Published in final edited form as:

Epilepsy Behav. 2019 June ; 95: 87–94. doi:10.1016/j.yebeh.2019.03.046.

Functional Brain Connectivity in a Rodent Seizure Model of Autistic-Like Behavior

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Abstract

Objective: There is increasing evidence that Autism Spectrum Disorder (ASD) is a disorder of functional connectivity with both human and rodent studies demonstrating alterations in connectivity. Here we hypothesized that early-life seizures (ELS) in rats would interrupt normal brain connectivity and result in autistic-like behavior (ALB).

Methods: Following 50 seizures adult rats were tested in the social interaction and social novelty test and then underwent qualitative and quantitative intracranial EEG monitoring in the medial prefrontal cortex (PFC) and the hippocampal subfields, CA3 and CA1.

Results: Rats with ELS showed deficits in social interaction and novelty and compared to control rats had marked increases in coherence within the hippocampus (CA3-CA1) and between the hippocampus and PFC during the awake and sleep states indicating hyper-connectivity. In addition, sleep spindle density was significantly reduced in rats with ELS. There were no differences in voltage correlations and power spectral densities between the ELS and control rats in any bandwidths.

Conclusion: Taken together these findings indicate that ELS can result in ALB and alter functional connectivity as measured by coherence and sleep spindle density. These findings implicate altered connectivity as a robust neural signature for ALB following ELS.

Keywords

epilepsy; sociability; power spectrum; development; coherence; voltage correlations

1. Introduction

Autism Spectrum Disorder (ASD) is a group of complex neurodevelopmental disorders, characterized by deficits in social communication and interaction and restricted, repetitive, and stereotyped patterns of behavior. The symptoms are present from early childhood and impair everyday functioning. Individuals with ASD have co-occurring intellectual disability,

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language disorder, and epilepsy at higher rates than the general population [1]. A singular pathophysiological mechanism is unlikely to be responsible for the autistic phenotype. While genetics play an important role in ASD, underlying causal genetic variants have been defined in only a small subset of cases. In addition, the genes so far identified tend to be biologically pleiotropic and drive multiple diverse functions across developmental time and anatomical distribution [2]. In addition, environmental and other non-genetic factors can contribute to the development of autistic symptomatology [3]. The etiological heterogeneity associated with ASD raises the question as to how such a disparate group of etiologies can result in a recognizably consistent phenotype of impaired social communication and behavior, which can often be devastating.

There is increasing data indicating the common neurophysiologic underpinnings of individuals with ASD, regardless of etiology, is aberrant functional connectivity, defined as the “temporal correlations between remote neurophysiological events” [4–6]. While functional MRI and diffusion tensor imaging have been widely used to assess spatial connectivity between brain regions in ASD [7–12], neuroimaging studies lack temporal resolution. EEGs and magnetoencephalography have been widely used to assess connectivity over time and during different states. A particularly useful EEG feature is coherence, a measure of connectivity between brain regions. Coherence is a measure of “coupling” oscillations and therefore provides a dynamic link between brain areas required for the integration of distributed information [13, 14]. In an EEG study using a large cohort of children with ASD, developmental delay without ASD and neurotypical children, brain connectivity, as measured by coherence, distinguished children with ASD from both neurotypical and developmentally delayed children [15]. Children with ASD had increased coherence which was most prominent during slow wave sleep (SWS). In addition, this same cohort of children with ASD had reduced sleep spindle density when compared to the other groups [16].

Rodent studies have paralleled some of these findings in children. In rat pups with early-life seizures (ELS) and autistic-like behavior (ALB), EEG recordings from hippocampus and prefrontal cortex (PFC) demonstrated marked increases in coherence at all bandwidths compared to controls [17]. While alterations in coherence were seen during the awake state, alteration of coherence during sleep has not been studied in a model of ASD. Studying sleep is important since sleep EEG not only reflects the maturation of the brain [18] but also allows for examination of dynamic neural networks in the absence of external stimuli.

