Published in final edited form as:

Exp Neurol. 2008 October ; 213(2): 397–404. doi:10.1016/j.expneurol.2008.07.004.

GABA_A receptor-mediated excitation in dissociated neurons from human hypothalamic hamartomas

Jie Wu^{1,*}, Jamie DeChon¹, Fenqin Xue², Guohui Li¹, Kevin Ellsworth¹, Ming Gao¹, Qiang Liu¹, Kechun Yang¹, Chao Zheng¹, Ping He³, Jianglong Tu¹, Do Young Kim¹, Jong M. Rho¹, Harold Rekate⁴, John F. Kerrigan¹, and Yongchang Chang²

¹Division of Neurology, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, 350 West Thomas Road, Phoenix, AZ 85013-4496

²Division of Neurobiology, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, 350 West Thomas Road, Phoenix, AZ 85013-4496

³Haldeman Laboratory of Molecular and Cellular Neurobiology, Sun Health Research Institute, 10515 West Santa Fe Drive, Sun City, AZ 85351-3020

⁴Division of Neurosurgery, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, 350 West Thomas Road, Phoenix, AZ 85013-4496

Abstract

The cellular mechanisms underlying intrinsic epileptogenesis in human hypothalamic hamartoma (HH) are unknown. We previously reported that HH tissue is composed predominantly of GABAergic neurons, but how GABAergic-neuron-rich HH tissue is intrinsically epileptogenic is unclear. Here, we tested the hypotheses that some HH neurons exhibit immature features and that GABA excites these neurons via activation of GABA_A receptors (GABA_ARs). Gramicidin-perforated and cell-attached patch-clamp recordings were performed using freshly-dissociated HH neurons to evaluate GABA_AR-mediated currents, Cl⁻ equilibrium potentials, and intracellular Cl⁻ concentrations. Single-cell RT-PCR and immunocytochemical techniques were used to examine cation-Cl⁻ co-transporter (NKCC1 and KCC2) gene and KCC2 protein expression and molecular markers of maturation. From a total of 93 acutely-dissociated HH neurons from 34 patients, 76% were small (soma: 6–9 μm) and 24% were large (soma: >20 μm) in size. Under gramicidin-perforated patch recording conditions, GABA_AR activation depolarized/excited large but hyperpolarized/inhibited small HH neurons in most cases. Compared to small HH neurons, large HH neurons exhibited more positive Cl⁻ equilibrium potentials, higher intracellular Cl⁻ concentrations, lower KCC2 expression, and an immature phenotype, consistent with GABA_AR-mediated excitation. Taken collectively, we provide novel evidence for and mechanistic insights into HH epileptogenicity: GABA_AR-mediated excitation.

*Please send all correspondence to: Jie Wu, M.D., Ph.D., Director, Neural Physiology Laboratory, Division of Neurology, NRC 445, Barrow Neurological Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ 85013-4496. Tel: (602)-406-6376. Fax: (602)-406-7172. E-mail: Jie.Wu@chw.edu

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DISCLOSURE STATEMENT

None of the authors has any financial conflict of interest with the present study—no financial support has been or will be received in conjunction with the generation of this paper.

Keywords

cation-Cl⁻ co-transporters; GABA_A receptor; gelastic seizures; hypothalamic hamartoma; patch-clamp

INTRODUCTION

Human hypothalamic hamartoma (HH) is a developmental malformation occurring in the region of the tuber cinereum and inferior hypothalamus. This lesion is associated with a range of neurological and endocrine disorders, including intractable epilepsy, cognitive impairment, behavioral disturbances, and central precocious puberty (Berkovic et al., 1988; Deonna and Ziegler, 2000; Freeman, 2003; List et al., 1958; Quiske et al., 2007; Weissenberger et al., 2001). The epileptic syndrome in HH patients is often characterized by gelastic seizures beginning in early infancy, followed by the development of additional seizure types (Berkovic et al., 1988; Valdueza et al., 1994). Gelastic seizures associated with HH are typically refractory to standard anti-epilepsy drugs (AEDs) and non-pharmacological therapies (Andermann et al., 2003; Leal et al., 2002).

Ictal recordings from implanted electrodes in HH patients, including electrode contacts placed directly into HH tissues, have revealed that gelastic seizures arise from the lesion itself (Castro et al., 2007; Kuzniecky et al., 1997; Munari et al., 1995). The notion that intrinsic propensity resides within HH tissues has been further supported by neurosurgical treatments that have led to dramatic improvements in seizure control (Procaccini et al., 2006; Rosenfeld et al., 2001). Thus, it is now widely accepted that HH is intrinsically epileptogenic (Berkovic et al., 2003). However, the cellular mechanisms underlying seizure genesis within HH are unknown.

