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Abstract

EXTRACELLULAR GLUTAMATE, GLUTAMINE, AND GABA IN THE HIPPOCAMPUS AND CORTEX OF REFRACTORY EPILEPSY PATIENTS.

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Antiepileptic drug (AED) resistance affects one third of patients with epilepsy and is associated with significant disability. Brain microdialysis studies on the epileptogenic hippocampus of patients with medication refractory epilepsy have identified elevations in extracellular glutamate, the primary brain excitatory neurotransmitter, both acutely during seizures and chronically during the interictal periods. Whether extracellular glutamate, along with the metabolites glutamine and the inhibitory neurotransmitter GABA (gamma-aminobutyric acid), are elevated in other cortical regions is unknown. In addition, the effect of the administration of AEDs on the extracellular levels of these neurochemicals in patients with medication-refractory epilepsy is also unknown.

Microdialysis samples were obtained from probes coupled to the EEG depth electrodes and implanted in the cortex and hippocampus of 81 awake patients with medication-refractory epilepsy undergoing intracranial electroencephalographic (EEG) monitoring. Probes were classified according to location and seizure activity into cortical or hippocampal *non-epileptic*, *epileptogenic*, *propagated, non-localized* or *lesion* sites. Samples were collected during the interictal period, in all subjects on their full AED dose during the first couple of days of their hospitalization, and then again (in a subset of 38

patients) after their AEDs were tapered. The samples were analyzed with high performance liquid chromatography (HPLC) for glutamate, glutamine and GABA levels. Data were log-transformed and compared by ANOVA or multiple t-tests with a Bonferroni correction, where appropriate.

In the cortex, glutamate was significantly higher in epileptogenic (mean ± SEM, 17.3 ± 5.1 μM), propagated (25.8 ± 4.0 μM), non-localized (43.9 ± 9.9 μM), and lesion (46.9 ± 9.0 μM) sites compared to non-epileptic cortex (2.6 ± 0.3 μM). In the hippocampus, glutamate was significantly higher in the epileptogenic (10.3 ± 1.9 μM) and propagated sites (33.0 ± 13.8 μM) than non-epileptic sites (2.8 ± 0.5 μM). Glutamine was not significantly different between sites in both the cortex and hippocampus. In the cortex, GABA was significantly elevated in propagated (1503 ± 273 nM) and lesion (827 ± 183 nM) sites compared to non-epileptic sites (265 ± 62 nM). In the hippocampus, GABA was elevated in the propagated (1079 ± 395 nM) compared to non-epileptic sites (391 ± 169 nM).

There were no significant differences in glutamate, glutamine, or GABA between the hippocampus and cortex within non-epileptic, epileptogenic, or propagated sites, which enabled cortical and hippocampal probes to be combined. Glutamate was now found to be significantly elevated in propagated compared to non-epileptic sites ($p = 0.0001$). GABA was significantly elevated in epileptogenic compared to non-epileptic sites ($p = 0.011$) and in propagated compared to epileptogenic sites ($p = 0.028$).

After AED taper, there were no significant changes in glutamate in any site, although it decreased non-significantly in non-localized sites ($p = 0.090$). Glutamine decreased significantly in lesion sites ($p = 0.0095$). GABA declined significantly in non-

localized sites ($p = 0.014$). When all sites were examined together, there were overall significant decreases in glutamine ($p = 0.0011$) and GABA ($p < 0.0001$).

In conclusion, elevations in glutamate and GABA extend beyond epileptogenic sites in patients with refractory epilepsy. Levels of glutamate, glutamine, and GABA were comparable between the hippocampus and cortex. AEDs may increase extracellular levels of glutamine and GABA but are inefficient in reducing glutamate to normal levels in these patients, which may relate to AED resistance.

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Background

Introduction

Epilepsy, a disorder characterized by repeated unprovoked seizures, affects 1-3% of the population (1). Epilepsy is broadly classified as localization-related or generalized, depending on whether it originates in one hemisphere or involves the cortex bilaterally, respectively. Epilepsy can be further characterized as idiopathic, which suggests a genetic basis and is not associated with a structural abnormality; symptomatic, in which a structural cause is identified; and cryptogenic, in which a structural cause is suspected but is not identifiable with current modalities. The initial treatment for epilepsy consists of administration of antiepileptic drugs (AEDs), which are thought to mitigate epileptic activity by either reducing neuronal excitation or increasing inhibitory tone. Failure to respond to the first AED heralds poor seizure control, since the probability of response to successive AEDs follows the law of diminishing returns: 47% of patients will respond to their first AED, 13% will respond to the second, and 4% respond to the third or more (2). Despite the development of new AEDs, 25-30% of patients remain refractory to medications (2). Surgery is a consideration for a subset of patients with intractable localization-related epilepsy, and seizure freedom is achieved only in two-thirds of mesial temporal and in one half of neocortical resections (3). For the remaining patients, no cure is available. Therefore, it is necessary to gain a better understanding of drug-resistant epilepsy to guide future therapies.

Medication-Resistant Epilepsy:

Drug-resistant epilepsy is a significant public health concern. It is associated with a mortality rate five times greater than the general population (4) and with psychiatric comorbidities, social disability, and a reduced quality of life (5). The type of epilepsy predicts drug-responsiveness. Greater seizure control is achieved in patients with idiopathic generalized epilepsy (86%) compared to patients with generalized symptomatic or cryptogenic epilepsy (26%), symptomatic partial epilepsy (35%), and cryptogenic partial epilepsy (45%) (6). The presence of identifiable lesions decreases drug-responsiveness dramatically to 24% in cerebral dysgenesis, 11% in hippocampal sclerosis alone, and 3% in dual pathology (the presence of hippocampal sclerosis in conjunction with another lesion). Although most drug resistance becomes apparent soon after a medication is initiated, some patients exhibit deterioration years after a successful drug response (7). Other poor prognostic indicators include early age of onset, developmental delay, multiple seizures prior to treatment, failure of early treatment to control seizures, complex febrile seizures, status epilepticus, and generalized electroencephalographic (EEG) activity (2, 8).

Molecular theories on medication resistance fall into two main categories: 1) reduced sensitivity of the target to the medication and 2) poor concentration of medication at the target site (9). Alterations in sodium channels and GABA receptors induced by epileptic changes or genetic mutations underlying epilepsy may affect drug binding and efficacy. For example the changes in $GABA_A$ receptor configuration in epileptic rat hippocampi may mediate phenobarbital resistance (10, 11), and alterations in

sodium channel properties may underlie carbamazepine resistance (12). Although its role in resistance is uncertain, the molecular target of levetiracetam is decreased in patients with hippocampal sclerosis and in chronically epileptic rats (13). The transporter hypothesis, which is better studied due to previous work on cancer chemotherapy resistance, posits that the expression of efflux transporters, such as p-glycoprotein (P-gp) and multi-drug resistance proteins (MRPs), actively prevent drug accumulation in the brain parenchyma. The overexpression of drug transporters in lesions and other epileptic foci may explain why symptomatic focal epilepsies are more drug-resistant than idiopathic epilepsies, in which the anomalies are more evenly distributed (14).

