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FULL-LENGTH ORIGINAL RESEARCH

Nodule excitability in an animal model of periventricular nodular heterotopia: c-fos activation in organotypic hippocampal slices

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Summary

Objective: **Aberrations in brain development may lead to dysplastic structures such as periventricular nodules. Although these abnormal collections of neurons are often associated with difficult-to-control seizure activity, there is little consensus regarding the epileptogenicity of the nodules themselves. Because one common treatment option is surgical resection of suspected epileptic nodules, it is important to determine whether these structures in fact give rise, or essentially contribute, to epileptic activities.**

Methods: **To study the excitability of aberrant nodules, we have examined c-fos activation in organotypic hippocampal slice cultures generated from an animal model of periventricular nodular heterotopia created by treating pregnant rats with methylazoxymethanol acetate. Using this preparation, we have also attempted to assess tissue excitability when the nodule is surgically removed from the culture. We then compared c-fos activation in this in vitro preparation to c-fos activation generated in an intact rat treated with kainic acid.**

Results: **Quantitative analysis of c-fos activation failed to show enhanced nodule excitability compared to neocortex or CA1 hippocampus. However, when we compared cultures with and without a nodule, presence of a nodule did affect the excitability of CA1 and cortex, at least as reflected in c-fos labeling. Surgical removal of the nodule did not result in a consistent decrease in excitability as reflected in the c-fos biomarker.**

Significance: **Our results from the organotypic culture were generally consistent with our observations on excitability in the intact rat—as seen not only with c-fos but also in previous electrophysiologic studies. At least in this model, the nodule does not appear to be responsible for enhanced excitability (or, presumably, seizure initiation). Excitability is different in tissue that contains a nodule, suggesting altered network function, perhaps reflecting the abnormal developmental pattern that gave rise to the nodule.**

KEY WORDS: **c-fos, Dysplasia, Epileptogenicity, Nodular heterotopia, Organotypic slice cultures, Seizure initiation.**

Cortical dysplastic lesions are now recognized as a common feature of brain tissue involved in epileptic discharge.[1,2] Focal cortical dysplasia (FCD) comes in many forms, and may arise from a variety of "insults" that alter the development and/or organization of the tissue, including genetic mutations and/or external trauma.[3,4] The abnormal regions of the brain may include pathologic cell types (e.g., balloon cells) or simply "normal" ectopic neurons. Given this variability, it is really not possible to make any general statements about the role of these abnormalities in the

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generation of epileptic activities. Yet, there has been a pressing practical need to assess the "epileptogenicity" of these lesions, since surgical management of epileptic brain regions often involves decisions about the antiepileptic efficacy of removing the dysplastic brain region.

To contribute to this discussion, we have been studying a rat model of cortical dysplasia induced by treating pregnant dams with the drug methylazoxymethanol acetate (MAM). The brains of the offspring include ectopic cell regions—the result of aberrant neuronal migration during brain development—resembling periventricular nodular heterotopia (PNH) in humans.[5,6] Although PNH in humans most often occurs as a result of a genetic mutation in the filamin A (*FLNA*) gene,[7] aberrant gray matter nodules appear as a result of various mutations and developmental abnormalities.[8,9]

Several studies on patients with PNH have supported the view that the nodular dysplastic tissue provides an epileptic trigger.[10–13] In both human and animal model studies, abnormal cell properties that might affect excitability have been found not only within the nodule, but also in perinodular tissue.[14,15] These findings have led investigators to entertain the idea that epileptogenicity in dysplastic brain is a result of circuitry reorganization (i.e., new/abnormal connectivity) rather than—or in addition to—epileptogenic properties of cells within the region of dysplasia.[16–20]

In our previous work with the MAM model,[21–23] we attempted to characterize the relative excitability of the nodular tissue relative to the surrounding brain. Those studies failed to show that the nodule was more sensitive, or that epileptic discharges in the nodule led epileptic activities in the surrounding neocortex or underlying hippocampus. However, our technical approaches limited our conclusions because of the following: (1) in acute brain slices which included nodular tissue, key pathways for seizure initiation and spread may have been disrupted; and (2) in intact animals, stereotaxically guided electrode placement restricted our recordings to a very limited population of cells.

To overcome these technical difficulties, we have turned to organotypic slice cultures[24,25] generated from MAM-exposed rat pups, and used c-fos immunochemistry as a surrogate marker for neuronal excitation.[26–29] To help interpret these in vitro analyses, we have coupled these culture studies with parallel investigations in the intact animal, using c-fos to evaluate cellular excitability in MAM-exposed rats injected with kainic acid to induce epileptic activity. The organotypic slice culture studies presented herein provide no evidence that the periventricular nodular heterotopia (PNH) is more excitable than the surrounding tissue.

Methods

Preparation of organotypic hippocampal slice cultures (OHSCs)

Pregnant Sprague–Dawley rats were injected intraperitoneally with either 25 mg/kg MAM or sodium chloride (NaCl) at embryonic day 15 (E15). Hippocampal slice cultures were prepared as described previously[24] and adapted in our laboratory[25] to include the cortex (see Data S1). Slice cultures were maintained in a humidified incubator at 37°C (5% CO_2) for up to 12 days in vitro. At 9–11 days in vitro (DIV), OHSCs were exposed for 60 min to either artificial cerebrospinal fluid (ACSF with 6 mM potassium) or 40 μM (bicuculline methiodide [BMI]; #14343; Sigma-Aldrich, St. Louis, MO, U.S.A., in ACSF with 6 mM potassium). After 1 h, OHSCs were prepared for immunocytochemistry. Culture experiments were repeated at least twice and always involved a set of OHSCs from more than one rat pup (see Results for numbers); in each set of BMI treatments, a set of "control" cultures was exposed to ACSF.

MAM treatment results in typical histopathologic features, including disruption of neocortical lamination and development of PNH adjacent to CA3 (Fig. 1A,B). Slices from MAM pups were chosen for further analyses/experiments if a PNH could be visualized under a dissection microscope. The presence of the nodule was confirmed by immunocytochemistry with neuron-specific nuclear protein (NeuN) antibody. Slices from control pups (dam injected with NaCl at the same gestational age) were chosen if the shape of the hippocampus was similar to the shape of the hippocampus in the previously chosen MAM cultures.