Recently, we described two major types of HH neurons (Coons et al., 2004,2007;Wu et al., 2005): small-sized (6-9 μ m) and large-sized (>20 μ m); and the vast majority of small cells are GABAergic (Wu et al., 2005). We also have demonstrated that small HH neurons express functional GABA_A receptors (GABA_ARs) (Wu et al., 2005,2007).

The most prevalent inhibitory neurotransmitter in the mammalian central nervous system is GABA, and derangements in GABAergic function have been repeatedly implicated in epileptogenesis (Ben-Ari and Holmes, 2005). One notable attribute of GABAergic neurons is the fact that GABA_AR activation in immature and some mature neurons results in cellular membrane depolarization and/or excitation (Ben-Ari, 2002; DeFazio et al., 2002; Gullledge and Stuart, 2003; Khazipov et al., 2004; Stein and Nicoll, 2003), an effect that is believed to contribute to seizure activity (Ben-Ari, 2006).

While the ontogeny of HH is unknown, it is possible that this lesion may be comprised of neurons that have not fully differentiated and thus may exhibit immature features. In the present study, we hypothesized that GABA_AR-mediated excitation might occur in some HH neurons. We tested the first hypothesis using gramicidin-perforated patch and cell-attached patch recordings, and additionally studied the molecular substrates of GABA-induced excitation and HH neuronal maturation using single-cell RT-PCR and immunocytochemical techniques. Our data demonstrate that most large HH neurons exhibited an overall phenotypic profile consistent with immaturity and that GABA_AR-mediated excitation may in part contribute to HH epileptogenicity.

MATERIALS AND METHODS

Informed consent for the use of post-surgical tissue for research purposes was obtained with a protocol approved by the Institutional Review Board at the Barrow Neurological Institute and St. Joseph's Hospital and Medical Center, Phoenix, AZ.

Patient profile

HH tissue specimens for this study were obtained from 34 patients undergoing surgical resection at our institution between June 2003 and April 2007. The mean age of patients at the time of surgery was 11.1 years (range: 0.7 to 36.8 years). There were 15 females (44%). All patients had intractable epilepsy that was refractory to at least 3 AEDs. Of these patients, 26 (77%) had onset of seizures before one year of age, including 15 (44%) with seizure onset during the first month of life. At the time of surgery, 31 (91%) had multiple daily seizures, while the remaining 3 patients had at least one seizure per week. Eight patients (24%) had only gelastic seizures at the time of resection, while 26 (77%) had multiple seizure types. More than one AED was being taken by 25 (74%) patients. All patients (100%) had gelastic seizures at some time during their clinical course.

Eighteen (53%) patients had mental or developmental retardation (full-scale intelligence quotients or estimated developmental quotients <70). A history of central precocious puberty was present in 13 cases (38%). Two patients (6%) had Pallister-Hall syndrome. Three cases (9%) were previously treated with gamma knife radiosurgery, and 3 cases (9%) had previously undergone surgery with subtotal resection of HH. Classification of HH anatomic subtypes (Delalande and Fohlen, 2003) revealed that 3 patients had Type I (9%), 20 Type II (59%), 5 Type III (15%), and 6 Type IV (17%). The mean lesion volume was 4.47 cm³ (range: 0.23 to 38.40 cm³). Surgical resection of HH was by a transcallosal, interforneal approach in 17 (50%) patients, and transventricular, endoscopic approach in the other 17 (50%) patients. Pathology confirmed the diagnosis of HH in all cases.

