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Metalloproteinase inhibition prevents inhibitory synapse reorganization and seizure genesis

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ABSTRACT

The integrity and stability of interneurons in a cortical network are essential for proper network function. Loss of interneuron synaptic stability and precise organization can lead to disruptions in the excitation/inhibition balance, a characteristic of epilepsy. This study aimed to identify alterations to the GABAergic interneuron network in the piriform cortex (PC: a cortical area believed to be involved in the development of seizures) after kindling-induced seizures. Immunohistochemistry was used to mark perineuronal nets (PNNs: structures in the extracellular matrix that provide synaptic stability and restrict reorganization of inhibitory interneurons) and interneuron nerve terminals in control and kindled tissues. We found that PNNs were significantly decreased around parvalbumin-positive interneurons after the induction of experimental epilepsy. Additionally, we found layer-specific increases in GABA release sites originating from calbindin, calretinin, and parvalbumin interneurons, implying that there is a re-wiring of the interneuronal network. This increase in release sites was matched by an increase in GABAergic post-synaptic densities. We hypothesized that the breakdown of the PNN could be due to the activity of matrix metalloproteinases (MMP) and that the prevention of PNN breakdown may reduce the rewiring of interneuronal circuits and suppress seizures. To test this hypothesis we employed doxycycline, a broad spectrum MMP inhibitor, to stabilize PNNs in kindled rats. We found that doxycycline prevented PNN breakdown, re-organization of the inhibitory innervation, and seizure genesis. Our observations indicate that PNN degradation may be necessary for the development of seizures by facilitating interneuron plasticity and increased GABAergic activity.

Introduction

Appropriate brain function relies on highly interconnected and organized networks of interneurons that control the activity of projection (excitatory) neurons that distribute information between neuronal assemblies. Any aberrant re-organization of these networks can and often leads to disorders of brain function. Although epilepsy is a diverse disorder with over 40 recognized types (McCormick and Contreras, 2001), all forms are characterized by highly synchronised and unprovoked bursts of neuronal activity that represent a breakdown in normal neuronal connectivity. Epileptogenesis is the process by which the brain is altered from a normal neural network to a hyper-excitable, epileptic one (McIntyre et al., 2002). It also seems clear that seizures and/or numerous other perturbations like head injury can induce re-wiring exacerbating epileptiform activity. In the case of brain injury seizures may only occur after a latent period lasting months or years (Pitkanen and Lukasiuk, 2011). Thus, it is hypothesized that there are progressive changes in neuronal structure, connections, and functions that ultimately create an imbalance of excitation and inhibition leading to seizures (McNamara, 1994; Morimoto et al., 2004; Racine et al., 2002; Tasker et al., 1996). No matter what the etiology, the prevention and treatment of epilepsy requires an understanding of how the brain mal-adapts, creating "wiring" patterns that support seizures.

The kindling model of epilepsy represents a progressive and permanent development of convulsive motor seizures that arise from daily electrical stimulations of subcortical brain areas, often hippocampus or basal lateral amygdala (Goddard, 1967). The kindling model is not associated with significant cell loss and therefore represents fundamental alterations in network behaviour that may include changes in cellular excitability, synaptogenesis, and/or metabolic activity as well as other outcomes. The same cellular mechanisms that establish and maintain kindling in animals have been suggested to be involved in partial epilepsy in humans (Adamec and Stark-Adamec, 1983; Sato et al., 1998). In particular aberrant plasticity has been postulated to underlie the formation of epileptic foci in the temporal lobe (Wilczynski et al., 2008). Therefore, the identification of mechanisms that are engaged during the kindling procedure that governs this maladaptive plasticity may give clues to a better understanding of the epileptogenesis.

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One important process that occurs during brain development is the formation of an extracellular matrix (ECM) that is essential for many processes including proliferation, migration, synaptogenesis, synaptic stability, and cell signaling (Dityatev and Schachner, 2003). The ECM of the nervous system is comprised of a complex mixture of proteoglycans, glycoproteins, tenascin, fibronectin, and hyaluronan (Deepa et al., 2006; Galtrey and Fawcett, 2007). A component of the ECM is perineuronal nets (PNNs), which form structures that surround cell bodies, dendrites, and axon initial segments leaving open "holes" at sites of synaptic contacts (Wang and Fawcett, 2012; Zaremba et al., 1989). PNNs are found in virtually all regions of the central nervous system but predominantly surround inhibitory GABAergic neurons. A high percentage of PNNs are found around interneurons that express the calcium binding protein parvalbumin, as well as being present in other subtypes (Bruckner et al., 1997; Dityatev et al., 2010). Although PNNs are generally thought to be stable structures, various perturbations can cause their breakdown. For example, the breakdown of PNNs has been reported in the kainic acid model of epilepsy (McRae and Porter, 2012; McRae et al., 2012). However, the effect of this breakdown on synaptic wiring or how degradation may be prevented has not been explored. To this end we have investigated whether PNN degradation occurs in the kindling model of epilepsy and if so whether decreased PNNs correlate with changes in inhibitory synapse "wiring patterns". We also investigated whether the inhibition of matrix metalloprotease (MMP) activity prevents the breakdown of PNNs and subsequent re-wiring.