Lesions of the PNH

In a subset of experiments, cultures from MAM-exposed pups were given lesions to remove the nodule; discrete lesions were made using vacuum suction through a glass pipette. In NaCl cultures, a similar-sized lesion was made superior-lateral to the hippocampus, mimicking the location and trauma of the PNH in MAM tissue. Lesions were made at DIV8; 2 days later (at DIV10), OHSCs were exposed for 60 min to either high-potassium ACSF (with 6 mM potassium) or 40 μM BMI in ACSF with 6 mM potassium). Following these treatments, OHSCs were prepared for immunocytochemistry.

Immunocytochemistry

Slice cultures were processed for immunocytochemistry using a modification of the avidin-biotin complex (ABC) peroxidase technique[30] as previously used in this laboratory[25] (see Data S1. Methods).

In vivo kainic acid experiments

Pups were generated as described earlier, and allowed to mature to 12 weeks of age. Animals were injected with either 12 mg/kg kainic acid (#K0250; Sigma-Aldrich) in NaCl or an equal volume per weight of NaCl vehicle. For the NaCl group, we waited 30 min after injection before starting anesthesia (4% isoflurane followed by deep anesthesia with 100 mg/kg pentobarbital) and perfusion (4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 15 min) for brain fixation; animals injected with kainic acid

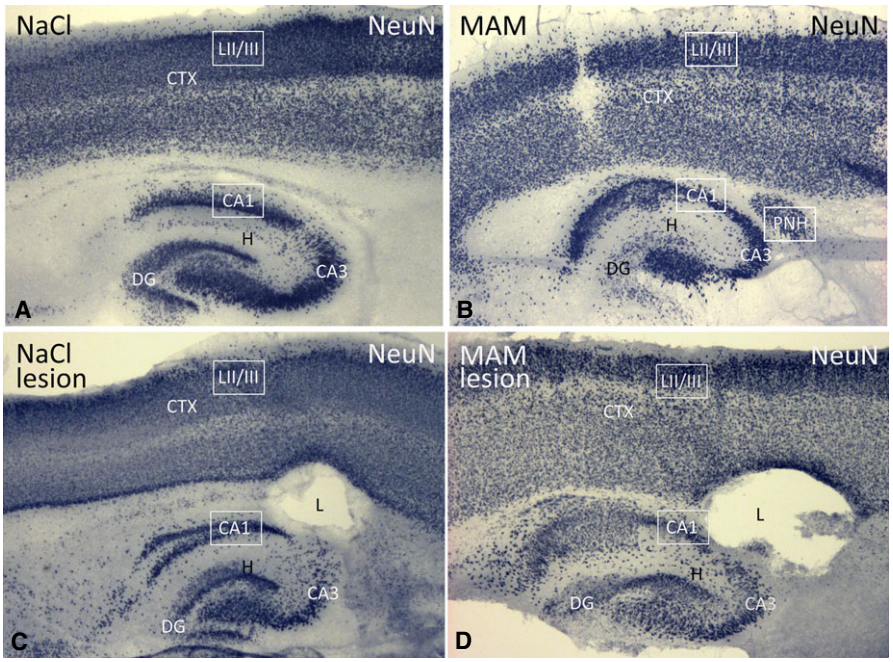

Figure 1.
(**A**, **B**) Periventricular nodular heterotopia (PNH) in the organotypic hippocampal slice cultures (OHSCs) generated from immature rats exposed to (MAM) in utero. (**A**, **B**) Photomicrographs of NeuN-labeled OHSCs at 9 DIV prepared from P8 rat pups (**A**—NaCl control; **B**—MAM exposed at E15). MAM exposure results in characteristic histopathologic features including disruption of neocortical lamination and development of PNH adjacent to the CA3 region of hippocampus. Boxed areas indicate regions used for quantitative cell counts. CTX, neocortex; LII/III, cortical layers 2/3; CA1 and CA3, hippocampal regions CA1 and CA3; DG, dentate gyrus; H, hippocampus; PNH, periventricular nodular heterotopia. (**C**, **D**) NeuN-stained OHSCs, showing the placement of the PNH lesion (L) in a culture from a MAM-exposed rat pup (**D**) and the "control" lesion in a culture (from NaCl-exposed pup) with no PNH (**C**).

were perfused either 10 or 60 min after they first displayed unilateral forelimb clonus. Following perfusions, the brains were immediately extracted and postfixed in the same cold fixative for 2 h. They were then rinsed twice in 0.1 M PB for 5 min each. Brains were transferred to 10% sucrose in 0.1 M PB for 1 h at 4°C and then transferred to 30% sucrose solution in 0.1 M PB at 4°C for 48 h. The brains were then submerged in dry ice to flash freeze. Intact brains were stored at −80°C until processed for immunocytochemistry. Transverse serial sections were cut at 40 μm on a sliding microtome and placed in a 10% sucrose solution. Sets of sections containing the PNH, CA1, and neocortex (CTX) (MAM tissue) or CA1 and CTX only (control tissue) were selected for further processing. Sections were then processed for immunocytochemistry as described earlier for the OHSCs.

As was the case for our analysis of organotypic hippocampal slice cultures, statistical analysis of intact kainate-treated animals was based on counts of c-fos–labeled neurons and neuronal activity level (number of pixels activated within labeled cells divided by the number of labeled neurons). The following experimental variables were included in the analysis: (1) animal type (non-MAM vs. MAM); (2) treatment (KA-treated, sacrificed at 10 min after seizure onset; KA-treated, sacrificed at 60 min after seizure onset); and (3) region of interest (CA1, CTX, and PNH). We also compared animals receiving NaCl injections to those receiving KA injections, but did not quantify the differences, since c-fos labeling was very low in NaCl-injected animals; KA caused clear neuronal activation in both the 10 and 60 min postseizure animals.