Acute dissociation of single neurons from human HH tissues

Acute enzymatic/mechanical dissociation of HH neurons was carried out based on our recently published protocols (Wu et al., 2005, 2007). Briefly, fresh HH tissue sections obtained at the time of surgery were immediately placed in ice-cold (2–4°C) dissection solution, which consisted of (in mM): 136.7 NaCl, 5 KCl, 0.1 NaH₂PO₄, 9.84 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 16.6 glucose and 21.9 sucrose, and were continuously bubbled with carbogen (95% O₂/5% CO₂; pH 7.4) during delivery from the operation room to the research laboratory (within 5 min). This Ca²⁺-free dissection solution has been shown to maintain the viability of neuronal tissue (Ishihara et al., 1995). The tissue sections were quickly sliced into several smaller pieces (~300–400 μm thick) using a vibratome (Vibratome Company, St. Louis, MO, USA) and were bubbled with carbogen at 35°C for 30 min in an incubation solution (in mM: 124 NaCl, 5 KCl, 24 NaHCO₃, 1.3 MgSO₄, 1.2 KH₂PO₄, 2.4 CaCl₂, 10 glucose) and then further incubated at room temperature (22 ± 1°C) for at least 1 h. Thereafter, tissue sections were treated with the same incubation solution containing 4–6 mg/mL papain (Sigma Chemical Co., St. Louis, MO, USA) at 31°C for 50–60 min. Tissue fragments were then washed twice with oxygenated incubation solution, and each fragment was then singly transferred to a 35-mm culture dish filled with oxygenated standard extracellular solution, which contained (in mM): 150 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 10 HEPES (pH adjusted to 7.4 with Tris-base). Each tissue section was then mechanically dissociated using fire-polished micro-Pasteur pipettes. Isolated, single cells usually adhered to the bottom of the dish within 30 min and maintained both good morphology and function for 2–6 h.

Patch-clamp recordings from acutely-dissociated HH neurons

Acute enzymatic/mechanical dissociation of HH neurons was carried out as previously described (Wu et al., 2005,2007). The gramicidin-perforated patch-pipette solution contained (in mM): 150 KCl, 4 MgCl₂ and 10 HEPES; adjusted to a pH of 7.2 (using Tris-OH). The junction potential between the pipette solution and the external solution was 4 mV and was corrected post-hoc. Gramicidin was dissolved in methanol (10 mg/mL as a stock) and diluted with patch-pipette solution immediately before use to a final concentration of 150 µg/mL. GABA-induced currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Whole-cell access resistance less than 60 MΩ was accepted for experiments. The series resistance was not compensated in this study. Typically, data were acquired at 10 kHz, filtered at 2 kHz, displayed and digitized on-line (Digidata 1322 series A/D board, Axon Instruments), and stored to a hard drive. Data acquisition and analyses of whole-cell currents were done using Clampex 9.2 and Clampfit 9.2 (Axon Instruments), respectively, and results were plotted using Origin 5.0 (Microcal, North Hampton, MA, USA). The standard external solution (in mM: 150 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 10 HEPES; pH adjusted to 7.4 with Tris-base) was used for perforated whole-cell patch recordings and also as pipette solution for cell-attached patch recording of extracellular action potentials (pipette potential was held at 0 mV). All drugs used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Tocris Cookson, Inc (Ellisville, MO, USA). All experiments were performed at room temperature (22 ± 1°C).

Single-cell RT-PCR

Single-cell RT-PCR was performed using SuperScript III Platinum CellDirect RT-PCR kits (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Briefly, using freshly-dissociated HH neurons not previously exposed to electrophysiological recordings, single-cell content was harvested by suction into pipettes filled with recording solution (~3 µL), and both the harvested content and depleted cells attached to the pipettes were immediately transferred to an autoclaved 0.2-mL PCR tube containing 10 µL of cell resuspension buffer and 1 µL of lysis enhancer. In most cases, following dissociation the single cells were lifted off the bottom of 35-mm culture dishes using pipettes prior to suction of cell content. The potential contaminating genomic DNA was removed by DNaseI digestion at 25°C for 6 min. After heat-inactivation of DNaseI at 70°C for 6 min in the presence of EDTA, RT was performed by adding a reaction mix with oligo(dT)20 and random hexamers and SuperScript III enzyme mix and then incubating at 25°C for 10 min and 50°C for 50 min. The reaction was terminated by heating the sample to 85°C for 5 min. The PCR primers for GAPDH, GABA_AR subunits (α1, α4, γ2), and the cation-Cl⁻ co-transporters NKCC1 and KCC2 were designed using Primer3 (Rozen and Skaletsky, 2000) with annealing temperature ~60°C (nearest neighbor). The PCR was performed with 20 µL of hot-start Platinum PCR Supermix (Invitrogen), 3 µL of cDNA template from the RT, and 1 µL of gene specific primer pairs (5 pmole each) with the following thermocycling parameters: 95°C for 2 min (95°C for 30 sec, 60°C for 30 sec, and 72°C for 40 sec) × 70 cycles, 72°C for 1 min. The PCR product was resolved on 1.5% TBE-agarose gels and photographed using a gel documentation system.

