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# Electrical Stimulation and Glutamate in the Hippocampus of Epilepsy Patients

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Electrical Stimulation and Glutamate in the  
Hippocampus of Epilepsy Patients

A Thesis Submitted to the  
Yale University School of Medicine  
in Partial Fulfillment of the Requirements for the Joint Degree of  
Doctor of Medicine and Masters of Health Science

By  
Gabriel Antonio Widi

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## ELECTRICAL STIMULATION AND GLUTAMATE IN THE HIPPOCAMPUS OF EPILEPSY PATIENTS.

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Electrical brain stimulation has been proposed as a promising treatment option for patients with medically resistant epilepsy disorder. Glutamate levels in the epileptogenic human hippocampus are elevated interictally and increase with seizures. Fifty Hz stimulation is a candidate therapeutic stimulation that is also used for clinical cortical mapping. We examined the effects of 50 Hz stimulation on glutamate efflux in the hippocampus of patients with medically refractory epilepsy. Subjects ( $n = 10$ ) underwent intracranial EEG (icEEG) evaluation for possible therapeutic resection. Electrical stimulation was delivered through implanted hippocampal electrodes ( $n = 11$ ) and microdialysate samples were collected every 2 mins. Basal interictal glutamate was measured with the zero-flow microdialysis method. Stimulation of the epileptogenic hippocampus induced significant glutamate efflux at the time of stimulation ( $p = 0.005$ ,  $n = 10$ ) that was significantly related to the basal glutamate concentration ( $R^2 = 0.81$ ,  $p = 0.001$ ). During stimulation, four patients experienced seizures and two had auras. No change in glutamate level was observed in the group of patients who experienced a seizure ( $p = 0.47$ ,  $n = 4$ ). Conversely, a significant increase in glutamate was observed in the patients that did not experience a seizure ( $p = 0.005$ ,  $n = 7$ ). Basal glutamate levels were significantly higher in the no seizure group ( $p = 0.04$ ,  $n = 5$ ) than in the stimulated seizure group ( $n = 4$ ). Fifty Hz stimulation of the epileptogenic hippocampus can cause significant glutamate efflux and may produce seizures or auras. The degree of stimulated glutamate elevation is related to the basal glutamate concentration but not to the induction of seizures. Electrical stimulation at 50 Hz may exacerbate interictal glutamate dysregulation in the epileptogenic hippocampus and may not be optimal for seizure control.

## Introduction

### *Medication Resistant Temporal Lobe Epilepsy*

Epilepsy is a common disorder affecting approximately 15 per 1000 people, causing a significant amount of morbidity and mortality [1]. Medial temporal lobe epilepsy (MTLE) is the most common form of epilepsy affecting about 2.5 million persons in the United States with a worldwide incidence of 4.8-7.8/1000 [2]. In about 40% of patients with TLE the seizures are medically intractable [3]. The disease is characterized by partial seizures, which may or may not be secondarily generalized. Seizure activity in MTLE involves a medial temporal lobe/limbic network [4]. Included in this network are the hippocampus, amygdala, entorhinal cortex, lateral temporal neocortex, medial thalamus, and inferior frontal lobes [4]. The underlying pathology of MTLE is most often mesial temporal sclerosis (MTS), which is defined by hippocampal neuronal loss and gliosis [5]. Patients with MTLE are refractory to anti-epileptic drugs (AEDs) in about 75% of cases [5] and may continue to have disabling seizures or medication-related side effects such as nausea, drowsiness, and weight gain [6]. Resective surgery is considered when such seizures have a focal origin, and when presurgical evaluation clearly demonstrates that the epileptogenic zone, i.e. the volume of brain tissue at the root of the generation of seizures, can be removed without causing additional, unacceptable neurological or cognitive deficits. Although anterior temporal lobectomy in persons with MTS can achieve one- to two-year remission in less than 85% of cases, resection of a well-defined neocortical lesion achieves one-year remission in only 56% [7]. Three to four year follow up after neocortical or medial temporal lobe resection demonstrates cumulative relapse rates up to 25%, though whether these relapses occur more often after medial temporal lobe resections has not been shown consistently [8-12]. Depending on the localization of the seizure onset zone, conventional resective surgery is unsuitable in at least one third of medically refractory patients because of the involvement of memory, language, visual, sensory, or motor cortices, or because of the multifocal, bilateral or generalized expression of the seizure disorder. Furthermore, surgical resection is not advisable in patients with bilateral temporal foci of seizure spread or a unilateral focus of seizure origin spreading to surrounding cerebral regions of the dominant hemisphere, because both bilateral conventional and unilateral extensive temporal lobectomies can produce devastating cognitive deficits of language and memory. Pharmacological

advances are unlikely to decrease the number of such drug-resistant epileptic patients in the near future [13], thus creating a need for better alternatives to AEDs and surgery.

Vagus nerve stimulation (VNS) as an adjunct to medical therapy is a reasonable option for patients with refractory epilepsy who are not suitable for surgical resection and can result in a 50% reduction in seizure frequency [14]. Nevertheless, most of these patients will not be seizure-free. In the controlled trials leading to market approval, adults with partial onset seizures treated with optimal VNS stimulation parameters had a median seizure reduction of 25–30%, and < 2% experienced seizure freedom at one year [15]. Given the limitations of currently available therapeutic options, new approaches for treating epilepsy are necessary. Due to the remarkable success of deep brain stimulation (DBS) for movement disorders [16, 17], combined with its advantages of titratability, reversibility, and low risk of complications [18, 19], research into implantable deep brain devices for treating refractory epilepsy has increased tremendously [20, 21].

#### *Brain Stimulation Offers A New Therapeutic Option*

Electrical stimulation of the human nervous system has been used successfully for diagnostic and therapeutic purposes in a variety of neurologic diseases. Low-frequency stimulation of the spinal cord [22] or of the thalamus [23] for the treatment of pain has proven beneficial. DBS is now a substitute for the ablative lesioning methods used in stereotactic and functional neurosurgery for the treatment of movement disorders and offers a reversible and adjustable intervention in all places where permanent ablative lesion of neuronal structures, such as nuclei of the basal ganglia, had proven some therapeutic efficiency [24]. DBS was first applied to the thalamus to mimic thalamotomy for the treatment of tremor, then to the subthalamic nucleus (STN) and the pallidum to treat the tremor, akinesia, rigidity and dyskinesias associated with advanced forms of Parkinson's disease [25]. The number of syndromes treated with DBS is growing, and now includes depression [26, 27], dystonias [28], epilepsy [29-31], obsessive compulsive disease [32], cluster headaches [33], and experimental approaches are being made in the field of obesity and food intake control [34]. In epilepsy, the therapeutic effect of electrical stimulation have been variable, and so far there is no consensus about where to stimulate and how [30]. Currently, there is a need to study the effects of electrical stimulation on excitability in the epileptic brain to better inform and develop a

rational stimulation paradigm. Different hypotheses have been proposed based on the clinical observation that high frequency DBS may mimic ablative lesioning methods performed in stereotactic and functional neurosurgery [35]. Stimulation-induced disruption of unopposed network activity is one hypothesis that appears to be consistent with available data [36, 37]. DBS at high frequencies has also been postulated to block cortical epileptiform activity [38-40]. How electrical stimulation affects neurotransmitter release in the human adult brain is completely unknown.

#### *Glutamate is a Primary Neurotransmitter and Neurotoxin*

Glutamate is the major excitatory neurotransmitter in the brain and has long been implicated in the initiation and spread of seizure activity [41]. In normal healthy brains, the extracellular concentration of glutamate is kept within low safe levels by efficient reuptake mechanisms at a baseline level of one to two micromolar ( $\mu\text{M}$ ) [42]. Synaptic transmission increases glutamate transiently to one millimolar ( $\text{mM}$ ), activating postsynaptic glutamate receptors. Primarily glial, and to some extent neuronal sodium-dependent excitatory amino acid transporters (EAATs) then quickly and efficiently remove glutamate from the synaptic cleft, returning its concentration to normal baseline levels [43]. In pathological conditions, abnormal elevation of extracellular glutamate may result in neurotoxicity, cell death, and impaired function [44]. The pathogenesis of glutamate-induced excitotoxic cell death occurs by way of an acute sodium-dependent phase followed by a delayed calcium-dependent phase [45]. The acute component occurs within minutes of glutamate binding to the *N*-methyl-D-aspartic acid (NMDA) receptor. The overabundance of glutamate hyperactivates the NMDA receptor, causing sodium influx associated with passive influx of water and chloride with resultant neuronal dendritic swelling. The delayed component occurs hours post-exposure and is triggered by excessive calcium influx, which produces a cascade-like effect leading to cell death [45, 46]. This excessive calcium influx has been implicated in the glutamate-induced injury of hippocampal neurons [47].

Animal studies have shown that the administration of glutamate or glutamate analogues to the hippocampus triggers seizures, while co-injection of glutamate antagonists suppresses seizure generation [48, 49]. A variety of established and potential antiepileptic drugs including phenobarbital, carbamazepine [50], lamotrigine [51, 52], vigabatrin [52], diazepam [53], valproate and its derivatives [54], adenosine-1

receptor agonists [55], kainate receptor antagonists [56], group I metabotropic glutamate receptor antagonists [57], topiramate [58], angiotensin IV and somatostatin [59] suppress pilocarpine-induced increases in hippocampal extracellular glutamate. Increases in hippocampal extracellular glutamate [60-69] have been observed during different types of chemically and electrically induced seizures in rats. However, others have found no change or even a decrease in hippocampal glutamate during seizures [70-74]. In a microdialysis study by Sierra-Paredes and co-workers, intrahippocampal microperfusion of 100  $\mu$ M picrotoxin (a GABA-A receptor antagonist) over 5 min resulted in limbic seizures and a concomitant decrease in hippocampal extracellular glutamate [75]. Conversely, Meurs and colleagues induced limbic seizures of comparable severity by intrahippocampal microperfusion of picrotoxin, pilocarpine (a muscarinic receptor agonist) and (R,S)-3,5-dihydroxyphenylglycine (DHPG) (a group I metabotropic glutamate receptor agonist) in rats and observed increased extracellular glutamate, GABA, and dopamine following administration of convulsant in all groups [76]. Furthermore, they found that this increase was related to seizure activity rather than to the pharmacological effects of the respective chemoconvulsants [76]. Methodological differences between studies such as the duration of time between probe insertion and start of dialysate collection or the duration of chemoconvulsant perfusion may give rise to contradictory results even when the same sampling technique is used and should be taken into account when comparing different studies of the effect of seizures on transmitter levels in the brain. In addition, regional differences in seizure-related alterations in extracellular glutamate and GABA concentration within the hippocampus have been described [69], as well as different time courses of hippocampal extracellular glutamate and GABA concentrations in maximal electroshock seizures in rats, with both increases and decreases at different intervals [66]. Such spatial and temporal variations in the response of extracellular amino acid levels to seizures within the hippocampus could account for some of the conflicting results of animal studies. Currently, the seizure related increases in extracellular glutamate similar to those observed in humans [67, 77] have been observed more reliably in chronic models of epilepsy, such as kindling with electrical stimulation [78], or intrahippocampal [67] or amygdalar kainite acid (KA) injection [79].

