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Research Paper

Gap Junctions Contribute to Ictal/Interictal Genesis in Human Hypothalamic Hamartomas

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ABSTRACT

Human hypothalamic hamartoma (HH) is a rare subcortical lesion associated with treatment-resistant epilepsy. Cellular mechanisms responsible for epileptogenesis are unknown. We hypothesized that neuronal gap junctions contribute to epileptogenesis through synchronous activity within the neuron networks in HH tissue. We studied surgically resected HH tissue with Western-blot analysis, immunohistochemistry, electron microscopy, biocytin microinjection of recorded HH neurons, and microelectrode patch clamp recordings with and without pharmacological blockade of gap junctions. Normal human hypothalamus tissue was used as a control. Western blots showed increased expression of both connexin-36 (Cx36) and connexin-43 (Cx43) in HH tissue compared with normal human mammillary body tissue. Immunohistochemistry demonstrated that Cx36 and Cx43 are expressed in HH tissue, but Cx36 was mainly expressed within neuron clusters while Cx43 was mainly expressed outside of neuron clusters. Gap-junction profiles were observed between small HH neurons with electron microscopy. Biocytin injection into single recorded small HH neurons showed labeling of adjacent neurons, which was not observed in the presence of a neuronal gap-junction blocker, mefloquine. Microelectrode field recordings from freshly resected HH slices demonstrated spontaneous ictal/interictal-like discharges in most slices. Bath-application of gap-junction blockers significantly reduced ictal/interictal-like discharges in a concentration-dependent manner, while not affecting the action-potential firing of small gamma-aminobutyric acid (GABA) neurons observed with whole-cell patch-clamp recordings from the same patient's HH tissue. These results suggest that neuronal gap junctions between small GABAergic HH neurons participate in the genesis of epileptic-like discharges. Blockade of gap junctions may be a new therapeutic strategy for controlling seizure activity in HH patients.

1. INTRODUCTION

Human hypothalamic hamartomas (HHs) are developmental malformations occurring in the ventral hypothalamus. HHs are associated with neurological and endocrine disorders, including intractable seizures, cognitive impairment, behavioral disturbances, and central precocious puberty (Berkovic et al., 1988; Kerrigan et al., 2005). The epileptic syndrome in HH patients is characterized by laughing (gelastic) seizures, often beginning in early infancy. Most patients with gelastic seizures have a progressive natural history, and later develop additional seizure types and cognitive and psychiatric comorbidities (Berkovic et al., 1988; Prigatano et al., 2008). Seizures associated with HH are usually refractory to standard anti-epilepsy drugs, but surgical treatment can be effective (Mittal et al., 2013). Multiple clinical studies have demonstrated that HHs are intrinsically epileptogenic (Kuzniecky et al., 1997; Munari et al., 1995). However, cellular and molecular mechanisms underlying epileptogenesis within HH lesions remain incompletely understood (Wu et al., 2015).

Gap junctions are cell-to-cell channel-forming structures formed by specialized proteins (connexins) in the plasma membranes of adjacent cells. They allow direct electrical coupling and chemical communication between almost all cell types in the central nervous system. Many different connexin proteins have been identified; most are specific for different cell types in the brain, including neurons and glia (Nakase et al.,

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2004; Rozental et al., 2000). Connexin-36 (Cx36) is predominantly expressed in gamma-aminobutyric acid-ergic (GABAergic) interneurons (Cruikshank et al., 2005; Sohl et al., 2005). In the rat, Cx36 shows a high level of expression in the hypothalamus, including the mammillary bodies (Condorelli et al., 2000). Gap junctions play an important role in locally synchronizing GABAergic neuron activity, including action-potential firing and sub-threshold changes in transmembrane potential resulting from inhibitory and excitatory post-synaptic potentials (Cruikshank et al., 2005). Gap junctions contribute to oscillating field potentials and enable GABAergic entrainment of principal (projection) neuron behavior within normal networks (Sohl et al., 2005).

In view of these functional features, gap junctions likely contribute to the pathogenesis of epilepsy, particularly with respect to enhancing synchronous activity of neuronal subgroups within epileptic networks (Carlen et al., 2000; Dudek et al., 1998; Traub et al., 2004). This concept is supported by in vitro and in vivo epileptic animal models, in which the pharmacological blockade of gap junctions significantly reduces seizure occurrence (Carlen et al., 2000; Traub et al., 2004). Interictal epileptiform discharges (in the form of fast oscillations) can be abolished in freshly resected human epileptic tissue slices with carbenoxolone, a non-specific gap-junction blocker (Roopun et al., 2010). However, the understanding of gap junctions in human epileptogenesis is still limited and the potential for gap-junction blockers as therapeutic agents for human epilepsy remains effectively unexplored.

We hypothesized that neuronal gap junctions have a mechanistic role in synchronizing neuronal firing and contributing to seizure onset in human HH. We evaluated the role of gap junctions in epileptogenesis using multiple experimental approaches including Western-blot, immunohistochemical staining, electron microscopy and electrophysiology in HH tissue surgically resected from patients with treatment-resistant gelastic seizures. We found that HH tissue expressed significantly higher level of Cx36 and Cx43 compared to normal control hypothalamic tissue, and pharmacological block of gap junctions significantly reduced seizure-like discharges in HH slices.