In addition, new insight into the function of sleep states makes it imperative that the brain's activity during sleep in neurodevelopmental disorders not be overlooked. While the exact function of sleep is unknown, the strongest evidence from human and animal experimental studies suggests that sleep's major role is to regulate brain plasticity [19]. SWS is implicated in learning and memory throughout the lifespan and a prevailing hypothesis has been that SWS is essential for the consolidation of memories temporarily laid down during waking hours [20]. Given that there are both age- and state-dependent differential effects of sleep on neuronal responses and processes, further evaluation of sleep coherence is clearly important to our understanding of neurodevelopmental disorders.

The goals of this study were to determine if adult rats with prior ELS have ALB and alterations in functional connectivity and if hyper-coherence as seen in previous rodent models and children is present across sleep and awake states. We used multi-site electrophysiology in the ELS model of postnatal acquired epilepsy to study its resulting effects within and between brain regions that are critical for cognition, the PFC and CA1 and CA3 regions of the hippocampus. To parallel the human studies, we assessed EEG measures during the awake state, SWS and rapid eye movement (REM) sleep.

2. Material and methods

Overview of Experiment

To assess seizure-induced alterations in brain oscillatory activity male rat pups were subjected to 50 flurothyl-induced seizures from postnatal (P) day 5–14 (roughly corresponding to the first year of life in humans) [21]. This procedure in rats and mice has been shown to result in ALB when the animals are studied following the seizures [17, 22]. To test for ALB the sociability and social novelty tests were assessed at P60.

Between P70–P80 rats were implanted with intracranial electrodes in the right prelimbic region of the medial prefrontal cortex (PFC) and hippocampal subfields, CA1 and CA3. One to two weeks later EEG recordings were carried out during epochs of sleep and wakefulness.

2.1. Animals

Sprague-Dawley rats were obtained from Charles River (Saint-Constant, QC). Animals were housed individually under USDA and AAALAC-approved conditions, with a 12hr light/dark cycle, a temperature of 26°C, and ad libitum access to food and water. Rats were weaned a postnatal (P) day 21. All procedures were approved by the University of Vermont's Institutional Animal Care and Use Committee and conducted in accordance with guidelines from the National Institutes of Health. All experiments were performed in accordance with the guidelines provided by the National Institute of Health and University of Vermont for the humane treatment of animals. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Male and female Sprague-Dawley rats ($n = 8$; 4 male, 4 female) were subjected to 5 flurothyl-induced seizures daily from P5 to P14 for a total of 50 seizures using previously described methods [23–26]. A 10% flurothyl solution (Bis(2,2,2-trifluoroethyl) ether), an inhaled convulsive agent, was delivered to the pups, which were placed in a plastic container located in an airflow hood. Flurothyl (0.1 ml) was injected slowly onto filter paper placed on the inside of the container where it evaporated. Pups were removed from the flurothyl after approximately 2 min when tonic extension of both forelimbs and hindlimbs was observed. Littermate control (CTL) pups ($n = 5$; 4 male, 1 female) were handled and removed from the dam during the time of the seizure to control for the effects of maternal separation stress.

2.2 Behavioral Studies

Social Interaction and social novelty tests.—The three chamber sociability test was used to test social behavior in the seizure and CTL rats [17, 27–29]. The social test apparatus

consists of a wooden box with removable plastic partitions separating the box into three chambers. The size of the entire box was 122×41 cm (5002 cm^2) with the middle chamber area of 1148 cm^2 (28×41 cm) and two side chambers of 1927 cm^2 (47×41 cm). The height of the walls was 43 cm. An aluminum metal cylinder (11 cm in height, bottom diameter 11 cm) with grating (1 cm apart) with a lid was placed in each of the end chambers. On the first day the rat was placed in the center chamber for 5 min and then the doors to the side chamber were open for 5 min to allow the rat to explore. Following this phase the rat was removed from the chamber and returned to its home cage for 5 min. The test rat was then placed in the middle chamber with the dividers closed to allow it to explore the middle chamber for five minutes. After this 5 min habituation period, an unfamiliar rat of the same sex was placed in one cylinder box while a marble block (object) was placed in the other cylinder. The doors were then opened and the animal was allowed 15 min to explore. Following this 15 min period, the test rat was removed from the box and placed in its home cage for 5 minutes. The object was then substituted with a new (novel) rat of the same sex of the test rat. The test rat was then placed in the center of the maze and allowed another 15 min to explore. Animals were videotaped during all sessions. Measurements of the corresponding amount of time spent in each chamber were done for both the sociability and social novelty tests by an investigator blinded as to group.