Along the same lines, Wilson and colleagues report that the glutamate increase during seizures in the human hippocampus is similar to the glutamate rise seen in the chronic kainate rat model of epilepsy [67]. Elevated serum basal glutamate has also been noted in several familial epilepsies [80, 81]. Further,



*in vivo* microdialysis studies of MTLE patients show that the extracellular concentration of glutamate rises in the epileptogenic (and sclerotic) hippocampus, compared to the non-epileptogenic side, during spontaneous seizures and remains elevated for at least 15 min after the electrographic seizure ends [77]. The regulation of glutamate in the epileptogenic human hippocampus appears impaired not only during seizures but also during the interictal period, as the baseline extracellular glutamate is chronically elevated [82], glutamate-glutamine cycling is decreased [83] and glutamate reuptake is possibly impaired [84-87]. Furthermore, this chronic elevation in glutamate may be linked to neurotoxicity, as we recently found that higher glutamate levels in the epileptogenic hippocampus are related to decreased hippocampal volume on MRI [88] and to increased neuronal cell loss [89]. Extracellular glutamate concentrations also increase significantly in the epileptogenic hippocampus during the performance of complex figure memory tasks, suggesting that the epileptic brain is particularly susceptible to the cognitive activation-induced glutamate release [90]. Hippocampal sclerosis is also believed to play a key role in the generation of seizures in MTLE [91, 92]. In the sclerotic hippocampus, changes in NMDA and AMPA receptors are observed [93, 94] and intracellular recordings from dentate granule cells in the human hippocampus reveal a glutamate-dependent hyperexcitability in these neurons [95]. As the above findings indicate, sclerotic hippocampi seem to have an impaired glutamate uptake capacity in comparison to nonsclerotic hippocampi.

Previous groups [96, 97] have postulated a reduction in glutamate transporters to explain the elevation of extracellular glutamate in the epileptogenic and sclerotic hippocampus [77, 82] during seizures. However, other studies have offered little support for the role of glutamate transporters in this phenomenon based on the expression of transporter proteins [98, 99]. On the other hand, Eid and colleagues propose that astrocytic glutamate uptake may be influenced by changes in the metabolic state of the glial cell rather than glutamate transporter protein levels. Specifically, the down-regulation of glutamine synthetase (an enzyme that converts glutamate to glutamine in an energy-dependent fashion) in astrocytes in sclerotic areas of the hippocampus may lead to an accumulation of glutamate in involved areas [98]. Given that glutamate transport in astrocytes is an active process, abnormal energy metabolism could also be a contributory factor but little is known about the energetic state of sclerotic astrocytes to support this hypothesis [100]. Another plausible explanation for the elevated glutamate levels in TLE with sclerosis is that neuronal glutamate uptake may be impaired, though this idea is refuted by Peghini and

colleagues [101]. In their study of EAAC1 (a neuronal glutamate transporter)-deficient mice (EAAC1<sup>-/-</sup>), they showed that there is no neurodegeneration, apoptosis or reactive astroglia (an expected finding with impaired glutamate clearance) even at greater than 12 months of age. These animals did not exhibit any spontaneous epileptic activity nor did they exhibit a reduced threshold to pentamethylenetetrazole (PMT) challenge [101]. Astrocyte-dependent glutamate release has also been postulated to explain the increased levels of glutamate in sclerotic seizure foci [102]. Tian et al. provide evidence to support this hypothesis by showing that in hippocampal slice models of seizure activity, calcium dependent glutamate release from astrocytes rather than neuronal glutamate release predominantly underlies the paroxysmal depolarization shifts that characterize seizure activity [102]. In human TLE, such calcium dependent glutamate release from astrocytes could be triggered by inflammatory factors such as interleukin 1 $\beta$  and chemokines in sclerotic hippocampi [91].

#### *The Hippocampus is a Therapeutic Target for Brain Stimulation*

The hippocampus is a structure often involved in seizure genesis [103] in TLE and is also often selected as a stimulation target for the treatment of refractory epilepsy. Patients with complex partial seizures (CPS) arising from the hippocampus who undergo resective surgery of the epileptic focus have a positive outcome [8, 104-107], though relapse in patients with initially good results may occur. Most seizure recurrences are within 6 months postoperatively [108], but many occur up to a decade later [109]. A review of studies with 1–5 year follow-up [7] reported freedom of disabling seizures in 63.% of patients (95% CI = 60, 66) and identified a trend for better outcomes in more recent reports. Compared with Engel et al., long-term follow-up studies (i.e.  $\geq 5$  years) of temporal lobe epilepsy surgery reveal a slightly lower seizure free rate (median 58% in studies using author defined outcomes, and 65% in those using Engel's class I “seizure free” classification) and a narrower range of seizure-free rates (59–89%) [110]. Thus, the hope for seizure freedom following initially good results is tempered by the increasing risk of recurrence over time. For a number of patients, surgical resection is not even an option. Temporal lobectomy or hippocampectomy may cause memory deficits [111], or even amnesia [112], particularly in patients with bilateral hippocampic surgery [113]. Instead, these patients are either excluded from resective procedures, or surgery is performed under high risk with incomplete resections and residual seizures. The need for

non-resective therapies is clear and has ushered in the use of neurostimulation for consideration in these cases.

A number of anatomic targets have been stimulated, including the thalamic centromedian nucleus (CMN) [114-116], vagus nerve [117-119], and cerebellum [120-122]. Stimulation of these targets has improved secondary seizure generalization, but has shown variable results in patients with CPS. Stimulation of the anterior nucleus of the thalamus (ANT) has been proposed [123]; however, preliminary reports show significant, but limited effects on seizure control. In these preliminary studies, basic mechanisms underlying the beneficial therapeutic effect of hippocampal stimulation on seizures were also investigated [124-127]. Velasco et al. report the case of a patient with intractable temporal lobe epilepsy and normal MRI in whom depth-electrode recording showed bilateral independent hippocampal foci [125]. This patient received chronic bilateral hippocampal stimulation of biphasic 130 Hz pulses (450  $\mu$ sec duration, 400  $\mu$ A) for 8 months, and this produced sustained seizure control without undesirable effects on language and memory. Similarly, Vonck et al. (2002) report three CPS patients with DBS electrodes in the amygdalo-hippocampal junction that received biphasic 130 Hz pulses as well, showing a 50–90% seizure frequency decrease, while Téllez-Zenteno's group report a 15% seizure frequency reduction in four patients with continuous monopolar stimulation of the hippocampus at 190 Hz [128, 129]. In another study by Velasco et al. (2000), ten patients were transiently implanted with bilateral hippocampal or unilateral subdural basal temporal electrodes before a planned temporal lobectomy. AEDs were discontinued from 48 h to 72 h before 2–3 weeks of continuous 130 Hz electrical stimulation, delivered 23 h per day [130]. In seven patients with stimulation electrode in the hippocampal formation or parahippocampal gyrus who experienced no interruption during the stimulations, clinical seizures stopped and the number of interictal EEG spikes at the focus decreased after 5–6 days. No evident therapeutic response was found in three patients when stimulation was either interrupted or given outside the hippocampus.

In most of these studies, electrical stimulation of the hippocampus was tried on a short-term basis (<1 month) during preoperative intracranial EEG assessment. Emerging reports of chronic hippocampal stimulation (3 to 6 months) demonstrate similar benefits to those documented with short-term stimulation, without evidence of adverse effects [126, 128, 131]. However, most published studies include neither

blinding nor controls. This is important because, with few exceptions, [122, 132] DBS studies that use blinded assessments or controls report little or no effect of stimulation [133-135]. Also, reports of seizure improvement following electrode implantation for intracranial EEG monitoring [136] raise the possibility that implantation itself can confound stimulation studies. Given these limitations, the only way to determine the efficacy of hippocampal neurostimulation for seizure control is through randomized controlled trials with blinded outcome assessment. One example is a recent double-blinded controlled study by Velasco et al. that reported 5 of 9 patients demonstrated a significant decline in seizure occurrence within 1-2 months of hippocampal stimulation at 130 Hz [132]. The remaining four patients exhibited a delayed response, with only a slight decrease in seizure frequency evident after 6-8 months of hippocampal stimulation. Although seizure reduction occurred in all patients, the observation was made that not all MTLE patients respond in the same manner, as a difference between patients with normal MRIs and those with hippocampal sclerosis was noted: the five responders to hippocampal stimulation had a normal MRI while the four non-responders demonstrated hippocampal sclerosis. For patients with normal MRI, this observation implies the possibility of having a non-lesional neurosurgical alternative, such as DBS, since it has been reported that these patients have a less favorable outcome and a higher risk of memory impairment after temporal lobectomy [111, 112]. In patients with hippocampal sclerosis, partial and delayed response in seizure reduction could explain the limited results found in the Téllez-Zenteno et al. (2006) study because one inclusion criterion in this report was MRI-demonstrated hippocampal sclerosis. In these patients, seizure reduction was partial and occurred after eight months of electrical stimulation. Based on these observations, Velasco et al. speculate that in order to achieve a satisfactory response to stimulation it is important that the neuronal network be preserved in the stimulated area [132]. They support this hypothesis by reasoning that the severe neuronal reduction that accompanies MTS may represent a less satisfactory tissue for modulation with stimulation [132]. This line of thought is supported by a previous report from the same group [127] linking the response to subacute electrical stimulation with high GABA levels in the stimulated tissue. GABA levels were significantly higher in specimens with higher cell counts derived from patients with minimal or no MTS. Our research indicates that chronically elevated glutamate levels linked to neuronal cell loss [89], may be an additional obstacle for treatment with DBS. Another possibility is that sclerotic tissue has different conductance properties than normal tissue and

may require stimulation at different charge density [132].