Localization-Related Epilepsy:

The term *localization-related epilepsy* suggests that a discrete site of seizure onset is identifiable, either through imaging or electrographic study. These epilepsies can be broadly divided into mesial temporal lobe (MTL), lesional, and nonlesional cortical onsets. The MTL is the most common site of localization-related epilepsy and is associated with mesial temporal sclerosis (MTS) in 70% of patients (15). MTS is characterized by hippocampal cell loss, atrophy, and gliosis (15). Causes of lesional epilepsy include tumors, vascular malformations, infarct- or trauma-related encephalomalacia, and developmental anomalies (16). Focal developmental malformations are due to abnormal neurogenesis, neuronal migration, or cortical organization and include focal cortical dysplasia, heterotopia, polymicrogyria, and schizencephaly (17). In addition to their pathological correlates, MTS and lesional epilepsy can often be identified preoperatively through MRI (18). Nonlesional cortical epilepsy, also referred to as cryptogenic or non-substrate-directed partial epilepsy, is not

anatomically distinct from surrounding brain on MRI but may be identified by abnormal physiology detected on nuclear medicine studies, such as PET or SPECT (16). Histologically, nonlesional epilepsy may demonstrate gliosis, focal cell loss, and developmental anomalies, but sometimes no abnormality can be perceived (19).

Theories of Epileptogenesis: Focus or Network

The extent of brain tissue necessary for seizure generation is a matter of controversy. Whether epileptogenesis is a product of a discrete "focus", a larger "irritative zone", or a distributed property of a wider network has been disputed (20). The theory proposed by Penfield and Jasper in the 1950s that the ictal onset zone alone is necessary for epileptogenesis (21) continues to guide to the extent of surgical resection. In the 1990s Tailarch and Bancaud proposed that epileptogenesis involves the area of immediate seizure propagation in addition to the ictal onset zone (22). More recently, the late Susan Spencer proposed that epileptogenesis is distributed equally through a wider network rather than being localized to one discrete area, which implies that disruption of the network at any point can abolish epileptogenesis (23). Of these epileptogenic networks, the best characterized is the medial temporal network, which involves connections between the hippocampus, amygdala, temporal pole, temporal neocortex, and orbitofrontal cortex (24). Others include the medial occipital/lateral temporal and superior parietal/medial frontal networks (23). Knowledge of the necessary constituents of epileptogenesis has treatment implications. For example, the presence of seizure termination outside of the onset site predicts a poor outcome following resection of the epileptogenic focus alone, which has been proposed to be due to globally reduced

inhibitory tone (25). Consequently, a better understanding of the elements of seizure propagation is needed.

Identification of Seizure Focus: Role of Medication Withdrawal

Before resective surgery can be considered for patients with medication-refractory epilepsy, inpatient continuous audio-visual EEG recording (AV-EEG), with either scalp or intracranial electrodes (icEEG) depending on the complexity of localization, is employed to identify the site of seizure onset. In this procedure, patients are admitted on their full outpatient AED dose, and medications are then gradually withdrawn to increase the habitual seizure frequency in order to shorten the recording period. In one study on patients with TLE undergoing medication taper during AV-EEG monitoring, the first habitual seizure was recorded after a mean of 4.4 days, and 7.4 days were required to record three habitual seizures (26). In another study examining AED taper, the first CPS was recorded after a mean of 3.2 days, and the mean monitoring period lasted 6.4 days (27).

The increase in seizure frequency is most prominent immediately after drug withdrawal and depends on the half-life of the AED (28). The increase in seizure frequency does not require a significant drop in AED levels, as the first CPS is often recorded when drug levels in the blood are sub-therapeutic rather than minimal (27). Since AED administration leads to a greater reduction in seizure frequency in patients with temporal lobe epilepsy (TLE) than extra-temporal epilepsy, TLE patients often requires a greater AED dose reduction to obtain an adequate number of seizures for localization during monitoring (26).

For most medications, including commonly-used carbamazepine and valproate, medication taper enhances seizure propagation but does not change the characteristic patterns of seizure onset (29), which is an important consideration when the results of the electrographic study guide the extent of surgical resection. In contrast withdrawal of barbiturates and benzodiazepines can cause atypical seizures that are falsely localizing (30), so doses of these two classes of medications are left unchanged during taper protocols. However, in patients with previously undiagnosed multifocal epilepsy, the taper of non-benzodiazepine/barbiturate AED may also appear to alter seizure onset patterns, because it unmasks atypical seizures arising from other onset foci (30). Clinical medication taper effects vary with each medication, which may influence the design of a taper protocol. For example, discontinuation of carbamazepine (31) and its metabolite oxcarbazepine (32) dramatically increase seizure frequency, including generalized tonic-clonic seizures, acute due to a rebound phenomenon, while tapering of phenytoin and valproate do not exhibit this marked effect (32).

While AED-taper-related changes have not been previously been studied by microdialysis in humans, electrographic studies at our center have identified a decrease in interictal spike frequency and interictal EEG Teager energy after medication withdrawal. The reduction in interictal spike frequency is a global phenomenon, not restricted to epileptogenic areas, related to drug taper seen in both hippocampal- and neocortical-onset epilepsies that may reflect a reduction in inhibitory tone (33). In contrast to spikes, high frequency oscillations do increase after medication taper and appear related to seizure susceptibility (34). Teager energy, which measures high-frequency energy and is positively correlated to increased extracellular glutamate (35), has been found to decrease

both globally and within epileptogenic sites during AED taper, suggesting that AEDs may have anti-seizure effects unrelated to reductions in excitation and overall signal energy (36).

In Vivo *Microdialysis*

Technique:

Microdialysis was first introduced in the 1970s for neurochemical research in animals. This innovation enabled *in vivo* studies of the extracellular concentrations of ions, drugs, and neurotransmitters in freely-moving animals, in contrast to previous analytic methods that required animal sacrifice for tissue extraction (37, 38). In the 1990s microdialysis was extended to investigations in humans and has enabled real-time sampling of the extracellular milieu in trauma, stroke, tumor, and epilepsy. In brief, a microdialysis probe tipped with a semi-permeable membrane (usually 5-20 kDa) is implanted in the brain region of interest. It is then infused with a solution isotonic to the surrounding extracellular fluid (ECF), enabling solutes to diffuse into the fluid according to their concentration gradients, and the dialysate is collected at regular time intervals and analyzed by high-performance liquid chromatography (HPLC), mass spectroscopy, or enzymatic reaction for the molecule of interest. At a constant infusion rate, the concentration of a solute within the dialysate is proportional to its concentration in the brain ECF, a ratio termed the *fractional recovery*. Properties of the diffusing molecule, blood brain barrier integrity, inflammation surrounding the probe, temperature, flow rate, and attributes of the probe can influence the fractional recovery, which can be increased by decreasing flow rates and lengthening the dialysis membranes (37, 39). The true

concentration of the substance in the brain ECF can be extrapolated from the concentration in the dialysate using either the zero-flow (40) or the no-net-flux (41, 42) methods. Another advantage of microdialysis is its ability to follow concentrations in real-time; the temporal resolution of microdialysis can be enhanced by increasing the perfusate flow rate and shortening the collection intervals.

Microdialysis Studies in Epilepsy Patients:

In human epilepsy studies the microdialysis catheter can be coupled to a depth electrode and implanted into hippocampal or cortical areas of interest, allowing correlation of the local neurochemical changes with the electrical activity. Because of the invasive nature of this technique, all of the human microdialysis data have been obtained in patients with medication-refractory epilepsy who are undergoing other neurosurgical procedures. In the first reported human microdialysis study, Carlson et al. intraoperatively sampled the epileptogenic frontal cortex of an anesthetized patient undergoing resective surgery and noted elevations in extracellular glutamate, aspartate, glycine, and serine during a period of status epilepticus partialis (43). Ronne-Engstrom et al. performed intraoperative microdialysis on seven anesthetized patients and observed similar neurochemical changes after electrically stimulating the epileptogenic site (44). Thomas et al. found that extracellular levels of glutamate, GABA, and aspartate measured intraoperatively in the anterior hippocampus of patients with MTLE correlated with electrical activity (45) but were unable to appreciate differences between the hippocampus and adjacent lateral temporal lobe (46).

