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INITIATION AND SPREAD OF EPILEPTIFORM DISCHARGES IN THE METHYLAZOXYMETHANOL ACETATE RAT MODEL OF CORTICAL DYSPLASIA: FUNCTIONAL AND STRUCTURAL CONNECTIVITY BETWEEN CA1 HETEROTOPIA AND HIPPOCAMPUS/NEOCORTEX

N. TSCHULUUN, J. H. WENZEL, K. KATLEBA AND P. A. SCHWARTZKROIN*

Department of Neurological Surgery, University of California, Davis, Medical Neuroscience Building, Room 612G, 1515 Newton Court, Davis, CA 95616, USA

Abstract—Neuronal migration disorders (NMDs) are often associated with medically intractable epilepsy. In utero injection of methylazoxymethanol acetate into pregnant rats gives rise to dysplastic cell clusters ("heterotopia") in hippocampus (and nearby regions), providing an animal model of NMD. In the present study, we have examined the structural and functional integration of hippocampal heterotopic cells into circuits that link the heterotopia with surrounding "normal" brain. Bi-directional morphological connectivity between the heterotopia and hippocampus/neocortex was demonstrated using the neurotracer, biotinylated dextran amine. Single cell recordings in hippocampal slices showed that heterotopia neurons form functional connections with the surrounding hippocampus and neocortex. However, simultaneous field recordings from the CA1 heterotopia, normotopic hippocampus, and neocortex indicated that epileptiform discharges (spontaneous events seen in slices bathed with high $[K^+]_o$ and bicuculline) were rarely initiated in the heterotopia (although the heterotopia was capable of generating epileptiform discharges independently of normal brain regions). Further, in most of the experiments, the aberrant connectivity provided by CA1 heterotopia failed to function as a "bridge" for epileptiform discharges to propagate directly from low-threshold hippocampus to neocortex. These data do not support the hypothesis that NMDs (heterotopic cell populations) serve as a focus and/or trigger for epileptiform activity, and/or facilitate propagation of epileptiform events. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: MAM, cortical dysplasia, CA1 heterotopia, epileptiform activity, aberrant connectivity, seizure propagation.

Neuronal migration disorders (NMDs), caused by failure of newly formed cells to migrate into proper positions in the cortex, are frequently associated with epilepsy (Schwartzkroin and Walsh, 2000; Porter et al., 2002). Morphologically, NMDs are often characterized by clusters of displaced cells ("heterotopia") in the cortex and/or cytologically abnormal cells of both neuronal and glial phenotypes (Taylor et al., 1971; Palmini et al., 1991; Spreafico et al., 1998). Clinical electrographic studies have suggested that heterotopia are sometimes sites of seizure genesis. The results of surgical resection of morphologically abnormal tissue have been used to support this view of heterotopia as sites of seizure initiation, since resection is sometimes an effective means for controlling medically intractable forms of epilepsy associated with NMDs (Palmini et al., 1991; Guerrini et al., 1992; Hirabayashi et al., 1993).

Several animal models have been developed to study morphological and physiological aspects of NMDs (for review see Chevassus-au-Louis et al., 1999a). Perhaps the best-studied among non-genetic models is the methylazoxymethanol acetate ("MAM") model. Pregnant rats treated with MAM, a DNA alkylating agent, give birth to offspring that exhibit many of the characteristics of NMDs. In utero exposure, at gestational day 15, leads to the formation of heterotopic cell clusters (similar to those seen in human periventricular nodular pathology) in cortex, the periventricular region, hippocampus, and white matter (Singh, 1977; Chevassus-au-Louis et al., 1998; Baraban et al., 2000). In this MAM model, heterotopic cells display abnormal cellular morphology (Singh, 1980), reduced inhibitory drive (Calcagnotto et al., 2002; Pentney et al., 2002), and defective potassium currents (Castro et al., 2001), features that might well give rise to CNS hyperexcitibility. Indeed, although MAM animals do not usually exhibit spontaneously occurring seizures, they have been shown to display lower thresholds for convulsant agents (e.g. flurothyl: Baraban and Schwartzkroin, 1996; kainic acid: Germano and Sperber, 1997) as well as other epileptogenic stimuli (e.g. kindling: Germano et al., 1998).