Suppression of hippocampal epileptiform activity by electrical stimulation has also been shown in studies using *in vivo* and *in vitro* animal models and human hippocampal slices [137]. Low-frequency stimulation (LFS) of the hippocampus can lead to long-term depression (LTD) in experimental animals, and a possibly similar effect of low-frequency stimulation has been demonstrated in resected human temporal lobe specimens [30]. On the other hand, high-frequency stimulation (HFS) might produce local inhibition of the epileptogenic zone. Another possible explanation for the anti-seizure effect of HFS of the hippocampus is that it is mediated through activation or inhibition of downstream structures, rather than the stimulated region itself [128, 138]. Electrical stimulation of the hippocampus for therapeutic reasons in patients with medically refractory epilepsy [125, 138] may also induce glutamate release [139], which has the potential to exacerbate the excitotoxicity and epileptogenicity of the stimulated site [49, 85, 140, 141]. In rats, electrical stimulation of the hippocampus is reported to cause a rapid transient increase in glutamate concentration and concomitant enhancement in synaptic efficacy [139]. Other studies indicate that repeated increases in glutamate, whether by direct microinjection [142, 143] or by application of high potassium [144], can increase seizure frequency and lead to a kindling-like state that may contribute to further elevation in basal glutamate [79]. Thus, it is important to investigate if and how electrical stimulation affects glutamate release in the human hippocampus. Such information may elucidate mechanisms underlying electrical stimulation in humans and may also provide insight into its effects on seizure control, neuronal preservation and therefore the clinical outcome in these patients.

#### *High Frequency Brain Stimulation for Seizure Control*

Electrical brain stimulation at high frequencies (50 - 130 Hz) has been studied in a number of human trials. Open label studies investigating high frequency stimulation (HFS) of the CMN [116, 125, 145-147], hippocampus [125, 126, 128-130, 132], STN [148, 149], ANT [123, 150-152], and cerebellar dentate nucleus (CDN) [146] have shown promise; however, only one [122, 132] of these techniques have been effective in blinded trials [153, 154]. Velasco et al. demonstrated in a double-blinded randomized control study of hippocampal stimulation at 130 Hz in nine patients, that seizure frequency was significantly decreased in five of nine patients with four of them remaining seizure free at 18 months of

follow-up [132]. Other multicenter prospective randomized trials of scheduled (open-loop) chronic ANT stimulation [154] and of intracranially implanted responsive (closed-loop) neurostimulation in persons with medically intractable localization related epilepsy are now under way [154].

Brain stimulation at 50 Hz is a candidate method for seizure control [123, 155-157]. This form of stimulation is used extensively for functional brain mapping of medically refractory epilepsy patients evaluated for surgical resection. In clinical practice, subdural cortical stimulation with brief electrical bursts at 50 Hz can disrupt or induce the relevant behavior and in some cases, if the epileptogenic site is stimulated directly, may trigger seizure. Systematic studies in a limited number of refractory epilepsy patients have reported that subdural cortical stimulation at 50 Hz may suppress epileptic activity at the site of stimulation. Kinoshita et al. showed in an open label study that 50 Hz (bipolar, biphasic square wave pulses of 0.3 ms duration 1-15mA for 5 s) stimulation of epileptic cortex in four patients with medically intractable focal epilepsy suppressed interictal spikes and reduced EEG power at the site of stimulation [157, 158]. Moreover, they showed that 50 Hz stimulation of both epileptic and non-epileptic areas suppressed electrographic fast activities in the stimulated area, confirming previous observations [158, 159]. These findings are in concordance with previous studies showing the suppression of afterdischarges (ADs) by brief bursts of responsive cortical stimulation at 50 Hz. (biphasic 0.3 s, 50 Hz pulse presented for 0.3–2 s) of subdural electrodes during functional brain mapping in patients with medically resistant epilepsy [155, 156].

Fifty Hz stimulation has also been shown to decrease epileptic activity *in vivo* and *in vitro* animal models of epilepsy. Bikson et al. found that sinusoidal high frequency electric fields at 20-50 Hz ( $144 \pm 36$  mV) induced across the CA1 and CA3 regions in rat hippocampal slices suppressed zero-calcium, low-calcium, picrotoxin, and high potassium epileptiform activity for the duration of the stimulus and for up to several minutes following the stimulus by inducing potassium efflux and depolarization block [160]. Durand and colleagues replicated these findings using 50 Hz sinusoidal stimulation of the CA1 region of the rat hippocampus and added that 50 Hz stimulation to the alveus suppressed both the evoked potentials in the cell bodies of neurons and the compound action potentials from their axons [161]. These findings could not be attributed to desynchronization or damage and were associated with increased extracellular potassium concentrations. Instead, their data give insight into the effects of 50 Hz stimulation on neuronal

elements and how stimulation can block axonal activity through non-synaptic mechanisms. Similarly, 50 Hz stimulation (biphasic square wave pulses lasting 0.2–0.4 ms) of rat neocortical slices treated with either bicuculline or magnesium-free extracellular solution prematurely terminated ~50% of seizure-like events [162]. In addition, partial pharmacological blockade of ionotropic glutamate receptors was sufficient to suppress epileptiform discharges and enhance the antiepileptic effects of 50 Hz stimulation, suggesting that the antiepileptic effects of stimulation were mediated mostly by short-term synaptic depression of excitatory neurotransmission [162]. Several other *in vitro* and *in vivo* animal studies investigating similar HFS from 100-130 Hz in the rat hippocampus [163], STN [36, 40, 164], mammillary nuclei [165], substantia nigra pars reticulata (SNr) [166, 167], and ANT [168-170] have also shown decreased seizure activity.

#### *How High Frequency Brain Stimulation Affects Neurotransmitter Release is Largely Unknown*

Our knowledge of how 50 Hz electrical stimulation affects neurotransmitter efflux in an *in vitro* or *in vivo* model for epilepsy is minimal. Klancnik et al. stimulated Schaffer collateral-commisural fibers from the rat hippocampus continuously at 50 Hz (mean 230  $\mu$ A, 100  $\mu$ s square pulse width) for four minutes, inducing the tetrodotoxin-sensitive release of aspartate, glycine, cysteine sulphinic acid, and homocysteic acid, supporting the role for these endogenous amino acids in synaptic transmission in the hippocampus [171]. Ghijsen and colleagues [172] reported that 50 Hz stimulation (300  $\mu$ A, biphasic stimuli, 200  $\mu$ s<sup>2</sup> pulse width of 3-s duration applied at 20-s intervals during a 4-min period) of hippocampal slices in a rat kindling model achieved by daily titanic stimulation of the Schaffer-collateral fibers showed transient increase in GABA in the presence of a GABA receptor blocker. Since similar studies investigating neurotransmitter efflux in response to 50 Hz and other similar HFS do not exist, we must look to the movement disorder literature to further our understanding. Mantovani et al. showed that HFS at 130 Hz on human neocortical slices induced increased GABAergic activity, confirming earlier findings in the rat caudate-putamen [173-175]. Meanwhile, an *in vivo* model developed by Hiller et al. showed that HFS at 124 Hz significantly increased basal GABA outflow from the CN of freely moving rats without affecting glutamate levels [176]. HFS (130 Hz) has also been shown to induce glutamate release in the rat globus pallidus (GP) and SNr as well as GABA release in the SNr [177]. On the other hand, Iremonger et al.

studied HFS in the rat primary motor cortex and ventro-lateral thalamus and contend that HFS (125-185 Hz) of the subcortical white matter tracts projecting to the rat motor cortex caused depression of excitatory synaptic currents in postsynaptic neurons through neurotransmitter depletion [178].

#### *Microdialysis for Glutamate Levels in the Epileptogenic Hippocampus*

Maintenance and regulation of the composition of the brain extracellular fluid is critical for normal neuronal transmission. Through the use of microdialysis, we can explore the *in vivo* neurochemistry of the conscious human brain by continuously sampling ECF for extended periods of time. The microdialysis method is widely used in animal models of disease, including epilepsy, movement disorders, ischemia, trauma, subarachnoid hemorrhage and so forth, to measure for drug levels, neurotoxins, and neurotransmitters in the ECF. Clinical applications for microdialysis in humans include the monitoring and/or study of brain neurochemistry in relation to trauma, ischemia and hemorrhage during neurointensive care, in brain tumors in the neurosurgical setting, and in intracranial EEG monitoring for epilepsy patients to identify a seizure focus [179, 180]. In patients undergoing presurgical evaluation with intracranial EEG, microdialysis catheters are implanted into the area of interest so that ECF may be sampled and analyzed for composition and concentrations of neurotransmitters [67, 77]. Chemicals in the brain ECF diffuse down a concentration gradient across a semi-permeable membrane into the perfusion fluid inside the catheter, which is collected for analysis. As the perfusing fluid passes through the dialysis probe, the concentration gradient generated between the regional brain ECF and the intraluminal fluid drives molecules smaller than the molecular cut-off of the membrane into the probe. The amount of brain chemical recovered through the outlet represents a fraction of its actual concentration. The efficiency of recovery depends on factors such as the molecular weight, charge, uptake and metabolism of the measured compound, diffusion, tortuosity factors in the interstitial microenvironment, length and composition of the dialysis membrane, flow rate, temperature and composition of the perfusion fluid. Usually, higher relative recovery is obtained at lower perfusion rates and with longer membranes. The collected dialysate can be either frozen at  $-80^{\circ}\text{C}$  for future analysis or analyzed on-line using HPLC, capillary electrophoresis, mass spectroscopy or enzyme-based methodologies. The microdialysis probe can be combined to the depth electrodes for simultaneous electrophysiological recording. Microdialysis typically samples neurochemical changes related to volume



transmission in the extrasynaptic space and only indirectly provides information on changes within the synapse [181-183]. The extrasynaptic concentration of a transmitter reflects a balance between its rates of neuronal and glial (vesicular and non-vesicular) release and reuptake. The extracellular levels may also reflect extrasynaptic spillover or transmitters that may have escaped uptake and degradation by the synapse. As such, measured ambient neurotransmitter levels may be most relevant for the volume transmission and function of the extrasynaptic receptors [184]. Microdialysis catheters have also been employed concurrently with bilateral depth electrode placement in the mesial temporal lobe of conscious epileptic patients prior to resective surgery [67, 77, 185] to show extracellular glutamate surges in the epileptogenic hippocampus at the onset of clinically observed seizures.