During and Spencer, the first investigators to perform microdialysis in awake patients, measured the extracellular concentrations of glutamate and GABA in epileptic

and non-epileptic hippocampi during spontaneous seizures (47). They observed a rise in glutamate levels in the epileptic hippocampus immediately preceding the seizure, which was followed by a dramatic increase during the seizure and continued post-ictal elevation. During and Spencer also measured an increase in GABA during the seizure that, interestingly, was even greater in the contralateral non-epileptic than ipsilateral epileptic hippocampus. Wilson et al. found that glutamate and GABA in the hippocampi of awake patients with MTLE rose similarly with seizures (48); although, the rise in glutamate was not as great as that reported by During and Spencer, and they did not discern any differences between the epileptogenic and contralateral hippocampi.

Cavus and colleagues (35, 49-52) have focused on the basal levels of extracellular glutamate, glutamine and GABA during the interictal period, six hours away from seizure activity, of awake medication-resistant epilepsy patients undergoing intracranial EEG evaluation. They have found that the epileptogenic hippocampus has significantly higher basal glutamate levels than the non-epileptic hippocampus and that this is associated with a low glutamine/glutamate ratio and impaired glucose metabolism (49). Furthermore, the elevated glutamate in the hippocampi correlated with the degree of hippocampal atrophy (50), decreased hippocampal neuronal count (52) and was associated with poor cognitive functioning (53).

Elevated hippocampal basal glutamate was also found to be correlated with EEG Teager energy, a measure of high-frequency EEG power, and to be associated with hyperexcitability measured in *ex vivo* tissue preparations (35). In contrast, the results related to the cortical glutamate were more mixed. In the initial study, with limited cortical sampling, glutamate in the epileptogenic cortex was not significantly elevated

compared to the non-epileptic cortex and did not have the same metabolic associations as in the epileptogenic hippocampus (49). Later preliminary data collected from a similar patient population, suggested that glutamate is elevated in regions of seizure propagation as well as in onset sites (35).. More limited information has been obtained regarding the basal levels of the inhibitory neurotransmitter GABA. In conjunction with magnetic resonance spectroscopy (MRS), Pan, Cavus, et al. have found that while in the epileptogenic hippocampi higher basal GABA is correlated with impaired mitochondrial function, as determined by the ratio of N-acetyl aspartate to creatine, in the non-epileptogenic hippocampi, the increased GABA is correlated with improved mitochondrial function (51).

Microdialysis Studies in Animal Models of Epilepsy:

Results from microdialysis studies in animal models of epilepsy have been less consistent than the findings in humans. Some studies show an increase in glutamate during seizures (48, 54-59), while others indicate no change or even a decrease in glutamate (60-63). Many studies in rodents find an increase in GABA after seizure onset (59, 64, 65), although some have also shown a steady decrease (55, 66) or a brief increase followed by sustained decrease (67). This discrepancy may reflect the differences between chronic epilepsy in humans and the seizure models developed in animals and variations in the timing of microdialysate collection relative to convulsant infusion, as well as methodological differences between microdialysis in experimental animals and humans (including the development of substantial gliosis around chronically-implanted microdialysis catheters in animal models) (68). For example, pilocarpine injection is associated with an initial decrease in glutamate, which is followed by a sustained rise

associated with seizure activity, suggesting that the decrease is an artifact of the convulsant action (64). Wilson et al. were able to reproduce the ictal increases in glutamate and GABA seen in humans using a chronic rat kainate epilepsy model (48). Other chronic epilepsy models have borne out similar findings (69, 70).

Basal neurotransmitter concentrations have also been examined in animal epilepsy models. Kaura et al. noted elevated basal ECF glutamate and glutamine and reduced basal GABA compared to controls in both the electrically-stimulated and contralateral amygdalae of kindled rats (71). Zhang et al. observed that basal glutamate remained elevated in the hippocampi of kindled rats for one to three months after the last electrical stimulation (72).

Origins and Significance of Extracellular Glutamate, Glutamine, and GABA

Whether the neurotransmitters detected in microdialysis studies are of neuronal, glial, or combined origin is controversial. The degree to which the extracellular concentrations of neurochemicals represent neuronal synaptic spillover or glial release is unknown (73). The glutamate and GABA measured by microdialysis has been also proposed to represent volume transmission, a form of non-synaptic communication between neurons and astrocytes through the extracellular space (74). In earlier studies the insensitivity of extracellular glutamate to blockade of voltage-dependent sodium and calcium channels has suggested a non-neuronal source (73, 75), but the appearance of ^{13}C-enriched glutamate in the non-stimulated rat brain after injection of labeled glucose has also suggested at least a partial neuronal origin (76). Basal extracellular GABA is also insensitive to sodium channel blockade, suggesting a non-vesicular source (77).

Increasing evidence points to an astrocytic contribution to the elevated neurotransmitters observed in the ECF. Elevated extracellular glutamate and GABA may result from a reversal of astrocytic uptake due to changes in the ionic gradients that govern their respective cotransporters, specifically under conditions of energy deprivation (78). In the rat striatum, non-vesicular release by the cysteine-glutamate antiporter, which is predominantly glial, is thought to be the largest contributor to extracellular glutamate (79). This cysteine-glutamate antiporter is subject to feedback regulation by metabotropic glutamate receptors and the sodium-dependent glutamate transporter (79). Although originally thought to be limited to neurons, astrocytes can also release both glutamate (80) and GABA (81) in a calcium-dependent manner.

It is postulated that extrasynaptic glutamate and GABA reflects an interaction between neurons and astrocytes that serve to modulate neuronal transmission through extrasynaptic receptors (74). Neuronal glutamate release, by activating astrocytic receptors, may stimulate astrocytic glutamate release, leading to an overall amplification of extracellular glutamate (82). This astrocytic glutamate release can induce paroxysmal depolarization in neurons, which has been proposed to contribute to seizure propagation (83).

Finally, it is possible that the ictal or interictal increases in extracellular glutamate are due to changes in blood-brain barrier (BBB) permeability. BBB permeability is regulated by glutamatergic NMDA receptors (84) and permeability has been shown to increase in kainate-treated rats during seizures, coincident with the period of glutamate elevation (85). This suggests the possibility that elevated glutamate is a result of increased passage of its precursor, glutamine, through a leaky blood brain barrier.

Glutamate:

Under normal conditions astrocytes tightly regulate the concentration of synaptic and extracellular glutamate through transport-mediated uptake. Extracellular glutamate is usually maintained at a concentration of approximately 1-2 μM, but can reach up to 1 mM within the activated synapse (86). Dysregulation of extracellular glutamate probably increases susceptibility to seizures rather than provoking seizures directly. Chronic infusion of 1 mM glutamate in rats does not lead to electrographic changes, but an infusion of 200 μM glutamate before the convulsant picrotoxin lowers the seizure threshold and lengthens the seizure duration (87).

Transporter dysfunction may contribute to the abnormal extracellular glutamate concentrations seen in epilepsy. While histologic studies on glutamate transporters in human TLE have been inconclusive (88), possibly confounded by the degree of neuronal loss, genomic and proteomic analysis of resected human neocortical epileptogenic tissue has identified reduced glutamate transporter expression (89). Reductions in transporters have been noted in animal models, including dogs with idiopathic familial epilepsy (90) and in a rat chronic seizure model (91). Inhibition of glutamate transport expression in rats results in elevated extracellular glutamate and is associated with the development of epilepsy and neuronal degeneration (92), which has implicated a role for glutamate transporter dysfunction in the pathogenesis of epilepsy. Elevated glutamate may impair its own clearance, since glutamate promotes the formation of reactive oxygen species (ROS), which can further impede glutamate transporter function (93). Although it is not known if this is related to transporter dysfunction or perturbed intracellular processes, an abnormal bioenergetic state, as measured by levels of PCr/ATP on magnetic resonance

spectroscopy, is associated with elevated glutamate (35). Therefore, increased energy demand or decreased energy availability may promote excitability.

