Electrophysiological Properties and Subunit Composition of GABA<sub>A</sub> Receptors in Patients With Gelastic Seizures and Hypothalamic Hamartoma

Jie Wu, Yongchang Chang, Guohui Li, Fenqin Xue, Jamie DeChon, Kevin Ellsworth, Qiang Liu, Kechun Yang, Nasim Bahadroani, Chao Zheng, Jianliang Zhang, Harold Rekate, Jong M. Rho, and John F. Kerrigan

1Divisions of Neurology, 2Neurobiology, and 3Neurosurgery, Barrow Neurological Institute, St. Joseph’s Hospital and Medical Center, Phoenix, Arizona

Submitted 13 February 2007; accepted in final form 7 April 2007

INTRODUCTION

Hypothalamic hamartomas (HHs) are developmental malformations occurring in the ventral hypothalamus. These lesions are 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 et al. 2003; Iannetti et al. 1992; Kerrigan et al. 2005; List et al. 1958; Weissenberger et al. 2001). The epileptic syndrome in HH patients is often characterized by gelastic (i.e., laughing) seizures beginning in early infancy, followed by the development of additional seizure types in conjunction with cognitive decline and neurobehavioral problems (Berkovic et al. 1988; Kerrigan et al. 2005; Valdueza et al. 1994). The gelastic seizures are usually refractory to standard antiepileptic therapies (Andermann et al. 2003; Arzimanoglou et al. 2003).

Importantly, HH lesions are known to be intrinsically epileptogenic based on seizure recordings using intracranial electrodes and functional imaging studies (Berkovic et al. 2003; Kuzniecky et al. 1997; Leal et al. 2002; Munari et al. 1995; Palmini et al. 2002). This concept has also been supported by recent advances in surgical techniques, allowing for safe resection of the HH through transeptal or transventricular endoscopic approaches, leading to significant improvements in seizure control and possibly neurocognitive outcome (Ng et al. 2006; Polkey 2003; Rekate et al. 2006; Rosenfeld et al. 2001).

The cellular mechanisms underlying seizure onset within HH tissue are unknown. The histopathology of HH lesions consists of clusters of small (8–12 μm) neurons and limited numbers of larger neurons (Coons et al. 2007; Mottolese et al. 2001; Mullatti et al. 2003). Although the abundance and size of the clusters vary from case to case, the presence of these neuronal clusters seems to be an invariant feature of HH lesions associated with epilepsy (Coons et al. 2004). We recently characterized the basic electrophysiological properties and cellular phenotype of small HH neurons acutely dissociated from surgical specimens (Wu et al. 2005). We discovered that freshly isolated HH cells exhibited a neuronal phenotype with spontaneous, sustained rhythmic firing. Most of these cells were immunoreactive to glutamic acid decarboxylase 67 (GAD67) and also expressed functional GABA<sub>A</sub> and GABA<sub>B</sub> receptors (Wu et al. 2005).

GABA, the major inhibitory neurotransmitter in mature mammalian brain, plays an important role in the modulation of neuronal excitability. Moreover, GABA is critically involved in early brain development and is an important determinant of neurobehavioral function. The physiological consequences of GABA action are mediated by three subclasses of receptors: GABA<sub>A</sub> and GABA<sub>B</sub> receptors, which are ligand-operated chloride channels; and the G protein–coupled metabotropic GABA<sub>B</sub> receptor (MacDonald and Olsen 1994). Epileptic seizures may arise from impairment of normal GABAergic transmission, either by genetic alterations in subunit structure or by exogenous application of GABA<sub>A</sub> receptor antagonists.

Address for reprint requests and other correspondence: J. Wu, Neurophysiology Lab., Div. of Neurology, Barrow Neurological Inst., St. Joseph’s Hospital and Medical Center, Phoenix, AZ 85013-4496 (E-mail: Jie.Wu@chw.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Translational Physiology

WU ET AL.

In contrast, drugs that enhance GABAergic transmission are often used for the treatment of epilepsy (Jones-Davis and MacDonald 2003). Furthermore, in animal models of epilepsy and in tissue from patients with temporal lobe epilepsy (TLE), a loss of subsets of hippocampal GABAergic neurons can be observed (Kumar and Buckmaster 2006; Ratte and Lalacille 2006), highlighting the important role that GABA plays in modulation of network excitability. Neurodegeneration-induced loss of GABA receptors can also be accompanied by a marked change in the expression of GABA_A receptor subunits in the dentate gyrus and other parts of the hippocampus, which may lead to altered physiological and pharmacological properties of GABA_A receptors (Sperk et al. 2004). Such GABAergic mechanisms may be highly relevant for seizure genesis, augmentation of endogenous protective mechanisms, and resistance to antiepileptic drug therapy (Avoli et al. 2005).

