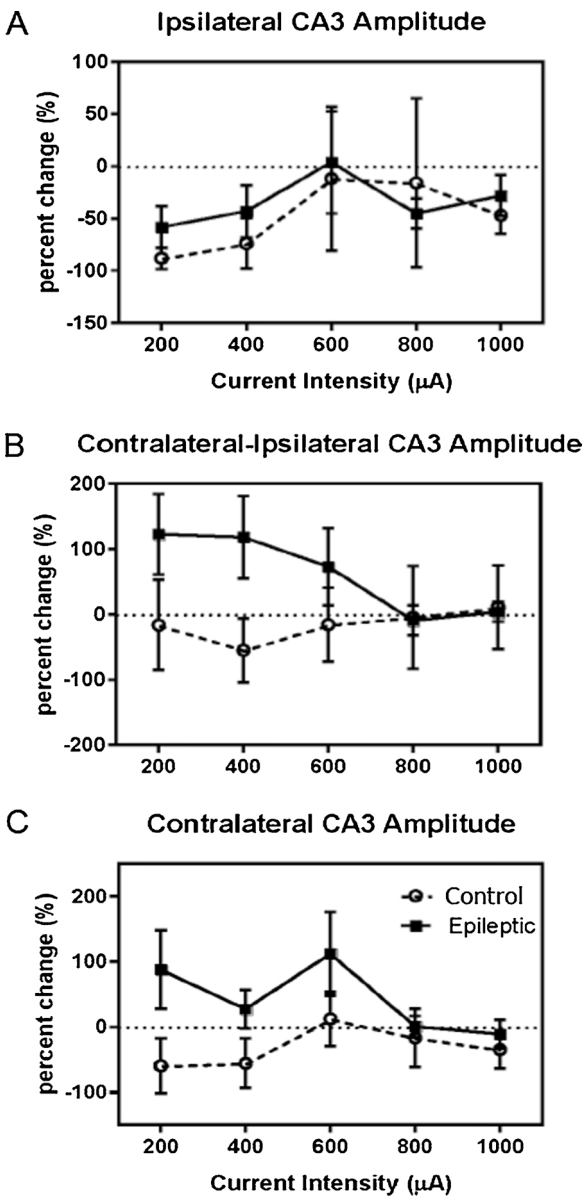

Fig. 4. Percentage change of CCEP amplitude in epileptic and controls. All animals received stimulations at current intensities ranging from 200-1000 μA before KA or normal saline injections and after the emergence of SRS in epileptic animals and a comparable duration in controls. The average number of stimulations was 83.6 ± 1.7 and did not differ among trials. **(A)** Percentage change in amplitude responses in the ipsilateral CA3. **(B)** Percentage change in amplitude responses of the referenced contra- to ipsilateral CA3. **(C)** Percentage change in amplitude responses in the contralateral CA3.

CCEP amplitudes without latency changes near ictal onset zones compared to other brain regions, which could reflect higher excitability of the epileptogenic cortex [28]. However, the fact that ipsilateral CA3 CCEPs (also an epileptogenic zone) did not change in amplitude after emergence of spontaneous seizures in our study, and that contralateral CCEP latency decreased and its slope increased suggest that the increased contralateral amplitude is due to improved interhemispheric connectivity that does not include the ipsilateral hippocampus as a node. However, to better explore the mechanism of this CCEP increase, future studies may record from more brain regions and utilize Granger causality analysis or other cross-correlation approaches [29].