Immunocytochemical analyses

To determine NKCC1 and KCC2 protein expression in HH neurons, immunocytochemical experiments were performed. The primary antibodies used in the present study were goat anti-NKCC1 and goat anti-KCC2 (C-14 and R-14, respectively; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Secondary antibodies were both donkey anti-goat, one conjugated to a Texas Red fluorophore and the other conjugated to FITC (Santa Cruz Biotechnology, Inc.). Antibodies were diluted and applied to samples in HBSS containing 5% bovine serum albumin (Sigma-Aldrich). Single HH cells were fixed with 4% paraformaldehyde for 15 min at 21°C

and rinsed three times with PBS. Nonspecific binding was blocked by incubation with PBS containing 8% normal donkey serum for 1 h. Primary antibodies were applied to HH cells which were then incubated overnight at 4°C. Sections were rinsed thoroughly with PBS following primary antibody treatment and then incubated in secondary antibody for 1 h at room temperature. Following secondary antibody treatment, sections were thoroughly rinsed with PBS and post-fixed by immersion in methanol for 5 min at -20°C, and then mounted in ProLong (Invitrogen). Fluorescence intensity of the two types of HH neurons stained with the FITC-labeled KCC2 antibody was measured. Fluorescence mean intensity in soma (marked by a red dashed circle, Fig. 3C) of each neuron was measured using ImageJ (NIH) and normalized to the average of its surrounding background.

To assess the maturational state of HH neurons, HH tissue was harvested and sectioned (25 μ m) by cryostat for additional immunocytochemical studies. Cells from HH tissues were isolated as previously described (Wu et al., 2005,2007). The cells and the sections were fixed with 4% paraformaldehyde and blocked with 10% goat serum for 30 min. The primary antibodies used were mouse anti-polysialylated form of the neural cell adhesion molecule (PSA-NCAM) IgM (MAB5324, 1:400; Chemicon, Temecula, CA, USA) as an immature neuronal marker and rabbit anti-MAP-2 (sc-20172, 1:400; Santa Cruz Biotechnology, Inc.) as a mature neuronal marker. Fluorescent-labeling 488 (green) or 594 (red) secondary antibodies against rabbit IgG, mouse IgG or IgM were applied (1:1000; Invitrogen). Counterstaining with 4',6'-diamidino-2-phenylindole hydrochloride (DAPI; sc-3598, Santa Cruz Biotechnology, Inc.) was performed to identify cell nuclei.

Statistics

All statistical results are presented as mean $\pm$ SEM. The grouped Student's *t* test was used to compare two groups of data. Fisher's exact test was used for nonparametric data comparisons. Significance was set at $p < 0.05$.

RESULTS

GABA_AR-mediated excitation in HH neurons

From a total of 93 acutely-dissociated HH neurons that were characterized using electrophysiological recordings for this study, 76% (71 neurons from 34 patients) were small (Fig. 1Aa; soma: 6-9 μ m), while 24% (22 neurons from 19 patients) were large (Fig. 1Ba; soma: >20 μ m) in size. Each cell type exhibited distinct electrophysiological and phenotypic characteristics (Table I). Using gramicidin-perforated patch recordings ($n = 37$ small HH neurons from 20 patients with an average age of 10.5 ± 2.0 years and $n = 8$ large HH neurons from 6 patients with an average age of 9.5 ± 2.5 years, $p > 0.05$), bath-application (via a U-tube) of the GABA_AR agonist muscimol (30 μ M) induced depolarization in 38% of small HH neurons (14/37; Fig. 1C), but elicited membrane depolarization in 88% of large HH neurons (7/8; Fig. 1Bb,C). The 14 small HH neurons that exhibited muscimol-induced depolarization were obtained from 8 patients with an average age of 9.8 ± 2.3 years. Muscimol hyperpolarized 23 small HH neurons obtained from 12 patients with an average age of 11.6 ± 2.4 years. The difference between the average ages of patients when comparing muscimol-induced depolarization and hyperpolarization in small HH neurons was not significant ($p > 0.05$). The 7 large HH neurons that were depolarized by muscimol were obtained from 5 patients with an average age of 7.8 ± 2.5 years, while muscimol hyperpolarized only 1 large HH neuron from an 18-year-old patient, and the difference in the average age of patients (7.8 ± 2.5 , $n = 7$ vs. 18, $n = 1$) was not significant ($p > 0.05$). The total numbers of cells and their responses to muscimol are summarized in Fig. 1C. Similarly, during cell-attached patch recordings, muscimol suppressed spontaneous action potential firing in most small HH neurons (24/25 from 8 patients with an average age of 9.6 ± 3.4 years; Fig. 1Da), but facilitated action potential

firing in most large HH neurons (3/4 from 4 patients with an average age of 6.3 ± 1.5 years; Fig. 1*Db*). These results indicate that in most acutely-dissociated cells, the activation of GABA_ARs inhibited small but excited large HH neurons.

