Neurobiology of Disease 78 (2015) 35–44

Contents lists available at ScienceDirect

Neurobiology of Disease

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

Molecular alterations in areas generating fast ripples in an animal model of temporal lobe epilepsy

Kellen D. Winden [a,b,c], Anatol Bragin [c,e], Jerome Engel [c,d,e,f], Dan H. Geschwind [a,b,c,f,g,*]

[a] Interdepartmental Program for Neuroscience, University of California, Los Angeles, Los Angeles, CA, USA
[b] Program in Neurogenetics, University of California, Los Angeles, Los Angeles, CA, USA
[c] Department of Neurology, University of California, Los Angeles, Los Angeles, CA, USA
[d] Department of Neurobiology, University of California, Los Angeles, Los Angeles, CA, USA
[e] The Brain Research Institute, University of California, Los Angeles, Los Angeles, CA, USA
[f] Semel Institute for Neuroscience and Human Behavior, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA, USA
[g] Department of Human Genetics, University of California, Los Angeles, Los Angeles, CA, USA

ARTICLE INFO

Article history:
Received 22 October 2014
Revised 26 January 2015
Accepted 13 February 2015
Available online 25 March 2015

Keywords:
Epilepsy
Fast ripples
Microarray
Coexpression network analysis

ABSTRACT

The molecular basis of epileptogenesis is poorly characterized. Studies in humans and animal models have identified an electrophysiological signature that precedes the onset of epilepsy, which has been termed fast ripples (FRs) based on its frequency. Multiple lines of evidence implicate regions generating FRs in epileptogenesis, and FRs appear to demarcate the seizure onset zone, suggesting a role in ictogenesis as well. We performed gene expression analysis comparing areas of the dentate gyrus that generate FRs to those that do not generate FRs in a well-characterized rat model of epilepsy. We identified a small cohort of genes that are differentially expressed in FR versus non-FR brain tissue and used quantitative PCR to validate some of those that modulate neuronal excitability. Gene expression network analysis demonstrated conservation of gene co-expression between non-FR and FR samples, but examination of gene connectivity revealed changes that were most pronounced in the cm-40 module, which contains several genes associated with synaptic function and the differentially expressed genes *Kcna4*, *Kcnv1*, and *Npy1r* that are down-regulated in FRs. We then demonstrate that the genes within the cm-40 module are regulated by seizure activity and enriched for the targets of the RNA binding protein *Elavl4*. Our data suggest that seizure activity induces co-expression of genes associated with synaptic transmission and that this pattern is attenuated in areas displaying FRs, implicating the failure of this mechanism in the generation of FRs.

Introduction

The epileptogenic regions of both patients with temporal lobe epilepsy (TLE) and animal models of TLE display abnormal electrophysiological oscillations between 250 and 600 Hz, which have been termed fast ripples (FRs) (Bragin et al., 1999; Staba et al., 2002). Within the dentate gyrus of animals with TLE, FRs can appear before the onset of epilepsy and have not been observed in non-epileptic animals, suggesting that they may reflect pathological mechanisms involved in epileptogenesis (Bragin et al., 2000). In patients with epilepsy, FRs have been shown to reliably demarcate the seizure onset zone (Jacobs et al., 2008). In addition, surgical resection of regions generating FRs is a better predictor of seizure freedom than resection of the seizure onset zone itself (Jacobs et al., 2010), demonstrating that FRs may play a role in ictogenesis. Therefore, understanding the cellular and molecular alterations that occur in areas generating FRs may provide insight into the key processes of epileptogenesis and ictogenesis in TLE.

In the hippocampus of non-epileptic animals, there are physiological oscillations between 120 and 200 Hz that occur during immobility or slow wave sleep (Buzsaki et al., 1992). Therefore, it has been suggested that FRs are related to these sharp-wave ripple oscillations. However, physiological sharp-wave ripples have been shown to be dependent on inhibitory post-synaptic potentials (Buzsaki et al., 1992; Ylinen et al., 1995), whereas areas generating FRs are enlarged when inhibitory transmission is blocked (Bragin et al., 2002). Recent evidence suggests that FRs represent abnormal population spikes within the dentate gyrus (Bragin et al., 2011; Ibarz et al., 2010). However, the cellular dysregulation and network abnormalities that cause this behavior are unknown.

Studies examining the molecular mechanisms underlying FRs have been difficult because FRs are not diffusely distributed throughout the epileptic hippocampus, but are generated by small clusters of neurons surrounded by large areas of tissue that do not generate FRs. However,

* Corresponding author at: Daniel H Geschwind, UCLA Neurogenetics Program, 2309 Gonda, 695 Charles E. Young Dr. South, Los Angeles, CA, USA.
E-mail address: dhg@mednet.ucla.edu (D.H. Geschwind).
Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2015.02.011
0969-9961/

advances in applying functional genomics techniques on limited biological samples provide the ability to study molecular processes within electrophysiologically identified neuronal clusters (Bragin et al., 2004). Therefore, we designed our study to compare gene expression in areas of the dentate gyrus that generated FRs to neighboring regions that did not display abnormal activity. After isolating these regions, we examined the gene expression changes between FR and non-FR generating areas, and we found that there were differences in the molecular networks between these regions, indicating changes in gene expression associated with synaptic transmission that are regulated by seizure activity.