Quantitative assessments

For each slice culture, photomicrographs of representative regions of the neocortex (CTX) and hippocampus (CA1) and nodule (PNH) were taken at 40× (Fig. 2). Micrographs were taken when crisply stained nuclei appeared (indicating NeuN or c-fos immunoreactivity in the nucleus) during focusing from the top of the culture. The CTX region consisted of cortical layers I–III overlying the lateral tip of the dentate granule cell layer, and the hippocampal region consisted of the middle portion of pyramidal cell layer in region CA1 (see boxed areas in Fig. 1A,B). The images were evaluated using ImageMeterPro software (www.flashscript.biz). A red-green-blue (RGB) channel threshold was set for each immunocytochemical treatment group, based on the faintest neuron found within the group (Fig. S1). Each neuron within an image was manually

highlighted and ImageMeterPro determined how many pixels within the highlighted box were above the preset threshold, and therefore considered "activated." For each image of c-fos–stained cultures, we quantified the number of activated neurons (i.e., neurons expressing c-fos) and the number of pixels within each activated neuron above the RGB threshold. Baseline neuronal cell density was calculated based on counts of NeuN-stained sister sections. The evaluation and quantitative analysis of OHSCs were performed by one investigator on coded cultures (so that the investigator was blinded to treatment). In c-fos–immunoreacted OHSCs, a c-fos–positive neuron was defined as a cell with a large cell body with smooth, round edges of the nucleus (thus excluding astrocytes—which did not stain for NeuN or c-fos); small triangular-shaped cells showing 3′3-diaminobenzidine (DAB)-positive reaction were excluded from the analyses as presumably c-fos-immunoreactive microglia (these cells did not show immunoreactivity for NeuN). Degenerating neurons were recognized/excluded by their characteristic histopathology. The total number of c-fos–positive neurons per culture was separately counted in the CTX, CA1, and PNH (for MAM cultures).

For each experimental animal in the kainic acid experiments, photomicrographs of the representative regions were taken at 40× magnification (as done for the hippocampal slice cultures—see above), from sections immunoreacted against c-fos and/or NeuN, which displayed the cortical region, CA1 subfield, and PNH (MAM tissue) or CA1 and CTX region (control tissue) at the same section level. To ensure comparable conditions for the immunostaining of the different experimental animal groups, sections from all the different groups were parallel-processed for immunocytochemistry. For the quantitative evaluation, four photomicrographs representing comparable qualities of the staining patterns were taken from each region and experimental condition, and immunopositive neurons were counted and analyzed using the same ImageMeterPro software (www.flashscript.biz) as used for the slice-culture tissue. c-fos immunoreacted sections were used to determine the number of activated neurons and the degree of activation (pixels per neuron). NeuN–immunostained sections were used for neuronal cell counts; cresyl violet–stained sections were used for comparison and to determine the general pathology of the brains.

Statistical analysis

In our statistical analysis, we compared the number of c-fos-positive cells and c-fos levels (pixels per cell) across brain regions (PNH, neocortex [CTX], hippocampal CA1 region [CA1]), treatment condition (bicuculline or ACSF applications in culture; kainate or saline in intact animals), and culture type (non-MAM and MAM) or lesion status (lesioned or intact). To compare number of c-fos–positive cells and c-fos levels (pixels per cell), we used a linear mixed-effects model with a random intercept to model animal-to-animal variability. This analysis method is similar to the repeated measures analysis of variance (ANOVA) commonly employed to account for correlation of repeated measurements within an animal (indeed, the repeated-measures ANOVA is a special case of a linear mixed model, but more flexible and powerful). For the study on slice-culture activation (Table S1), depending on the comparison, the factors in the statistical analysis included the following: treatment (bicuculline [BMI] or ACSF application), region (PNH, CTX, and CA1), treatment by region; or culture type (MAM, non-MAM), and culture type by region interaction. For the study on PNH lesioning (Table S2), the linear mixed model included the following as experimental factors: types across cultures (non-MAM intact, non-MAM lesioned, MAM intact, MAM lesioned), region (CA1 and CTX), and their interactions. For the in vivo kainate study (Table S3), the experimental factors included treatment (kainate, 10 min survival; kainate, 60 min survival), region (CA1, CTX, and PNH), and animal type (MAM or non-MAM). We summarize the results of these analyses by presenting estimates, standard errors, and p-values. The quantity "estimate" represents the model-based estimate of the difference between two comparison groups. For example, in Table S2B, the estimate "−1131.13" represents the estimate of the difference in means of the outcome (here average pixels activated per cell) between the ACSF and BMI groups (ACSF minus BMI). Because of the large number of comparisons in these studies, we used the false discovery rate (FDR) adjustment to account for multiple comparisons. FDR is an alternative error control procedure for dealing with large numbers of group comparisons.[31] Significant p-values after FDR adjustment are indicated by asterisks through the tables of results. All analysis was conducted in SAS version 9.3 (SAS Institute, Inc., Cary, NC, U.S.A.).

Results

Slice cultures with PNH

Neuronal density in the brain regions of interest was evaluated by quantifying the number of NeuN-positive neurons; in most cases, data from two counting frames per region were averaged to obtain a value for a given culture. In four BMI-treated MAM cultures, there were no significant differences in neuronal density across the PNH, neocortex, and hippocampal CA1 (two tailed *t*-test, two-samples with unequal variance). Similarly, in four non-MAM BMI-treated cultures, there was no significant difference in neuronal density between the CTX and CA1. For CTX and CA1, there were no statistically significant differences in neuronal density between non-MAM and MAM cultures.

Slice culture activation

We used neuronal c-fos labeling as our marker of neuronal activation, and quantified the number of c-fos–labeled neurons within each counting frame, as well as the number