Whether functional GABA_A receptors in patients with gelastic seizures and HH also show abnormalities in pharmacology and/or subunit composition is unknown. In this study, we investigated the detailed pharmacology and subunit composition of native GABA_A receptors found on single HH neurons freshly dissociated from surgically resected human HH tissue using patch-clamp, immunocytochemical, and RT-PCR techniques. We also compared these properties to GABA_A receptors in normal human hypothalamic tissues and HH tissue from patients with gelastic seizures using a Xenopus oocyte expression system.

METHODS

Informed consent for the use of postsurgical tissue for research purposes was obtained with a protocol approved by the Institutional Review Board of the Barrow Neurological Institute and St. Joseph’s Hospital and Medical Center, Phoenix, AZ. Experiments using Xenopus oocytes were carried out in accordance with the Guidelines for Animal Experimentation, Barrow Neurological Institute.

Patient profile

Tissue samples were obtained from 14 patients having undergone surgical resection at our facility between May 2003 and October 2005. There were 8 male and 6 female patients, and the mean age at the time of surgery was 12.2 yr (range = 2.1–30.6 yr). All patients had medically refractory epilepsy, with onset of gelastic seizures occurring at <2 yr of age. Of the 14 patients, 8 (57%) had onset of gelastic seizures at <1 mo of age. All patients had daily seizures of multiple types before surgical treatment. Nine of 14 (64%) patients had developmental retardation, and 8 patients had a prior history of central precocious puberty. Two patients in this series had Pallister-Hall syndrome. The mean HH lesion volume was 3.93 cm³ (range = 0.47–15.70 cm³). The anatomic subtypes of HH lesions were assigned according to the classification system proposed by Delalande and Fohlen (2003). There were 3 type I lesions (21%; parahypothalamic), 8 type II lesions (57%; small intrahypothalamic), and 3 type IV lesions (21%; giant). Surgical resection of HH tissue was accomplished by a transcerebral approach in 9 (64%) patients and a transventricular, endoscopic approach in 5 (36%) patients. Pathological examination of resected tissue confirmed the diagnosis of HH in all cases.

Acute dissociation of HH neurons from HH tissue

Acute enzymatic/mechanical dissociation of HH neurons was carried out using our previously 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) that contained (in mM) 136.7 NaCl, 5 KCl, 0.1 NaH₂PO₄, 9.84 HEPES, 16.6 glucose, and 21.9 sucrose, and were continuously bubbled with carbogen (95% O₂-5% CO₂; pH 7.4) during delivery (within a 5-min window) from the operating room to the research laboratory. 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 (~350–400 µm thick) using a vibratome (Vibratome Company, St. Louis, MO) and were bubbled with carbogen at 35°C for 30 min in an incubation solution containing (in mM) 124 NaCl, 5 KCl, 24 NaHCO₃, 1.3 MgSO₄, 1.2 KH₂PO₄, 2.4 CaCl₂, and 10 glucose, and further incubated at room temperature (22 ± 1°C) for ≥1 h. Thereafter, tissue sections were treated with the same incubation solution containing 4–6 mg/ml papain (Sigma) at 31°C for 50–60 min. Tissue fragments were washed twice with oxygenated incubation solution, and each fragment was singly transferred to a 35-mm culture dish filled with oxygenated standard culture medium solution containing (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 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.

Immunocytochemical analyses of dissociated HH neurons

To confirm the neuronal phenotype of HH cells, GAD67 expression in dissociated neurons was determined using immunocytochemical techniques as previously reported (Wu et al. 2005). The antibody used in this study was a mouse anti-GAD67 primary (MAB5406, Chemicon International, Temecula, CA); the secondary antibody used was Cy3-labeled goat anti-mouse (Jackson ImmunoResearch, West Grove, PA). The antibodies were diluted and applied to samples in PBS containing 5% bovine serum albumin (Sigma). Immunolabeled, frozen sections were imaged using an Axiosplan fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Single HH cells were fixed with 4% paraformaldehyde for 15 min at 4°C and rinsed three times with PBS. Nonspecific binding was blocked by incubating the cells in PBS containing 0.1% Tween-20 and 3% normal goat serum for 20 min. Primary antibodies were applied to HH cells either singly or as combination cocktails, and the treated cells were incubated either for 2 h at room temperature or overnight at 4°C. Sections were rinsed thoroughly with PBS after primary antibody treatment and were incubated in secondary antibody for 1 h at room temperature. After secondary antibody treatment, sections were thoroughly rinsed with PBS, postfixed and dehydrated by immersion in methanol for 5 min at −20°C, and mounted in ProLong (Invitrogen, Carlsbad, CA).