**Statement of Purpose**

In this study, we used microdialysis probes coupled to hippocampal depth electrodes to investigate the effect of 50 Hz electrical stimulation on glutamate release in refractory epilepsy patients. In brief, we used 50 Hz stimulation because this frequency is employed extensively and with relative safety for functional brain mapping of epilepsy patients evaluated for surgical resection. In clinical practice, subdural cortical electrode stimulation with brief bursts of electrical pulses at 50 Hz can disrupt or induce site-specific behavior and, in some cases, if the epileptogenic zone is stimulated directly, trigger seizures. Cortical stimulation at 50 Hz has also been proposed as a candidate method for seizure control, as it suppresses stimulus-induced ADs [155, 156], interictal spike frequency and EEG power in the epileptic cortex of refractory epilepsy patients [157-159]. Moreover, studies in rat brain slices have suggested that stimulation at 50 Hz can suppress epileptiform activity through various mechanisms, including depolarization block [160], short-term excitatory synaptic depression [162], and increase in GABA release [172]. Our previous work indicates that extracellular glutamate in the epileptogenic hippocampus is chronically elevated, suggesting that glutamate regulation is impaired [82]. We hypothesized that stimulation would induce further changes in glutamate at these sites. Therefore, we investigated the relationship between basal glutamate, as measured with quantitative zero-flow intracranial microdialysis, and stimulus-induced glutamate efflux.

## Methods

### *Subjects*

Subjects were patients with medication-resistant CPS undergoing phased clinical evaluation for possible resective surgical treatment. In Phase I, patients are admitted to a hospital-based epilepsy unit for continuous audio-video and scalp electroencephalogram (EEG) monitoring to record interictal epileptiform transients and ictal patterns during at least three typical seizures. Patients have brain magnetic resonance imaging (MRI) interictal and ictal single-photon emission computed tomography (SPECT), interictal positron emission tomography (PET), and extensive neuropsychological assessment. Phase II evaluation consists of the intracarotid amobarbital procedure to determine lateralized memory function and hemispheric dominance for language. Those patients in whom the seizure focus is not localized by Phase I evaluation, or if there was discordant findings, are offered intracranial EEG monitoring (icEEG) (Phase III study). This phase of the evaluation involves continuous audiovisual monitoring and EEG recording from a combination of depth, subdural strip, and grid electrodes implanted intracranially to target brain regions suspected of being involved in seizure generation or propagation. Patients undergoing Phase III evaluation were invited to participate in the microdialysis study between 2000 and now. Patients who consented to participate in the microdialysis and electrical stimulation studies approved by the Yale University School of Medicine Human Investigations Committee were stereotactically implanted with microdialysis probes with combined depth electrodes targeting the suspected epileptic hippocampus. icEEG Recordings from the two depth electrode contacts flanking the microdialysis membrane were used to determine whether the microdialysis catheter was within or outside an epileptogenic area. The epileptogenic area was defined as the site of seizure origin in at least one seizure, whereas the non-epileptogenic sites were either not involved or were only secondarily involved (propagated) during seizures.

Ten patients selected randomly (four male and six female, age  $34.75 \pm 13.63$ , mean  $\pm$  SD, see Table 1) with eleven hippocampal probes were stimulated at 50 Hz per protocol. Nine patients had one hippocampal probe stimulated, while one subject had two ipsilateral hippocampal probes stimulated. Two patients with probes that were not accurately placed in the hippocampus or with probes near tumor or dysplasias were excluded. The demographic information of participating patients was obtained by medical chart review and then recorded in a common database. Table 1 summarizes patient information regarding

gender, duration of epilepsy, probe location, and classification of disease state as epileptogenic versus non-epileptogenic of patients included in the final analysis. MRI findings of the hippocampus ipsilateral to the probe are reported, as well as pathology at the probe site following resection if available. The data represented in Table 1 were collected by M. Cassaday, D. Ocame, and S. Forselius and myself.

**Table 1.** Subject characteristics. Data was collected from 10 subjects implanted with 11 probes (\*subject #3 had two probes stimulated).

| Pt | Sex | Age (yrs) | Epilepsy Duration (yrs) | Probe Placement            | Disease   | Stimulated Sx | Clinical MRI       | AEDs          |
|----|-----|-----------|-------------------------|----------------------------|-----------|---------------|--------------------|---------------|
| 1  | F   | 49        | 24                      | L Hipp                     | Epi       | Sz            | Normal             | LEV           |
| 2  | M   | 23        | 23                      | R Hipp                     | Epi       | Sz            | Normal             | DPH, TPM      |
| 3* | M   | 47        | 26                      | L Ant Hipp<br>L Post Hipp  | Epi       | Aura<br>Sz    | L Hipp<br>Atrophy  | TPM           |
| 4  | M   | 17        | 7                       | R Post Hipp                | Epi       | Aura          | R Hipp<br>Atrophy  | CBZ, DPH      |
| 5  | F   | 52        | 17                      | R Ant Hipp                 | Epi       | None          | BL Hipp<br>Atrophy | DPH           |
| 6  | F   | 17        | 8                       | L Hipp                     | Epi       | None          | L Hipp<br>Atrophy  | LEV, CBZ      |
| 7  | M   | 40        | 23                      | L Hipp                     | Epi       | None          | L Hipp<br>Atrophy  | LTG, DPH, TPM |
| 8  | F   | 38        | 36                      | R Ant Hipp^ R<br>Post Hipp | Epi       | None          | R Hipp<br>Atrophy  | PB            |
| 9  | F   | 43        | 20                      | L Hipp                     | Epi       | None          | Normal             | CBZ, LTG      |
| 10 | F   | 24        | 12                      | R Hipp                     | Non-epi** | Sz            | Normal             | DPH           |

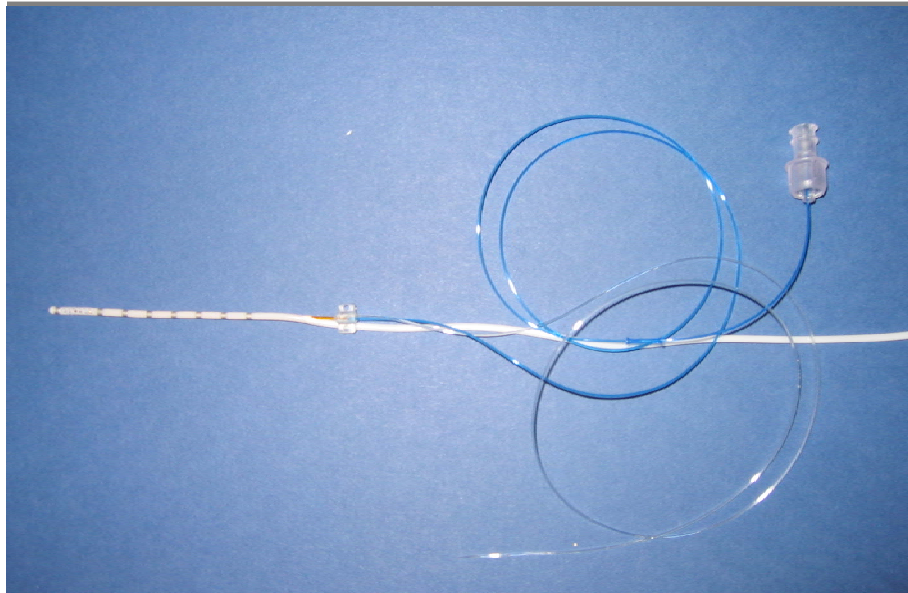
Abbreviations: AEDs, anti-epileptic drugs; Ant, anterior; Epi, epileptogenic; Hipp, hippocampus; L, left; Non-epi, non-epileptogenic; Post, posterior; R, right; Sx, symptoms; Sz, seizure; Yrs, years. AED abbreviations: CBZ carbamazepine; CLZ clonazepam; DPH phenytoin; GBP gabapentin; LTG lamotrigine; LEV levitiracetam; OXC oxcarbazepine; PB phenobarbital, TPM topiramate; ZNS zonisamide. ^ (Not Stimulated) \*\* focus in R Parietal cortex

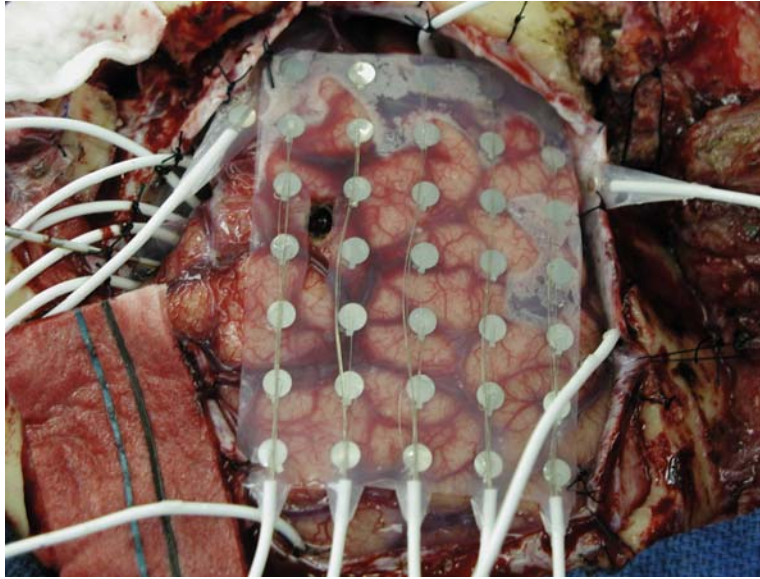
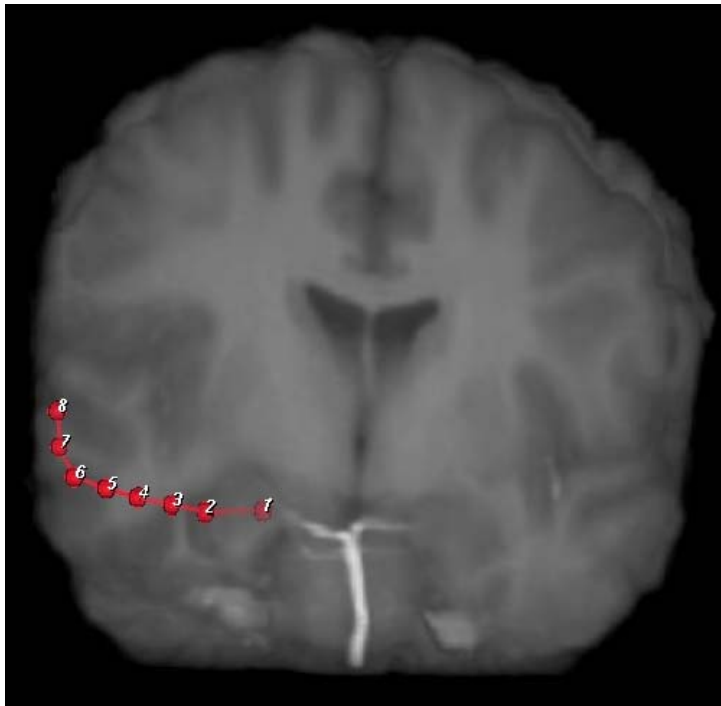
### *General Procedures*