Typically, the MAM-induced heterotopia is integrated into the dorsal hippocampus, interrupting the CA1 cell band. Interestingly, CA1 heterotopic cells do not express a marker characteristic of hippocampal cell types, and have been hypothesized to be cells destined for neocortex. Further, they form synaptic connections outside the heterotopia and outside the hippocampus (Colacitti et al., 1998; Chevassus-au-Louis et al., 1998). Electrophysiological studies carried out on brain slices through the heterotopic region have shown that cells in the CA1 heterotopia are functionally connected to both hippocampus and neocortex, and can relay stimulus-elicited responses from hip-

*Corresponding author. University of California Davis, One Shields Avenue, Davis, CA 95616, USA. Tel: +1-530-754-5029; fax: +1-530-754-8976.
E-mail address: paschwartzkroin@ucdavis.edu (P. A. Schwartzkroin).
Abbreviations: ACSF, artificial cerebrospinal fluid; BDA, biotinylated dextran amine; BMI, bicuculline methiodide; BSA, bovine serum albumin; CD, cortical dysplasia; DAB, 3,3′-diaminobenzidine; DMSO, dimethylsulfoxide; EPSP, excitatory post-synaptic potential; IPSP, inhibitory post-synaptic potential; MAM, methylazoxymethanol acetate; NMD, neuronal migration disorder; PB, phosphate buffer; s., stratum; TB, Tris buffer; TBS, Tris-buffered saline.

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pocampus into neocortex. Based on those findings, it has been proposed that the CA1 heterotopia may serve as a "bridge" between the hippocampus and the overlying neocortex (Chevassus-au-Louis et al., 1998), a pathway by which hyperexcitability generated in the low-threshold hippocampus might spread directly to neocortex, bypassing the normal anatomical route (e.g. through subiculum and entorhinal cortex). This hypothesized role of CA1 heterotopias has significant clinical implication for epileptogenicity and seizure spread.

In the present study we addressed two major questions: 1) How are heterotopia integrated functionally and structurally into surrounding tissue? and 2) Does abnormal connectivity provided by hippocampal heterotopia contribute to aberrant propagation of epileptiform discharges? Using anatomical and physiological techniques, we show that CA1 heterotopia form bidirectional connectivity with the hippocampus and neocortex. Although this aberrant circuit is functional, it does not seem to play a powerful role in the spread of epileptiform discharges directly from hippocampus to the overlying neocortex.

EXPERIMENTAL PROCEDURES

All procedures were carried out in accordance with NIH animal use guidelines, and were approved by the UC Davis Institutional Animal Care and Use Committee. Experimental procedures were designed to minimize the number of animals used and to minimize animal suffering.

MAM administration

Pregnant Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) were injected with either 25 mg/kg MAM (MRI, Kansas City, MO, USA), 25 mg dissolved in 1 ml 0.9% sodium chloride (NaCl) or the vehicle NaCl alone. A single i.p. injection of MAM was administered at gestational day 15. There were no significant differences in gestational parameters (i.e. pup weights, litter size [seven to13 pups]) between litters from MAM- and NaCl-injected rats.

Surgical procedures and injections of the neurotracer, biotinylated dextran amine (BDA)

Nine adult rats (2–6 months of age), exposed to MAM in utero, were prepared for tracer injection under 2% isoflurane anesthesia. All animals underwent identical surgical procedures (i.e. anesthesia, intubation, ventilation, fixation in a stereotaxic frame and craniotomy). The neuronal tracer BDA (NeuroTrace BDA-10,000 Neuronal Tracer Kit; Molecular Probes, Eugene, OR, USA) was used for bidirectional (i.e. anterograde and retrograde) labeling. Somata at the site of a BDA injection take up the dye and transport it, anterogradely, to their axons and terminals (Brandt and Apkarian, 1992). In addition, BDA labels distant cell bodies and dendritic structures via retrograde transport from terminals within the injection site (Reiner et al., 2000). Injections (10% BDA in 0.9% NaCl) were made bilaterally, at two sites per side, either into the neocortex (five animals: 0.5 and 1.5 mm caudal from bregma, 2.0 and 3.0 mm lateral from the midline, 0.7 and 0.8 mm deep from the cortical surface, respectively) or into the hippocampus (four animals: 1.8 and 3.2 mm caudal from bregma, 2.0 and 2.5 mm lateral from the midline, 2.0 and 2.3 mm deep from the cortical surface, respectively). Injections (0.5 μl per site) were delivered via a Hamilton syringe (30 gauge; Hamilton, Reno, NV, USA) over the course of 5 min (0.1 μl/min). At the conclusion of all injections, anesthesia was discontinued and the animal removed from the stereotaxic device; rats were hydrated and warmed, and returned to their cages once they had recovered normal locomotor function. After a survival time of 8–10 days, animals were perfused with a paraformaldehyde–glutaraldehyde solution, and brains were further processed with a histochemical procedure to visualize the BDA tracer (see below).

