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Loss of cholecystokinin-containing terminals in temporal lobe epilepsy

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ABSTRACT

Altered GABA-mediated inhibition is proposed to play a role in the pathogenesis of epilepsy. Previous studies have demonstrated a loss of somatostatin-containing GABAergic interneurons innervating granule cells in epileptic animals. However, the reorganization of synapses between interneurons and granule cells has not been investigated. We studied synapse organization in an animal model of temporal lobe epilepsy (TLE) using continuous hippocampal stimulation. The distribution of axon terminals and inhibitory synapses on granule cell dendrites was studied using a combination of immunohistochemistry and pre-embedding electron microscopy techniques. A whole-cell patch-clamp technique was applied to study the functional changes in GABAergic input from different interneurons. In epileptic animals, the density of cholecystokinin (CCK)-immunoreactive (IR) fibers and α2 subunit containing $GABA_A$ receptors in the inner molecular layer of the dentate gyrus was reduced. Quantitative immuno-electron microscopy study revealed that the ratio of CCK-containing symmetric synapses to the total symmetric synapses was reduced. The frequency of GABAergic synaptic currents (sIPSC) was decreased and their amplitude was increased. The inhibitory effect of the activation of cannabinoid 1 (CB1) receptors was also reduced in epileptic animals. Isolation of CCK- and parvalbumin (PV)-containing GABAergic inputs by N- and P/Q-type calcium channel blockers respectively suggested that GABA release from CCK-containing interneurons was selectively reduced in epileptic rats. This study found that there was a loss of CCK-containing GABAergic synapses to granule cells both morphologically and functionally. These studies add to our understanding of the mechanisms that contribute to altering GABAergic inhibition of granule cells in TLE.

Introduction

In animal models of temporal lobe epilepsy (TLE), GABAergic inhibition is altered (Joshi and Kapur, 2012). Inhibitory interneurons in the hippocampus play a crucial role in generating inhibitory effects (Hefft and Jonas, 2005). GABAergic terminals are abundant throughout the dentate gyrus, with the highest densities in the outer third of the molecular layer and slightly lower densities in the inner two-thirds of this layer (Houser, 2007). The cell bodies and proximal dendrites of dentate granule cells are innervated by a dense plexus of GABAergic terminals arising from cholecystokinin (CCK) and parvalbumin (PV)-containing basket cells; distal dendrites are innervated by somatostatin (SOM)-positive terminals (Houser, 2007). Some somatostatin-containing interneurons are lost in animal models of TLE (Best et al., 1993; Buckmaster and Dudek, 1997; Houser, 1991; Sloviter, 1987; Sun et al., 2007a). Similar findings are reported in TLE patients (de Lanerolle et al., 1989; Maglóczky et al., 2000; Mathern et al., 1996). However, it is unknown whether CCK, PV, or SOM terminals in the hippocampal dentate gyrus are reorganized in epileptic animals.

PV and CCK basket cells are two major inhibitory interneurons that connect at perisomatic and proximal dendritic inhibitory synapses in the dentate gyrus. Their terminals contain the GABA synthetic enzyme glutamic acid decarboxylase (GAD) and form symmetric inhibitory contacts primarily on the cell bodies and shafts of apical dendrites of the granule cells (Kosaka et al., 1984). It has been proposed that PV interneurons operate as clocks for cortical network oscillations, whereas CCK interneurons function as a plastic fine-tuning device in the hippocampus (Freund and Katona, 2007). CCK basket cells influence their target cells by activating α2 subunit containing $GABA_A$ receptors (Nyíri et al., 2001). The α2 subunit containing $GABA_A$ receptors were absent or expressed at very low levels at synapses formed by PV interneurons (Freund and Katona, 2007). Cannabinoid receptor type I (CB1) and M1/M3 acetylcholine receptors are expressed in CCK- but not PV-containing terminals (Freund and Katona, 2007; Katona et al., 1999). CCK-containing basket cells express N-type calcium channels, whereas PV-containing cells express P/Q-type calcium channels (Hefft and Jonas, 2005; Poncer et al., 1997; Wilson et al., 2001). These specific biological markers can be used to distinguish these two types of interneurons.