Estimation of intracellular Cl⁻ concentrations in HH neurons

Since GABA_ARs are Cl⁻ channels, their inhibitory or excitatory effect is determined principally by the Cl⁻ equilibrium potential. To determine whether the intracellular Cl⁻ concentration was higher in large compared to small HH neurons, we evaluated current-voltage (I-V) relationships for GABA_A receptor-mediated currents using gramicidin-perforated patch recordings. Figure 2*A* shows that GABA (*a*) or muscimol (*b*) induced similar current responses under our experimental conditions. Figure 2*B* shows typical cases of 100 μM GABA-induced currents at different holding potentials in small (*a*) or large (*b*) HH neurons. The reversal potentials (V_{revs}) of GABA-induced currents were -56.3 ± 4.6 mV and -36.2 ± 1.9 mV for small ($n = 7$) and large ($n = 7$) HH neurons, respectively ($p < 0.01$). Similar to GABA, muscimol-induced currents showed V_{revs} of -59.4 ± 3.9 mV and -35.7 ± 2.8 mV for small ($n = 5$) and large ($n = 2$) HH neurons, respectively. Based on the Nernst-equation, the known external Cl⁻ concentration (161 mM), and the measured V_{revs} of GABA-induced currents, the calculated intracellular Cl⁻ concentrations were 19.2 ± 3.7 mM and 39.1 ± 2.8 mM ($p < 0.01$) in small and large HH neurons, respectively. These results indicate that relatively high intracellular Cl⁻ concentrations underlie GABA-induced excitation in most large HH neurons.

NKCC1 and KCC2 mRNA expression, and KCC2 protein expression, in HH neurons

The relative levels of expression and activity of the inwardly-directed Na⁺-K⁺-2Cl⁻ (NKCC1) and outwardly-directed K⁺-Cl⁻ (KCC2) co-transporters principally determine intracellular Cl⁻ concentrations in neurons. Thus, in parallel experiments, we examined NKCC1 and KCC2 mRNA expression in HH neurons using single-cell RT-PCR and immunocytochemical techniques. Data were obtained using HH neurons from 7 patients with an average age of 10.1 ± 4.3 years. In 24 large HH neurons, only 4 (17%) showed positive KCC2 mRNA expression, while in 32 small HH neurons, 13 (41%) showed positive KCC2 mRNA expression (Fig. 3*A-C*). All of these neurons expressed GAPDH and the GABA_A γ2 subunit as positive control PCR products. The fraction of large HH neurons showing positive KCC2 mRNA expression was lower compared to the fraction of small HH neurons ($p < 0.05$). However, mRNA expression of NKCC1 and GABA_A α1 and α4 subunits was not different between large and small HH neurons ($p > 0.05$; Fig. 3*B*). Immunocytochemical labeling revealed that large ($n = 6$) HH neurons expressed lower levels of KCC2 protein compared to small ($n = 11$) HH neurons (Fig. 3*C,D*). These results suggest that in large HH neurons, low KCC2 expression is associated with relatively high intracellular Cl⁻ concentrations, which would lead to Cl⁻ efflux and neuronal depolarization upon GABA_A activation.

Immature features of HH neurons

Our results thus far suggest that most large HH neurons exhibit functionally-immature features, such as low KCC2 expression, high intracellular Cl⁻ concentrations, and GABA_A receptor-mediated excitation. We then asked whether HH neurons that exhibit GABA_A receptor-mediated depolarization express a molecular marker of immaturity. To address this question, isolated HH neurons were immunolabeled (double-stained) using the immature neuronal marker PSA-NCAM and the mature neuronal marker MAP-2. These experiments revealed that most large-sized HH neurons exhibited immunoreactivity to PSA-NCAM, while most small HH neurons exhibited stronger immunopositive labeling of MAP-2 (Fig. 4*A*). In order to further confirm the presence of immature neurons in HH tissues, 25-μm sections of HH were labeled with anti-PSA-NCAM antibody. Immunofluorescent studies revealed that PSA-NCAM-positive neurons were large in size (indicated by red arrows) while immunonegative neurons were small (indicated by

yellow arrows; Fig. 4B). Cell nuclei were identified with DAPI counterstaining. All of these results were obtained using HH tissues from 2 patients with an average age of 6.8 ± 2.7 years. These results confirmed that most large HH neurons exhibit an immature phenotype while most small HH neurons are likely mature.