Microdialysis probes coupled to depth electrodes (Spencer probe; Ad-Tech Instrument, Racine, WI) were implanted stereotactically in the suspected epileptogenic hippocampus using a stereotactic neuronavigation device (BrainLAB, Westchester, IL). All surgeries were performed by Drs. Dennis Spencer, Kenneth Vives, and members of the Yale-New Haven Hospital Department of Neurosurgery. The design of the earlier Spencer probe has been modified [186]. Briefly, the probes are CMA/20 custom-modified concentric and flexible microdialysis probes (0.67 mm diameter, 70 mm length, 10 mm membrane with 20 kDa cut-off CMA/20 (CMA, North Chelmsford, MA) which allow stable flow, recovery, and dialysate collection for one-two weeks, and usually throughout the duration of intracranial EEG monitoring. The dialysis probe is inserted into a polyurethane/silastic flexible depth-electrode (1 mm i.d., Ad-Tech Instrument Co., Racine, WI), which has perforations between contacts 1 and 2 to allow for fluid exchange with the membrane. The total diameter of this combination microdialysis/depth electrode (Spencer probe) is 1.85 mm. The probes are sterilized by gamma radiation and flushed with sterile artificial extracellular fluid (AECF) to ensure patency prior to insertion. Following surgery, three-dimensional co-registered CT and MRI is used to verify probe location (See Figure 1. A-C). After one to two days of recovery, patients are transferred to the epilepsy monitoring unit and the AEDs are tapered to allow for spontaneous seizures. Patients usually undergo continuous audiovisual and icEEG monitoring from one to two weeks. Once a satisfactory number of spontaneous habitual seizures are obtained, the AEDs are reinitiated and all intracranial probes are removed. Whether the microdialysis membrane was in the hippocampus of seizure origin (epileptogenic hippocampus) is determined based on the consensus interpretation of the collected icEEG data by epileptologists experienced in intracranial electrophysiology and confirmed by the long-term outcome of resective surgery..

**Figure 1.** (A) Spencer depth electrode with inserted microdialysis catheter prior to intracranial implantation. (B) Intraoperative implantation of depth electrodes with attached microdialysis catheters and placement of subdural grid electrodes into the temporal cortex of a patient undergoing Phase III evaluation. (C) Magnetic resonance image (MRI) from a patient with one Spencer probe implanted in the right hippocampus. Depth electrode contacts 1 through 8 are visible (highlighted in red). The platinum contacts of the depth electrode generate significant artifact and appear much larger than their actual diameter of 1mm. The microdialysis membrane, which is not visible on magnetic resonance imaging, lies between depth electrode contacts 1 and 2.

**A**



*B**C*

### *Zero-Flow Microdialysis*

The zero-flow microdialysis method estimates the steady state or basal concentration of the measured substrate in the extracellular fluid (ECF) [187], and has previously been described [82]. In brief, the study was conducted two to five days after implantation, in the afternoon, when patients were quietly resting, at least six hours from any intracranially recorded seizure activity, and  $168 \pm 81$  hrs (mean  $\pm$  SDEV) before the stimulation study, to avoid the effects of acute probe implantation, anesthesia, ictal activity, behavioral stimuli, or food intake [188-190]. Patients were on a combination of AEDs (Table 1). Sterile artificial cerebrospinal fluid (composition: 135 mM NaCl, 3 mM KCl, 1 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.2 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  in 1 mM sodium phosphate buffer, pH 7.4) was infused using portable CMA 107 syringe pumps (CMA, North Chelmsford, MA) at a rate of 2.0  $\mu\text{l}/\text{min}$  for one hour to reach steady state. Dialysis samples (20  $\mu\text{l}$ ) were collected at progressively decreasing flow rates (2.0 – 1.5 – 0.5 and 0.2  $\mu\text{l}/\text{min}$ ), allowing a period of 60 - 90 min for equilibration after each rate change. Chemical substrates in the hippocampal interstitial fluid diffuse across the dialysis membrane into the artificial CSF within the probe, which is then collected via the outlet tubing on the microdialysis probe into microvials. The experiment was completed in approximately six hours. Samples were stored on dry ice and then at  $-80^\circ\text{C}$  for later analysis of neurometabolite concentration using high-performance liquid chromatography (HPLC). The basal levels at steady state (i.e. at 0.0  $\mu\text{l}/\text{min}$ ) were estimated using regression analysis to fit a second order polynomial extrapolated to a flow of zero (i.e.  $x=0$ ). The zero-flow studies for this set of patients were collected by M. Cassaday, D. Ocame, S. Forselius, and G. Widi.

### *Electrical Brain Stimulation*

To decrease the probability of stimulus-induced seizures, electrical stimulation was applied when the clinical monitoring was completed and patients were restarted on their AEDs, usually on the day before electrode removal. The two contacts (contacts 1 and 2) flanking the microdialysis probe on the tip of the hippocampal depth electrode were stimulated in bipolar fashion using a Grass S12 constant current square wave generator (Grass Instrument Division, Astro-Med Inc, West Warwick, RI). A series of single pulse stimulations were used to determine the stimulation current for the 50 Hz stimulus train with a maximum stimulation current defined as one that does not produce any afterdischarges or uncomfortable subjective

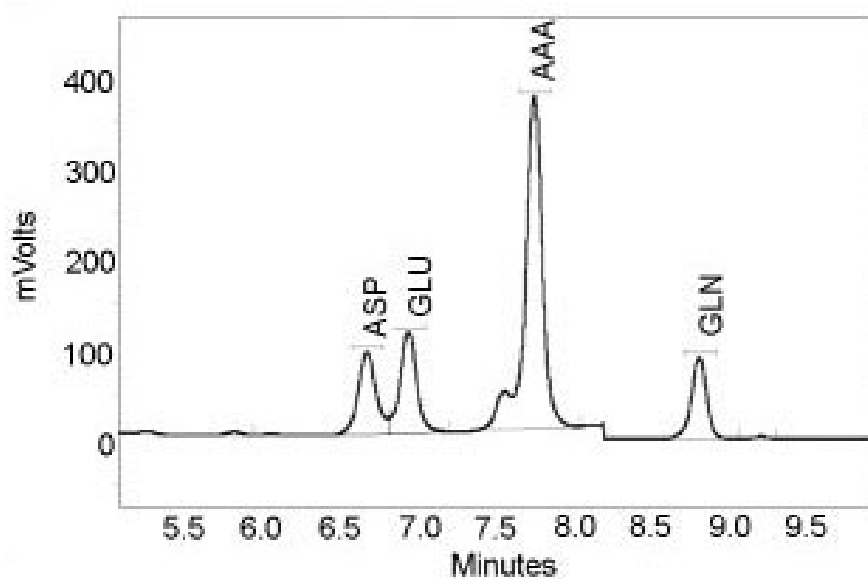


sensations, up to 10 mA. After a 20 minute baseline period, three 50 Hz trains (biphasic square wave pulses, 0.2 msec per phase) of 5 second duration were applied with 5 second interval (total pulse number 750). Microdialysate from stimulated hippocampal probes was collected using the CMA/170 microdialysis fraction collector (CMA, North Chelmsford, MA) every 2 mins at a flow rate of 2.0  $\mu$ l/min. The samples were stored initially at -4 °C and then at -80 °C. The icEEG data from this group of patients was not available for later analysis. R. Duckrow performed the electrical stimulation while G. Widi and D. Ocame collected the microdialysis data.

#### *High Performance Liquid Chromatography Analysis of Glutamate Levels*

Microdialysate samples were analyzed for glutamate levels using the HPLC method modified from the method previously described by Bourdelais and Lakivas [191]. In brief, 1  $\mu$ l of patient sample is added to 9  $\mu$ l of an internal standard of alpha-aminoadipic acid (AAA). This mixture is derivatized by adding 20  $\mu$ l of an O-phthaldialdehyde. After eight minutes, 20  $\mu$ l of the derivatized sample is injected onto the column (3  $\mu$ m Phase II ODS column, 3.2 x 100 mm cartridge, Bioanalytical Systems, Inc., West Lafayette, IN). The mobile phase consists of 0.1M acetic acid (pH 6.0) with a 12 to 20% acetonitrile gradient at a 1 ml/min flow rate. Within 30 minutes, chromatograms demonstrate adequate separation showing glutamate approximately at 6.9 minutes and AAA at 7.7 minutes (see Figure 2). The excitation and emission wavelengths on the fluorescence detector (Shimadzu Scientific Instruments, Columbia, MD) are set at 338nm and 425nm, respectively. The sensitivity limit for glutamate is 0.1, based on a signal-to-noise ratio of 10:1. Peak areas of the neurometabolite on chromatograms are then compared with external standards to determine the concentration in the samples using EZCHROME elite software from ESA (Chelmsford, MA). HPLC analysis for this set of patients was kindly performed by M. Cassaday, D. Ocame, and G. Widi.

**Figure 2.** High-performance liquid chromatogram sample showing the peak separations for aspartate (asp,  $2.0\mu\text{M}$ ), glutamate (glu,  $1.7\mu\text{M}$ ), the internal standard  $\alpha$ -aminoadipic acid (AAA), and glutamine (gln,  $80.5\mu\text{M}$ ). (Reprinted with permission from Cavus et al, *Annal Neurol* 2005.)