Patch-clamp recordings

Perforated-patch whole cell recordings, combined with a U-tube fast application system (allowing for both quick application and removal of drug), were used following previously described techniques (Wu et al. 2004, 2005). The perforated-patch pipette solution contained (in mM) 150 CsCl, 4 MgCl₂, and 10 HEPES, adjusted to a pH of 7.2 using Tris-OH (CsCl electrode). In some experiments, a potassium gluconate solution (in mM) 130 K⁺-gluconate, 5 KCl, 5 MgCl₂, 10 HEPES, pH 7.2 with TrisOH was used. The junction potential between the pipette solution and the external solution was 4 mV for the CsCl electrode and 14 mV for the K⁺-gluconate electrode (calculated with Clampex 9.2, Axon Instruments, Foster City, CA). Typically, cells were voltage-clamped at −64 mV, and corrections were made for junction potentials post hoc. Amphotericin B was dissolved in dimethylsulfoxide (DMSO; 40 mg/ml as a stock) and diluted with internal (patch-pipette) solution to a final concentration of 200–250 µg/ml immediately before use. GABA-induced currents...
were recorded using an Axopatch 200B amplifier (Axon Instruments). Cells with a whole cell access resistance < 60 MOhm were used for experiments. Series resistance compensation was not performed in this study. Typically, signals were filtered at 2 kHz, displayed and digitized on-line at 10 kHz (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 9.0 (Microcal, North Hampton, MA). All experiments were performed at room temperature. All drugs used in this study were purchased from Sigma and Tocris Cookson (Ellisville, MO).

**RNA isolation and RT-PCR**

Precautions were taken throughout all RT-PCR experiments to both ensure a ribonuclease-free environment and avoid PCR product contamination. After surgical resection, each HH tissue fragment (~20–50 mg) was immediately placed into liquid nitrogen. The tissue was homogenized with a microtube pestle in 10× volume of QIAzol Lysis reagent (QIAGEN, Valencia, CA). The homogenate was passed through a 22-gauge needle five times. After removing insoluble cell debris by centrifugation at 12,000 g for 10 min, 0.2 volume of chloroform per amount of QIAzol used was added to the supernatant. After mixing and incubation (10 min at room temperature), the homogenate was centrifuged at 4°C and 12,000g for 15 min, and the RNA-containing upper aqueous phase was carefully transferred to a new tube. The total RNA was precipitated by isopropanol (Sigma). The RNA pellet was washed with 75% ethanol and dissolved in RNase-free water. The genomic DNA was removed from the RNA sample by DNase I digestion (16 min at 25°C). After heat inactivation of DNase I in the presence of EDTA, the total RNA was further purified by RNeasy Mini cleanup (QIAGEN) following the manufacturer’s protocol. The total RNA concentration was determined by an Eppendorf BioPhotometer spectrometer (Brinkmann Instruments, Westbury, NY). First-strand DNA was synthesized by reverse transcription from 20 ng HH tissue total RNA, hypothalamic total RNA (Ambion, Austin, TX) or total RNA purified from control hypothalamus (Harvard Brain Tissue Resource Center, Cambridge, MA) using Superscript III First-strand Supermix (Invitrogen) with oligo (dT)20 and random hexamers following the manufacturer’s protocol. After cDNA synthesis, reactions were stored at –80°C for ≈1 wk before PCR. The PCR primers for GABAA receptor subunits (α1–6, β1–3, γ1–3, δ, ε, θ) and GAD67 were designed using the Primer 3 internet server (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to amplify the subunit-specific region in the intracellular loop between the third and fourth transmembrane domains of each GABA receptor subunit. Each PCR was performed with 1 μl of the cDNA (RT product) template, subunit specific primer pair [with 60°C annealing temperature (nearest neighbor), 5 pmol each], and hot-start Platinum PCR Supermix (Invitrogen) for 40 cycles (95°C for 30 s, 60°C for 30 s, and 72°C for 40 s). The PCR product was resolved on 2% TBE-agarose gels and photographed using a gel documentation system.

**FIG. 1.** GABAA receptor–mediated currents in dissociated human hypothalamic hamartoma (HH) cells. A: acutely dissociated single cells from human HH tissues. After acute dissociation, most of the single HH cells were small and contained 2–3 thin processes. Cells (Aa–Ad) are representative examples of small HH cells from 4 different HH patients showing homogeneous morphology. B: perforated patch-clamp (amphotericin B) recording under current-clamp mode shows spontaneous action potential firing in a small HH cell. C: after patch-clamp recording in (B), the fluorescent dye lucifer yellow was delivered into the cell. Left: phase-contrast picture of recorded HH cell. Middle: lucifer yellow-labeled HH cell. Right: GABAA–positive reaction of the same HH cell. Scale bar indicates 50 μm. D: at a holding potential (Vh) of –60 mV, GABA induced an inward current (a), which was mimicked by the selective GABAA receptor agonist muscimol (b) and abolished by the selective GABAA receptor antagonist bicuculline (BMI; c). E: kinetic analyses of I_{\text{GABA}}. Bar graphs represent the average rising time (τrising), current density (pA/pF), desensitization time constant (τdesensitization) and deactivation time constant (τdeactivation) from 10 HH cells and vertical bars represent means ± SE.
Microtransplantation of GABA<sub>A</sub> receptors into Xenopus oocytes and two-electrode voltage-clamp recordings