DISCUSSION

The novel and important finding of the present study is that activation of GABA_ARs induced neuronal excitation in the majority of large neurons dissociated from human HH tissues after surgical resection. This GABA_AR-mediated excitation is likely a consequence of a reversed Cl⁻ electrochemical gradient, as indicated by the more positive Cl⁻ equilibrium potential and higher intracellular Cl⁻ concentration in these large neurons compared to smaller neurons that were hyperpolarized following GABA_AR activation. Additionally, we provide evidence that most large HH cells expressed the immature neuronal marker PSA-NCAM, whereas the majority of small HH neurons were preferentially labeled with the mature marker MAP-2. Collectively, our data indicate that HH tissues, which are comprised of large populations of clustered small, GABAergic neurons, also contain large, immature-like neurons, which may play a role in the intrinsically epileptogenic nature of the lesion.

A key variable related to GABA_AR-mediated neuronal excitation is the intracellular Cl⁻ concentration, which is determined principally by the relative expression and activity of the cation-Cl⁻ co-transporters NKCC1 and KCC2 (Ben-Ari, 2002; Ben-Ari and Holmes, 2005; Delpire, 2000; Owens and Kriegstein, 2002). In immature brain, neurons predominantly express NKCC1 and to a lesser extent KCC2 (Ben-Ari, 2002). Under these conditions, there is a higher than normal concentration of intracellular Cl⁻, such that GABA_AR activation induces Cl⁻ efflux and consequent membrane depolarization or neuronal excitation. The phenomenon of GABA_AR-mediated excitation is believed to play an important role in some forms of epilepsy (Cohen et al., 2002; Cossart et al., 2005; Jin et al., 2005). For example, Cohen et al. (2002) reported that epileptic activity in slices prepared from surgically-resected human epileptic temporal lobe tissues was critically dependent on GABA_AR-mediated depolarization based on sensitivity to blockade of depolarizing post-synaptic potentials before and during interictal spikes by a GABA_AR antagonist. Recently, GABA_AR-mediated excitation also was identified in human temporal lobe epileptic tissues (Huberfeld et al., 2007) and in human cortical dysplastic tissues (Cepeda et al., 2007). Collectively, these studies suggest the altered connectivity associated with abnormal temporal lobe and/or seizure activity caused neuronal deafferentation, conversion of neurons to an 'immature state', decreased the ability of neurons to prevent excess Cl⁻ accumulation, and led to excitatory effects induced by GABA. This mechanism also has been suggested to operate in other pathological conditions (Jin et al., 2005; Nabekura et al., 2002; Payne et al., 2003).

In the present study, we found that small HH neurons exhibited a more positive resting membrane potential compared to large HH neurons (Table I). Although we cannot definitively determine whether the measured resting membrane potential evaluated using perforated-patch recordings reflected the "natural" membrane potential as described by Tyzio et al. (2003), our data obtained from cell-attached patches confirming that GABA_AR activation inhibited most small but excited the majority of large HH neurons (Fig. 1D) supports our hypothesis. We also found that both small and large HH neurons exhibited relatively low levels of NKCC1 protein expression compared to KCC2 (data not shown), while large neurons expressed relatively low levels of KCC2 compared to small neurons (Fig. 4). The cause of the unexpected finding of low NKCC1 protein expression in large HH neurons is unknown. One possible explanation is the current lack of a highly-selective NKCC1 antibody. On the other hand, we found that the NKCC1 inhibitor bumetanide failed to alter V_{rev}s of GABA_AR-mediated currents in oocytes microtransplanted with HH tissues (Supplementary Fig. 1). These results suggest large HH

neurons may be functionally immature since a lower expression of KCC2 has been described for immature neurons (Ben-Ari, 2002; Rivera et al., 1999), and this low level of KCC2 expression/function may play a key role in GABA_AR-mediated excitation in HH. In support of this, other investigators have reported that some HH neurons exhibit an immature phenotype based on immunocytochemical evidence (Fukuda et al., 1999). Our demonstration of strong immunoreactivity of large HH neurons to the immature neuronal marker PSA-NCAM (Fig. 4) further strengthens the hypothesis that large HH neurons that depolarize in response to GABA_AR activation are developmentally immature. The present data also are complemented by our recent report that activation of GABA_ARs induced Ca²⁺ channel activity in large HH neurons using HH slices (Kim et al., 2007).