*Statistical Analysis*

The timing of microdialysate samples from the stimulation study was corrected for dead volume (14 mins at 2  $\mu$ l/min). The change in glutamate concentration in response to the stimulation was expressed as % change from baseline and was analyzed using repeated measures ANOVA and Tukey-Kramer's post-hoc test (SAS statistical program, version 5, SAS Institute, Cary, NC). Since basal glutamate data was not normally distributed, log transformation was used to achieve normality. Linear regression on log-transformed data was used to analyze the relationship between basal interictal glutamate levels and the peak glutamate level at the time of stimulation. In addition, to increase the confidence in our statistical model, a non-parametric test (Spearman's rank correlation test) was used to correlate the raw basal glutamate levels to the raw post-stimulus glutamate levels. The significance level was set at 0.05 and all data are reported as mean  $\pm$  standard error of the mean (SE). This investigator organized the dataset, and performed all statistical analyses in conjunction with I. Cavus.

## Results

In nine out of ten cases, the hippocampus studied with electrical stimulation was either the site of seizure genesis or propagation (epileptogenic hippocampus). Of those nine probes in the epileptogenic hippocampus, six were placed within an atrophic hippocampus and three were within a normal-appearing hippocampus, as determined by the clinical MRI interpretation (Table 1). The epileptogenic hippocampus was resected in six cases, and spared in three cases to avoid functional impairment (Table 2). From the six resected hippocampi, three showed minimal cell loss and three showed sclerosis on subsequent hispathological examination. The three cases that showed sclerosis also had marked atrophy on MRI, while the three cases that showed minimal cell loss were normal on MRI. Of patients with hippocampal resection, three were seizure-free and three had a significant decrease in seizures at one-year post-operative follow-up (Table 2). In patient number 10, seizures originated from parietal cortex and did not involve the hippocampus (non-epileptogenic hippocampus).

**Table 2.** Seizure localization, surgery, pathology, and follow-up at one year post-operatively.

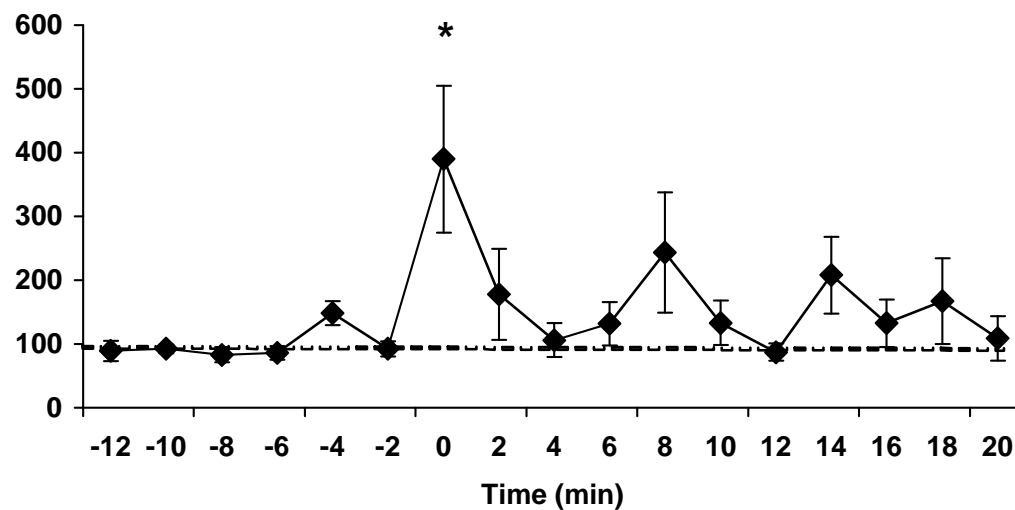
| Pt | Localization  | Resection             | Hipp Pathology | Sz Freq Pre-Op<br>(per month) | Total Sz at 1 Yr<br>Follow-Up |
|----|---------------|-----------------------|----------------|-------------------------------|-------------------------------|
| 1  | L Hipp        | L AMTL                | Min cell loss  | 10                            | 4                             |
| 2  | R Hipp        | R AMTL                | Min cell loss  | 2                             | None                          |
| 3  | Multifocal    | None – VNS<br>offered | N/A            | 8                             | 1                             |
| 4  | R Med Hipp    | R AMTL                | Sclerosis      | 13                            | 4                             |
| 5  | R Post Hipp   | R AMTL                | Sclerosis      | 3                             | 2                             |
| 6  | L Ent Ctx     | None*                 | N/A            | 7                             | N/A                           |
| 7  | L Ent Ctx     | L AMTL                | Sclerosis      | 12                            | None                          |
| 8  | Post temp ctx | R post temp<br>neoctx | N/A            | 8                             | None                          |
| 9  | L Ent Ctx     | L AMTL                | Min cell loss  | 30                            | None                          |
| 10 | R Par Neoctx  | R lat temp-<br>occip  | N/A            | 40                            | None                          |

*Abbreviations: AMTL, Anterior medial temporal lobectomy; BL, bilateral; Ent Ctx, entorhinal ctx; Freq, frequency; Hipp, hippocampus; lat, lateral; L, left; med, medial; min, minimal; N/A, Not Applicable; Neoctx, neocortex; Par, parietal; post, posterior; prop, propagated; occip, occipital lobe; R, right; temp, temporal cortex; Sz, seizure; Yr, year. \* - Resection not performed because of risk of memory impairment - VNS offered instead.*

### *50 Hz Stimulation of the Human Epileptogenic Hippocampus Induces Glutamate Efflux*

Stimulation at 50 Hz (stimulation strength 6 - 10 mA) caused an increase in glutamate efflux in the epileptogenic hippocampus ( $F(16,143) = 2.338, p = 0.005, n = 10$ , Figure 3). This increase was significant only at the time of stimulation ( $p < 0.05$ , Tukey's post-hoc test), when it reached  $390 \pm 115\%$  of baseline and then declined to pre-stimulation levels within the next few minutes. In six patients, the glutamate levels were elevated two-fold over baseline for 4 minutes following stimulation. However, the analysis of the group data showed significant elevation only at the time of stimulation. In one patient (patient #10), stimulation of the non-epileptogenic hippocampus triggered a brief habitual seizure, but had no discernable effect on glutamate efflux (data not shown). In another patient (patient #8) with probes in the ipsilateral anterior and posterior hippocampus, stimulation of the posterior hippocampus did not cause change in glutamate levels at the anterior probe indicating that the effect of stimulation on glutamate efflux in the posterior-anterior axis of the hippocampus can be local. The distance between these two probes was approximately 14.1 mm (Figure 4A). As seen in Figure 4B, the 50 Hz stimulation induced glutamate efflux 30 times greater than baseline only at the stimulated posterior probe site, with no change in the nearby anterior hippocampal probe. Although glutamate efflux in the sclerotic hippocampus ( $250 \pm 188\%, n = 3$ ) was greater than the hippocampus exhibiting minimal cell loss ( $206\% \pm 88\%, n = 3$ ), this difference was not significant ( $p = 0.733$ ), possibly due to the small number under analysis.

**Figure 3.** Fifty Hz electrical stimulation (at 0 min) of the epileptogenic hippocampus induced significant glutamate efflux at the time of stimulation compared to baseline (\* $p < 0.05$ ,  $n = 10$ , Tukey's post-hoc test). Microdialysis samples were collected every two minutes at 2  $\mu\text{l}/\text{min}$  flow rate from 10 patients.



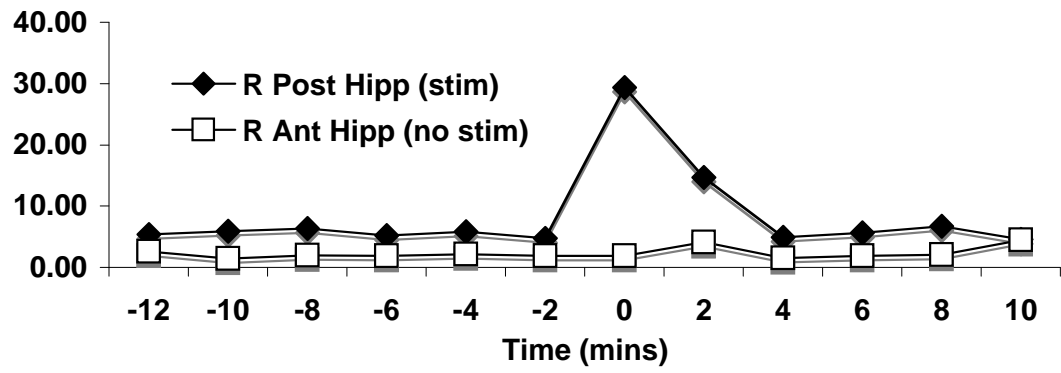
**Figure 4.** (A) MRI image of a patient who had two ipsilateral probes implanted in the right anterior and posterior hippocampus. The two probes were 14.1 mm apart and were both classified as epileptogenic with the seizure onset site in the right posterior temporal lobe. Only the posterior probe was stimulated. (B) Simultaneous microdialysis from the anterior (not stimulated) and posterior (stimulated) hippocampus probes shows that the stimulus-induced glutamate release is limited to the stimulated probe. Abbreviations: Ant-Anterior, Hipp-Hippocampus, Post-Posterior, R-Right, Stim-Stimulated, and Temp-Temporal.

A

**Rig ht Ant Hipp Probe (no stim)**

**Rig ht Post  
Hipp Probe  
(sti m )**

QuickTime™ and a  
TIFF (Uncompressed) decompressor  
are needed to see this picture.