Excessive levels of glutamate are associated with neurotoxicity (94). Glutamate concentrations of 50-100 μM are toxic to neuronal cell cultures when applied for 5 minutes (95). However, a higher glutamate concentration is required for toxicity *in vivo* in the presence of intact reuptake mechanisms (96). Glutamatergic excitotoxicity has acute and chronic components. The acute phase, which is sodium-dependent, is characterized by neuronal swelling that can progress to cell rupture if unchecked. The chronic phase, which is calcium-mediated, is associated with mitochondrial dysfunction leading to impaired glutamate reuptake and free radical damage (94). Excitotoxicity is enhanced in states of metabolic stress, such as ischemia or hypoglycemia (97), but is diminished in immature brains, possibly because the cell death pathway is undeveloped (56). In a 4-aminopyridine rat model of epilepsy, the chronic elevation of glutamate for over 60 minutes has been demonstrated to result in an NMDA-receptor mediated neurodegeneration (98). In humans Cavus et al. have found that basal microdialysate glutamate levels greater than 5 μM are correlated with hippocampal atrophy, which may be evidence of glutamatergic neurotoxicity in human epilepsy (50). A similar glutamate concentration (5.8 μM) was indentified in the CSF of patients with ALS and found to be neurotoxic in cell culture (99).

Glutamine:

Although there is no evidence that glutamine participates in neuronal signaling, it is an important metabolic precursor of glutamate and GABA through the glutamate-glutamine and GABA-glutamine cycles (51). In neurons the enzyme glutaminase

converts glutamine to glutamate and ammonia. After being released from neurons, glutamate is transported from the synapse into astrocytes, where it is converted to glutamine by glutamine synthetase (GS). Glutamine enters neurons, where it is converted to glutamate to complete the cycle. In GABAergic neurons, glutamate is converted to GABA via the enzyme glutamic acid decarboxylase (GAD). The regulation of glutamate and glutamine is closely coupled to the oxidative metabolism of glucose (100).

The ratio of glutamine to glutamate is lower in the epileptogenic compared to non-epileptic hippocampus, where glutamate is increased while glutamine is unchanged. This was seen in both the extracellular (49) and intracellular (101) hippocampal glutamate-glutamine ratio. A low glutamine-glutamate ratio suggests that glutamate is not being adequately converted to glutamine, as a result of dysfunction in both glutamate transport and GS. There is evidence of decreased GS in hippocampi affected by MTLE (102), which is proposed to lead to glial accumulation of glutamate. Interruption of glutamate-glutamine cycling could also vesicular GABA content (103). The resulting increased glutamate and decreased GABA may explain why blockade of glutamine synthetase in rats induces seizures (104). The epileptic brain may also preferentially convert excess glutamine to glutamate instead of GABA, as has been shown in tissue slices from the rat neocortical undercut epilepsy model (105).

GABA:

As the primary inhibitory neurotransmitter, GABA is involved in modulating seizures. Animal models with reduced GABA, either due to deficient precursors or impaired GAD, are prone to seizures (106). However, blockade of GABA synthesis will not increase excitability without simultaneous network activation (107). Epileptic patients

have reduced brain tissue GABA measured by magnetic resonance spectroscopy (MRS) compared to controls, and this deficiency extends to sites remote from the seizure focus (107). Low levels of brain GABA on MRS in patients with complex partial seizures are associated with a greater propensity to seize (107, 108), and increases in GABA after the addition of AEDs predict better seizure control (109). This association may not apply to patients with idiopathic generalized epilepsy, who exhibit lower brain GABA than those with complex partial seizures (107), even when receiving optimal seizure control from their AEDs (108).

This reduction in GABA may be related to impaired transport. The population of GABAergic neurons is relatively intact in hippocampal sclerosis (110), and no significant changes in GAD, which synthesizes GABA, or GABA-transaminase, which catabolizes GABA, have been detected in the setting of chronic seizure activity (111). While GABA transporters usually remove GABA from the ECF, these transporters can operate in reverse, especially when intracellular GABA is elevated or under conditions of increased energy expenditure, such as seizures (112). Changes in GABA transporter function have been noted in humans with epilepsy and animal models. Microdialysis studies by During et al. in the hippocampi of patients with intractable TLE and in amygdala-kindled rats observed a reduction in transport-mediated glutamate-induced GABA release in both species, which was associated with reduced GABA transporter levels in the rat tissue (113). This may explain why their earlier-noted seizure-induced rise in extracellular GABA was greater in the contralateral than epileptogenic hippocampus (47). Forward and reverse transport of GABA is impaired in human tissue obtained from patients with mesial temporal sclerosis and in kainate-treated rats (114). Dysfunctional GABA

transport may diminish the compensatory increase in GABA in response to seizures, tipping the balance towards increased excitability and reduced inhibition.

Several lines of evidence also indicate that within the epileptic brain sites, GABA may promote enhanced excitability rather than inhibition. In the healthy adult brain, the inhibitory function of GABA is dependent on a negative reversal potential for Cl^-, which is regulated by the K+/Cl cotransporter (KCC2). However, addition of GABA to injured neurons in cell culture has been noted to induce outward Cl- flux and depolarization that could persist for two weeks (115). Decreased KCC2 function associated with a reversal in GABA potential and enhanced excitability has been also observed in rat hippocampal dentate granule cells after pilocarpine-induced status epilepticus (116), and may contribute to epileptogenesis.

AED effects on Glutamate, Glutamine, and GABA

Although antiepileptic drugs often have multiple effects, their activity is broadly mediated through three main mechanisms: blockade of voltage-gated sodium and calcium channels, which limits action-potential-related neuronal vesicular glutamate release; blockade of glutamate receptors; and potentiation of GABAergic inhibition, through increasing GABA availability or modulating GABA receptors (117, 118). While much is known about their actions on neurons and within the synapse, less is known about their effects on extracellular neurotransmitter levels, especially in humans.

MRS Studies on AED Effects

Due to its non-invasive nature, magnetic resonance spectroscopy (MRS) has been employed in a number of *in vivo* human and animal studies to measure the effects of

AEDs on brain tissue levels of glutamate, glutamine, and GABA. While this process does not discriminate between intracellular and extracellular neurochemicals, most of the neurochemicals measured reflect the intracellular concentration of the neurochemicals, due to the negligible volume of the extracellular space. Elevated intracellular glutamate on MRS has been observed in the surgically resected human epileptogenic hippocampi (119) and cortex (120). As mentioned earlier, low brain GABA on MRS is associated with increased seizure frequency, presumably reflective of decreased inhibitory tone (107). An MRS study by Petroff and coworkers found increased glutamate or decreased GABA in 9 of our 14 patients with refractory epilepsy (121), suggesting that high brain glutamate and low brain GABA on MRS may be markers of poor response to AEDs. Effects of several AEDs on brain glutamate and GABA levels has also been examined using MRS. While phenobarbital (PB) and primidone (PMD) reduced the glutamate levels in epileptic patients, carbamazepine (CBZ), phenytoin (PHT), and gabapentin (GBP) had no effect (122). On the other hand, topiramate (TPM) (123), GBP (124), and vigabatrin (VGB) (125) increased the GABA levels in epileptic patients. TPM, GBP, and lamotrigine (LTG) also increase brain GABA levels in healthy subjects (126).