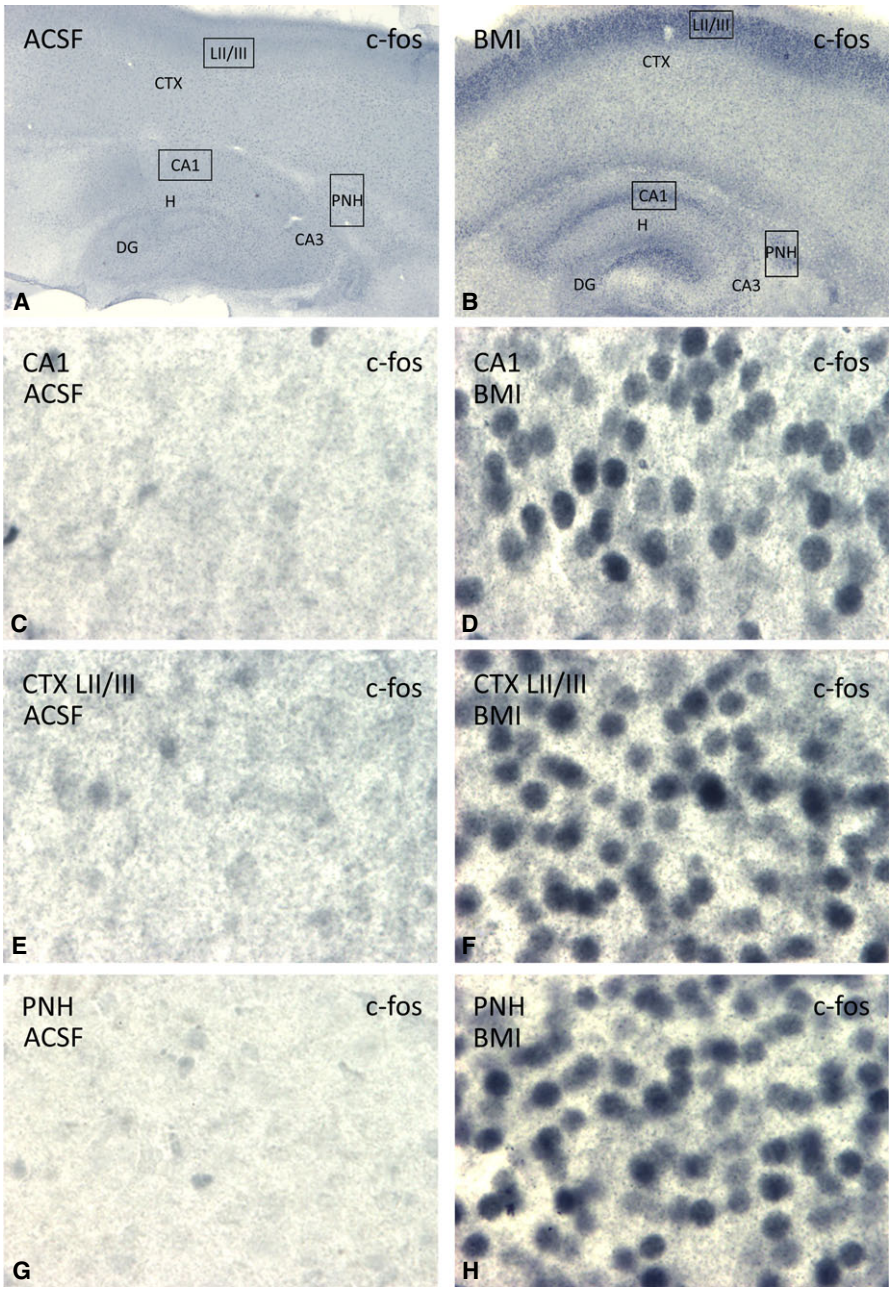

Figure 2.
c-fos labeling in OHSCs from MAM rats. (**A**, **B)** Low-power micrographs of the cultures, including PNH, CA1, and CTX. (**C**–**H)** Higher magnification micrographs from each region of interest (CA1—**C**, **D**; CTX—**E**, **F**; PNH—**G**, **H**). Left column provides baseline c-fos levels in cultures treated with ACSF, and right column shows c-fos labeling in cultures exposed to bicuculline.

of pixels per labeled neuron. Observations were made on cultures derived from 10 MAM-exposed rat pups. The experimental variables considered in our analysis were treatment (ACSF and BMI) and region of interest (CA1, CTX, and PNH). A summary of these data is provided in Figure 3A,B (see also Table S1A).

When comparing number of c-fos–positive neurons in MAM cultures treated with ACSF (three cultures from two pups) versus BMI (13 cultures from 10 pups), we found the expected differences: BMI-treated cultures showed dramatically higher numbers of c-fos–labeled neurons (statistically significant) in CA1 ($p = 0.0057$) and CTX ($p = 0.0008$), but not in the PNH ($p = 0.061$) (Table S1B). Although the number of ACSF-treated cultures was small, the number of c-fos–labeled neurons in these cultures was consistently very low (close to zero); we therefore invested our efforts in

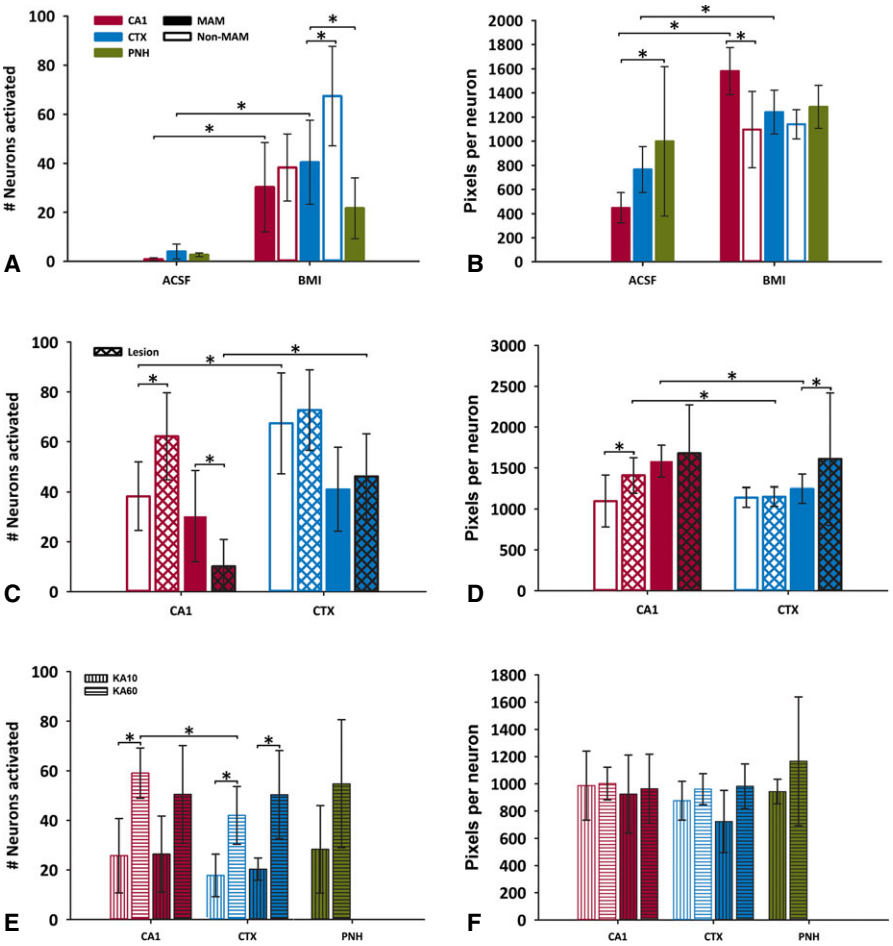

Figure 3.