2.3 Electrophysiology

P70 rats underwent electrode implantation into the prelimbic cortex of the PFC and hippocampal subfields CA1 and CA3 using a custom designed head stage. Two $72 \mu\text{m}$ diameter, insulated nichrome electrodes (California Fine Wire, CA) with tips separated by ~ 0.5 mm were implanted at each location. The rats were anesthetized with inhaled isoflurane and placed in a stereotaxic frame. The skull was exposed and four screws inserted, two anterior to the left and right ends of bregma and two over the left and right cerebellum. The three pairs of electrodes were stereotaxically placed using coordinates from bregma, sagittal suture and skull surface using Sherwood and Timiras [30]: PFC: 3.2 mm anterior to bregma; 0.8 mm lateral, mm below skull; CA1: -3.2 mm posterior to bregma, 2.0 mm lateral, 2.1 mm; CA3 -3.2 mm posterior to bregma, 3.0 mm lateral, -3.1 mm below skull. A right cerebellar screw was used for grounding while two wires were placed in over the left cerebellum and used as reference electrodes. All implants were fixed to the skull via the skull screws and grip cement. The wound was sutured and a topical antibiotic was applied. Platinum iridium EMG electrodes were implanted in the trapezius muscles bilaterally. The interval between surgery and the beginning of the EEG recordings was a minimum of 1 week.

The implant was connected to a pre-amplifier with a cable connecting the rat to an amplifier and analog/digital board. Signals were filtered wideband (1–9000 Hz) and sampled at 30K Hz and recorded on a Neuralynx (Montana) system. A light diode was attached to the headstage to allow tracking of movements. EEG recordings were obtained from the rats during foraging for food pellets in a cylindrical recording chamber for 30 minutes for 4 days. Rats were food deprived for 24 hours prior to the foraging sessions then allowed to eat ad libitum for one hour after the foraging. Sleep studies were done during the early afternoon

for 2–3 days. The rat was placed in the recording chamber and left undisturbed for two hours in the dark.

During foraging for food pellets the EEG predominantly consisted of theta activity with an active EMG pattern. Slow wave sleep (NREM sleep) demonstrated a high amplitude EEG consisting primarily of delta activity with intermixed faster frequencies and sleep spindles and a low voltage EMG. REM sleep consisted of a lower voltage EEG pattern with abundant theta frequencies and an inactive EMG pattern.

Field Recordings: All EEG analyses were performed using NeuroExplore 5 software (Nex Technologies, Colorado Springs, CO). EEG bandwidths were defined as delta (δ ; >0 - <4 Hz); theta (θ ; 4 to <10 Hz); alpha (α ; 10 to <20 Hz); beta (β ; 20 to <25 Hz); slow gamma (γ ; 25 to <50 Hz); and fast gamma (γ ; 50 to 100 Hz) [17, 31]. Oscillatory properties using local field potentials (LFPs) were calculated for each animal during the awake state, SWS and REM. Sleep spindles were analyzed during SWS from the PFC electrodes.

Coherence.—Phase coherence quantifies the consistency of the relative phase between two simultaneous signals that have the same frequency [32]. Coherence was computed between two simultaneously recorded EEG signals from different brain regions in each frequency band. The Fast Fourier Transform (FFT) of the EEGs (after detrending and applying Hanning tapering) were calculated for 1–100 Hz. The number of frequency values was 65536 with a 50% window overlap. Then, individual and cross-densities were calculated: $P_{xx} = \text{FFT}(X) * \text{Conj}(\text{FFT}(X))$; $P_{yy} = \text{FFT}(Y) * \text{Conj}(\text{FFT}(Y))$; $P_{xy} = \text{FFT}(X) * \text{Conj}(\text{FFT}(Y))$ where X and Y refer to electrodes in different brain regions. P_{xx} , P_{yy} and P_{xy} values were averaged across all intervals and coherence values calculated as: $\text{Mean}(P_{xy}) * \text{Mean}(P_{xy}) / (\text{Mean}(P_{xx}) * \text{Mean}(P_{yy}))$. Coherence values for delta, theta, alpha, beta, and gamma for 60 sec of artifact free epochs were calculated between: i) the PFC and CA1; ii) PFC and CA3; and iii) CA3 and CA1.