AED effects measured by MRS may differ between humans and animal models. In a rat forebrain MRS study, GBP and pregabalin (PGB) decreased brain glutamate but did not affect brain GABA (127), while in humans GBP increased GABA (124, 126) but did not affect glutamate (122). An MRS evaluation of human and rat neocortical slices treated with GBP and PGB showed that PGB increased GABA in both the human and rat slices while GBP raised GABA only in the human tissue (128). It is important to emphasize that MRS results may not be analogous to microdialysis. While MRS results

reflect mostly the intracellular levels of the measured substrate, microdialysis samples the extracellular, and even more specifically, the extrasynaptic space. Therefore results obtained with one method can not be extrapolated to the other method. For example, gabapentin has not been shown to increase extracellular basal or stimulated GABA in the rat substantia nigra (129) even though it raises intracellular GABA.

Microdialysis Studies of AED Effects in Animal Models

Effects on Evoked Neurotransmitter Release

The effects of AEDs on both the extracellular basal (interictal) and evoked (seizure-induced) levels of glutamate and GABA have been examined with microdialysis in rodent models in multiple studies, with some conflicting results. The effects of each medication on microdialysate glutamate and GABA concentrations are summarized by study for evoked conditions in Table 1 and for basal conditions in Table 2. The notable findings are summarized below. Multiple medications, including CBZ, diazepam (DZP), LTG, levetiracetam (LEV), PB, PHT, TPM, valproate (VPA), and zonisamide (ZNS), attenuated the seizure-induced rise in glutamate, which may reflect their effectiveness as anticonvulsants. With the exception of VGB and VPA, both of which inhibit GABA degradation, these medications also decreased the seizure-associated rise in GABA. Although many mechanisms overlap, CBZ, LTG, PHT, TPM, VPA, and ZNS block voltage-depended sodium channels; LEV inhibits release of synaptic vesicles; and DZP, PB, and TPM enhance chloride influx at GABA receptors (117, 118, 130), which may explain their reductions in presynaptic glutamate and GABA release.

Studies by different research groups on the same AED may have contradictory results that may be related to the animal model used, the relationship of medication administration to seizure, and the timing of microdialysate sample collection. For example, while one group (131) found no change in veratridine-evoked glutamate release after VPA administration, others (67, 132) observed a decrease in electroshock- and pilocarpine-evoked glutamate. The anatomical location sampled by the microdialysis probe may also affect the results. For example, CBZ, LTG, and oxcarbazepine (OXC) reduced evoked glutamate in the rat cortex but had no effect in the striatum (133).

Effects on Basal Neurotransmitter Levels

The effect of AEDs on basal extracellular glutamate and GABA levels is less consistent (Table 2). Multiple medications, including CBZ, DZP, and PHT, that have been demonstrated to diminish evoked neurotransmitter release appear to have no effect on basal levels. Inactivation of sodium channels by CBZ and PHT is use-dependent (130) and the reduction in presynaptic glutamate release by DZP may be depolarization-related (134). Even those AEDs that share a similar mechanism of action may have different effects on extracellular neurotransmitters. Ahmad et al. found that CBZ, LTG, and PHT affected basal and evoked glutamate and GABA differently, even though all three AEDs block sodium channels, which may suggest that their antiepileptic properties are mediated by other unknown mechanisms (135). Another possibility, suggested by Yoshida and Okada, is that the basal and evoked release of extracellular neurotransmitters are modulated by two different voltage-sensitive calcium channel complexes, which may explain why ZNS increases basal GABA but blocks evoked glutamate and GABA (136, 137).

TGB, VPA, VGB, and ZNS increase basal GABA, which may reflect their mechanisms of action on GABA reuptake and degradation. TGB inhibits GABA uptake; VPA increases GABA synthesis and decreased its metabolism; VGB prevents GABA degradation; and ZNS increases GABA release (118). TPM, VPA and VGB can either increase or decrease basal glutamate depending on the dosage, anatomical site, and animal model studied. This may be related to the disinhibition of feedback control of glutamate levels (138).

It is important to note that findings from animal models may not apply to humans and that multiple factors, such as dose and duration of administration, may modify drug activity. Different effects may occur at different doses. Peritoneal administration of VPA into rats at 100 mg/kg, which approximates the dose in humans, reduces hippocampal basal extracellular GABA by half, while 400 mg/kg valproate doubles it (139). The duration of AED administration may affect outcome, which is important because, in humans these medications are intended for chronic use. After chronic administration, LTG was less effective in reducing basal glutamate but more effective in attenuating the evoked glutamate increase in the rat hippocampus than when given acutely (140). Another concern in humans is the effect of multiple AEDs, which is especially relevant for the medication-refractory population, and several studies have addressed this in animals. Acute co-administration of VPA with LTG was more effective in decreasing basal glutamate and increasing basal GABA in the rat hippocampus then either medication alone (131). In some cases, the synergistic effects are different from the individual medication effects. The addition of a non-anticonvulsant dose of FBM to LTG

in mice increased whole-brain GABA and decreased evoked seizure activity, even though LTG alone decreased whole-brain GABA (141).

Table 1: Rat microdialysis studies of AED effects on evoked extracellular glutamate and GABA.

AED	Dose (mg/kg)	Animal Model	Probe Site	GLU	GABA	Reference
CBZ	5	Pilocarpine	Hipp	↓	↓	(142)
CBZ	10-20	Veratridine	Hipp	↔	↓	(135)
CBZ	30	Veratridine	Hipp	↓	N/A	(133)
DZP	5	Pilocarpine	Hipp	↓	↔	(134)
GBP	100	K+	S. N.	N/A	↔	(129)
LEV	100	K+, $FeCl_3$	Hipp	↓	N/A	(143)
LTG	10	Pilocarpine	Hipp	↓	↓	(64)
LTG	15	Veratridine	Cortex	↓	N/A	(133)
LTG	10-20	Veratridine	Hipp	↓	↓	(135)
LTG+ VPA	10+300	Veratridine	Hipp	↔	↑	(131)
OXC	60	Veratridine	Cortex	↓	N/A	(133)
PB	15	Pilocarpine	Hipp	↓	↓	(142)
PHT	20-40	Veratridine	Hipp	↔	↔	(135)
PHT	20	Max E-shock	Hipp	↓	↔	(67)
TPM	200	Pilocarpine	Hipp	↓	↓	(144)
VPA	300	Veratridine	Hipp	↔	↑	(132)
VPA	400	Pilocarpine	Hipp	↓	↓	(132)
VPA	400	Max E-shock	Hipp	↓	↑	(67)
VGB	30	Pilocarpine	Hipp	↓	↑	(145)
ZNS	N/A	K+	Hipp	↓	↓	(137)

AEDs are generally administered intraperitoneally or orally, except for N/A doses, which are infused hippocampally. Abbreviations: CBZ, carbamazepine; DZP, diazepam; GBP, gabapentin; LEV, levetiracetam; LTG, lamotrigine; Max E-shock, maximal electroshock; PB, phenobarbital, PHT , phenytoin; S. N., substantia nigra; TPM, topiramate; VPA, valproic acid; VGB, vigabatrin, ZNS, zonisamide.

Table 2: Rat microdialysis studies of AED effects on basal glutamate and GABA.

