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Mechanisms of Intrinsic Epileptogenesis in Human Gelastic Seizures with Hypothalamic Hamartoma

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SUMMARY

Human hypothalamic hamartoma (HH) is a rare developmental malformation often characterized by gelastic seizures, which are refractory to medical therapy. Ictal EEG recordings from the HH have demonstrated that the epileptic source of gelastic seizures lies within the HH lesion itself. Recent advances in surgical techniques targeting HH have led to dramatic improvements in seizure control, which further supports the hypothesis that gelastic seizures originate within the HH. However, the basic cellular and molecular mechanisms of epileptogenesis in this subcortical lesion are poorly understood. Since 2003, Barrow Neurological Institute has maintained a multidisciplinary clinical program to evaluate and treat patients with HH. This program has provided a unique opportunity to investigate the basic mechanisms of epileptogenesis using surgically resected HH tissue. The first report on the electrophysiological properties of HH neurons was published in 2005. Since then, ongoing research has provided additional insights into the mechanisms by which HH generate seizure activity. In this review, we summarize this progress and propose a cellular model that suggests that GABA-mediated excitation contributes to epileptogenesis in HH lesions.
mechanical and unnatural [1,17] and usually does not result in feeling positive emotions [10,14,18], it therefore seems possible that seizure activity originating within HH excites this system. Initiated by abnormal electrical activity spreading rostrally and dorsally to the areas in the neighboring limbic system, and caudally to the brainstem, the HH can induce physiological and psychophysiological manifestations of laugh attacks [15].

Neuropathology of HH

Histological examination of surgically resected HH tissue from patients with treatment-resistant epilepsy shows clusters of small neurons intermixed with glia and relatively sparse large neurons [19,20]. Clusters of small HH neurons are highly variable with regard to their size and abundance within the tissue, but appear to be a universal feature of HH microarchitecture [19]. Approximately 90% of the total neuron population consists of small HH neurons which have an interneuron phenotype with small round soma and bipolar or unipolar morphology with largely unbranched and aspiny dendrites [19,21,22]. Small HH neurons express glutamic acid decarboxylase (GAD), the synthetic enzyme for GABA, and are therefore likely to be GABAergic interneurons with local network connections [23,24].

Large HH neurons make up approximately 10% of the total neuron population in HH tissue and are dispersed throughout the tissue, although often situated at the edges of the small neuron clusters [19,22]. These cells have features consistent with projection-type neurons, including pyramidal morphology, abundant Nissl substance, and multiple processes, often with a single large proximal dendrite [19,21,22]. Dendrites are more likely to be branched and spiny. At least some large HH neurons express markers consistent with excitatory neurotransmitters, such as VGLUT2.

Epileptogenesis of Human Gelastic Seizures

The mechanisms underlying the epileptogenesis of gelastic seizures are unknown. Several hypotheses relevant to epileptogenic mechanisms can be postulated. (1) Local irritation of adjacent normal structures: HH, especially large-size tumors, mechanically affect and distort local anatomy, including the mammary body, which in turn may alter the excitability of hypothalamic networks. Anatomical analysis showed that HH lesions within the third ventricle alter the excitability of hypothalamic networks. Anatomical analysis showed that HH lesions within the third ventricle may alter the excitability of hypothalamic networks. Anatomical analysis showed that HH lesions within the third ventricle may alter the excitability of hypothalamic networks. (2) Abnormalities of hormone expression: HH cells express neuropeptides which may contribute to seizures in other conditions [29,30]. Hormones secreted by the hypothalamus and pituitary have been identified during gelastic seizures [31]. However, experimental evidence supporting a role for endocrine mechanisms in seizure generation within HH tissue is lacking. (3) HH is intrinsically epileptic: Ictal EEG recordings from electrode contacts placed directly into HH tissue identify seizure onset within the lesion itself [14,32]. Direct electrical stimulation of these electrodes within the HH can evoke typical gelastic seizures [15]. Functional imaging at the time of gelastic seizure activity demonstrates activation of the HH lesion with increased perfusion on single photon emission computed tomography (SPECT) or increased glucose utilization with fluorodeoxyglucose positron emission tomography (FDG-PET), consistent with the behavior of a seizure focus [15,17,33,34]. Perhaps most importantly, recent advances in surgical techniques, allowing for safe resection of the HH, can result in immediate disappearance of gelastic seizures in a majority of patients [8,9,35]. Taken together, these studies suggest that gelastic seizures originate within the HH itself, and it is now widely accepted that HH tissue is intrinsically epileptogenic [7,11,36].