MEMBRANE PROTEIN PREPARATION. Membrane proteins from both HH tissues and normal hypothalamus were prepared according to a protocol described previously (Miledi et al. 2006). Briefly, frozen tissues from liquid nitrogen were homogenized with a glass homogenizer in glycine buffer (in mM: 200 glycine, 150 NaCl, 50 EGTA, 50 EDTA, 300 sucrose; pH 9) plus 20 μl of protease inhibitor cocktail (Sigma). The homogenate was centrifuged for 15 min at 9,600 g and 4°C. The supernatant was further centrifuged for 2 h at 100,000 g using a Beckman L-7 Ultracentrifuge with a SW61 Ti rotor at 4°C. The pellet was washed and resuspended in 5 mM glycine solution and used immediately for injection or stored at −80°C in aliquots for later use.

OOCYTE PREPARATION AND PROTEIN INJECTION. Female Xenopus laevis (Xenopus I, Ann Arbor, MI) were anesthetized using 0.2% MS-222. The ovarian lobes were surgically removed from the frogs and placed in an incubation solution that consisted of (in mM) 82.5 NaCl, 2.5 KCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 1 Na<sub>2</sub>HPO<sub>4</sub>, 0.6 theophylline, 2.5 sodium pyruvate, 5 HEPES, 50 mg/ml gentamycin, 50 U/ml penicillin, and 50 μg/ml streptomycin; pH 7.5. The frogs were allowed to recover from surgery before being returned to the incubation tank. The lobes were cut into small pieces and digested with 0.08 Wunsch U/ml liberase blendzyme 3 (Roche Applied Science, Indianapolis, IN) with constant stirring at room temperature for 1.5–2 h. The dispersed oocytes were thoroughly rinsed with incubation solution. Stage VI oocytes were selected and incubated at 16°C before injection. Micropipettes used for injection were pulled from borosilicate glass (Drummond Scientific, Broomall, PA) using a Sutter P87 horizontal puller, and the tips were broken with forceps to ~40 μm in diameter. Membrane protein was drawn up into the micropipette and injected into oocytes using a Nanoject microinjection system (Drummond Scientific) at a total volume of ~60 nl.

TWO-ELECTRODE VOLTAGE-CLAMP RECORDINGS. One to 3 days after injection, an oocyte was placed in a small-volume chamber and continuously perfused with oocyte Ringer solution, which consisted of (in mM) 92.5 NaCl, 2.5 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 5 HEPES; pH 7.5. The chamber was grounded through an agarose bridge. The oocytes were voltage-clamped at −60 mV to measure GABA-induced currents using GeneClamp 500B (Axon Instruments).

DATA ANALYSIS. The dose-response relationship of GABA-induced current of microtransplanted receptors was fit with a least-squares algorithm, and the Hill equation was used to derive both EC<sub>50</sub> (the GABA concentration required for inducing half-maximal current) and Hill coefficient values and maximum current. The maximum current was used to normalize the dose-response curve for each individual oocyte. The average of the normalized currents for each GABA concentration was used to plot the data.

Statistical analysis

All statistical results are presented as means ± SE. The Student’s t-test was used to compare two groups of data and significance was set at P < 0.05.

RESULTS

GABA<sub>A</sub> receptor–mediated currents in single HH neurons

Most (~90%) dissociated HH neurons were small (6–9 μm) and contained one to three processes (Fig. 1A). Under perforated patch-clamp conditions in current-clamp mode, most of these small HH neurons exhibited spontaneous action potential firing (7–14 Hz) at the resting potential of –56 ± 4 mV (Fig. 1B; n = 24). In some experiments, after perforated patch-clamp recording, the fluorescent dye lucifer yellow (0.5 mg/ml in the pipette) was delivered to the recorded neuron by con-
GABAergic receptors on HH neurons. Under perforated patch-clamp conditions (using a CsCl electrode), in voltage-clamp mode, GABA (100 μM) induced an inward current \( I_{\text{GABA}} \) (Fig. 1Da) at the holding potential \( (V_H) \) of −60 mV [chloride equilibrium potential \( (E_{\text{Cl}}) = −0.4 \text{ mV} \)], which was mimicked by the GABA\(_A\) receptor agonist muscimol (Fig. 1Db) and inhibited by the selective GABA\(_A\) receptor antagonist bicuculline methiodide (BMI; Fig. 1Dc). Kinetic analyses showed that \( I_{\text{GABA}} \) exhibited a relatively slow desensitization. The rising time \( (T_{\text{rising}}) \), current density \( (\text{pA/pF}) \), decay time constant \( (\tau_{\text{desensitization}}) \), and deactivation time constant \( (\tau_{\text{deactivation}}) \) of 100 μM GABA-induced whole-cell current are shown in Fig. 1, Eb–Ec. These results suggest that \( I_{\text{GABA}} \) was mainly mediated by GABA\(_A\) receptors.