Histogram summaries of c-fos activation, showing number of neurons activated (see Methods) (left column) and pixels/activated neuron (right column). Means and standard deviations are provided in Tables S1–S3, as are the comparison measures on which significant differences (asterisks) were based. (**A**, **B**) c-fos activation by bicuculline, in MAM and non-MAM cultures, for CA1, CTX, and PNH. Legend: red bars, CA1; blue bars, CTX; green bars, PNH; solid bars, MAM cultures; open bars, Non-MAM cultures. (**C**, **D**) Comparison of cultures with and without lesion of the PNH (or control lesion in non-MAM cultures). Legend: as in **A**, **B**; cross-hatched bars, lesion. (**E**, **F**) c-fos labeling in tissue from rats exposed to kainic acid. We compared labeling at 10 versus 60 min after seizure onset. Legend: as in **A**, **B**; vertical stripes, KA10; horizontal stripes, KA60.

assessing the question of whether there were regional differences in c-fos labeling in cultures exposed to BMI. Within the BMI-treated MAM cultures, the CTX showed a significantly higher number of c-fos–labeled neurons than the PNH ($p = 0.0014$) (Table S1C); the CA1 was not significantly different from the PNH or CTX. Given that the neuronal density across regions was not different, this difference in c-fos labeling is not simply a function of regional differences in cell density.

We used pixels/labeled cell as a reflection of neuronal activity level (total number of pixels activated within labeled cells in the region of interest, normalized by the number of labeled neurons). As expected, with BMI treatment, all regions of MAM cultures showed a highly significant increase in neuronal activity compared to ACSF-treated cultures (CA1, $p < 0.001$; CTX, $p = 0.0021$; PNH, $p = 0.05$) (Table S1B), with the largest difference seen in hippocampal CA1; with BMI treatment, CA1 neurons showed a significantly higher level of activation than CTX ($p = 0.0003$) and PNH ($p = 0.0009$) neurons. Of interest, in ACSF-treated MAM cultures, the PNH showed a significantly higher neuronal activity level than CA1 ($p = 0.004$) (not significantly higher than CTX, although trending in that direction) (Table S1B).

Slice cultures with PNH lesions

To evaluate the potential contribution of the PNH to activity/excitability in cortical and hippocampal regions of organotypic cultures, we attempted to lesion (i.e., remove) the PNH through "surgical intervention" (Figs. 1C,D and 4). In the results reported below, we evaluate number of c-fos–labeled neurons and activity/labeled cell (activated

pixels) in the CA1 and CTX regions of cultures exposed to BMI. We based our lesion analysis on six MAM cultures (from four pups) in which the PNH was judged to be "completely" lesioned (counts from cultures in which we judged the PNH lesion to be incomplete are not included in this analysis). To control for the mechanical effects of the lesioning process, we also made "control" lesions in cultures from rats not exposed to MAM (i.e., without the PNH; 12 cultures from five pups); these lesions were placed in approximately the same position that the PNH would normally occupy in cultures from MAM-exposed rats. In both culture types, only two regions (CA1 and CTX) were quantified. In addition, we compared these lesion groups to CA1 and CTX measures from intact MAM cultures (13 cultures for CA1 counts/14 cultures for CTX counts, from eight pups) and intact non-MAM cultures (18 cultures from six pups). The overall descriptive statistics are shown in Figure 3C,D (see also Table S2A).

CA1 in MAM cultures with the nodule completely removed had significantly lower numbers of c-fos–labeled neurons than CA1 in intact (nonlesioned) MAM cultures ($p = 0.0223$) (Table S2B). The opposite lesion effect was seen in CA1 in the comparison between lesioned non-MAM cultures and intact non-MAM cultures ($p = 0.0007$). In CTX, there was no difference in numbers of c-fos–labeled neurons due to lesioning, for either MAM or non-MAM cultures. Cell activity level (number of pixels/labeled cell) in CA1 in lesioned non-MAM cultures was significantly higher than CA1 in intact non-MAM cultures ($p = 0.0133$); activity level in CTX of lesioned MAM cultures was significantly higher than in CTX from intact MAM cultures ($p = 0.0311$) (Table S2B).

In lesioned MAM cultures, the number of c-fos–positive neurons was significantly lower in CA1 hippocampus than in CTX ($p = 0.0003$) (Table S2C). A similar pattern was also observed in intact non-MAM cultures ($p < 0.0001$). In contrast, the number of pixels per labeled neuron was higher in CA1 than in CTX in both lesioned non-MAM ($p = 0.0315$) and intact MAM ($p = 0.0037$) cultures.

Kainate in intact rats

A summary of the results from our analysis of kainate-treated rats is shown in Figure 3E,F (see also Table S3A). We first compared the KA10 group to the KA60 group (Fig. 5) to determine if neuronal activation was increased with a longer time postseizure (Table S3B). In non-MAM animals, both the CTX ($p = 0.0144$) and CA1 ($p = 0.0027$) showed significantly more c-fos–labeled neurons in the KA60 group ($n = 7$) than in the KA10 group ($n = 3$); there was no difference, however, in the number of pixels activated per labeled neuron. In MAM animals ($n = 4$ for KA10; $n = 5$ for KA60), only the CTX showed a time-dependent increase in number of labeled neurons ($p = 0.0298$); there was no significant difference in CA1 or PNH. As in non-MAM animals, all three regions in MAM animals showed insignificant changes in number of pixels activated per labeled neuron.