Roles of Intrinsic Neuron Firing in Seizure Genesis

Altersions in neuronal network input–output relationships underlie various nervous system disorders. An increase in neuronal network output is thought to underlie seizure genesis in areas such as the hippocampus. The input–output relationship of neuronal networks is dependent on both their synaptic connectivity and on the intrinsic properties of their neuronal elements [37]. Accumulating lines of evidence suggest that intrinsic neuronal properties play an important role in seizure genesis. It has long been postulated that the inherent firing properties of neurons can be linked to the epileptogenicity of brain tissue [38–40]. In most human epileptic tissue preparations in vitro, the prevalent neuronal firing pattern is that of spontaneous, regular action potential generation. Surprisingly, overt action potential bursts are rarely observed from human neocortical slices without injection of depolarizing current [41–43]. In cortical dysplasia (CD), cytomegalic neurons (but not balloon cells) act as epileptic “pacemakers” and likely contribute to seizure genesis [44–46].

Electrophysiological microelectrode recordings have demonstrated that small HH neurons have intrinsic pacemaker-like firing behavior [21,23,24,47]. Field potential recording shows the seizure-like discharges in most HH slices tested (Figure 1A). Patch-clamp recording in cell-attached mode shows spontaneous action potential (AP) firing (Figure 1B) in a small neuron from a HH slice (soma diameter <16 μm). In the same HH neuron, whole-cell patch-clamp recording (current-clamp) shows the similar intracellular AP firing pattern (Figure 1C). These small neurons in HH slices demonstrate diverse firing patterns with average firing frequency of 10.5 ± 0.8 Hz at the resting membrane potential (RMP) of −53.2 ± 1.1 mV [23,24,47]. In 64 small HH neurons recorded from the tissue slice, derived from a total of 35 different patients, regular firing was observed in 63%, irregular firing in 28%, and burst firing in 9% of the recorded neurons [21]. These findings were verified with single-cell microelectrode recordings of small HH neurons in the freshly resected, perfused tissue slice, with RMP of 53.9 ± 0.9 mV and spontaneous firing frequency of 6.6 ± 1.0 Hz [21,48]. In acutely dissociated single HH neurons (small sized, Figure 1Da), there was spontaneous AP firing (Figure 1Db), which stopped in the presence of tetrodotoxin (TTX), a voltage-dependent sodium channel blocker, but show no change
in firing behavior when exposed to pharmacological blockade of GABA-A and ionotropic glutamate receptors, suggesting the intrinsic pacemaker-like firing [23]. Hyperpolarization of small HH neurons with current injection demonstrates “sag-like” membrane depolarization returning the cell to the RMP, consistent with an h-current as seen in other pacemaker cells [23]. As previously mentioned, small HH neurons express glutamic acid decarboxylase (GAD) with interneuron-like morphology, and therefore likely express GABA as their primary neurotransmitter [23,24]. Taken together, these findings suggest that spontaneous firing is an intrinsic membrane property of small HH neurons.

Conversely, in both the acutely dissociated single-cell preparation and in the tissue slice, microelectrode recordings from large HH neurons (soma diameter >16 μm) showed that these neurons are usually firing at much lower frequency or even quiescent at rest with RMP of −60.8 ± 1.7 mV (dissociated cell) and −65.1 ± 1.7 mV (tissue slice) [21,47] (Figure 2A,B in right panel).

**GABA-Mediated Excitation during Normal Development and in Epilepsy**

Gamma-aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the central nervous system of vertebrates, exhibiting binding to ligand-gated (GABAA and GABAC) and G-protein-coupled (GABAB) receptors [49]. The GABAA receptor is highly permeable to chloride ions, and its activation tends to hyperpolarize the membrane potential through the influx of these negative ions [50,51]. Deficits in GABAA receptor-mediated inhibition are a major mechanism contributing to seizure genesis in several forms of epilepsy [52].

In contrast to the hyperpolarizing (inhibitory) effect on normal mature neurons, GABA can exert an excitatory role on immature neurons [53,54]. Ben-Ari and colleagues reported that activation of GABA receptors on immature neurons in neonatal hippocampal slices induced membrane depolarization [55]. In these immature neurons, the reversal potential for Cl− was more positive than the resting membrane potential, suggesting that the intracellular Cl− concentration was higher in these neonatal neurons compared to mature neurons. GABA produces depolarization and increased concentration of intracellular calcium in immature neurons in a variety of brain regions, including hippocampus, neocortex, and hypothalamus [53]. GABA-induced excitation likely plays an important role in activity-dependent neuronal development [56]. Notably, GABAergic excitation is also observed in several highly selected circumstances in normal mature neurons [54,57–67].