Materials and methods

Animals and surgery

Male Sprague–Dawley rats from Charles River weighing approximately 200 g at the time of initial surgery were used. They were housed in standard plastic cages with free access to food and water under a continuous 12 h/12 h light/dark schedule. For electrode implantation animals were anesthetized with ketamine (100 mg/kg, i.p.)/domitor (10 mg/kg, i.p.) and implanted with two bipolar stimulating/recording electrodes bilaterally in the basolateral amygdala using the following coordinates: 2.6 mm posterior to bregma, 4.5 mm lateral to midline and 8.0 mm ventral (Paxinos and Watson, 1986). Electrodes were made from two twisted strands of diamel-insulated nichrome wire with a diameter of 0.127 mm. They were attached to male Amphenol pins. Once implanted the electrodes were secured to the skull with jeweler screws and the electrode assembly was fixed to the skull using dental acrylic cement (McIntyre and Molino, 1972).

Kindling procedure

The kindling procedure began at least 10 days after surgery. An afterdischarge threshold (ADT) was determined in each amygdala by delivering a 3 s 60-Hz sine wave stimulus of progressive intensity (25, 50, 75, 100, 250, 500, 750 μA) until an afterdischarge and a behavioral response were triggered (McIntyre and Plant, 1993). The rats were stimulated unilaterally daily until three generalized stage 5 seizures occurred. The stimulated side of the brain (ipsilateral) was considered the "kindled" tissue, whereas the unstimulated side of the brain is referred to as the contralateral tissue. The contralateral tissue was evaluated since some kindling effects have been observed on this side even without direct stimulation. A minimum of 10 days were passed post-kindling before kindled rats were sacrificed. Statistical differences in kindling outcomes were analyzed by a non-parametric Mann–Whitney test.

Immunohistochemistry

All rats used in this study were deeply anesthetised with ketamine (100 mg/kg, i.p.)/domitor (10 mg/kg, i.p.) and perfused intracardially with heparinized saline, followed by LANA's fixative (4% paraformaldehyde, 20% picric acid). The brain was quickly removed and post-fixed in LANA's fixative for 24 h, then transferred to a 30% sucrose phosphate buffer for 48 h. Brains were then flash frozen in −80 °C isopentane for 50 s and stored at −20 °C until sectioning. The tissue was sectioned on a cryostat at −15 °C in the coronal plane into 40 μm sections. Free-floating sections were placed in tissue culture dishes with a cryoprotectant solution of 35% sucrose and 35% ethylene glycol in 0.1 M phosphate buffer, and stored at −20 °C until they were ready to be processed.

Slices were washed twice for 5 min in 0.5% Triton X-100, then blocked with 10% donkey or goat serum in 0.025% Triton X-100 in 1% BSA in 1 × PBS for 1 h at room temperature. Slices stained with aggrecan (AGG) antibodies were pre-treated with 0.2 U/ml chondroitinase ABC (Sigma-Aldrich, St. Louis, MO) at 37 °C for 40 min prior to blocking. The primary antibody solutions were diluted in 1% BSA in 1 × PBS and pipetted into tissue culture dish wells. The sections were incubated overnight at 4 °C. The sections were then washed twice for 5 min in 0.5% Triton X-100, followed by an application of secondary antibody diluted in 1% BSA in 1 × PBS and incubated for 1 h at room temperature under low-light conditions. The sections were then washed 3 × 10 min in 1 × PBS. To reduce lipofusion autofluorescence, the sections were placed in 1% Sudan Black B dissolved in 70% ethanol for 1.5 min (Schnell et al., 1999). The sections were then washed twice for 1 min in 70% ethanol, and then in 70% ethanol for 2 min. This was followed by 2 washes in 1 × PBS for 5 min each. All washes and incubations for immunohistochemistry took place on an agitator to ensure thorough washes and maximal antibody binding. The sections were wet mounted onto Fisher SuperFrost Plus slides and mounted with glass coverslips in Prolong gold anti-fade with DAPI mounting medium (Molecular Probes, Eugene, OR). Slides were stored protected from light at −20 °C until they were imaged.

Image acquisition and analysis

Confocal images were taken on an Olympus IX 60 inverted microscope outfitted with a Perkin Elmer spinning disk confocal attachment with either a 60× (Numerical aperture = 1.4) immersion oil, 40× (N.A. = 0.75), 20× (N.A. = 0.5), or 10× (N.A. = 0.3) objective. The microscope was equipped with a Hamamatsu Orca ER CCD camera (1300 × 1030 pixels; pixel size 6.5 μm) and images were acquired using Velocity software. Each image shown represents a stack of 10 images spaced 0.2 μm apart in the z-plane, for each wavelength.

For each test animal three fields of 800 × 600 pixels were counted by the observer who was blinded to the experimental group being quantified.

PNN visualization and quantification

Lectin from *Wisteria floribunda* agglutinin (WFA) and AGG primary antibodies were used to stain PNNs in the piriform cortex (PC). To image the PNNs, three pictures were taken in layer 3 of the PC with a 20× objective. For WFA stained PNNs we assigned the nomenclature "grade 1" to identify fully formed PNNs and "grade 2" to identify diffusely formed PNNs. This classification was used as we noticed that image stacks of PNNs varied in their appearance between those that had extensive localizations around cell body and proximal dendrites and those where the staining was less extensive (please see Supplemental Fig. 1 for examples of these differences). The total number of PNNs in each grade was compared between experimental groups as well as comparing ipsilateral and contralateral kindled tissues using a one-way ANOVA and Tukey's post hoc test. Significance level was set at $p < 0.05$.

WFA was co-localized with primary antibodies for three calcium binding proteins (CBPs); parvalbumin (PV), calbindin (CB), and calretinin (CR). To image the co-localization of PNNs with CBPs, three pictures were taken in layer 3 of the PC with a 20× objective. The