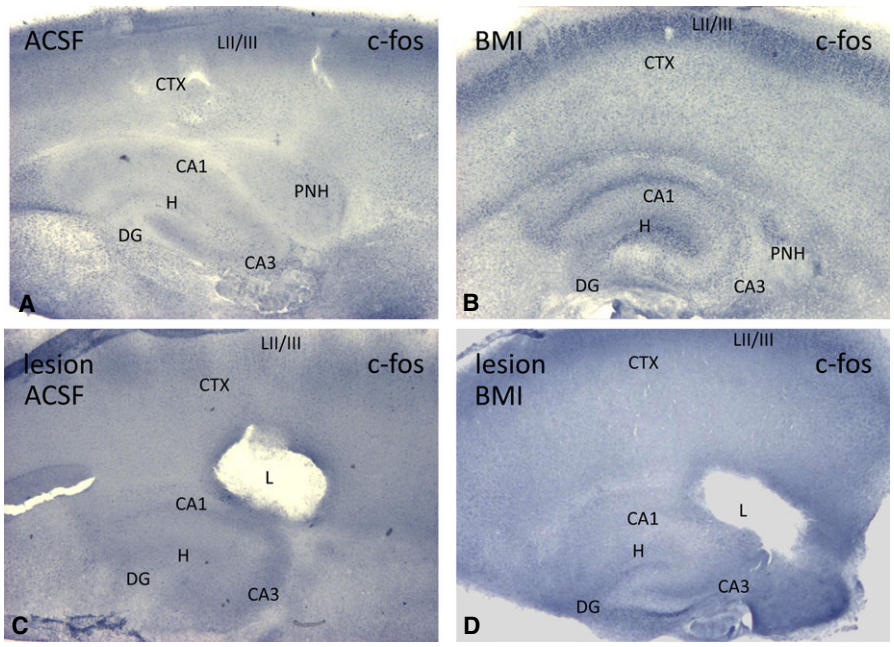

Figure 4.
Illustration of c-fos labeling in intact and lesioned cultures treated with ACSF versus bicuculline. (**A**, **B**) Intact (nonlesioned) cultures (**A**—ACSF; **B**— BMI); (**C**, **D**) lesioned cultures (**C**—ACSF; **D** —BMI). Lesions were made in cultures from MAM-exposed rat pups, with the PNH as the lesion target.
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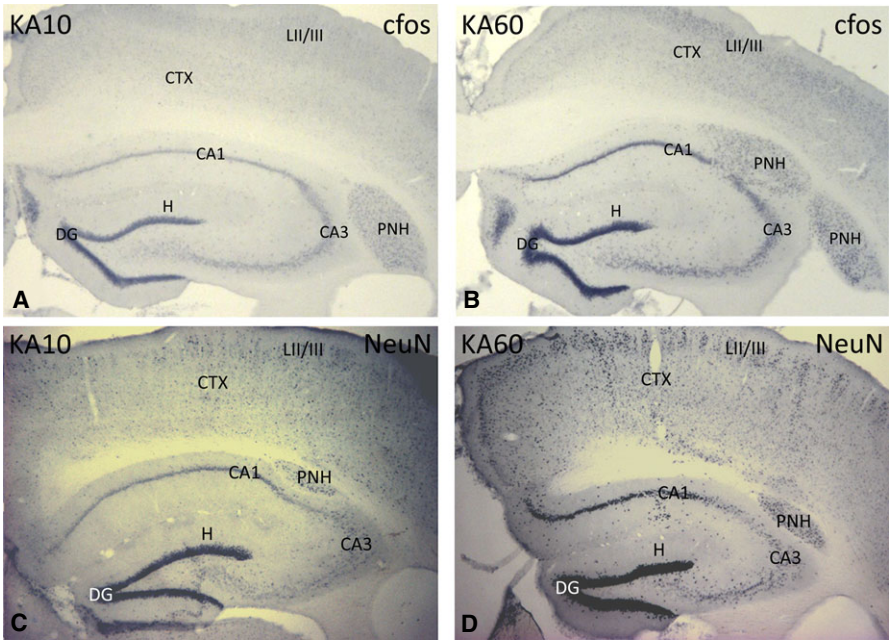

Figure 5.
Sections (NeuN labeling on left, c-fos labeling on right) from MAM-exposed rats treated with kainic acid and sacrificed at 10 min (KA10— **A**, **B**) and 60 min (KA60—**C**, **D**) after onset of seizure activity. Regions of analysis are the same as used for the analysis of OHSCs
(see Fig. 1).

We then looked within each group (KA10 and KA60, for both MAM and non-MAM) and compared activation across regions (Table S3C). At 10 min, there were no significant differences in number of c-fos-labeled neurons across brain regions (CTX, CA1, PNH) in MAM or non-MAM rats. In MAM rats, at 10 min, the number of pixels per labeled neuron in CTX was significantly lower than in both CA1 ($p = 0.0488$) and PNH ($p = 0.0349$). At 60 min in non-MAM rats, the CA1 showed a significantly higher number of c-fos–labeled neurons than the CTX ($p \leq 0.0001$). There were no differences in number of pixels per labeled neuron for any of the comparisons at 60 min.

When we compared non-MAM animals to MAM animals for each time point and region, we found no statistically significant differences between any of the groups, for either number of c-fos–labeled neurons or number of activated pixels per labeled neuron (Table S3D).

Discussion

The data from these experiments on organotypic slice cultures suggest that the presence of a nodule affects the bicuculline-induced excitability of both the cortex and the hippocampus, but in somewhat different ways. The cortex showed a higher number of neurons activated (i.e., c-fos–labeled) by the bicuculline treatment; in contrast, the CA1 hippocampus showed a greater pixel/labeled neuron level. Thus, although the PNH itself does not appear to be highly excitable, the question of whether its presence alters the excitability of the surrounding tissue remains unresolved. It is for that reason that we initiated the lesion studies—to see if removal of the PNH would "normalize" the excitation of surrounding CA1 and/or CTX. Surprisingly, when we lesioned the PNH in these cultures from MAM-treated rats (an intervention that we predicted would reduce excitability), the pixel/labeled neuron level rose in cortex (but not in CA1); however, the number of activated (i.e., c-fos–labeled) neurons was lower in CA1 (an intuitively less surprising result), but not in cortex. It is worth noting that the opposite effect—an increase in c-fos–labeled neurons—was seen in CA1 of lesioned non-MAM tissue (compared to intact non-MAM cultures), suggesting that the effect observed in MAM tissue was not simply a result of lesion-induced injury. Similarly, we saw an increase in CA1 cell excitability (pixels/labeled neuron) in lesioned non-MAM cultures compared to intact non-MAM cultures. Given the low sample numbers in these experiments, the high variability, and the numerous but inconsistent differences across groups (including changes in non-MAM tissue subject to lesion manipulations), the lesion approach to date does not resolve this question about the excitability effects of PNH presence.