One of the important limitations of studies involving surgically-resected human epileptic tissue is the lack of absolute controls. For obvious reasons, an ideal control tissue for HH experiments cannot be obtained. Although it is natural to assume that HH arises from the hypothalamus, the precise tissue of origin and its ontogeny remain unknown. Previous studies have demonstrated that some HH neurons contain GnRH-secreting vesicles (Inoue et al., 1995), and importantly, GABA also has been reported to induce excitation of some GnRH-releasing neurons (DeFazio et al., 2002). Furthermore, the microanatomy and connectivity of the cellular elements within HH tissues have yet to be elucidated. In light of these observations, our data are consistent with the notion that HH may (at least in part) be of hypothalamic origin (Freeman, 2004).

To address the issue of an ideal control for our studies, we performed microtransplantation experiments using *Xenopus* oocytes to compare normal human hypothalamic tissues (preserved as autopsy specimens) to surgically-resected HH tissues. In these experiments, we found that the V_{rev} s of GABA-induced currents in microtransplanted HH tissue membrane fractions were more positive compared to those found in oocytes injected with normal human hypothalamic tissues (Supplementary Fig. 1). This indirect evidence supports our belief that some HH neurons exhibit immature features and maintain relatively high intracellular Cl⁻ concentrations. Based on our RT-PCR and immunocytochemical experiments, we believe that the observed positive shift in the Cl⁻ equilibrium potential in oocytes microtransplanted with HH tissues is consistent with relatively lower KCC2 expression (compared to microtransplanted control tissues) since natively-expressed K⁺-Cl⁻ co-transporters in oocytes (i.e., KCC1 or KCC3) can only be activated by hypotonicity (Mercado et al., 2001). Although NKCC1 and KCC2 are the principal regulators of neuronal Cl⁻ concentrations in most cases, other contributors also may play important roles. For example, the Na⁺-independent Cl⁻/HCO₃⁻ exchanger AE3 that is expressed in neurons (Raley-Susman et al., 1993) could mediate Cl⁻ uptake on the basis of its driving force. In addition, the Na⁺-dependent anion exchanger NDCBE (NDAE) also appears to play a role in cellular Cl⁻ homeostasis (Romero et al., 2004). The present study cannot exclude any contributions made by these exchangers.

Our results from oocyte recordings using HH tissue membrane fractions cannot distinguish between large or small neurons, and even though the value of the positive shift in the Cl⁻ equilibrium potential between HH and control tissues (Supplementary Fig. 1C) was smaller than that between large and small dissociated neurons (Fig. 2), the difference (10.6 mV vs. 20.1 mV) remained statistically significant ($p < 0.05$). Therefore, data from our oocyte experiments are consistent with a higher possibility of GABA-induced excitation occurring in HH. However, it is noted that there are age differences between HH and control tissues for our microtransplantation experiments, which may account for the shifted V_{rev} s of GABA-induced currents, although our patch-clamp data using dissociated HH neurons did not show there was any correlation between the V_{rev} s of GABA-induced currents and the ages of HH patients (Supplementary Table I).

Despite the lack of detailed information regarding the microanatomy and connectivity of HH tissues, our current and previously-published data provide evidence for a possible pathogenic mechanism involving GABA-induced neuronal excitation that may contribute to seizure genesis. Previously, we found that the majority (~90%) of HH neurons freshly dissociated from human HH tissues were small (6-9 μm) in size and immunoreactive to both the neuron-specific marker NeuN and glutamic acid decarboxylase 67 (GAD67), suggesting most of these cells were mature, GABAergic interneurons (Coons et al., 2007; Wu et al., 2005, 2007). Interestingly, most of these small HH neurons, which appear variably but universally in clusters or nodules (Coons et al., 2007), exhibited intrinsic rhythmic action potential firing (Wu et al., 2005), which could provide GABAergic input to large and/or adjacent small neurons. Our current preliminary data showing a positive reaction of large, but not small, HH neurons for the vesicular glutamate transporter 2 (data not shown) suggests large HH neurons are likely excitatory, projecting neurons (Freneau et al., 2001). Thus, endogenous GABA that activates GABA_ARs on large HH neurons could come from small, pacemaker-like, GABAergic HH neurons.

If it is assumed that small HH neurons are local GABAergic interneurons, that large HH neurons are ganglia-like, possibly projecting neurons, and that small GABAergic HH neurons synaptically innervate large HH neurons, then the following conceptual model of intrinsic seizure genesis can be hypothesized: spontaneous rhythmic activity of small, GABAergic HH neurons provides a tonic release of GABA onto their principal target cells - large projecting HH neurons - and synchronized GABAergic innervation excites projecting neurons, leading to an increase in neuronal output (perhaps excitatory) to structures adjacent to HH, thereby propagating network discharges that are manifested as seizure activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Normal human hypothalamic tissues (as controls) were provided by the Harvard Brain Tissue Resource Center, which is supported in part by a PHS grant (R24 MH 068855). This work was supported by NS-056104 (J.W., Y.C.) and grants from the Foundation and Women's Board of the Barrow Neurological Institute (J.W., J.F.K.).