The present study investigated the IR distribution of SOM, PV, and CCK terminals, as well as the α2 subunit of $GABA_A$ receptor in the

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dentate gyrus using fluorescent microscopy. There was reduced staining of CCK and the α2 subunit in the inner molecular layer in epileptic animals. Pre-embedding electron microscopy experiments demonstrated a reduction of CCK-positive symmetric synapses in epileptic animals. Furthermore, postsynaptic CCK-containing GABAergic currents recorded from dentate granule cells demonstrated reduced perisomatic inhibition in epileptic animals. This suggested that loss of CCK-containing GABA interneuron terminals and synapses in the dentate gyrus may cause impairment of inhibitory function in the neuron circuit, which then leads to seizure discharge in patients.

Materials and methods

Animal model

Animals were handled according to AALAC Animal Care and Use Guidelines and a protocol approved by the University of Virginia Animal Care and Use Committee. Status epilepticus (SE) was induced in adult male Sprague–Dawley rats (2–4 month old) using the previously described continuous hippocampal stimulation method (Lothman et al., 1989). Briefly, bipolar electrode was stereotactically implanted in the hippocampus (AP − 3.6; ML − 4.9; DV − 5.0 to dura). Stimulations were delivered 1–2 weeks after surgery. Approximately 4–6 weeks after stimulation, animals developed spontaneous limbic seizures, which were detected by continuous EEG monitoring and direct observation of a behavioral seizure (Bertram et al., 1997). Animals with 3 month recurrent spontaneous seizures and age-matched controls were used for this study.

Immunohistochemistry for neuropeptide-IR interneurons

The procedures for tissue preparation were described in detail previously (Sun et al., 2004). Briefly, animals were anesthetized with an overdose of pentobarbitone sodium and perfused through the ascending aorta with 50–100 ml of 0.9% NaCl followed by 350–450 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and post-fixed in the same fixative for 2 h at 4 °C. After overnight incubation in a solution of 30% sucrose in 0.1 M PB for cryoprotection, the brains were separated and blocked as previously described (Sun et al., 2007a). The brains were frozen by immersion in −70 °C isopentane and the ventral hippocampus was sectioned (40 μm thick) horizontally.

The sections were processed for free floating immunohistochemistry, as described in detail previously (Sun et al., 2004; Sun et al., 2007a). Following 3 washes in 0.1 M PB, the sections were incubated with blocking solution containing 5% normal goat serum, 2% bovine serum albumin (BSA; Jackson Immunoresearch Laboratories, Inc., West Grove, PA), and 0.1% Triton X-100 in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 1 h. The tissue sections were placed on a shaker and incubated with the primary antibodies at 4 °C for 72 h, and then incubated in the dark with goat anti-rabbit or goat anti-mouse secondary antibodies conjugated with Alexa Fluor 488 or 594 (Molecular Probes, Eugene, OR, 5 μg/ml) for 1 h at room temperature. All antibodies were diluted with a solution containing 1% BSA in 0.1 M PBS. The sections were quickly rinsed 3 times and followed by 6 washes after the incubation with primary and secondary antibodies. Then, sections were mounted on a slide with Gel/Mount (Foster City, CA) and covered with a coverslip. After air-drying for approximately1 h, the edges of the coverslips were sealed with clear nail polish. We have characterized all antibodies (Mangan et al., 2005; Sun et al., 2007a).

Images of tissue sections were captured on a CoolSnap cf CCD camera (Roper Scientific Photometrics) mounted on an Eclipse TE200 microscope (Nikon) equipped with a mercury lamp and filters for detecting fluorescence, driven by Metamorph imaging software (Universal Imaging Corp., Downingtown, PA). Three 40 μm squares per section were randomly chosen to measure optical density. Data were analyzed using Prism 4.0 software (GraphPad Software, Inc. San Diego, CA). Unless specified otherwise, all values are reported as the mean ± standard error of the mean (SEM), and an unpaired Student *t*-test was used to determine significance.

