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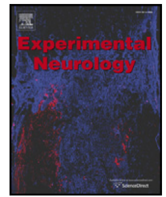

Research Paper

Progressive neuronal activation accompanies epileptogenesis caused by hippocampal glutamine synthetase inhibition

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ABSTRACT

Loss of glutamine synthetase (GS) in hippocampal astrocytes has been implicated in the causation of human mesial temporal lobe epilepsy (MTLE). However, the mechanism by which the deficiency in GS leads to epilepsy is incompletely understood. Here we ask how hippocampal GS inhibition affects seizure phenotype and neuronal activation during epilepsy development (epileptogenesis). Epileptogenesis was induced by infusing the irreversible GS blocker methionine sulfoximine (MSO) unilaterally into the hippocampal formation of rats. We then used continuous video-intracranial electroencephalogram (EEG) monitoring and c-Fos immunohistochemistry to determine the type of seizures and spatial distribution of neuronal activation early (1–5 days postinfusion) and late (16–43 days postinfusion) in epileptogenesis. Early in epileptogenesis, seizures were preferentially mild (stage 1–2), activating neurons in the entorhinal-hippocampal area, the basolateral amygdala, the piriform cortex, the midline thalamus, and the anterior olfactory area. Late in epileptogenesis, the seizures were generally more severe (stages 4–5) with neuronal activation extending to the neocortex, the bed nucleus of the stria terminalis, the mediodorsal thalamu\s, and the central nucleus of the amygdala. Our findings demonstrate that inhibition of GS focally in the hippocampal formation triggers a process of epileptogenesis characterized by gradual worsening of seizure severity and involvement of progressively larger neuronal populations over a period of several weeks. Knowledge about the underlying mechanism of epileptogenesis is important because such knowledge may result in more specific and efficacious treatments of MTLE by moving away from large and poorly specific surgical resections to highly targeted surgical or pharmacological interventions of the epileptogenic process.

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1. Introduction

Mesial temporal lobe epilepsy (MTLE) is one of the most common types of medication refractory epilepsies. For up to one-third of patients with MTLE, current antiepileptic drugs do not adequately control seizures, or cause side effects that limit their use. Because the seizures are thought to originate from mesial (i.e. limbic) structures within the medial temporal lobe – particularly the hippocampal formation, entorhinal cortex and amygdala – unilateral resection of these structures along with adjacent white matter tracts and neocortical areas has remained the standard of care for medication refractory MTLE for over half a century (Falconer and Taylor, 1968; Spencer et al., 1984). While such surgery leads to short-term cessation of seizures in >75% of cases, the procedure can result in complications and adverse outcomes (Hader et al., 2013). Moreover, the seizures will eventually recur in approximately 50% of patients who become seizure free after surgery (Foldvary et al., 2000; Jeha et al., 2006; Yoon et al., 2003), suggesting that the causative mechanism of MTLE is not removed by current neurosurgical approaches, but remains latent elsewhere in the brain. Knowledge about the causative mechanism of epilepsy is important because such knowledge is expected to result in more specific and efficacious treatments of MTLE.

There is increasing evidence to suggest that the enzyme glutamine synthetase (GS) is implicated in the causation of epilepsy. GS is deficient in hippocampal astrocytes in patients with medication refractory MTLE (Eid et al., 2004; van der Hel et al., 2005) and in astrocytes in the amygdala in patients with neocortical epilepsies (Steffens et al., 2005). Because GS is necessary for the conversion of glutamate and ammonia to glutamine, its deficiency is expected to cause an increase in glutamate and ammonia in astrocytes lacking the enzyme. It is possible that the high levels of glutamate and ammonia in astrocytes can enter the extracellular compartment of the brain via several mechanisms and lead to

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seizures, oxidative/nitrosative stress, inflammation, and cellular injury (Bezzi et al., 1998; Danbolt, 2001; Olney et al., 1972; Robert et al., 2015; Tian et al., 2005).

We have previously shown that inhibiting GS by chronic infusion of the irreversible enzyme blocker methionine sulfoximine (MSO) into the hippocampal formation or amygdala of rats, leads to a sequence of events similar to those experienced by some patients with MTLE. I.e. an initial episode of prolonged, low-grade seizures; a clinically silent latent period; and eventually, recurrent seizures that increase in severity as the disease evolves over time. The MSO-treated animals recapitulate many other features of human MTLE such as perturbations in the homeostasis of neurotransmitter glutamate, loss of hippocampal neurons, changes in monocarboxylate transporters, and comorbid depressive features (Eid et al., 2008; Gruenbaum et al., 2015; Lauritzen et al., 2012). We now use c-Fos staining in the MSO-model of MTLE to assess how hippocampal GS inhibition affects neuronal activation during epileptogenesis. Our working hypothesis is that epileptogenesis caused by hippocampal GS inhibition is characterized by activation of progressively larger neuronal populations over a period of weeks, suggestive of a process of evolving neuronal plasticity.

2. Materials and methods

2.1. Chemicals and animals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted. Male Sprague Dawley rats were used in this study (200 to 250 g; Charles River Laboratories, Wilmington, Mass.). Rats had free access to food and water and were housed on a 12 h/12 h light/dark cycle, with lights on from 7 a.m. to 7 p.m. The animal care and use procedures were approved by the Institutional Animal Care and Use Committee of Yale University. All experiments were performed in accordance with current guidelines.