Recent studies, examining the functional alteration of GABAA receptors, have demonstrated that this functional switch can also been found in some epileptic conditions [61–63]. For instance, Cohen and colleagues reported that the interictal epileptic activity in slices prepared from surgical resection of human temporal lobe was substantially dependent on GABAA receptor-mediated depolarization. This was determined by observing cells with depolarizing IPSPs firing before and during interictal spikes that were effectively silenced by a GABAA receptor antagonist [61]. These authors postulated that the altered connectivity was associated with the abnormal temporal lobe. This resulted in neuronal deafferentation, causing neurons to switch to an immature state, in which GABAA receptors mediate remarkable depolarization [61]. Khalilov et al. [64], described a unique triple compartment chamber that accommodates two intact hippocampi and their commissures, allowing for application of a convulsive drug to one hippocampus to determine the consequences of the propagation of seizures on the other side of the control hippocampus. Using this novel preparation, they demonstrated that after establishing the propagation of 7–10 seizures (induced by chemical stimulation of one side of the hippocampus) and then interrupting the connections between the two hemispheres, the control hippocampus still maintained spontaneous epileptic activity, indicating that the
“seizure-induced seizures”. Moreover, the application of a GABA<sub>A</sub> receptor antagonist before the formation of seizures induced an epileptogenic focus, which generated seizures, while the same procedure blocked ongoing seizures once the mirror focus had been established. This suggests that the secondary seizures were mediated by GABA<sub>A</sub> receptor-induced excitation [64]. Direct measurement of Cl<sup>-</sup> reversal potential indicated a shift toward a more positive value, causing GABA to depolarize and excite these neurons [64]. Therefore, seizures appear to reduce the ability of neurons to prevent excess chloride accumulation, which in turn results in a quasi-permanent accumulation of chloride intracellularly, leading to the excitatory effects of GABA. This mechanism has been suggested to operate in a variety of pathological conditions [62,65,66].

**Role of GABA<sub>A</sub> Receptor-Mediated Excitation in HH Epileptogenesis**

Most large HH neurons have a functionally immature response with depolarization and increased firing when exposed to GABA agonists [21,47,67]. Gramicidin-perforated patch microelectrode recordings of acutely dissociated single HH neurons were studied in both large and small HH neurons (total of 62 cells derived from 34 patients) [47]. Bath application of muscimol (selective GABA<sub>A</sub> receptor agonist) resulted in depolarization of 83% of large HH neurons and 24% of small HH neurons (Figure 2C,D).

The functional difference between large and small HH neurons correlates with differences in intracellular chloride concentration, consistent with reversal of the chloride gradient in large HH neurons. GABA-induced transmembrane currents were evaluated at different holding potentials, establishing a reversal potential of −36.2 ± 1.9 mV for large HH neurons (predicting an intracellular Cl<sup>-</sup> concentration of 39.1 ± 2.8 mM) and −59.4 ± 3.9 mV for small HH neurons (predicted intracellular Cl<sup>-</sup> concentration 19.2 ± 3.7 mM) [47].

These findings were confirmed in a complementary recording platform with single-cell patch-clamp recordings in perfused, surgically resected HH tissue slices [21,67]. Utilizing either whole-cell or gramicidin-perforated patch recordings, administration of muscimol resulted in a depolarizing response in 70%
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of large HH neurons, whereas the mature hyperpolarizing response was observed in 100% of small HH neurons. The depolarizing response of large HH neurons was blocked with coadministration of bicuculline. Depolarizing behavior once again correlated with estimates of intracellular chloride concentration. Large HH neurons that depolarized to muscimol (70% of large HH neurons) had a reversal potential of $-30 \pm 2$ mV (predicted intracellular Cl$^-\text{ concentration } 40.2 \pm 4.3$ mM), whereas large HH neurons with a hyperpolarizing response (30%) had a reversal potential of $-64 \pm 3$ mV (intracellular Cl$^-\text{ concentration } 11.4 \pm 0.8$ mM) [21]. Bicarbonate efflux and activation of L-type calcium channels contribute to membrane depolarization of large HH neurons [67].