Concentration-response relationship of \( I_{\text{GABA}} \)

To explore the pharmacological properties of dissociated HH neurons, the concentration-response relationship of GABA-induced responses was examined. As shown in Fig. 2 (CsCl electrode; \( E_{\text{Cl}} = −0.4 \text{ mV} \)), when the concentration of GABA was increased, the amplitude of \( I_{\text{GABA}} \) increased (Fig. 2A), thereby generating a sigmoidal-shaped concentration-response curve (Fig. 2B). Using 10 HH neurons from seven patients, the average EC\(_{50}\) and Hill coefficient of \( I_{\text{GABA}} \) were 6.8 μM and 1.9, respectively. These results suggest that functional GABA\(_A\) receptors possessed a relatively high (i.e., low micromolar) affinity for GABA on dissociated HH neurons, and two GABA molecules are likely required to open GABA\(_A\) receptor/Cl\(^-\) channels.

Current-voltage (I-V) relationship of \( I_{\text{GABA}} \)

To assess the I-V relationship of \( I_{\text{GABA}} \), two experimental protocols were employed. First, GABA (100 μM)-induced currents in small HH neurons were recorded at different \( V_H \) (from −60 to +20 mV; Fig. 3Aa). As shown in Fig. 3Aa, the I-V curve was linear, and \( I_{\text{GABA}} \) reversed from an inward to an outward current near a \( V_H \) of 0 mV. Second, a ramp-pulse was applied before and during exposure to GABA (100 μM). In this experiment, voltage-dependent Na\(^+\) and Ca\(^+\) channels were blocked by adding TTX (0.5 μM) and La\(^{3+}\) (10 μM) into the external solution, and voltage-sensitive K\(^+\) channels were blocked by the high concentration of Cs\(^+\) in the pipette solution. Figure 3Ba shows an example of a ramp-pulse experiment. A ramp-pulse (from −100 to +60 mV, 400 ms) was applied before I) and during 2) exposure to GABA. The intersection of the two ramp currents before and during exposure to GABA indicates the reversal potential (Fig. 3Bb). These results suggest that functional GABA\(_A\) receptors on HH neurons exhibited a linear I-V relationship and that the reversal potential was close to the \( E_{\text{Cl}} (-0.4 \text{ mV}) \) when the external and internal chloride concentrations were similar.

Pharmacologic antagonism of \( I_{\text{GABA}} \)

The concentration-inhibition relationship of BMI-induced inhibition was further examined. As shown in Fig. 4A, 100 μM BMI GABA was inhibited by BMI in a concentration-dependent manner. The IC\(_{50}\) value and Hill coefficient of BMI-induced inhibition were 33.7 μM and 0.9 (n = 6 from 4 HH patients), respectively (Fig. 4B). Figure 4C shows that inhibition of \( I_{\text{GABA}} \) by BMI was independent of the membrane potential (K-glucuronate electrode; \( E_{\text{Cl}} = −60 \text{ mV} \)).

Allosteric modulation of \( I_{\text{GABA}} \)

The effects of several GABA\(_A\) receptor allosteric modulators were next examined to better characterize \( I_{\text{GABA}} \). Figure 5A shows a typical case recorded from the same HH neuron, in which 3 μM \( I_{\text{GABA}} \) exhibited low sensitivity to diazepam and zinc, but higher sensitivity to pentobarbital and pregnanolone. Normalized to 3 μM \( I_{\text{GABA}} \) (as 100%), the currents induced by GABA plus diazepam (1 μM), pentobarbital (30 μM), pregnanolone (1 μM), and zinc (100 μM) were 176 ± 19% (P <
0.001; n = 19 neurons from 10 patients), 300 ± 47% (P < 0.01; n = 10 neurons from 7 patients), 248 ± 40% (P < 0.01; n = 7 neurons from 6 patients), and 78 ± 7% (P < 0.05; n = 10 neurons from 7 patients), respectively (Fig. 5B). These results suggest that GABA_A receptors expressed on small HH neurons exhibited low sensitivity to benzodiazepine and zinc but were more sensitive to pentobarbital and neurosteroid modulation.