AED	Dose (mg/kg)	Animal Model	MD Probe Site	GLU	GABA	Reference
CBZ	10-20	Normal	Hipp	↔	↔	(135)
DZP	5	Normal	Hipp	↔	↔	(134)
GBP	100	Normal	S. N.	N/A	↔	(129)
LEV	40, 80	Normal	Hipp, Frontal CTX	↔	↔	(146)
LTG	10	Normal	Hipp	↔	↔	(64)
LTG	20	Normal	Hipp	↓	↔	(135)
LTG+ VPA	10+300	Normal	Hipp	↓	↑	(131)
PB	15	Normal	Hipp	↔	↔	(142)
PHT	20-40	Normal	Hipp	↔	↔	(135)
PHT	20	Normal	Hipp	↔	↔	(67)
TGB	11.5, 21	Normal	Basal ganglia	N/A	↑	(147)
TPM	200	Normal	Hipp	↑	↓	(144)
TPM	40	Normal	Hipp	↔	N/A	(148)
TPM	40	SER	Hipp	↓	N/A	(148)
VPA	300	Normal	Hipp	↔	↑	(131)
VPA	400	Normal	Hipp	↑	↔	(132)
VPA	400	Normal	Hipp	↔	↑	(67)
VPA	400	Normal	Hipp	↔	↑	(139)
VGB	N/A	Normal	Striatum	↑	↑	(138)
VGB	30	Normal	Hipp	↓	↔	(145)
VGB	1000	Normal	Frontal CTX	N/A	↑	(149)
VGB	1000	Normal	Hipp	N/A	↔	(149)
ZNS	N/A	Normal	Hipp	↔	↑	(137)

AEDs are generally administered intraperitoneally, except for N/A doses, which indicates hippocampal or striatal infusion. "Normal" model indicates rat not otherwise predisposed to epilepsy before convulsant administration. Abbreviations: CBZ, carbamazepine; CTX, cortex; DZP, diazepam; GBP, gabapentin; LTG, lamotrigine; PB, phenobarbital, PHT , phenytoin; SER, spontaneously epileptic rat; S.N., substantia nigra; TGB, tiagibine; TPM, topiramate; VPA, valproic acid; VGB, vigabatrin, ZNS, zonisamide.

In Vitro Studies on the AED Effects of Neurotransmitter Release

AED effects on neurons have also been explored *in vitro* in synaptosomes and brain tissue slices. Synaptosomes may accurately reflect AED effects on neurotransmitter release, but may not be comparable to microdialysis results, since the degree of synaptic contribution to the neurotransmitter levels in the ECF is disputed (73). CBZ, PHT, LTG, and OXC, which block sodium channels, reduce glutamate release in isolated veratridine-stimulated nerve endings, while TPM and PB had minimal effects on glutamate (150, 151). Under resting conditions, CBZ, PHT, LTG, OXC, and TPM did not affect basal glutamate release from synaptosomes (151). VPA increased GABA levels in synaptosomes and tissue slices (152), similar to its findings in microdialysis studies. In rat entorhinal cortex slices, LTG (153) and PHT (154) decreased basal glutamate and increased basal GABA release. However in rat amygdala slices, LTG at similar concentrations slices *decreased* spontaneous and evoked GABA release (155). Analysis of whole-brain homogenate, which is similar in principle to MRS, may also not accurately predict microdialysis results. TPM (156) and LEV (157) administration had no effect on whole brain glutamate or GABA in rats.

AED Effects on Neurotransmitter Transport and Metabolism

In addition to their direct action on neuronal transmitter release, AEDs may also exert their effects through regulation of neurotransmitter transport and synthesis. LEV (143), VPA (158), ZNS (159) have been shown to upregulate glutamate transporters; for LEV this upregulation has been shown to be associated with a reduction in evoked extracellular glutamate (143). While LEV has been reported to upregulate the GABA

transporter GAT-3 (143), VPA (158) and ZNS (159) were reported to downregulate GABA transporters. Although GABA transporter regulation was not directly correlated with levels of extracellular GABA in those studies, there is evidence that VPA (67, 131, 139) and ZNS (137) increase basal GABA. Mechanisms underlying GABA increases vary by AED: TGB inhibits GABA reuptake (160), VGB blocks the degradative enzyme GABA-transaminase (161), and VPA stimulates GABA synthesis while decreasing catabolism (162).

AEDs also affect glutamine metabolism, either by increasing precursor or interacting with synthetic enzymes. This is important, because glutamine is in balance with glutamate and GABA, through the pathways discussed earlier. TPM (163), VPA (121) and VGB (164) increase brain glutamine in human *in vivo* MRS studies. In the case of VPA, the increase in whole-brain glutamine may result from drug-induced hyperammonemia, due to disruption of the urea cycle, which stimulates GS and inhibits glutaminase in the brain (165). However, a rat microdialysis study did not find any change in glutamine after VPA administration (139), which suggests either that rodents respond differently to VPA or that the intracellular increase in glutamine seen on MRS is not detected extracellularly. Other causes of increased glutamine may include normalization of glutamate-glutamine cycling (164) or changes in GABAergic neurotransmission (163). Finally, some drugs affect GS directly. Chronic administration of PHT, CBZ, PB, FBM, and TPM in mice has been shown to reduce GS activity, while VPA, VGB, LTG, GBP, TGB, and LEV were not found to affect GS (166).

Summary of AED effects:

The effects of antiepileptic drugs on glutamate and GABA have been explored through MRS in humans, microdialysis in rats, and in isolated synaptosomes. The findings of each of these methods may be different from microdialysis in humans: MRS assesses whole-brain concentrations, which are predominantly intracellular; animal models may poorly approximate results in humans; and synaptically-released neurotransmitter may not be a significant constituent of microdialysate. Also, it is important to recognize that drug effects on neurochemicals during seizures may be different from drug effects under basal conditions. For example, CBZ and PHT, which block sodium channels, suppress evoked glutamate and GABA in rats (67, 142), but do not decrease intracellular glutamate in humans (122) or reduce basal glutamate in synaptosomes (151) or in animal microdialysis (135). Medications that increase GABA appear to have consistent effect across different studies. VGB is shown to increase GABA in human MRS (125), synaptosomes (167), and in rat evoked (145) and basal (149) microdialysis studies. VPA increases GABA release in synaptosomes (152) and basal and evoked rat microdialysis (67), but did not have an effect on whole-brain GABA in a human MRS study (121). It may also be possible that AEDs mediate their effects through other mechanisms that have not been previously explored, such as decreasing the excitability of astrocytes (83).

AED Distribution and Resistance

Microdialysis studies on AED Levels in the Brain

In addition to detecting changes in neurotransmitter concentrations, microdialysis can be also used to measure the levels of the AEDs. Since microdialysis measures the concentration of free, unbound AED in the brain, it assesses the fraction of whole-brain drug that is functionally significant (168). Drug concentrations are often estimated by their levels in serum or cerebrospinal fluid (CSF), but these do not always reliably predict ECF drug levels, because the blood-CSF barrier is often more permeable that the blood-brain barrier (169). Early microdialysis studies in a limited number of subjects with drug-refractory epilepsy reported that the ECF concentrations of CBZ (170) and PHT (171) in the hippocampus reflected their unbound serum concentrations. However, a study in eight patients with head injury found that although in individual patients the serum PHT concentration correlated with the concentration in the ECF, this ratio was unique to each patient, and there was no universal ratio between serum and ECF concentrations (172). In humans with medically refractory epilepsy, the AED concentrations in the ECF have been shown to be lower than their respective concentrations in CSF and in the resected brain tissue (173). In animals, the ECF concentrations of LTG (169), PHT (174), and TGB (175) follow different kinetics than their serum concentrations, often enabling greater stability in their brain compared to serum concentrations.

Within the brain ECF, drug concentrations may differ between brain regions and even within the same region. Some AEDs, such as LTG (169), TGB (175), and LEV (146) are evenly distributed between frontal cortex and hippocampus in rats. However, PHT concentrations are higher in the hippocampus (176), and VGB is higher in the

frontal cortex (149). This may be due to differences in white matter content, regional blood flow, and drug transporter expression. The differences in concentration may affect the effects of AEDs on target neurotransmitters. In VGB-treated rats basal GABA increased five-fold in the frontal cortex but was unchanged in the hippocampus, reflecting their respective drug concentrations in these regions (149). Rambeck and colleagues intraoperatively examined the concentrations of CBZ, OXC, LTG, LEV, TPM, and PHT in the middle temporal gyrus of refractory epilepsy patients and found that concentrations could differ markedly within the same patient, even though the MD probes were located 0.6 cm apart (173). Although differences in probe fractional recoveries may account for these findings, these data may also suggest evidence for influence of drug efflux transporters on AED concentration.