Reversal of the chloride potential in large HH neurons is associated with altered expression of the cation–chloride transporters NKCC1 and KCC2 [21,47]. Single-cell reverse transcriptase-polymerase chain reaction (RT-PCR) shows reduced expression of KCC2 mRNA in large HH neurons relative to small HH neurons and immunocytochemical staining for KCC2 protein shows lower levels in large versus small HH neurons [21,47]. Bumetanide (an NKCC1 inhibitor) partially blocks the depolarizing and firing behavior of large HH neurons exposed to GABA agonists [21]. Altered cation–chloride transporter expression may relate to constitutive activation of the tyrosine kinase B (trkB) receptor complex and related downstream intracellular signaling pathways based upon Western blot analysis of surgically resected HH tissue (see also Semaan et al., present issue).

Network Activity within HH Tissue

Indirect evidence for functional network activity within HH is provided by high-density microelectrode arrays that provide two-dimensional field recordings of surgically resected HH tissue slices [68]. Perfused HH tissue slices generate spontaneous high-frequency oscillations (HFOs), including ripples (100–200 Hz) and fast ripples (200–600 Hz). Paired-pulse electrical stimulation shows potentiation (usually an expression of enhanced synaptic activity) with a 4-fold increase in single unit firing behavior. Pharmacological challenge with 4-aminopyridine (4-AP; a nonspecific potassium channel blocker) increases the abundance and burst duration of fast ripples [68]. Two-dimensional analysis of field potentials over brief time intervals (<200 ms) is consistent with spread of activity within small local regions, potentially correlating with the neuronal clustering characteristic of HH microanatomy.

In an effort to observe the activity of HH neurons within their completely intact (native) network, microelectrode field recordings were obtained from HH tissue via the working channel of a surgical endoscope immediately prior to surgical resection [69]. Analysis of single unit (single neuron) behavior confirmed spontaneous firing behavior in situ. For all neurons, the median firing frequency was 8.8 spikes/second, but at least two phenotypes were apparent with a slow-firing (median firing rate of 0.6 spikes/second) and fast-firing neuron populations (median firing rate 15.0 spikes/second). The fast-firing group of neurons was much more likely to have burst-firing features. Functional connectivity within a local network was examined by evaluating all possible neuron pairs (recorded within the same epoch) for time-linked firing behavior with the finding that 51% of all possible neuron pairs had a significant level of firing synchrony [69].

Mechanisms Responsible for Increased Synchrony

There are two basic cellular conditions required for every epileptic network; first, imbalance of excitatory and inhibitory influences in the direction of net excitation and second, hypersynchrony of neuronal firing [70]. The contribution of synchronized firing of inhibitory neurons within the reticular nucleus as a fundamental contributing factor to generalized spike-wave discharges in the thalamocortical circuit is well recognized [71].

GABA$\text{A}$ receptor-mediated synchronization also contributes to seizure-onset mechanisms in focal epilepsy, including mesial temporal sclerosis [72,73] and cortical dysplasia [74]. Cepeda and colleagues have recently reported postsynaptic evidence for pacemaker-like GABA-firing as a contributing factor for epileptic network synchronization in human surgically resected cortical dysplasia tissue [75]. With regard to synchronization of the ictogenic network in HH, GABA-mediated mechanisms are attractive candidates, in light of the high abundance of small HH interneurons, their spontaneous pacemaker-like firing behavior, and HH microanatomy, in which small HH neurons cluster in close proximity. These clusters, in association with large depolarizing pyramidal (projection-type) HH neurons, may be the “functional unit” for ictogenesis in HH [36]. As previously noted, there is indirect evidence for network activity within HH clusters as demonstrated by HFOs in HH tissue slices [68] and a high degree of firing synchrony of single units with in situ recordings from HH lesions [69].

A possible contributing mechanism to GABA-mediated synchrony is the phenomenon of GABA$\text{A}$ current rundown, which is determined experimentally by observing decreasing transmembrane current responses to repetitive (use dependent) GABA-ligand exposure. GABA$\text{A}$ current rundown has been related to temporal lobe epilepsy [76,77] and cortical dysplasia [78]. Functional rundown has also been described in GABA-related transmembrane currents in surgically resected HH tissue [79]. In two different experimental platforms derived from human HH, functional rundown was specific to GABA (not observed in response to repetitive exposure to glycine or glutamate) and was also specific to GABA$\text{A}$ receptors expressed on the surface of small HH neurons and not large HH neurons [79]. We believe that rundown is likely to contribute to the entraining of synchronous firing of small HH neurons. Further testing of this hypothesis would be enhanced by developing a computational model.