*B*

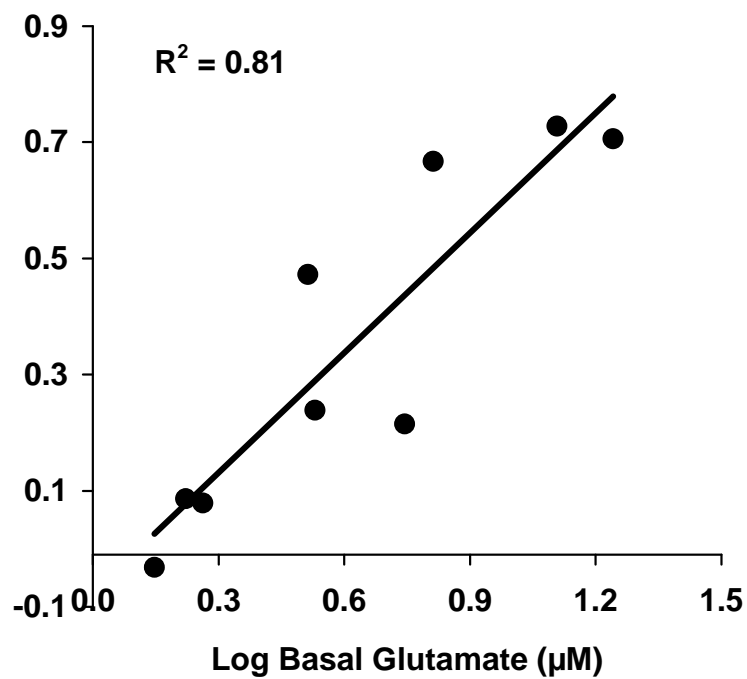


*Higher Basal Glutamate Levels are Related to Higher Stimulated Increase in Glutamate:*

We have previously reported that basal interictal extracellular glutamate concentration in the epileptogenic hippocampus are abnormally elevated [82]. Hypothesizing that the interictal impairment in glutamate regulation underlies its increase with stimulation, we examined the relationship between basal levels and peak glutamate at the time of stimulation. Basal (zero flow) glutamate levels in the epileptogenic hippocampus ranged from 1.67 to 17.46  $\mu\text{M}$  ( $6.56 \pm 5.68$ , mean  $\pm$  SDEV,  $n = 8$ , basal levels were not obtained for two probes), and in the non-epileptogenic hippocampus of one patient glutamate was 1.40  $\mu\text{M}$ , consistent with values reported earlier [82].

Linear regression analysis on log-transformed data showed that higher peak glutamate levels at the time of stimulation were significantly related to elevated basal glutamate levels ( $R^2 = 0.81$ ,  $p = 0.001$ , Figure 5). A non-parametric test on raw data revealed an even stronger correlation ( $R = 0.90$ ,  $p < 0.0009$ , Spearman's test). Thus, the higher the basal interictal levels in the hippocampus, the higher the glutamate peak is in response to 50 Hz stimulation, with 80 – 90 % variability in the stimulus-related glutamate increase being due to the degree of elevation in basal glutamate.

**Figure 5.** The increase in the glutamate efflux in response to 50 Hz stimulation was significantly related to the interictal basal glutamate levels measured with zero-flow microdialysis at the stimulation site. ( $p = 0.001$ ,  $n = 9$ , linear regression on log-transformed data).

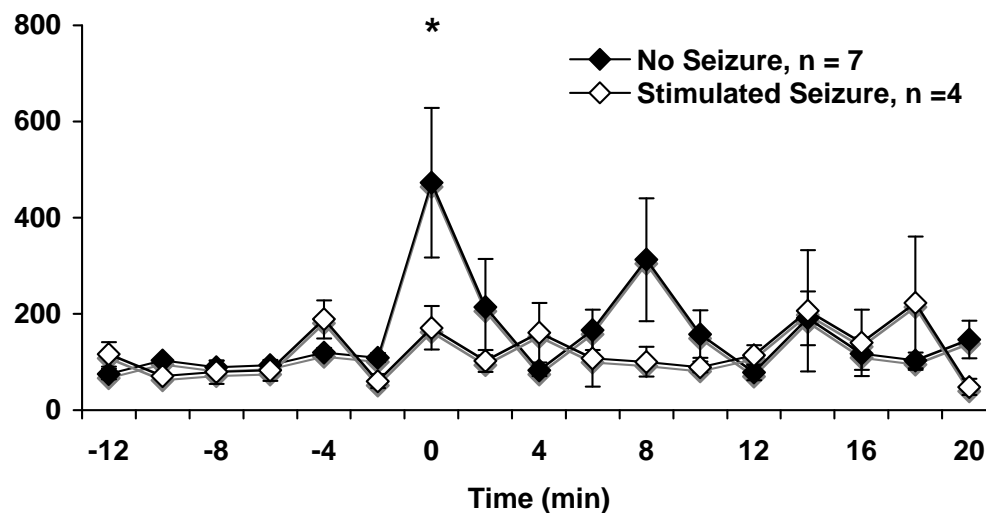


*Dissociation Between Stimulus-Induced Glutamate Efflux and Stimulus-Induced Seizures*

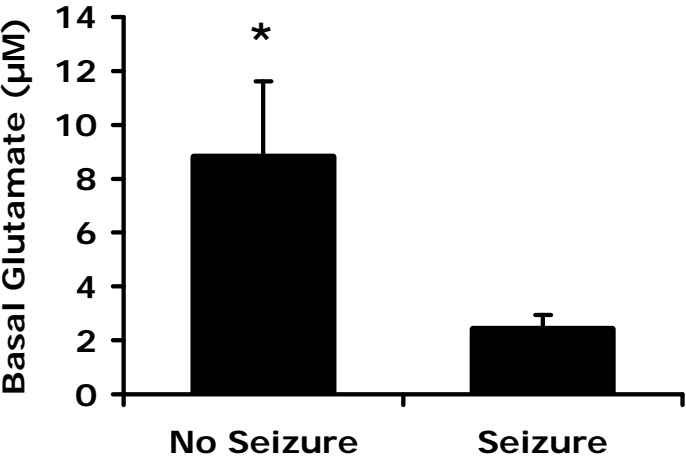
Stimulation of the epileptogenic hippocampus at 50 Hz triggered brief self-limited habitual seizures in three cases and aura in two (Table 1). In one patient (patient #10), stimulation of the non-epileptogenic hippocampus also caused a brief habitual seizure. To investigate if the stimulus-induced glutamate release was related to these triggered seizures, data from sites with seizures and with no seizures were analyzed separately. Subjects without stimulus-induced seizure had a significant change in glutamate efflux ( $F(16,98) = 2.38, p = 0.005, n = 7$ , Figure 6A) at the time of stimulation ( $p < 0.05$ , Tukey's test). Unexpectedly, there was no significant stimulus-induced glutamate efflux in subjects who experienced seizure ( $F(16,61) = 1.00, p = 0.47, n = 4$ , Fig. 6A). Consistent with our earlier result (see Figure 5), basal glutamate levels were significantly higher in the group that had stimulus-induced glutamate increase but no seizures ( $8.8 \pm 2.8 \mu\text{M}, n = 5, p = 0.04$ , t-test; Figure 6B) than in the group that had stimulated seizures but no significant change in glutamate ( $2.4 \pm 0.5 \mu\text{M}, n = 4$ ). Thus, these data indicated that the elevation in the interictal basal glutamate levels, but not the presence of seizures account for the increase in glutamate in response to stimulation. Interestingly, the stimulus-induced seizures also occurred in the hippocampi with relatively low – to - normal basal interictal glutamate levels.

**Figure 6.** Stimulation of the hippocampus evoked self-limited seizures in four out of ten patients. **A.** Significant glutamate efflux in response to stimulation (time 0 min) was observed in the group that did not experience seizures ( $n = 7$ ,  $p = 0.005$ , ANOVA), while glutamate levels did not change in the group that had stimulated-seizures ( $n = 4$ ,  $p = 0.47$ , ANOVA). **B.** The basal glutamate levels in the group that did not experience seizures ( $n = 5$ ) were significantly higher ( $p = 0.04$ ,  $t$ -test on log transformed data) than in the group that did experience stimulated seizures ( $n = 4$ ).

**A**



*B*



## Discussion

This is the first study to report the effects of electrical stimulation on glutamate release in the human hippocampus. Our subjects were awake patients who were undergoing intracranial EEG evaluation of their seizure focus. Stimulation of the epileptogenic hippocampus at 50 Hz caused large, transient glutamate efflux. In one case, where the non-epileptogenic hippocampus was stimulated, the stimulation did not cause any change in the glutamate levels, but evoked a brief, self-limited seizure. Our studies indicate that 50 Hz direct stimulation of the human hippocampus in patients with epilepsy can promote excitability, instead of controlling it [123, 155-157], as five out of ten patients experienced seizures or auras, despite being on their AEDs. However, in contrast to the spontaneous hippocampal seizures that are known to be associated with large increases in glutamate [67, 77, 192], the stimulated-seizures were not associated with significant glutamate efflux. Rather, the degree of stimulated glutamate increase was related to the elevation in the interictal basal glutamate level. The factors that contribute to the elevation in the interictal glutamate level in the human epileptogenic hippocampus are unknown. However, our study indicates that elevated basal glutamate predispose to higher stimulus-induced increase, possibly by affecting a shared mechanism of glutamate dysregulation.

Direct electrical stimulation of the hippocampus at 50 Hz was excitatory in six of ten patients; four had short habitual, self-limited seizures and two patients reported auras. Seizures were induced with stimulation of the epileptogenic as well as the non-epileptogenic (in one case) hippocampus. This differs from studies reporting that 50 Hz stimulation of the epileptogenic and non-epileptogenic cortex in refractory epilepsy patients suppresses EEG spike rate and power, suggesting that this stimulation form has an inhibitory effect on epileptiform activity [123, 155-158, 160-162]. Unfortunately, we have insufficient EEG data to determine the effects of 50 Hz stimulation on EEG spike rate and power in this group of patients. It is possible that the same stimulation frequency has different effects on excitability in the hippocampus and in the cortex, and this remains to be studied.

Recent work on *in vitro* models of epilepsy may provide new insights as to the antiepileptic mechanisms of 50 Hz stimulation in the hippocampus. Bikson et al. (2001) and Durand et al. (2006) stimulated the CA1 region of the rat hippocampus at 50 Hz (sinusoidal) and observed that low-Ca<sup>2+</sup>, picrotoxin, and high-K<sup>+</sup> induced epileptiform activity was suppressed by potassium efflux and

depolarization block [160]. Durand et al. (2006) expanded on these findings by citing that 50 Hz stimulation of the alveus in the rat hippocampus suppressed spontaneous and evoked electrical activity in the cell body and axons of surrounding neurons. In addition, Schiller and Bankirer (2007) found that 50 Hz stimulation (biphasic square pulses lasting 0.2-0.4 ms) of rat neocortical slices treated with either bicuculline or magnesium-free extracellular solution prematurely stopped ~50% of seizure-like events. Interestingly, the additional use of ionotropic glutamate receptor blockers suppressed the epileptiform discharges and enhanced the effects of 50 Hz stimulation, suggesting the possibility of short-term synaptic depression of excitatory transmission following stimulation [162]. Thus, it is apparent that different trains of stimulation (sinusoidal and square wave) could suppress epileptiform activity through different mechanisms *in vitro*. This antiepileptic effect was not observed *in vivo* in the present study even though a stimulation paradigm (50 Hz, biphasic square wave pulses, 0.2 ms per phase) similar to Schiller and Bankirer's (2007) was used. It is likely that the variability in response to stimulation may depend on other complex factors, such as the underlying pathophysiology of the stimulated site, network connectivity and recent history of the stimulated networks [132, 193].