Drug Efflux Transporters

The most commonly studied efflux transporters, P-glycoprotein (P-gp) and the multidrug resistance proteins (MRPs), are members of the adenosine triphosphate-binding cassette (ABC) family of transporters and are expressed heavily in excretory organs and in blood-tissue barriers, including the blood-CSF and blood-brain barriers (14, 177). These transporters have been shown to be over-expressed in epileptogenic tissues, such as in the hippocampal sclerosis (178, 179), focal cortical dysplasia (179, 180), and cortical tubers (181). It has been suggested that increased expression of drug transporters in epileptic regions may confer a cell survival advantage in the setting of impaired homeostasis, since apoptosis is rarer in epilepsy than would be expected (182).

It is unclear if the increased drug transporters are constitutive or are induced by drugs or seizures. While administration of some AEDs is associated with efflux

transporter induction in human cell lines (183) and human duodenum (184), no increases have been observed in non-epileptic rats treated with PB (185), PHT (185, 186), or CBZ (186). Increases in efflux transporters, however, have been noted after evoked seizures in rats (187, 188) and in human brain following a fatal attack of status epilepticus (189). Interestingly, in the human post-mortem study, efflux transporters were upregulated in the histologically normal brain as well as dysplastic focus (189). The seizure-induced expression of P-gp may be directly related to increased extracellular glutamate. Glutamate applied directly to isolated rat brain capillary endothelial cells has been shown to increase expression of P-gp (190), and blockade of glutamate receptors prevents *in vivo* P-gp accumulation in rat brain capillary endothelial cells after pilocarpine-induced seizures (191). P-gp has been shown to mediate glutamate efflux through the BBB in rats (192), and it is possible that the increased expression of P-gp is a defense against glutamatergic neurotoxicity, although its role in regulating extracellular glutamate in humans with epilepsy is unknown.

The therapeutic implications of these upregulations in P-gp and MRPs are currently under investigation. Neuroectodermal cell lines expressing *MDR1* have decreased intracellular phenytoin compared to MDR1-negative cells, suggesting that the overexpression of efflux transporters in epileptogenic tissue may prevent adequate AED concentration (178). In animal studies the effect of efflux transporters on AED levels by pharmacologic transporter blockade with verapamil or nimodipine for P-gp and with probenecid for MRP. Using these methods, inhibition of P-glycoprotein is associated with reduced brain ECF levels of CBZ, LTG, FBM, OXC, PB, and PHT in rats (193-196). LEV (197) and VPA (198) do not appear to be regulated by P-gp or MRPs in rats.

However, one must be cautious in applying animal data to humans. It has been shown that kidney cells transfected by human *MDR1* and *MRP2* are significantly less effective in transporting AEDs than those transfected by their rat counterparts, although this difference has not been examined in brain tissue (199). On the other hand, there is evidence in humans of improvement of refractory epilepsy after the addition of verapamil to the existing AED regimen (200, 201), although this may reflect an intrinsic antiepileptic property of verapamil-mediated calcium blockade rather than efflux transporter disruption. If efflux transporters are involved in AED medication refractoriness, it may explain why resistance to one antiepileptic drug is associated with resistance to others (202). Unfortunately, this will be difficult to prove in humans because microdialysis and tissue studies are conducted exclusively in drug-refractory patients, making it impossible to make comparisons with normal controls and patients with drug-responsive epilepsy (173).

Statement of Purpose

The technique of microdialysis in conscious human subjects undergoing intracranial EEG monitoring has made it possible to explore the neurochemical substrates (i.e. glutamate, glutamine, and GABA) of drug-resistant epilepsy, both ictally and interictally, and to characterize the differences between epileptic and non-epileptic brain regions. To date these extracellular neurochemicals have not been evaluated in regions of seizure propagation, MRI-identified lesions, and patients with non-localizable seizure onset. In addition the effect of antiepileptic medications on the extracellular levels of these neurochemicals has not been previously considered in humans. Due to the poor response of some epilepsies to treatment (2) and the significant costs associated with medication-resistance (4, 5), it is essential to better understand the neurochemistry and response to treatment of medication-refractory epilepsy. Since epileptogenesis may be distributed beyond the seizure onset site (22, 23) and electrographic (25) and metabolic abnormalities (203) distant from the seizure focus predict poor surgical outcome, it is important to study the extracellular milieu of the seizure propagation pathways. Although microdialysis studies on AED effects on glutamate and GABA have been conducted in animals, several of these studies report contradictory results and findings in animals may not apply to humans. Glutamate increases occur with seizures (47, 48) and elevated basal glutamate is associated with hippocampal atrophy (50), which implies that an understanding of the medication effects may guide further treatments directed specifically at extracellular glutamate (204).

This thesis will first explore the differences between epileptic, propagated, and non-epileptic hippocampus, cortex, and lesions with regard to the basal extracellular

levels of glutamate, glutamine, and GABA building on previous work by Cavus and colleagues that demonstrated a difference in glutamate between the epileptogenic and non-epileptic cortex and hippocampus (49). It is hypothesized that basal glutamate will be elevated in epileptogenic, non-localized, and lesion sites due to their association with seizure onset. Propagated sites, though exposed to frequent epileptic activity, presumably have intact glutamate regulation and are postulated to have only mild elevations in glutamate. If basal GABA levels reflect a response to frequent seizure activity, GABA would be expected to be most elevated in the propagated sites, which are involved in seizure spread after initiation elsewhere. GABA may be less elevated in epileptogenic sites, non-localized sites, and lesions, since the GABAergic response to frequent seizure activity may be impaired in these regions. Finally, GABA may be low in non-epileptic sites due to their lack of electrical activity and, presumably, of underlying pathology.

Finally, the effects of anti-epileptic drugs on these neurotransmitter levels will be explored by comparing the basal levels of glutamate, glutamine, and GABA, when the patients are their full or near-full dose of AEDS and after the AEDs are tapered, as per the inpatient monitoring protocol. It is hypothesized that medication taper will induce an increase in basal glutamate and a decrease in basal GABA, since many of these medications exert their anti-epileptic effects by reducing excitatory and enhancing inhibitory tone. If AEDs promote the production of glutamine over glutamate, then AED withdrawal may be expected to decrease basal glutamine. However, since all of our subjects have medically refractory epilepsy, in which the effects of AEDs are presumably impaired, then the taper effect may be less pronounced than expected and vary by site.

Methods

Patient Selection

All subjects were patients with medication-resistant complex partial seizures undergoing intracranial electroencephalographic evaluation for identification of their seizure focus prior to consideration for resective surgery. By this point these patients had already completed the less invasive stages of the three-phase workup algorithm (205). In Phase I, patients were evaluated as inpatients in the epilepsy unit via continuous audio-visual (AV) recording in concert with scalp EEG. These recordings were frequently accompanied by magnetic resonance imaging (MRI), ictal and interictal cerebral blood-flow evaluation with single-photon emission computed tomography (SPECT), cerebral metabolic assessment with positron emission tomography (PET), and neuropsychological evaluations in order to non-invasively identify the seizure focus. Phase II consisted of the intracarotid sodium amobarbital procedure, or Wada test, to identify the dominant hemispheres for language and memory in preparation for resective surgery. Those patients in whom a seizure focus could not be identified or for whom the collected data were inconsistent with regard to seizure onset localization were considered for a Phase III study, which consists of continuous AV-icEEG recording obtained from intracranial electrodes (IC) through a combination of subdural strip, grid, and depth electrodes implanted in regions of suspected seizure onset. Patients undergoing Phase III evaluation between 1998 and the present have been invited for participation in the microdialysis research protocols, as approved by the Yale Human Investigations Committee in compliance with the Declaration of Helsinki. Brain microdialysis data were collected only from consented subjects. Data for the basal neurochemical comparisons in the

hippocampus and cortex were collected between 1998 and 2008, corresponding to the period of neurochemical concentration analysis by HPLC. Data for the AED taper comparisons were collected between 2002 and 2008.