Synchrony within small HH neuron clusters may also be enabled by nonsynaptic mechanisms [80]. Neuronal gap junctions (connexin-36) are highly expressed within small HH neuron clusters and microelectrode field recordings of seizure-like discharges in surgically resected HH tissues slices are significantly reduced by pharmacologic exposure to gap junction blockers. Taken together, these data suggest the hypothesis that synchrony of pacemaker-like GABAergic neurons play a critical role in HH ictogenesis.
Summary of Cellular Model for Intrinsic Epileptogenesis of HH

There are two types of HH neurons, small and large-sized neurons [21,23–34,47]. Small HH neurons are mature GABAergic interneurons and exhibit pacemaker-like action potential firing, while large HH neurons are immature, glutamatergic projection-type neurons [21,23,47]. These small and large HH neurons are synaptically connected [22,47]. The activation of GABAA receptors on small HH neurons inhibits these small neurons, while the activation of GABAA receptors on large HH neurons leads to excitation [21,47,67].

Based on these observations, we propose a cellular model to interpret a possible mechanism of epileptogenesis within the HH lesion [36]. Here, we update this cellular model with currently published and unpublished data [68,69,79]. The specific elements of this model are illustrated in Figure 3. Nissl staining of a HH tissue section (A) revealed one large (red arrow) and numerous small (white arrows) neurons. Some small neurons are closely clustered (yellow arrows). Based on our experimental data, a schematic representation is shown (B) of how these small, GABAergic interneurons (black), local glutamatergic neuron (small, red) and projection glutamatergic neuron (large, red) work together to generate seizure activity, in which, small GABAergic HH neuronal quiescence may contribute to episodic occurrence of excitatory output from the HH. In addition to small GABAergic neurons, we also found a small portion of glutamatergic small HH neurons (VGLUT1 positive small neurons), which may participate in glutamatergic synaptic connections to GABAergic small neurons. The large HH neurons excited by GABA may participate in feedback to small HH neurons, thereby also affecting excitability of these small neurons (red lines). Finally, the neuronal type of gap junctions (GJ) between small GABA neurons plays a role in synchronizing these GABAergic neurons and drives a GABAergic network to generate seizure activity.

Methodological Issues

Investigational use of surgically resected human epileptic brain tissue is a powerful method for exploring the structural and electrophysiological properties of the neuronal networks responsible for human epilepsy [81,82]. Neuropharmacological probes can identify possible translational treatment strategies. Observations based upon human tissue can generate testable hypotheses that can be investigated with controlled experimentation in suitable animal models. However, the limitations of using surgically resected human material deserve mention. (1) The in vitro preparation (including slice, dissociated single neuron, or primary cell culture) using human brain tissue is a “reduced” system that does not possess the network connectivity present in the intact brain [83]. Thus, functional observations using any of these platforms cannot be expected to fully represent the behavior of epileptic tissue in situ. (2) The tools utilized to study surgically resected tissue usually differ from those available to study patients. For example, microelectrode field recordings derived from the surgically resected tissue slice have an uncertain relationship to the findings derived from scalp EEG. (3) There is generally an absence of

Figure 3 Cellular model for intrinsic epileptogenesis of HH tissue. (A) Panel A consists of photomicrograph of human HH tissue stained with Nissl technique. A large HH neuron (red arrow) is visualized, with numerous surrounding small HH neurons (white arrows). Small GABAergic HH neurons exhibit intrinsic pacemaker-like firing and provide GABA to their synaptic targets, including the large, glutamatergic projection HH neuron (VGLUT2 positive). Small HH neurons often occur in closely juxtaposed pairs and clusters (yellow arrows). It is likely that some glutamatergic small HH neurons (VGLUT1 positive) also innervate large HH neurons (B). In addition, between GABAergic small neurons, there is gap junction (GJ) expression (red arrows), which contributes to the synchrony of these pacemaker-like GABAergic neurons. Together, these HH neuronal networks may underlie the cellular mechanisms of seizure-genesis within HH lesion.
normal human control tissue. This is particularly true for HH research, where there is a very high premium on avoiding neurosurgical resection or injury to adjacent normal hypothalamus. Consequently, the use of ‘less abnormal’ adjacent cortex as control tissue, a strategy utilized for experimentation on surgically resected cortical dysplasia, is not available [82,84]. This lack of control tissue in HH research is particularly problematic for electrophysiological studies, whereas we have utilized age-matched human autopsy-derived hypothalamic tissue for selected neuropathological studies. We advocate utilizing the mammillary bodies for this purpose, as a large and generally recognizable hypothalamic nucleus within postmortem specimens that is always in close proximity to HH lesions that result in epilepsy [13].

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Conflict of Interest
The authors declare no conflict of interest.

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