2. Materials and Methods

2.1. Informed Consent

Written informed consent for use of post-surgical tissue for research purposes was obtained under protocols approved by the institutional review boards at Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, and Barrow Neurological Institute at Phoenix Children's Hospital (Phoenix, Arizona).

2.2. Patient Profile

Surgically resected HH tissue was obtained from 27 patients (15 males, 56%) treated between July 2003 and April 2013 (mean age at surgery, 10.3 years; range, 0.7–36.8 years). All patients had treatment-resistant epilepsy and a history of gelastic seizures. At the time of surgery, nine (33%) had only gelastic seizures, while 18 (67%) had multiple seizure types, including complex partial seizures (Kim et al., 2009), generalized tonic-clonic (nine), myoclonic (two), atonic (two), and infantile spasms (one). Twenty-five patients (93%) had at least one seizure per day, while two (7%) had at least one seizure per month. Intellectual disability or developmental retardation (intelligence quotient or developmental quotient <70) was present in 13 patients (48%) and prior history of central precocious puberty was present in nine (33%). None of the patients in this cohort had identified genetic syndromes. Classification of HH lesions according to the method of Delalande and Fohlen (Delalande et al., 2003) showed eight Type I (30%), nine Type II (33%), seven Type III (26%), and three Type IV (11%) lesions. Mean lesion volume was 2.7 cm^3 (range, 0.2–14.8 cm^3).

2.3. Control Tissue

Normal human hypothalamic control tissues were obtained from the U.S. National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders at the University of Maryland (Baltimore, MD).

2.4. Immunohistochemical Staining for Connexins in HH Tissue

HH tissue was sectioned into 4-μm slices. Tissue was deparaffinized and antigen retrieval was performed using 10 mM of sodium citrate buffer, washed with phosphate-buffered saline (PBS), blocked for 1 h at room temperature in 4% normal serum, 4% bovine serum albumin (BSA), and 0.4% Triton X in PBS. The tissue was incubated overnight at 4 °C with the primary antibody. Separate sections were stained for Cx36 1:150 (Abcam, Cambridge, MA) and Cx43 1:3000 (Abcam), each double-labeled with NeuN 1:500 (Millipore, Billerica, MA). Slides were incubated with goat anti-rabbit conjugated Alexa 594 and goat anti-mouse conjugated Alexa 488 (Invitrogen, Carlsbad, CA) secondary antibodies for 2 h at room temperature.

2.5. Western-blot Analysis of Connexin Expression in HH Tissue

Tissue samples were sonicated in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate, 50 mM Tris), and total protein was determined using BCA protein assays (Thermo-Scientific, Waltham, MA). Experiments were performed on the XCell II Blot Module (Invitrogen). From each sample, 20 μg of protein was loaded into a lane on a NuPage 4–12% Bis-Tris gel (Life Technologies, Carlsbad, CA), transferred to 0.2-μm polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), and processed with a blocking solution of 3% BSA and 5% nonfat dry milk in 1 × tris-buffered saline and Tween-20 (TBST). Rabbit anti-Cx36 (0.5 μl/ml; Invitrogen), rabbit anti-Cx43 (0.2 μl/ml; Invitrogen), and mouse anti-tubulin (1:10,000; Sigma-Aldrich, St. Louis, MO) were used as primary antibodies, and were visualized with horseradish peroxidase-conjugated anti-rabbit antibody (1:10,000; Invitrogen) or anti-mouse IgG antibody (1:10,000; Sigma-Aldrich). Signals were enhanced using enhanced chemiluminescence detection reagents (Thermo-Scientific) and detected on a BioSpectrum imaging system (UVP, Upland, CA). Densities were quantified using VisionWorks (UVP) and normalized relative to tubulin.

2.6. Electron Microscopy (EM) Analysis of Gap Junction Profiles in HH Tissue

Tissue processing: Up to three pieces of tissue (1–3 mm in diameter) were provided for ultrastructural examination, fixed overnight in 3% phosphate-buffered glutaraldehyde, post-fixed for 2 h with 2% phosphate-buffered osmium tetroxide, dehydrated with a series of graded ethanols and propylene oxide, and embedded in epoxy resin (LX-112, Ladd Research, Williston, VT). Thin tissue sections were cut with a diamond knife (Diatome AG, Biel, Switzerland), mounted on 150-mesh copper grids (Ted Pella, Redding, CA), and stained with lead citrate and uranyl acetate. Serial sections for three-dimensional reconstructions were mounted on polyvinyl formal-coated grids with a single slot (1 × 2 mm).

Image processing: Sections were examined with a Philips CM100 equipped with a CompuStage (FEI Company, Hillsboro, OR). Images were captured with an Orius SC1000 camera (Gatan, Pleasanton, CA).

2.7. Fresh HH Slice Preparation

Surgically resected HH slices were prepared based on our published protocol (Wu et al., 2005). Briefly, fresh HH-tissue sections obtained at the time of surgery were immediately placed in ice-cold dissection solution (2–4 °C), which contained (in mM) 136.7 NaCl, 5 KCl, 0.1 NaH_2PO_4,