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Fig 1.

Activation of GABA_ARs modulated HH neuron activity. Typical small (Aa) and large (Ba) neurons acutely dissociated from human HH tissues are depicted. Gramicidin-perforated patch-clamp recordings under current-clamp mode demonstrated that muscimol hyperpolarized small (Ab) but depolarized large (Bb) HH neurons. Ab: A representative trace from 23 small HH neuron recordings showing muscimol-induced hyperpolarization. Bb: A typical trace from 7 large HH neuron recordings showing muscimol-induced depolarization. C: Summary of the numbers of small and large HH neurons and their responses to the GABA_AR agonist muscimol using gramicidin-perforated recordings. D: Cell-attached patch recordings demonstrated suppression of spontaneous firing of small HH neurons (Da) but facilitation of large HH neuron firing (Db) by muscimol.

Fig 2.

I-V relationships for GABA-induced currents in small and large HH neurons under gramicidin-perforated patch recording conditions. Representative traces of GABA- and muscimol-induced currents recorded from a small HH neuron (A). GABA-induced currents were recorded from small (Ba) or large (Bb) HH neurons at different holding potentials (from -80 to -20 mV). Summary of I-V relationship curves are shown. The lines indicate the I-V curves of GABA-induced peak currents averaged from 7 small (○) or 7 large (▽) HH neurons (C). The vertical bars indicate $\pm$ SEM. All symbols were normalized to GABA-induced current at the holding potential of -80 mV (*). Based on the Nernst-equation and knowledge of both the external Cl^- concentration (161 mM) and the V_{rev} s of GABA-induced currents (small HH neurons: -56.3 ± 4.6 mV; large HH neurons: -36.2 ± 1.9 mV), intracellular Cl^- concentrations were calculated. Figure D shows the I-V curves of muscimol-induced peak currents averaged from 5 small (●) and 2 large (▼) HH neurons.

Fig 3. mRNA expression profiles and immunostaining for KCC2. *Ab*: Typical NKCC1, KCC2 and GABA_AR subunit mRNA expression profiles from a small (*Aa*, left) or a large (*Aa*, right) HH neuron assessed by single-cell RT-PCR, and these experiments were performed separately from patch-clamp recordings. *B*: Summary of mRNA expression from 32 small and 24 large HH neurons assessed for the indicated gene products from single, dissociated HH neurons. All of these neurons expressed GAPDH and the GABA_AR $\gamma 2$ subunit as positive control PCR products. *C*: Phase contrast (upper) and KCC2 immunostaining (lower) images of typical small (left) and large (right) HH neurons. *D*: Normalized staining intensity in small and large HH neurons reacting with anti-KCC2 antibody.

Fig 4.

Immature characteristics of large HH neurons. *A*: Small, acutely-dissociated neurons exhibited a positive reaction for the mature neuronal marker MAP-2, while large neurons exhibited a positive reaction for the immature neuronal marker PSA-NCAM. All immunolabeling experiments were repeated using tissues from 2 patients, and more than 10 cells were obtained for each observation. *B*: An HH tissue section was labeled using PSA-NCAM and showed a negative reaction in small HH neurons (indicated by yellow arrows) but a positive reaction (green) in 3 large HH neurons (indicated by red arrows, left panel). The middle panel shows DAPI staining identifying cell nuclei (blue), and the right panel represents a merged image of the left and middle panels.

Table I

Comparison of small and large HH neurons

	Small HH neurons	Large HH neurons
RP (mV)	-51.9 ± 1.3 , n=31 from 17 patients	$-60.8 \pm 1.7^{***}$, n=13 from 13 patients
Frequency (Hz)	9.9 ± 0.8 , n=31 from 17 patients	$3.7 \pm 0.4^{***}$, n=12 from 12 patients
Firing pattern	Regular	Irregular
Cm (pF)	6.8 ± 0.4 , n=32 from 19 patients	$16.8 \pm 1.9^{***}$, n=11 from 9 patients
Rm (MΩ)	1255 ± 157 , n=32 from 19 patients	$659 \pm 140^*$, n=11 from 9 patients
Muscimol	Depolarization: 24% (12/50)	Depolarization: 83% (10/12)
Muscimol	Hyperpolarization: 76% (38/50)	Hyperpolarization: 17% (2/12)

*
p<0.05***
p<0.001