While responsive neurostimulation requires electrode placement in the seizure-onset zone [30] and white matter tract stimulation has been suggested as a treatment for hippocampal seizures [31], we have been

investigating the LFS of the aPC as a possible treatment of focal epilepsy regardless of the localization of the seizure focus. The human CCEP data are obtained solely from patients with implanted intracranial electrodes for surgical management intractable epilepsy. These studies do not facilitate assessment of connectivity changes that are induced by epileptogenesis. However, future studies with chronically implanted electrodes (for both stimulation and recording) can facilitate assessment of CCEPs changes in the human brain in relation to seizure frequency or antiepileptic interventions. The PC is one such potential target because it has shown activation in multiple studies that corresponded to various spikes in patients with focal epilepsy [14]. The PC has extensive reciprocal connections with several other brain regions, including but not limited to, the amygdala, entorhinal cortex, hippocampus and subiculum, which may provide a positive feedback loop facilitating the spread and maintenance of epileptic discharges of limbic seizures [17,32,33]. The effective neuronal connectivity between the seizure onset zone and other key areas in the seizure network, notably areas that constitute the symptomatogenic zone, can be studied by CCEPs. While CCEPs cannot directly identify the actual anatomical pathway of the circuit, their changes over time may serve as biomarkers for epileptogenesis. Comparing human CCEPs with those we recorded in rats, it is important to mention that we have found consistent CCEP morphology despite varying current intensities of single pulse stimulation. In humans, morphological variability of CCEPs has been reported in the motor [34] and limbic [35] systems, leading to a wide range of the latencies of the earliest negative peak (N1). One may hypothesize that this variability is due to varying sizes of the electrical fields activating different populations of excitatory and inhibitory neurons, while in the small rat aPC, consistent neuronal populations could have been stimulated even at low current intensities. The consistency of the CCEP morphology in our experiment allowed straightforward assessment of both latency and slope.

In the current study, the amplitudes of the evoked responses in the contralateral hippocampus increased significantly in epileptic animals compared with the controls. The electric stimulation used for the purpose of obtaining evoked responses could possibly have influenced the plastic changes that would have occurred with the ipsilateral hippocampus. However, the contralateral amplitude increase could be the consequence of changes in synaptic, neurochemical, and/or expression of receptors and formation of new efferent outputs on downstream circuits in response to electrical stimulation of the pathologic neural network. The contralateral amplitude increases could also be possibly representing a mechanism that facilitates secondary generalization.

Neuronal network alterations due to axonal sprouting and synaptic reorganization are known to play a role in the contribution to pharmacoresistance [36]. Under physiological conditions, a relatively low percentage of CA3 pyramidal cells exhibit recurrent excitatory connections. However, under pathological conditions mossy fiber sprouting occurs in human focal specimens [37] and animal models of focal epilepsies. The formation of denser recurrent CA3-CA3 excitatory functional synapses and more extensive Schaffer collateral projections to CA1 [38] leads to an aberrant excitatory circuit [39]. Our current results showing an increase in the amplitudes of CCEPs could possibly relate to the aberrant formation of excitatory synapses not only in the hippocampus, but within a wider seizure network.

While the CCEP latency possibly reflects the speed of propagation determined primarily by the number of synapses, the amplitude reflects the size of the activated synchronous neuronal population in the hippocampus or enhanced signal amplification between the aPC and the hippocampus. The differences in latencies and amplitudes between the CCEP responses suggest that the short-range connections between the aPC and the ipsi-contralateral CA3 and the contralateral CA3 are denser pathways in the epileptic compared to those of the controls. This indicates stronger pathways which are more capable of transmitting high amplitude signals at faster rates (and may be associated with higher risks of secondary generalization). Also, the connections may vary in number and quality. For example, low quality neuronal connections with fewer axonal or dendritic connections may have a longer latency with lower peak amplitudes. Other metrics such as radial and axial diffusivity may impact the path quality resulting in the very weak correlation between these two measures of the CCEP response.