**Differences in GABA_A receptor function between normal human hypothalamus and HH tissue**

The above experiments have delineated the detailed functional properties of GABA_A receptors on dissociated HH neurons. The interesting but difficult question addressed in this study was whether these properties were indeed abnormal compared with normal human hypothalamic tissue. For obvious reasons, it was not possible to study normal human hypothalamus. However, to circumvent this intrinsic limitation, using a novel microtransplantation technique (Palma et al. 2004, 2005), we transplanted membrane proteins from normal human hypothalamic tissue (obtained from the Harvard Brain Tissue Resource Center), as well as from resected human HH tissue into *Xenopus* oocytes. In this manner, we were able to compare the functional properties of GABA_A receptors from normal (control) tissues and HH tissues in this expression system. As shown in Fig. 6, microtransplanted GABA_A receptors from both control (Fig. 6Aa) and HH (Fig. 6Ab) tissues were indeed functional, and the concentration-response relationship curves revealed an EC_{50} value of 53.7 ± 2.1 μM and a Hill coefficient of 1.2 for receptors from control (using 2 normal human tissues transplanted into 10 oocytes) and an EC_{50} value of 37.3 ± 6.0 μM and a Hill coefficient of 1.4 for receptors from HH (using 3 human HH tissues transplanted into 16 oocytes) tissues (Fig. 6B). Although the difference in EC_{50} values was statistically significant (P < 0.01), GABA_A receptor function was comparable. Figure 6C shows that GABA (30 μM)-induced currents exhibited low sensitivity to diazepam and zinc, were more sensitive to pentobarbital and pregnanolone, and there was no difference in modulation of GABA_A receptors from control versus HH tissues. Figure 6D summarizes the allosteric modulations of GABA (30 μM; as 100%)-induced currents using microtransplanted GABA_A receptors. These results suggest that in human HH tissue, functional GABA_A receptors show slightly high affinity for GABA, but exhibit normal allosteric modulation, results that are consistent with our prior patch-clamp recordings in single dissociated HH neurons (Fig. 5B).

**GABA_A receptor subunit expression in HH tissue**

We next explored the mRNA expression profiles of GABA_A receptor subunits in HH tissue using RT-PCR techniques and compared these results to those obtained using normal (control) human hypothalamic total RNA. Figure 7 shows a representative sample of mRNA expression data of GABA_A receptor subunits.
subunits from both HH and control tissues. Table 1 summarizes all cases of HH tissue samples from 14 patients and from 3 control tissues and reveals that most GABAA receptor subunits were expressed in both HH and control tissues. There was no difference in subunit mRNA expression when HH and control tissues were compared. The profile of GABA receptor subunit expression suggests that in human HH neurons, GABAA receptors on small HH neurons are comprised of several different subgroups based on GABAA receptor subunit composition. These structural differences also imply that there may be heterogeneity with respect to GABAA receptor function, pharmacology, and modulation.

**DISCUSSION**

In this study, we characterized for the first time the pharmacological and molecular features of functional GABAA receptors on single small neurons freshly dissociated from human HH tissues after surgical resection. This study was designed to evaluate the possibility that abnormal GABAA receptor structure and/or function may underlie in part the seizure propensity of HH, as has been reported for other epileptic tissues (Jones-Davis and MacDonald 2003; MacDonald and Olsen 1994; Sperk et al. 2004). Using cellular electrophysiological techniques, we showed that GABA-induced responses were mainly mediated through GABAA receptors and that these receptors showed slightly increased affinity for GABA. We also showed that GABAA receptors on HH neurons exhibited low sensitivity to benzodiazepine and zinc modulation but were more sensitive to pentobarbital and pregnanolone. More importantly, we compared functional GABAA receptors from human HH to normal human hypothalamic tissues and confirmed the similarities in pharmacosensitivity and subunit composition. Although our data are consistent with normally functioning GABAA receptors in patients with HH, this study provides a detailed profile of GABAA receptors on small HH neurons, which we believe is fundamental to a detailed mechanistic understanding of inhibitory neurotransmission in human HH tissue.

**Functional GABA<sub>A</sub> receptors on HH neurons**

It is well known that GABA<sub>A</sub> receptors play crucial roles in the mediation of fast synaptic inhibition in mature mammalian brain. In this study, we characterized functional GABA<sub>A</sub> receptors on dissociated HH neurons. Most acutely isolated HH neurons were small (6–9 μm) and GAD67-positive; these findings were consistent with a GABAergic phenotype and confirm our previous observations (Wu et al. 2005).

**FIG. 5.** Allosteric modulation of $I_{GABA}$. **A:** under voltage-clamp configuration, GABA (3 μM)-induced current exhibited little sensitivity to diazepam (DZP) and zinc, but was more sensitive to pentobarbital (PB) and pregnanolone (PGN). **B:** summary of the allosteric modulation in (A). Each column represents the average from 7 to 19 cells, and vertical bars represent means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.
was slow (>2,000 ms) and was fit with a single exponential function, suggesting that these receptors slowly desensitized in response to GABA. This kinetic feature also suggests the possibility that non-α1 GABA<sub>A</sub> receptor subunits may be expressed in HH neurons, and indeed, this was confirmed by our results from RT-PCR experiments. Also, the current density (pA/pF) was relatively high, suggesting that large numbers of functional GABA<sub>A</sub> receptors were expressed on HH neurons.