Pre-embedding electron microscopy

Pre-embedding electron microscopy study of CCK-positive terminals was performed following a previously published protocol (Sun et al., 2007a). Animals were transcardially perfused with Tyrode solution (Heck et al., 2002). A fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde (or 2% paraformaldehyde and 2% glutaraldehyde for the synaptic profile study) in 0.1 M PB was perfused until the effluent was clear. The brains were post-fixed in 4% paraformaldehyde for 2 h at 4 °C, and sliced in PB on a vibratome at a thickness of 60 μm. Sections were treated with 0.1% $NaBH_4$ at room temperature for 30 min, and then rinsed with PB. The sections were rinsed and treated in 1% bovine serum albumin (BSA) in PBS for 30 min. They were then incubated in a 1:50 dilution of monoclonal mouse anti-CCK antibody (Abcam Inc., Cambridge, MA) in PBS with 1% BSA and 0.05% sodium azide for 3 days at room temperature. Then, sections were rinsed and incubated in a biotinylated goat anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) for 2 h, followed by a 2 h incubation in HRP-conjugated avidin–biotin complex (ABC; Vector Laboratories). Immunoreactivity was visualized using diaminobenzidine (DAB, 0.03%) and H_2O_2 (0.001%). Deletion of the primary antibody eliminated all of the specific staining discernible at the electron microscopy level.

The sections were put in 1% osmium tetroxide in PB for 1 h, dehydrated, and flat-embedded in Epon 812 resin between two sheets of Aclar film (Electron Microscopy Sciences, Fort Washington, PA). After resin polymerization, a small area including the molecular layer, granule cell layer, and part of the hilus was dissected from the ventral hippocampus and mounted on capsules. Ultrathin sections at the interface of tissue and resin were collected and stained with uranyl acetate and lead citrate. Grids were examined on a Jeol JEM 1010 microscope, and images were captured by a 16 megapixel SIA-12C (sia-cam.com) digital camera coupled with MaxIm DL CCD software (Diffraction Limited, Ottawa, Canada).

For quantitative electron microscopy analysis with systematic sweeps, each synapse was located, and its length was measured with Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD). The type of synaptic contact (symmetric or asymmetric) and the type of postsynaptic element (dendrite shaft, spine, or soma) were evaluated by the criteria detailed in a previous study (Sun et al., 2007b). The length of the synapse was measured along the parallel-aligned plasma membranes. The areal density of synapses (N_A indicates the number of synapses per area) was calculated and used to find the volumetric density of synapses ($N_v = N_A$ / average synaptic length).

Whole-cell electrophysiological recording

Whole-cell patch-clamp recordings of GABA receptor currents (sIPSCs) from dentate granule cells (DGCs) in hippocampal slices were performed as previously described (Sun et al., 2007b). Adult male Sprague–Dawley rats were anesthetized with isoflurane and decapitated, brains were quickly removed, and then brains were sectioned with a 300 μm thickness using a Leica VT1200 slicer (Leica Microsystems, Wetzlar, Germany) in ice cold oxygenated slicing solution. The solution contained the following (in mM): 120 sucrose, 65.5 NaCl, 2 KCl, 1.1 KH_2PO_4, 25 $NaHCO_3$, 10 D-glucose, 1 $CaCl_2$, and 5 $MgSO_4$. The slices were then incubated at 32 °C for at least 1 h in oxygenated ACSF containing the following (in mM): 127 NaCl, 2 KCl, 1.1 KH_2PO_4, 25.7 $NaHCO_3$, 10 D-glucose, 2 $CaCl_2$, and 1.5 $MgSO_4$; osmolality was 290–300 mOsm in the chamber. Slices were then transferred to the recording chamber on the stage of an Olympus Optical BX51 microscope (Olympus, Tokyo).

Whole-cell patch-clamp recordings were performed under infrared differential interference contrast microscopy (Olympus) with a 40×