EEG Voltage Correlations.—This analysis calculates cross correlations from EEG in two areas and serves as a measure of modulation across the two electrode sites [32]. Cross-correlograms between LFPs in the PFC and CA1 and CA3 were obtained for 1 sec before and after the overlap and averaged over the 60 sec EEG recording (bin size = 3.4×10^{-4}). The EEG voltages within the specified bin size were calculated and then the correlations between the electrode pairs computed. If $x[i]$, $i=1, \dots, N$ is the reference voltage and $y[i]$, $i=1, \dots, N$ is another voltage, then $c[t]$ = Pearson correlation coefficients (PCC) between vectors $\{x[1], x[2], \dots, x[N-t]\}$ and $\{y[t+1], y[t+2], \dots, y[N]\}$. To assess modulation, the mean differences from the minimum and maximum correlation values over the 2 sec correlograms were calculated for each electrode pair during the three states (Fig. 1).

Sleep spindle density.—Spindle density in the PFC was calculated using two methods: 1) Automatic detection of the spindle band (12–20) Hz band. EEG activity in the 12–20 Hz was band-filtered during 10 min epochs of SWS (with filter band specified as [Main Band Min Freq, Main Band Max Freq]). Hilbert transform was then applied to the filtered signal. The jumps of the phase of the Hilbert transform from 360 degrees to zero were identified as starts of the oscillation cycles. The percentage of the spindle band to the

remaining activity was then calculated for each second. Figure 2 shows an example of sleep spindles with a corresponding spectrogram showing sleep spindles at ~12 Hz. 2. Manual counting. Spindle number per 10 min of SWS was calculated visually by one investigator (GLH) blinded to treatment group.

Histology.—Following all behavioral and electrophysiological testing, rats were sacrificed, brains removed and cut 40 μ m and stained with thionin for verification of electrode placement as previously described [17].

Statistics: The ANOVA was used to compare time spent with the rat, object or neither rat/object in the social interaction test, as well as time spent with novel or familiar rat or neither in the social novelty test. Tukey's multiple comparisons test was used to compare differences in the three locations (rat, object, neither or novel rat, familiar rat or neither). The t test was used to compare EEG measures in the ELS and CTL rats. False detection rate was assessed with the Holm-Sidak test with an alpha of 0.05. All p values reported were adjusted for false detection. Data are presented as mean $\pm$ standard error of the mean.

4. Results

All rats had tonic seizures with administration of the flurothyl. Four of the 10 (40%) rats died during or shortly after flurothyl administration while none of the CTL rats died. Behavioral testing was done on 6 ELS rats (3 σ , 3 φ) and 5 CTL (4 σ , 1 φ).

4.1 Behavioral Testing

Social interaction and novelty.—In the social interaction study the ELS rats behaved differently than the CTL rats ($F(2,15) = 23.3$, $p < 0.001$), with the CTL rats spending significantly more time than the ELS rats with the rat (CTL: 634.2 ± 51.16 sec; ELS = 464.5 ± 44.44 sec; $p = 0.033$). The CTL rat spent significantly more time with the rat than object ($p < 0.0001$) while there was no difference in the time ELS rats spent with the rat than the object ($p = 0.181$). CTL rats had significant differences in the amount of time spent with the familiar and novel rat ($F(2,15) = 12.74$, $p = 0.001$) with the CTL rats spending more time with the novel rat than the familiar rat, although the difference was not statistically significant ($p = 0.061$). In the social novelty test group difference were noted ($F(2,15) = 12.06$, $p = 0.008$) with the ELS group spending more time with the familiar than the novel rat ($p = 0.048$). These results demonstrate that following ELS, rats have deficits in social cognition.

4.2 EEG Findings.

Of the 6 ELS and 5 CTL rats that completed the behavioral testing, 5 ELS (3 male, 2 female) and 4 CTL (3 male, 1 female) rats had electrodes placed in the PFC, CA3 and CA1 regions. All analyses were conducted on artifact-free EEG.

Coherences.—A marked increase in coherence was noted in the ELS compared to CTL rats in almost all frequency bands in all three states (Fig. 4). At no frequency band or behavioral state were the coherences higher in the CTL than the ELS rats. The differences in

coherences were generally higher in all brain regions during SWS than during the awake and REM states.