Materials and methods

Animals

All procedures described in this study were approved by the University of California, Los Angeles, Institutional Animal Care and Use Committee. Wistar rats weighing 250–300 g were used for experiments. Twenty hours prior to the pilocarpine injection, each animal was injected intraperitoneally with 3 mg/kg of LiCl. Thirty minutes before pilocarpine injection, methylscopolamine (1 mg/kg) was injected subcutaneously, followed by 25 mg/kg subcutaneous injection of pilocarpine. Two hours after status began, it was terminated by intraperitoneal injection of pentobarbital (30 mg/kg). During the post status period, 2 cm^3 of Lactated Ringers solution was injected subcutaneously and repeated as necessary to maintain hydration. The following day, Lactated Ringers solution with 5% Dextrose was injected every 4–8 h until fluids were accepted by mouth. Beginning one week after status, animals were subjected to 24 hour video monitoring for identification of epileptic and non-epileptic rats. Seven rats with recurrent spontaneous seizures within 2–4 months after status epilepticus were used for electrophysiological experiments.

Electrophysiology

Rats were deeply anesthetized with 5% isoflurane, the brains were removed, and horizontal 400-μm-thick slices containing the EC, subiculum, and hippocampal formation in the horizontal plane were prepared using a Leica series VT1000S vibratome. The brains were cooled for 1 min in 4 °C artificial CSF (ACSF) containing (in mM): 126 NaCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 D-glucose, pH 7.4. Before recordings, the slices were incubated at 32 °C for 1–6 h in a 300 ml storage chamber filled with ACSF and continuously bubbled with a mixture of 95% O_2–5% CO_2.

Eleven slices from six animals were used for electrophysiological characterization. Before recording from each slice, a picture was taken of each slice and a 200 μm mesh was superimposed on each picture. This map of each slice was used to track the electrical response of each area to perforant path stimulation. Electrical activity was recorded using standard, extracellular, ACSF-filled glass microelectrodes (~10 MΩ impedance) in the DG granular cell layer at a depth of 200 μm in the middle of the slice. The stimulating electrodes (tungsten wires OD 50 μm) were placed in the perforant path. Rectangular pulses with 0.2 ms duration and currents ranging from 0.1 to 0.7 mA were delivered every 10 s for stimulation. Under visual control, the recording microelectrode was moved with 200 μm steps along the granular layer and evoked potentials were recorded after low-pass filtering at 3 kHz, with 10 kHz sampling rate using DataPac data acquisition software. Four different current amplitudes (0.1; 0.3; 0.5; 0.7 mA) were used to stimulate each point within the slice 10–15 times, and it was determined on-line whether responses contained single or multiple population spikes. FR generating areas were dissected based on the map of multiple population spikes elicited (number of population spikes ≥ 3), and dissection included an approximately 200 μm buffer area around the FR generating area. The area of dissection included the dentate gyrus granule cell layer and their dendrites. These pieces of tissue were all approximately 1 mm^2, which is consistent with our prior data demonstrating the size of FR generating areas (Bragin et al., 2002). An equivalently sized tissue area encompassing the same cellular regions in an area that did not generate multiple population spikes was also dissected from the same slice. After dissection, the pieces of tissue were immediately placed in microcentrifuge tubes on dry ice.

RNA extraction and microarray hybridization

Areas generating FRs and those that did not were isolated as above. Total RNA was extracted from each sample using the Qiagen miRNeasy kit, and its quality was checked using the Agilent 2100 Bioanalyzer (Agilent). RNA was amplified using a two-step protocol, TargetAmp 2-Round Aminoallyl-aRNA amplification kit (Epicentre). Amplified RNA was directly labeled with biotin using the Biotin-X-X-NHS reagent (Epicentre). This labeled RNA was hybridized to Illumina RatRef12 microarrays, and a total of 22 microarrays were used for this experiment (11 FR and 11 non-FR samples). Array hybridization and scanning were performed following the manufacturer's protocol at UCLA microarray core facility (http://microarray.genetics.ucla.edu/xowiki/).

Data normalization

Microarray data was imported into R (http://www.r-project.org/) and normalized using the Variance Stabilizing Transform and Robust Spline Normalization, which are a part of the lumi package in Bioconductor (http://www.bioconductor.org/). The Variance Stabilizing Transform was specifically designed to take advantage of the multiple technical replicates present on Illumina arrays, and Robust Spline Normalization is a simple scaling algorithm designed for use with Illumina data (Du et al., 2008). We then log transformed the data using Log_2. We limited subsequent analyses to genes that either met thresholds for mean expression in either the FR or non-FR samples (Expression > 5.6), or overall variance (variance > 0.02), which leads to the selection of 12,581 genes for further analysis.

Data analysis

Among the 12,581 genes that met the criteria for mean expression and variance, we limited this analysis to those with at least ten values with a detection p-value < 0.01 (10 out of 22 samples, >45%), which resulted in the analysis of 10,083 genes for differential expression. We used a Bayesian ANOVA to evaluate differential expression because it provides estimates of a gene's variance based on the variance of other genes with similar expression (Baldi and Long, 2001) and has been shown to be a robust method for determining differential expression in microarray experiments (Choe et al., 2005). Because the algorithm utilizes a composite of similarly expressed genes to estimate variance, we confirmed normality of all groups of genes with similar expression in the dataset using the Shapiro–Wilk test ($p < 2e-16$). Each FR sample was paired with an accompanying non-FR sample from the same slice, and these data were used in a paired Bayesian ANOVA to evaluate the differential expression. The threshold for significance was set at $p < 0.05$ and Bonferroni correction was used for multiple testing ($p < 5e-6$).

Weighted gene co-expression network analysis was performed as described previously (Oldham et al., 2006; Winden et al., 2009). Briefly, the top 15% of genes (among the 12,581 previously selected) with the highest variance in either the FR or non-FR datasets were selected, representing 2013 genes. Pearson correlations between all these genes were calculated, and this correlation matrix was scaled using an exponent (beta = 8) to best approximate scale free topology. This scaled matrix was then used to calculate the topological overlap matrix (Ravasz et al., 2002), which was used an input into the hierarchical clustering algorithm.