In vitro slice preparation and electrophysiology

Three to 5 week old male and female rats were used for electrophysiology experiments. Animals were briefly anesthetized with Halothane (Sigma Aldrich, St. Louis, MO, USA), decapitated and the brain quickly removed and put in ice-cold artificial cerebrospinal fluid (ACSF; consisting of 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 26 mM $NaCO_3$, 2 mM $CaCl_2$ and 10 mM dextrose). The brain was then blocked and glued (cyanoacrylic adhesive) to the stage of a Vibratome (Vibrotome 3000; Vibrotome Inc, St. Louis, MO, USA). Slices (400 μm thick), both in the transverse orientation (from dorsal hippocampus) and in the sagittal orientation, were cut in 1 °C oxygenated ACSF. Slices were subsequently incubated in ACSF at room temperature for at least 1 h before being transferred to an interface slice chamber for recording. Only slices with a visually apparent CA1 heterotopia were used for electrophysiology experiments. CA1 heterotopias could be reliably identified by the appearance of a pale mass that disrupted the normal CA1 pyramidal cell layer band. Heterotopias were confirmed after tissue processing for cell labeling with biocytin and Cresyl Violet staining.

Slices were transferred to a gas-interface slice-recording chamber (Fine Science Tools, Foster City, CA, USA) and perfused with ACSF at room temperature. All slices, from both MAM-exposed and control animals, were oriented in same way, with the neocortex facing the perfusion input port. This detail of procedure assured that localized initiation of burst discharge (see below) was not attributable to inadvertent slice placements with respect to regional exposure to the epileptogenic agent in the perfusion medium. Intracellular recordings were obtained to study the connectivity between heterotopic cells and the normal hippocampal CA1/CA3 or neocortical regions. Bipolar stimulating electrodes were placed in the stratum (s.) radiatum to stimulate Schaffer collaterals, or in the deep cortical layers (overlying the heterotopia), to study the projections from and to the hippocampus and cortex. Sharp electrodes, pulled from 1 mm O.D. filament borosilicate pipettes (Sutter Instruments, Novato, CA, USA), were used to record from cells in the CA1 heterotopia. Electrode tips were filled with 2% biocytin and the shaft with 3 M potassium acetate (electrode resistance 100–230 megohms). Brief (0.5 ms) current pulses (300–400 μA), delivered via the stimulating electrodes, elicited synaptic responses and/or antidromic spikes in the recorded cells. To prevent the spread of the stimulating current from neocortex into the heterotopia (and activating terminals directly), the current amplitude was adjusted by recording from the normotopic CA1 cells close to the heteropia before each experiment. Current amplitudes that did not elicit responses in normotopic CA1 cells were used to study the connectivity to and from neocortex. The voltage signal was recorded (Axoclamp 2B amplifier; Axon Instruments, Novato, CA, USA), digitized (Digidata 1200; Axon Instruments), and analyzed (Clampex 8). To determine the excitatory or inhibitory nature of postsynaptic potentials, membrane potential of the recorded cells was manipulated between −50 and −95 mV via intracellularly injected DC current. For intracellular labeling, biocytin was delivered into the recorded cell via hyperpolarizing current steps (700 ms, 1–2 nA), injected at 1 Hz for 5–20 min. Slices were then incubated at 32 °C for 1 h to allow biocytin to diffuse throughout the cell. Slices with biocytin-filled neurons were processed with avidin/horseradish peroxidase/3,3′-diaminobenzidine (DAB) and nickel enhancement to visualize labeled cells (see below).