In the intact rat kainic acid experiments, there was no consistent difference in excitability, at least based on the number of c-fos–labeled cells, between MAM and normal (non-MAM) rats. This result in the KA model is generally consistent with our previous electrophysiologic findings showing that the onset of epileptogenicity was not different between MAM and normal rats when animals were injected

with bicuculline.[23] The observation that numbers of activated neurons rose between 10 and 60 min in non-MAM (i.e., normal) rats indicates that cell activation at 10 min was submaximal (i.e., there was no "ceiling" effect). Of interest, in the MAM rats, optimal activation (as seen with c-fos) was already evident at 10 min; that is, there appeared to be no additional cells to activate at 60 min.

As indicated previously, there remains considerable uncertainty about whether dysplastic tissue is epileptogenic and can be considered the generator of epileptic activity in brains with focal dysplasia; that question is certainly unresolved. Clinical studies that involve surgical removal or radiation inactivation of suspected epileptogenic nodules have reported varied result,[32–36] perhaps reflecting the significant variability of these dysplastic disorders.

Given these clinical investigations and the results from both in vitro and in vivo animal model experiments, what more can we say now about nodular epileptogenicity? In general, our initial analysis of c-fos labeling in organotypic cultures appeared consistent with our previous electrophysiologic data from acute slices[22] and intact animals,[23] inasmuch as there was no indication that the nodule was more excitable than the surrounding tissue. That is, in no case was bicuculline- or kainate-induced activity in the PNH—as measured either with electrophysiologic methods or with c-fos staining—higher than in cortex or hippocampus (although in ACSF-treated cultures, PNH cellular excitability—reflected in c-fos labeling—appears higher than that in CA1 or CTX). In all cases, PNH activity rose, as expected, in response to the epileptogenic challenge.

What, then, is the advantage of the in vitro culture approach that we have introduced here? For both acute slices and intact animal experiments, a major interpretational problem has been the question of whether our recordings were appropriately "online." In acute slices, this issue arose with respect to the orientation of the slice and the possibility that key pathways were cut in the slicing process. In the intact animal, the problem was simply one of electrode placement, and the likelihood that our electrodes missed the relevant highly excitable regions of the PNH. These problems are eliminated in culture, since all the connectivity is intact (although admittedly not necessarily normal) and our "recording" method (c-fos labeling) sees the entire culture. Thus, we have reduced the possible spatial error, but in exchange, we have sacrificed the timing advantage inherent in an electrophysiologic approach. Another potential problem, of course, is the accuracy with which c-fos immunocytochemistry reflects cellular excitability. This question has been discussed at length in previous publications.[27,28] In particular, in assessing the excitability of dysplastic tissue, Chevassus-au-Louis et al.[29] previously used c-fos labeling to examine the activation of MAM-related heterotopic cell populations in rats treated with pentylenetetrazol. It is important to point out, however, that c-fos labeling—both in our experiments and in previous studies—shows only neuronal activation (i.e., excitability), and does not provide a measure of epileptiform activity or epileptogenicity.

We would conclude from our data that the PNH itself is not highly excitable, and thus is not likely to initiate epileptiform activity in this rat model of cortical dysplasia. Our lesion data are preliminary and must be interpreted with care, but are generally consistent with the view that removal of the PNH does not, in itself, reduce the excitability of the tissue. It is important to note, in that context, that even (or perhaps especially) in the organotypic culture model, tissue reorganization due to presence of the nodule has already occurred, and that removal of the nodule does not normalize this reorganization. Thus, as in the human epileptic condition associated with aberrant nodules, it may be "too late" to cure the seizure-sensitivity simply by removing the nodule. Other investigators have shown that "peridysplastic" tissue (that looks relatively normal) may indeed have epileptogenic properties.[37] And it certainly is the case that even when there is an apparently circumscribed lesion (as in PNH), the surrounding tissue is not entirely "normal," suffering from the same types of developmental irregularities that lead to the obvious dysplasia. Our decision to focus our lesion manipulations only on the circumscribed PNH (and not carry out lesions on, for example, intrahippocampal heterotopia[22,29] or potentially epileptogenic CA3 hippocampus), arose from the original rationale of these experiments: to assess the likelihood that a common surgical target (the PNH) is epileptogenic. The relative excitability of nodule/tuber and surround may well be model- and condition-specific; in other models of dysplastic lesions, and in various clinical presentations, the nodule (or other circumscribed aberrant tissue) may indeed offer a curative target.[38] Some recent publications suggest that dysplastic tissue (including cortical nodules) can itself be epileptogenic, and that removal is a useful treatment option.[32,33,36] In contrast, the recent work by Petit et al.,[39] on a "double cortex" model, is consistent with our result, showing that the "abnormal" tissue is not necessarily the site of seizure onset. Our study suggests that the aberrant nodule is not always an appropriate surgical target, and that evaluation of the treatment approach must be made on the basis of the specific abnormality.

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Disclosure

The authors have no conflicts of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