Enhanced affinity of GABA<sub>A</sub> receptors on HH neurons for GABA

Previous studies have shown that alterations in GABA<sub>A</sub> receptor function, pharmacological properties, and/or subunit composition contributes to, or is associated with, epileptogenesis in human and animal models (Jones-Davis and MacDonald 2003; MacDonald and Olsen 1994; Sperk et al. 2004). Indeed, in human epileptic tissue after surgical resection, investigators have identified a number of abnormalities (Palma et al. 2004, 2005; Wozny et al. 2003). For example, there is a lower affinity of GABA<sub>A</sub> receptors for the endogenous agonist GABA (Palma et al. 2005; Wozny et al. 2003). In contrast, we found that the affinity of GABA<sub>A</sub> receptors on small HH neurons for GABA was higher (EC<sub>50</sub> of 6.8 μM) than expected. Although we do not yet know the native subunit composition of GABA<sub>A</sub> receptors in HH tissue, this altered sensitivity could be explained by differential composition of subunits.

Enhanced affinity of GABA<sub>A</sub> receptors on HH neurons for GABA

It is known that GABA<sub>A</sub> receptors comprised of α4 or α6 subunits show a high affinity for GABA and a relative insensitivity to benzodiazepines (Santhakumar et al. 2006; Whitte-
more et al. 1996). In one study, α4-containing GABA_4 receptors exhibited a relatively higher affinity (~3-fold higher compared with α1-containing GABA_A receptors) for GABA (Whittemore et al. 1996). Our own data showed consistent expression of the α4 subunit (14 of 14 tissue samples, 100%), but not the α6 subunit (4 of 14, 29%; Fig. 7; Table 1). As such, the higher affinity of GABA_A receptors for GABA on small HH neurons may be caused by the presence of the α4 subunit. Certainly, the low sensitivity of GABA-induced currents to diazepam strongly supports this notion. However, at present, we have not yet confirmed such cell-specific expression patterns of GABA_A receptor subunits in HH tissue.

### Allosteric modulation of GABA_A receptors on small HH neurons

Differential assembly of GABA_A receptor subunits is also believed to underlie the heterogeneity of allosteric modulation (Mohler et al. 2005; Simeone et al. 2003). Chemical effectors acting at specific recognition sites can allosterically modulate the GABA_A receptor. Generally, benzodiazepines, barbiturates, and neurosteroids positively modulate, whereas zinc inhibits, the GABA_A receptor (MacDonald and Olsen 1994). Functional alterations in these modulatory mechanisms—caused by changes in subunit composition—have been described in both animal models of epilepsy and human epilepsy patients and are thought to play an important role in epileptogenesis (Jones-Davis and MacDonald 2003; Sperek et al. 2004).

We examined allosteric modulation of GABA_A receptors on HH neurons and found a generally low sensitivity to benzodiazepine modulation. In all cases, fGABA was sensitive to pentobarbital and pregnanolone but exhibited lower sensitivity to diazepam. Previous work had already shown that GABA_A receptors comprised of α4, β2, and γ2 subunits were insensitive to benzodiazepines compared with receptors composed of α1, β2, and γ2 subunits (Whittemore et al. 1996). We hypothesized that both the γ (likely γ2) and α4 subunits are likely present in functional GABA_A receptors on small HH neurons based on the following evidence: 1) the GABA-induced whole cell current decay constant (reflecting receptor desensitization) was relatively slow, 2) receptor affinity for GABA was relatively high, and 3) sensitivity to benzodiazepine modulation was relatively low. These pharmacological properties are characteristic of an α4β2γ2 subtype of GABA_A receptor (see review by Korpi et al. 2002). As expected, our RT-PCR experiments showed high expression ratios of both γ2 and α4 subunits, strengthening our suspicion that these specific subunits are widely expressed in small HH neurons. The function of α4-containing GABA_A receptors was postulated to play an important role in tonic inhibition because of the extrasynaptic location of these receptors. However, α4-containing GABA_A receptors located extrasynaptically mostly have an α4β2δ composition (Chandra et al. 2006), whereas in HH tissues, the δ subunit had a low level of expression. In this study, we were not able to test the GABA tonic effect using our enzymatically/mechanically dissociated, single HH neurons, but it is of interest to test this effect in future experiments using HH slice-patch recordings.