Medically-intractable multifocal seizures with frequent secondary generalization can be modeled with the KA rodent model [40]. We have shown that acute seizures in this model have both hippocampal and extrahippocampal origins [21]. Status epilepticus induced by KA, a glutamate analog, has been implicated in the damage of the hippocampus, PC, entorhinal cortex, amygdala, thalamus, and septal regions [41]. The PC also has a tendency to sustain neuronal injury due to repeated seizures [42] and glutamate-mediated excitotoxicity has been suggested as one of the underlying mechanisms. Amongst these brain regions, the hippocampus is the most important structure involved in temporal lobe epilepsy and shows prominent neuromorphological and electrophysiological changes such as hippocampal sclerosis related to kainate-induced excitotoxicity [43]. The KA model used for the current study is multifocal and primarily involves the hippocampi, specifically the CA3 region is known to be damaged following KA injections [22]. As recorded in our previous studies [44], we have placed the electrodes in the CA3 region and investigated the plastic changes in the connectivity between the PC and the hippocampi, using hippocampal evoked potentials of single-pulse stimulation of the aPC. Specifically, we studied the changes in waveform response in the CA3 regions in response to stimuli in the aPC.

Our results provide a rationale for further investigations of CCEPs as potential biomarkers of epileptogenesis. Future studies should include more such signal analysis for definitive conclusions. We have not studied the histopathological changes such as aberrant morphology, mossy fiber sprouting, neuronal loss or neurogenesis. Additional studies of the markers of synaptogenesis in excitatory neurons, altered regulation of glutamate transporters, and abnormal transcription of AMPA and GABA receptor subunits would help to understand the plastic changes involved in the increases in the CCEP amplitude leading to epileptogenesis.

5. Conclusions, future directions and clinical implications

The mechanisms of neural circuit modifications involved in epileptogenesis have not been completely elucidated. The PC may be a hub facilitating epileptogenesis and potentially contributing to the development of the intractable epilepsy, due to its complex connectivity with limbic and cortical neuronal circuits. Our results demonstrated that epileptogenesis result in plastic changes that may manifest as amplification of the amplitudes of the contralateral hippocampal evoked responses to single-pulse stimulation of the aPC. Further studies are required to determine the functional relevance of CCEPs generated by stimulation of the PC using various animal models of epilepsy and eventually study the alterations in tissue connectivity after administration of anti-seizure treatments.

The current study is focused on the stimulation of the aPC. Further investigation of the central and posterior PC would advance our understanding of the pivotal role of PC in facilitating epileptogenesis. Additionally, the PC is extensively connected with associative brain networks such as the prefrontal, amygdaloid, perirhinal and entorhinal cortices, which are also involved in epileptogenesis giving rise to several local recurrent circuits that may provide a substrate for seizure activity [17,45]. In the future, we plan to increase the sample size and stimulate the right (in addition to the left) aPC and try other animal models of epilepsy assessing whether a similar preservation of ipsilateral CCEPs and increase in contralateral CCEPs are present in the same animal. We implanted the aPC of only the left hemisphere. However, there are possible inherent differences between the hemispheres. For example, one study found that the right hemisphere in rats is larger in both weight and surface dimensions than the left

hemisphere [46], and another study that specifically evaluated the somatosensory cortex found size variations in the cortical areas that correspond to specific sensory functions between the two hemispheres of individual rats [47]. Therefore, future studies should attempt to implant both sides. Future work should also focus on multi-site recordings to advance our understanding of the circuit level morphological and functional changes. A future project could also demonstrate rearrangements of excitatory connectivity between PC and CA3 by using viral or bacterial tracers with immunostaining. By gaining a deeper understanding of the dynamic changes and the complex interactions of the pathways critical for epileptogenesis, together with anatomical precision provided by neuroimaging studies, CCEPs may be a reliable method towards the development of a seizure propensity biomarker and antiepileptic treatment strategies for intractable epilepsy.

Author contributions

MZK designed the experiments and outlined the manuscript. MDS generated the first draft. All authors contributed to conducting and analyzing the experiments and revising the manuscript.

Funding

The study was made possible by the startup funds from the George Washington University and pilot grant from Clinical and Translational Science Institute at Children's National in Washington, DC. Both sources of funding are for MZK.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.seizure.2020.07.008.

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