References

1. Schwartzkroin PA, Walsh CA. Cortical malformations and epilepsy. *Ment Retard Dev Disabil Res Rev* 2000;6:268–280.
2. Battaglia G, Clociaghi F, Finardi A, et al. Intrinsic epileptogenicity of dysplastic cortex: converging data from experimental models and human patients. *Epilepsia* 2013;54 (Suppl. 6):33–36.
3. Guerrini R, Dobyns WB, Barkovich AJ. Abnormal development of the human cerebral cortex: genetics, functional consequences and treatment options. *Trends Neurosci* 2008;31:154–162.
4. Marin-Valencia I, Guerrini R, Gleeson JG. Pathogenetic mechanisms of focal cortical dysplasia. *Epilepsia* 2014;55:970–978.
5. Battaglia G, Bassanini S, Granata T, et al. The genesis of epileptogenic cerebral heterotopia: clues from experimental models. *Epileptic Disord* 2003;5 (Suppl. 2):S51–S58.
6. Ferland RJ, Batiz LF, Neal J, et al. Disruption of neural progenitors along the ventricular and subventricular zones in periventricular heterotopia. *Hum Mol Genet* 2009;18:497–516.
7. Sheen VL, Jansen A, Chen MH, et al. Filamin A mutations cause periventricular heterotopia with Ehlers-Danlos syndrome. *Neurology* 2005;64:254–262.
8. Cardoso C, Boys A, Parrini E, et al. Periventricular heterotopia, mental retardation, and epilepsy associated with 5q14.3-q15 deletion. *Neurology* 2009;72:784–792.
9. Conti V, Carabalona A, Pallesi-Pocachard E, et al. Periventricular heterotopia in 6q terminal deletion syndrome: role of the C6orf70 gene. *Brain* 2013;136:3378–3394.
10. Battaglia G, Granata T, Farina L, et al. Periventricular nodular heterotopia: epileptogenic findings. *Epilepsia* 1997;38:1173–1182.
11. Kothare SV, VanLandingham K, Armon C, et al. Seizure onset from periventricular nodular heterotopias: depth-electrode study. *Neurology* 1998;51:1723–1727.
12. Sisodiya SM, Free SL, Thom M, et al. Evidence for nodular epileptogenicity and gender differences in periventricular nodular heterotopia. *Neurology* 1999;52:336–341.
13. Battaglia G, Franceschetti S, Chiapparini L, et al. Electroencephalographic recordings of focal seizures in patients affected by periventricular nodular heterotopia: role of the heterotopic nodules in the genesis of epileptic discharges. *J Child Neurol* 2005;20:369–377.
14. Battaglia G, Pagliardini S, Ferrario A, et al. AlphaCaMKII and NMDA-receptor subumit expression in epileptogenic cortex from human periventricular nodular heterotopia. *Epilepsia* 2002;43 (Suppl. 5):209–216.
15. Karlsson A, Lindquist C, Malmgren K, et al. Altered spontaneous synaptic inhibition in an animal model of cerebral heterotopias. *Brain Res* 2011;1383:54–61.
16. Colacitti C, Sancini G, Franceschetti S, et al. Altered connections between neocortical and heterotopic areas in methylazoxymethanol-treated rat. *Epilepsy Res* 1998;32:49–62.
17. Valton L, Guye M, McGonigal A, et al. Functional interactions in brain networks underlying epileptic seizures in bilateral diffuse periventricular heterotopia. *Clin Neurophysiol* 2008;119:212–223.
18. Archer JS, Abbott DF, Masterton RA, et al. Functional MRI interactions between dysplastic nodules and overlying cortex in periventricular nodular heterotopia. *Epilepsy Behav* 2010;19:631–634.
19. Kitaura H, Oishi M, Takei N, et al. Perventricular nodular heterotopia functionally couples with the overlying hippocampus. *Epilepsia* 2012;53:e127–e131.
20. Christodoulou JA, Barnard ME, Del Tufo SN, et al. Integration of gray matter nodules into functional cortical circuits in periventricular heterotopia. *Epilepsy Behav* 2013;29:400–406.
21. Baraban SC, Wenzel HJ, Hochman DW, et al. Characterization of heterotopic cell clusters in the hippocampus of rats exposed to methylazoxymethanol in utero. *Epilepsy Res* 2000;39:87–102.
22. Tschuluun N, Wenzel JH, Katleba K, et al. Initiation and spread of epileptiform discharges in the methylazoxymethanol acetate model of cortical dysplasia: functional and structural connectivity between CA1 heterotopia and hippocampus/neocortex. *Neuroscience* 2005;133:327–342.
23. Tschuluun N, Wenzel HJ, Doisy ET, et al. Initiation of epileptiform activity in a rat model of periventricular nodular heterotopia. *Epilepsia* 2011;52:2304–2314.
24. Stoppini L, Buchs PA, Muller D. A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* 1991;37:173–182.
25. Wenzel HJ, Tamse CT, Schwartzkroin PA. Dentate development in organotypic hippocqampal cultures from p35 knockout mice. *Dev Neurosci* 2007;29:99–112.
26. Morgan JI, Curran T. Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. *Trends Neurosci* 1989;12:459–462.
27. Liu Z, Yang Y, Silveira DC, et al. Consequences of recurrent seizures during early brain development. *Neuroscience* 1999;92:1443–1454.
28. Houser CR, Zhang N, Peng Z, et al. Neuroanatomical clues to altered neuronal activity in epilepsy: from ultrastructure to signaling pathways of dentate granule cells. *Epilepsia* 2012;53 (Suppl. 1):67–77.
29. Chevassus-au-Louis N, Rafiki A, Jorquera I, et al. Neocortex in the hippocampus: an anatomical and functional study of CA1 heterotopias after prenatal treatment with methylazoxymethanol in rats. *J Comp Neurol* 1998;394:520–536.
30. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981;29:577–580.
31. Storey JD. A direct approach to false discovery rates. *Journal of the Royal Statistical Society: Series B* 2002; 64:479–498.
32. Aghakhani Y, Kinay D, Gotman J, et al. The role of periventricular nodular heterotopia in epileptogenesis. *Brain* 2005;128:641–651.
33. Tassi L, Colombo N, Cossu M, et al. Electroclinical, MRI and neuropathological study of 10 patients with nodular heterotopia, with surgical outcomes. *Brain* 2005;128:321–337.
34. Stefan H, Nimsky C, Scheler G, et al. Periventricular nodular heterotopia: a challenge for epilepsy surgery. *Seizures* 2007;16:81–86.
35. Schmitt FC, Voges J, Buentjen L, et al. Radiofrequency lesioning for epileptogenic periventricular nodular heterotopia: a rational approach. *Epilepsia* 2011;52:e101–e105.
36. Agari T, Mihara T, Baba K, et al. Successful treatment of epilepsy by resection of periventricular nodular heterotopia. *Acta Med Okayama* 2013;66:487–492.
37. Ruppe V, Dilsiz P, Reiss CS, et al. Developmental brain abnormalities in tuberous sclerosis complex: a comparative tissue analysis of cortical tubers and perituberal cortex. *Epilepsia* 2014;55:539–550.
38. Wong M. Mammalian target of rapamycin (mTOR) inhibition as a potential antiepileptogenic therapy: from tuberous sclerosis to common acquired epilepsies. *Epilepsia* 2010;51:27–36.
39. Petit LF, Jalabert M, Buhler E, et al. Normotopic cortex is the major contributor to epilepsy in experimental double cortex. *Ann Neurol* 2014;76:428–442.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Quantitative assessment of c-fos–labeled neurons.

Table S1. Organotypic hippocampal slice culture (OHSC) c-fos activation.

Table S2. Organotypic hippocampal slice culture (OHSC) c-fos activation following periventricular nodular heterotopia (PNH) lesion.

Table S3. Periventricular nodular heterotopia (PNH) c-fos activation in intact, kainate-treated rats.

Data S1. Methods.