### Significance of GABA_A receptors in human HH tissue

Impaired GABA-mediated inhibition, leading to neuronal hyperexcitability, is widely implicated in the epileptogenic potential of certain seizure-prone structures such as the hippocampus (Tasker and Dudek 1991). In human TLE, abnormal GABA-mediated inhibition results from cell loss in selected regions of the hippocampus and from alterations in GABA_A receptor subunit composition in surviving cells (Loup et al. 2000). In focal cortical dysplasia (FCD), which represents regions of the hippocampus and from alterations in GABA mediated synaptic inhibition (Calcagnotto et al. 2005). Alterations in GABA_A receptor subunit composition and function have also been reported in animal models of TLE (Brooks-Kayal et al. 1998; Fritschy et al. 1999). Interestingly, in single dissociated dentate granule cells from both human and rodent epileptic hippocampus (Brooks-Kayal et al. 1998, 1999;
Porter et al. 2005), there was a high expression level of the GABA_4 receptor subunit. Despite these intriguing observations, however, it remains unclear how abnormalities in GABA-mediated inhibition—whether based on loss of inhibitory interneurons, altered pharmacosensitivity, or subunit composition of GABA_A receptors—result in network hyperexcitability and hypersynchrony, the two hallmark features of seizure activity. In contrast to many previous reports in other epileptic tissues, we found normally functioning GABA_A receptors in surgically resected human HH tissue. These receptors 1) were highly sensitive to GABA (EC_{50} of 6.8 μM); 2) exhibited low sensitivity to diazepam and zinb but were more sensitive to pentobarbital and pregabalin; 3) were composed of multiple GABA_A receptor subunits; and 4) showed similar properties compared with receptors from HH and normal human hypothalamic tissues (with the exception of EC_{50} values). Taken together, our results suggest that, unlike other epileptic tissues (e.g., from human TLE), the intrinsic hyperexcitability of HH tissue is not simply related to abnormal pharmacological properties and/or subunit composition of GABA_A receptors. Therefore how would normally functioning GABA_A receptors on small HH neurons contribute to seizure genesis? The answer might lie in the anatomical characteristics of HH tissue, much of which remains to be detailed. We have previously described a highly invariant feature: that is, cellular clusters of small neurons that exhibit spontaneous rhythmic activity and are largely immunoreactive to GAD67 (Coons et al. 2007; Wu et al. 2005). Although it remains unclear whether these spontaneously firing GABAergic neurons contribute to the genesis of gelastic seizures, it is possible that the sheer magnitude of this GABAergic activity could drive adjacent projection neurons in a hypersynchronous manner. We speculate that larger HH neurons, which are adjacent to clusters of small neurons, may represent such projection neurons (Coons et al. 2004, 2007; Fenoglio et al. 2007). There is a substantial body of evidence to suggest that enhanced (not diminished) GABAergic inhibition might be critical for network hypersynchrony, propagation of discharge, and ultimately electroclinical manifestations of seizure activity. Normally functioning GABA_A receptors would constitute the essential currency for such a physiological effect. For example, in the hippocampus, it is well known that each GABAergic interneuron synapses onto several thousand pyramidal neurons. As such, the activity of a single GABAergic interneuron could influence the collective output of thousands of principal cells. Both electrophysiological and computational studies have confirmed that increased GABAergic inhibition actually contributes to network hyperexcitability in the hippocampus (Aradi and Maccareferri 2004; Esclapez et al. 1997; Velazquez and Carlen 1999). It would be of interest to test whether GABA_A receptor activation in HH tissue contributes to neuronal hypersynchrony, thereby increasing the likelihood of network propagation and seizure generation (D’Antuono et al. 2004; Kumar and Buckmaster 2006). Fundamentally, however, a more detailed knowledge of the anatomical connectivity of HH neurons and their projections must first be obtained before more detailed cellular electrophysiological studies can be implemented. Alternatively, if such GABA-mediated hypersynchrony, which requires normally functioning GABA_A receptors, is not operant in HH tissue, GABA_A receptor activation might mediate membrane depolarization, not hyperpolarization—as has been described in immature neurons (Ben-Ari 2002; Gullelde and Stuart 2003; Stein and Nicoll 2003). GABA-mediated excitation of HH neurons could result in propagated excitation through as yet unidentified output pathways to extrahypothalamic structures.

Acknowledgments

The authors thank A. Ward and S. Snyder for help in coordinating the delivery of resected HH tissues from the operating room to the electrophysiology laboratory. Normal human hypothalamic tissues (as the control group) were provided by the Harvard Brain Tissue Resource Center, which is supported in part by Institute of Mental Health Grant R24 MH-068855.

Grants

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-056104 to J. Wu and Y. Chang and grants from the Foundation and Women’s Board of the Barrow Neurological Institute to J. Wu and J. F. Kerrigan.

References

Selective List CF, Dowman CE, Bagchi BK, Bebin J.
Gulledge AT, Stuart GJ.
Jones-Davis DM, MacDonald RL.
Jones-Davis MS, MacDonald RL. GABA(A) receptor function and pharmacology in epilepsy and status epilepticus. Curr Opin Pharmacol 3: 12–18, 2003.