Voltage Correlations.—Mean and peak correlations of voltage over a 2 sec window were compared in the CTL and ELS rats by comparing differences between minimum and maximum correlations for each state and each electrode position. No differences between CTL and ELS rats were apparent from visual or statistical analysis of the data (Fig. 5).

Sleep spindle density.—The spindle density during SWS was significantly higher in the CTL ($10.41 \pm 0.231\%$) than in the ELS ($8.737 \pm 0.138\%$) rats ($t = 6.591$, $p < 0.0001$). Likewise, a manual count of spindles showed a significantly greater number of spindles in the CTL than the ELS group (CTL: 87.2 ± 5.95 ; ELS: 53.4 ± 11.7 ; $p = 0.033$). No differences in spindle duration was found between the CTL and ELS rats (CTL: 0.846 ± 0.021 ; ELS: 0.891 ± 0.014 , $p > 0.05$).

Spectral power.—Other than for increased power in the theta band in ELS rats during the awake state, no significant differences in power across bandwidths in awake, SWS and REM were noted (Suppl. Figs. 1–3).

Histology.—Electrode placement was verified in the PFC and CA1 and CA3 regions.

5. Discussion

The primary finding in this study is that in a rodent model of ELS, adult rats demonstrate ALB with impairments in social interaction and social novelty. These ALBs occurred in rats showing significant alterations in connectivity with both intrahippocampal (CA3–CA1) and hippocampus–PFC networks having higher coherences than in CTL rats. The increases in coherence occurred at all frequencies and during all states (awake state, SWS, REM). While spectral coherence was markedly increased in the ELS rats, there were no differences between ELS rats and CTL rats in spectral power at any frequency during any of the three states. These findings show that following ELS the brain is hyper-connected during all states. In addition, compared to the CTL groups, rats with ELS had decreases in sleep spindle density, indicating a decrease in thalamocortical information flow.

Both the hippocampus and PFC are important in learning and memory and have been implicated in the pathophysiology of ASD [33–36]. While the hippocampus stores an explicit representation of space that is used as a “cognitive map” to guide goal-based behavior [37–41], the CA1–PFC pathway is crucial for goal-directed decision-making and providing abstract representations of goals and intentions [42–45]. During the awake state, the firing of action potentials is temporally coordinated through phase of local theta in both the CA3–CA1 [46–48] and CA1–PFC pathways [49–51]. This theta-entrained activity across neuronal circuits is critical for guiding the plastic changes that underlie the dynamic “back and forth” storage and retrieval of information across these networks [52–54]. Likewise, during SWS sleep there is delta-driven widespread synchronization of action potentials [55, 56]. High coherence values during both wakefulness and sleep are an indication of strong

phase linking between neuronal ensembles, indicating high connectivity between the brain regions that produce the EEG signals [57].

Since high coherence values are a marker of enhanced connectivity, it is difficult to explain why high coherences would be associated with the ALB associated with ELS. ELS can result in an imbalance of excitation/inhibition in the neocortex and hippocampus resulting in enhanced excitation that may predispose the developing brain to excessive connectivity [58–60]. High phase locking of neurons in multiple brain regions likely results in neurons in both structures firing with excessive synchrony with a diminished ability to develop localized functional ensembles [61]. In a recent study of fMRI in individuals at risk for psychosis it was found that high levels of hyper-connectivity in a circuit involving the cerebellum, thalamus, and cortex was predictive of impending full psychosis – hallucinations, delusions, and disorganized thought and behavior [62]. Neuronal synchrony in the brain is finely tuned and it is likely that functional “over-connectivity” may be as detrimental as “under-connectivity,” as a network that is over-connected at baseline may not be able to adapt to increased cognitive demand. We suggest that as with other electrophysiological processes there is an ideal “sweet spot” for dynamic coherence and that deviations in either a positive or negative direction can alter behavior adversely.