2.2. Intrahippocampal infusion of MSO or PBS

Nineteen rats were anesthetized with 1% to 2% Isoflurane (Baxter, Deerfield, Ill.) in O_2 and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, Calif.). A 30-gauge stainless steel cannula attached to a plastic pedestal (Plastics One, Roanoke, Va.) was introduced through a burr hole in the skull and into the right hippocampus, using the following coordinates with bregma as the reference: AP = −5.6 mm, ML = 5.3 mm, DV = −6.5 mm. The cannula was cemented to the skull using cyanoacrylate and connected via plastic tubing to a subcutaneously implanted Alzet osmotic pump (Model 2004, Durect Corp., Cupertino, Calif.). This pump holds a total volume of 200 μL and delivers a continuous flow of 0.25 μL/h for ~28 days (as per manufacturer's specifications). One set of pumps was filled with MSO (2.5 mg/mL; dissolved in Dulbecco's Phosphate Buffered Saline, PBS) to achieve a delivery of 0.625 μg of MSO per h ($n = 10$). Another set of pumps was filled with PBS and implanted in control animals ($n = 9$). Rats were operated on sequentially in 10 pairs of 2, each pair consisting of an MSO injected rat and a matched PBS control such that treatment and timing of fixation in relation to surgery could be maximally approximated in the control.

Four stainless steel epidural screw electrodes (Plastics One) were implanted to record cortical electroencephalogram (EEG) activity. Two electrodes (one in each hemisphere) were positioned in the epidural space overlying the parietal neocortex. A third electrode was positioned in the epidural space near lambda to serve as the reference. A fourth electrode was positioned over the occipital bone (not touching the dura) to serve as the ground. The female socket contacts on the end of each electrode were inserted into a plastic pedestal (Plastics One), and the entire implantation was secured by UV light cured acrylated urethane adhesive (Loctite 3106 Light Cure Adhesive, Henkel Corp., Rocky Hill, Conn.).

2.3. Video-intracranial EEG monitoring and seizure quantitation

The experimental setup for recording video-EEG was adapted from Bertram (2006). The rats were placed individually in custom-made Plexiglas cages. A spring-covered, 6-channel cable was connected to the electrode pedestal on one end and to a commutator (Plastics One) on the other. A second cable connected the commutator to the digital EEG recording unit (CEEGraph Vision LTM, Natus Bio-logic Systems Corp., Mundelein, Ill.). Digital cameras with infrared light detection capacity were used to record animal behavior (two cages per camera). The digital video signal was encoded and synchronized to the digital EEG signals. Seizures were identified by visual inspection of the EEG record. As detailed in Avoli and Gloor (1994) seizures were defined by EEG characteristics and not by the duration of the discharge. Specifically, seizures displayed distinct signal changes from background (interictal) activity. Such signal changes included sustained rhythmic or spiking EEG patters and a clear evolution of signal characteristics from onset to termination.

As timing of fixation following seizures was critical to the c-Fos staining method described below, the rats were followed in real time by a researcher blinded to their condition (MSO or PBS). Eight rats were monitored at a time, always consisting of 4 MSO rats paired with their matched PBS control. One set of MSO and PBS rats was monitored during the first five days of MSO infusion (early epileptogenesis group), and another set was monitored during days 15 to 43 after MSO infusion (late epileptogenesis group). When seizure activity was observed on the EEG and by monitoring rat behavior by the blinded researcher in real time, the time of the seizure was noted, and a second researcher immediately reviewed the video-EEG recordings to independently confirm the occurrence of, and stage of the seizure. The seizures were staged by behavioral analysis using a modification of Racine's criteria (Racine et al., 1973), as follows: stage I, immobilization, eye blinking, twitching of vibrissae and mouth movements; stage II, head nodding, often accompanied by facial clonus; stage III, forelimb clonus; stage IV, rearing; stage V, rearing, falling and generalized convulsions. The MSO seizure rat and its matched PBS control were transported together from the monitoring room to the main lab 45 min following the seizure for perfusion fixation, which occurred 1 h after the onset of the seizure.

2.4. Pilocarpine control

In order to confirm the efficacy of the c-Fos staining protocol, 1 rat was injected subcutaneously with 325 mg/kg pilocarpine dissolved in PBS. Within a few hours of injection several stage V seizures occurred. One hour after the onset of the first stage V seizure the rat was perfusion fixed and processed for c-Fos staining following the same protocol as for MSO/PBS infused rats.

2.5. Tissue preparation, immunohistochemistry and nomenclature

At 1 h after the observed seizure, the MSO seizure rat and its matched PBS control were anesthetized with Isoflurane and perfused transcardially with saline followed by 4% formaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4). The brains were removed and left in the same fixative overnight at 4 °C and then transferred to 20% sucrose (dissolved in PB) the next day. After at least 24 h in 20% sucrose, the brains were sectioned horizontally on a sliding microtome at 50-μm thickness. Every fifth section was mounted on gelatin-coated slides and stained with cresyl violet. The remaining four sets of sections were stored in FD Tissue Cryoprotection Solution (FD Neurotechnologies, Columbia, Md.) at −20 °C prior to staining. These sectioned brains were logged and randomly coded by one researcher in order to blind a second researcher to the condition of the rats. Blinding the primary researcher to both the condition (MSO, PBS, pilocarpine) and to the stage of seizure (stage 1 or stage 5) was critical due to the descriptive and subjective nature of the analysis.

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