Fifty Hz stimulation of the epileptogenic hippocampus also induced a large glutamate efflux at the stimulated site. Although in most patients this glutamate increase was relatively brief, returning to baseline within a couple of minutes, in few patients the glutamate levels remained elevated for up to 4-6 minutes following stimulation. Unexpectedly, this marked increase in glutamate was not due to the evoked seizures, as glutamate levels did not change significantly in the group that experienced seizures. Rather, the stimulus-induced glutamate increase was positively correlated with the elevation in the interictal basal glutamate concentration. We previously reported that the basal glutamate levels are abnormally high in the epileptogenic hippocampus of patients with refractory MTLE,[82], and are associated with decreased hippocampal volume [88] and neuronal cell count in the resected hippocampus [89]. Thus, the more sclerotic the hippocampus, the worse the extracellular glutamate regulation is during the interictal state as manifested by higher basal concentration and stimulated efflux. Whether the interictal accumulation of extracellular glutamate is a consequence of impaired glutamate reuptake [86, 96, 194] increased neuronal or glial release [83, 102, 195, 196] or slowed glial glutamate-glutamine metabolism in the human sclerotic hippocampus [83, 98] remains unresolved. However, the significant positive correlation between

stimulated and interictal glutamate levels likely reflects a shared mechanism where stimulation at 50 Hz exacerbates the already impaired glutamate regulation in the epileptogenic hippocampus. Fifty Hz stimulation did not, however, induce a significantly greater glutamate efflux in the sclerotic hippocampus than in the unaffected side. We believe that this finding may have been due the limited subject number, and in a larger study we would expect to find the glutamate surge following stimulation to be significantly higher as well as stay elevated significantly longer in duration in the sclerotic hippocampi given the aberrant glutamate handling of the affected site.

Our knowledge of how electrical stimulation affects neurotransmitter release is limited, and comes mostly from animal studies [197]. Because of restrictions related to clinical care, we could not investigate pharmacologically the mechanisms associated with stimulus-induced glutamate increase in our patients. In addition, our electrode configuration and implant procedures preclude us from determining the precise site of stimulation in the hippocampus. Since the distance between stimulated contacts is 13 mm, traversing the cephalo-caudal axis of the hippocampus, we assume that most of the hippocampal subfields have been stimulated. The increased glutamate release we observed may be due to impairment in one or several mechanisms regulating neurotransmitter metabolism and release that would be best studied using *in-vitro* preparations from human tissue or appropriate animal models. Stimulation could induce neuronal glutamate release that overwhelms an already impaired glial glutamate clearance mechanism, resulting in extrasynaptic glutamate spillover [198] that can be detected by the microdialysis probes [181-183]. Electrical stimulation can also trigger glial glutamate release, either directly or through neuronal activation [83, 98, 196, 199, 200], and may affect the glutamate – glutamine cycling, which is known to be impaired in the epileptic hippocampus [83, 98]. On the other hand, studies in rat hippocampal slices of the CA1 region have demonstrated that 100 Hz square pulses can deplete extracellular calcium and subsequently suppress presynaptic glutamate release [201]. Iremonger's group used similar HFS at 125 Hz with monophasic pulses to induce hippocampal LTD and decrease presynaptic glutamate release, as well as decrease excitatory currents in rat motor cortex [178]. The frequency of stimulation is likely one of the factors regulating neurotransmitter release. For example, subthalamic stimulation in a rat model of bradykinesia induced a frequency-dependent increase in microdialysate glutamate and GABA that reached a plateau after 130 Hz, which incidentally is also the therapeutic stimulation frequency range (~50-350 Hz)



for movement disorders [177]. Preliminary studies in epilepsy patients indicate that stimulation at 1 Hz suppresses the abnormally elevated extracellular glutamate at epileptogenic brain sites [202]. Future systematic studies will be needed to determine if stimulation methods that hold promise for seizure control [30, 129, 153, 203, 204], such as very low or very high frequencies, are also associated with suppression of glutamate release.

Spontaneous seizures in patients with intractable TLE are often associated with dramatic increases in hippocampal extracellular glutamate [67, 77, 192]. In contrast, the stimulus-evoked seizures in our patients did not cause significant change in glutamate levels. It is possible that spontaneous seizures are associated with more widespread, extensive and prolonged impairment in neurotransmitter regulation or metabolism, and therefore larger glutamate elevation. Alternatively, as suggested by our study, the degree of basal glutamate dysregulation at the epileptic site could also determine the degree of glutamate increase with seizures. Evidence for this is provided by studies in animals and in humans. Not all seizures are associated with a glutamate increase. Naïve rats generally show little glutamate elevation in response to a single acute electrical stimulation or administration of convulsants [67, 78, 79, 205, 206]. More reliable increases in glutamate are observed in chronic animal models of epilepsy where there is more extensive synaptic and cellular reorganization, such as kindling [67, 78, 79, 207] and with treatments that induce cytoskeletal disruption [207, 208]. Wilson and colleagues (1996) observed that patients with hippocampal sclerosis have a greater glutamate increase with seizures than patients with minimal hippocampal cell loss. In our study, only one of four patients with evoked seizures had hippocampal atrophy, and the basal glutamate levels in the seizure group were significantly lower, suggesting that the absence of glutamate increase with evoked seizures is in part due to relatively intact glutamate regulation. Any small, transient increases in glutamate may have been undetected by microdialysis, but may be measurable with more sensitive neurochemical measures, such as glutamate sensors (Day et al., 2006). Then again, the induction of seizures at sites of lower baseline extracellular glutamate – that are presumably more intact – is of interest. In clinical practice, early efforts using stimulation to localize habitual seizures [209, 210] were abandoned as seizures could be induced by the stimulation of non-affected sites. In addition, higher glutamate levels might protect to some degree against seizure induction as elevation in the extrasynaptic glutamate is reported to suppress the synaptic glutamate release by activation of the presynaptic

metabotropic glutamate receptors [211, 212]. However this regulation is to some extent impaired in the sclerotic hippocampus [211], where additional impairment in glutamate reuptake [93, 94], predisposing to greater glutamate neurotoxicity.

### *Confounding Factors*

Several factors limit the findings of the study. Methodological concerns include the technique of intracerebral microdialysis used to estimate extracellular glutamate concentrations in the conscious human hippocampus. It is important to note that microdialysis sampling reflects a pooled measure of extracellular fluid in the immediate vicinity of the catheter. In the epileptogenic hippocampus, the amount of sclerosis and atrophy may make it difficult to place the microdialysis probe directly in the most disease-affected regions. For instance, the functionally and anatomically different anterior and posterior hippocampus may be more involved in the patient's epilepsy, and depending on probe placement, basal glutamate levels and glutamate efflux following stimulation could vary. Since we did not do a sub-analysis on probe location within the hippocampus due to the small number of patients under analysis, our confidence in describing the extent of disease involvement in different hippocampal regions and how this relates to glutamate concentrations in their proximity is limited. Nevertheless, microdialysis provides the closest *in vivo* look at neurochemical activity of the epileptic human hippocampus. Another limitation to our findings is that we may only conclude that 50 Hz stimulation induces glutamate efflux in the epileptogenic hippocampus. We may not generalize to the non-epileptogenic hippocampus since only one non-epileptogenic hippocampus was studied. Furthermore, we may not generalize our findings from the hippocampus to other regions of the brain due to the great variation of neuronal architecture in different areas of the brain. In our experience, the changes in glutamate in response to 50 Hz stimulation of the epileptic cortex have been more variable. Indeed, because the investigational procedure employed in this study involves an invasive intracranial surgical procedure within a specific subset of patients with medically refractory epilepsy, there is a limited number of patients available for study, even in a large epilepsy center over the course of 5 years. The small patient size examined in this investigation may have prevented the emergence of statistically significant results that would have been realized in a larger study, and may have resulted in spurious results that would not be significant within a greater sample size. Lastly, since we do not have

EEG data from the stimulated probes at the time of stimulation, we could not study the effects of 50 Hz stimulation on interictal spiking (another surrogate marker for excitability) and relate these findings to basal glutamate levels at the probe site, which may have allowed us to further support our findings.

### *Clinical Implications*

Fifty Hz electrical brain stimulation is a candidate therapy for treatment of medically refractory epilepsy. In general, neurostimulation offers the advantage over traditional AED treatment because it is focal and offers the advantage over resective epilepsy surgery because it is reversible and modifiable. Although preliminary evidence studying efficacy have been favorable, stimulation parameters have often been chosen empirically or based on limited evidence from other disorders and from animal models, and much remains unknown regarding optimal stimulation parameters for seizure control. Furthermore, the neurotoxicity and excitability associated with high basal glutamate levels within the epileptogenic hippocampus underlie the importance of studying the effect of electrical brain stimulation on the brain's neurochemical environment. This study helps address this point by examining the effects of a candidate frequency on the neurochemical environment of its target. Overall, such studies are necessary in order to attain a greater understanding of how electrical brain stimulation affects the human brain. Such knowledge could then help design more rationale strategies for brain stimulation and optimize the treatment of medically refractory epilepsy.

## Conclusion

In summary, 50 Hz stimulation of the hippocampus in patients with medication-resistant TLE resulted in significant increase in extracellular glutamate concentration and in seizures in some patients. The magnitude of this glutamate increase was related not to the induction of seizures but to the elevation in interictal baseline glutamate, suggesting that the extent of glutamate dysregulation at baseline determines the degree of glutamate increase upon stimulation. This observation also suggests that shared mechanisms underlie the elevation in glutamate under interictal conditions and with stimulation. Studies in animal models indicate that repeated stimulations at sites where glutamate reuptake is impaired may lead to progressive, gradual accumulation in extracellular glutamate [49, 140, 141, 213], and promote further excitotoxicity and epileptogenicity. Therefore we conclude that stimulation of the epileptic hippocampus at 50 Hz may not be an optimal stimulation method for seizure control. Whether this stimulation method has different effects in other brain sites, or other stimulation methods have different effects remains to be studied.

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