Sleep spindles are measured by EEG as brief distinct bursts of activity in the frequency range of 12 to 20 Hz in rats. They have a characteristic waxing and waning shape and can be defined by their oscillatory frequency, amplitude, and density throughout the night. In addition, spindles are believed to play an important functional role in sleep-dependent synaptic plasticity and memory consolidation [63]. Spindles originate in the thalamic reticular nucleus [64] and reflect the integrity of the thalamocortical microcircuit. Spindle deficits have been reported in cohorts of adolescents and adults with schizophrenia [65–67] and recently in children with ASD [16]. In a sleep study including children with ASD, children with developmental delay but no ASD and neurotypical children, a strong relationship between sleep spindle density and nonverbal ratio IQ test and the Vineland Socialization domain scores was found, suggesting sleep spindles may play a role or be a biomarker of behavior in neurodevelopment disorders [16].

Another dimension of brain connectivity, amplitude or voltage correlation, is a measure of the co-modulation of the amplitude envelopes, i.e. power, of oscillations in the two brain areas [32, 68]. Amplitude correlation is also referred to as “power-to-power correlation” or “amplitude-amplitude coupling.” Voltage correlation marks the degree of association between amplitudes of the EEG across sites and does not calculate phase or the consistency of phase relationships seen with coherence. Amplitude correlations reflect state changes coupled across brain networks that are driven by neuromodulatory systems [69]. Studies have shown that amplitude correlations provide a measure of large-scale interactions between cortical areas that are only indirectly connected via polysynaptic pathways [70–73]. The lack of differences between the ELS and CTL indicates voltage modulation is not altered in adult rats with ELS.

While equating rat behavior to the complex symptoms of a child with ASD is difficult, the deficits in sociability and social novelty preference in rats have some similarities to the core

features that are seen in children with ASD. For example, children with ASD spend less time looking at people and longer times looking at objects compared to controls [74, 75]. Both the findings of increased coherences in three brain regions during awake, SWS and REM and decreased sleep spindles in SWS parallel findings in children with ASD [15, 16]. These studies indicate that the ELS rodent model recapitulates some of the features of the ASD condition in children and can provide insights into pathophysiological mechanisms.

The results of this study need further confirmation. The small number of animals did not allow us to determine if there are gender differences and future studies are necessary to determine if gender is a biological variable in this model. It is quite possible that there are gender differences in the model. For example, it is known that the relationship of sleep spindles with cognition may be sexually dimorphic [76, 77]. It is also important to note that although coherence values were higher in the ELS group than the CTL, this does not indicate cause and effect. Rather, hyper-connectivity and reduced sleep spindle density may serve as robust neural biomarkers for ALB following ELS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Supported by National Institute of Health, National Institute of Neurological Disorders and Stroke, grants NS108296 and NS108765.

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Highlights

- Early life seizures in rodents can lead to autistic-like behaviors
- Rats with autistic-like behaviors following early-life seizures have hyper-connected brains and reduced sleep spindle density
- Hyper-connectivity and reduced sleep spindle density may be a neural biomarker for autistic-like behaviors following early-life seizures

Fig. 1.

Example of voltage cross correlations during the awake and sleep states over 2 secs. Note the modulation by theta (~ 8 Hz) in the awake and REM states and by delta during SWS. Degree of voltage modulation was determined by subtracting the maximum (Max) and (Min) and averaging the differences in each state and brain region. In this example the reference variable is the PFC electrode and the comparison variable is the CA1 electrode.

Fig. 2. EEG with spindles (underlined) corresponding with a 12 Hz band in the spectrogram (arrows).

Fig. 3.

Social interaction and social novelty tests. In the social interaction study the ELS rats behaved differently than the CTL rats, with the CTL rats spending significantly more time than the ELS rats with the rat. The CTL rat spent significantly more time with the rat than object while there was no difference in the time ELS rats spent with the rat than the object. CTL rats had significant differences in the amount of time spent with the familiar and novel rat with the CTL rats spending more time with the novel rat than the familiar rat, although the difference was not statistically significant. In the social novelty test group difference were noted with the ELS group spending more time with the familiar than the novel rat. These results demonstrate that following ELS, rats have deficits in social cognition.

Fig. 4.

Coherences (mean±SEM) between PFC-CA1, PFC-CA3, CA1-CA3 in (A) awake, (B) SWS and (C) REM states. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Fig. 5. Examples of voltage correlations in three brain regions (PFC, CA1 and CA3) in ELS and CTL rats during three states. No differences were noted between the ELS and CTL groups in any brain region or state.