Microdialysis probes and procedures

Patients were implanted with microdialysis probes coupled to depth electrodes (Spencer probe, Ad-Tech Instrument Co, Racine, WI) under stereotactic guidance (BrainLab, Westchester, IL) in sites of suspected seizure onset, which include cortex, hippocampus, and lesions. All surgeries were performed by Drs. Dennis Spencer, Kenneth Vives, and members of the Yale Department of Neurosurgery. Two types of microdialysis probes have been used. Prior to 2003, the Spencer probe was employed, which consisted of a 10 mm membrane of 0.3 mm diameter with a 5 kDa cutoff, attached with epoxy adhesive to a flexible depth electrode. See (47, 49, 68) for details. More recently, a modified CMA/20 probe (CMA, North Chelmsford, MA) of 70 mm probe length with a 10 mm membrane of 0.67 mm diameter with 20 kDa cutoff has been used. See (50) for details. The CMA probe was inserted into the depth electrode (total diameter = 1.85 mm) and fluid exchange occurred through perforations located between electrical contacts 1 and 2. The location of each the probe was confirmed by a postoperative co-registered CT/MRI.

Following implantation surgery and 1-2 days post-op recovery in the intensive care unit, subjects were transferred to the epilepsy unit on their full outpatient AED dose for AV-icEEG monitoring. AED taper was then initiated in order to allow for the occurrence of spontaneous seizures. The nature and rate of the AED taper was dictated by

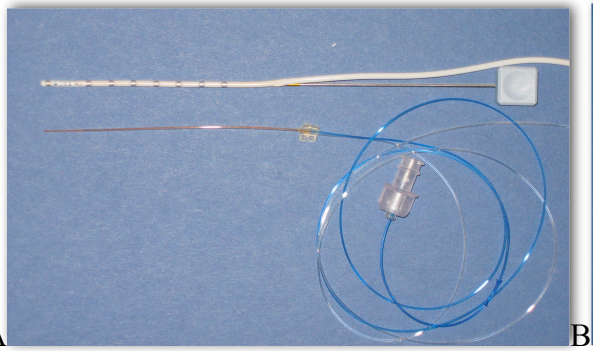
clinical needs. In order to avoid triggering atypical withdrawal seizures, benzodiazepines and barbiturates were not tapered (30). Usually, satisfactory icEEG data were obtained for sufficient seizure localization over 1-2 weeks. AEDs were then increased to their pre-op dose, and, in a subsequent surgery, all intracranial electrodes were removed. Usually resective surgery (Phase IV) was performed in another scheduled session.

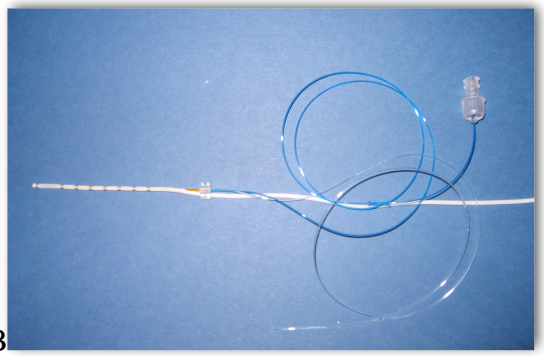
Classification of microdialysis probe sites:

Probes were classified according to their location in the *hippocampus* and *cortex* by the post-operative CT-MRI. Probes located in the thalamus and basal ganglia were excluded due to the lack of a sufficient number of similar probes for a group analysis. Following our microdialysis study group's methodology of averaging data from probes located in the same structure (50), which reflects the surgical convention of resecting the entire diseased hippocampus or lesion whenever possible, data from probes located in the same hippocampus or lesion with the same electrographic properties (i.e. non-epileptic, epileptogenic, and propagated) were averaged.

The epileptogenicity of the region sampled by the microdialysis probe coupled to a depth electrode was determined based on the conclusions of the Epilepsy Surgery Program conference, with decisions rendered by a consensus of epileptologists independent from the microdialysis program, incorporating all available clinical, icEEG, imaging and neuropsychological data. Probes were classified as *epileptogenic* if the seizure originated from either contact 1 or 2 of the depth electrode flanking the microdialysis membrane, *propagated* if the site was immediately involved after seizure onset by demonstrating abnormal rhythmic epileptiform activity before seizure

generalization, and *non-epileptic* if the site was not involved in seizure onset or any stage of propagation. If a single seizure onset could not be determined and patient was deemed to be non-localizable, then all probes for that patient were classified as *non-localized*. Cortical probes located in non-neoplastic structural abnormalities identified on MRI were classified as *lesion*, which included dysplasias, heterotopias, and cortical tubers. Six patients with tumors (astrocytoma or ganglioneuroma) were excluded. Probes of questionable anatomic location or electrical activity were excluded. Demographic and patient history data were obtained from a chart review and structured patient interview.

A 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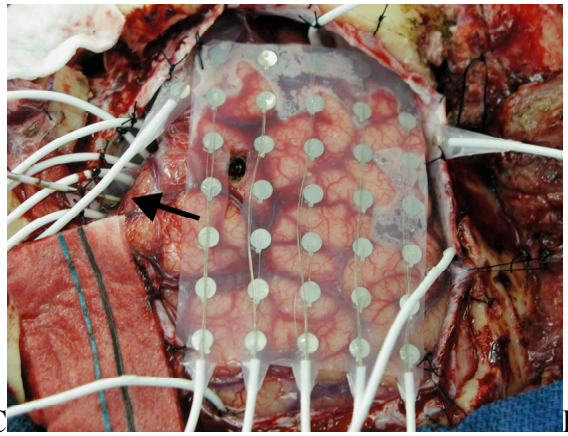
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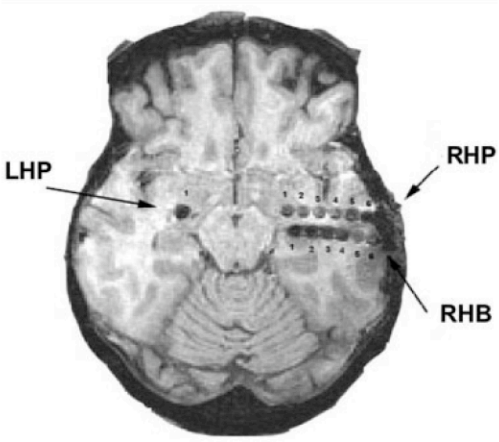

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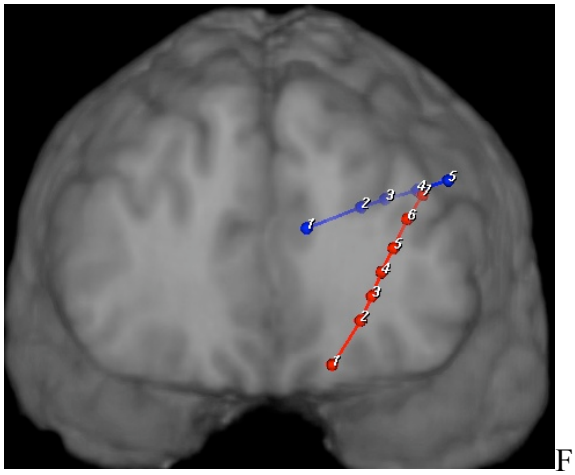

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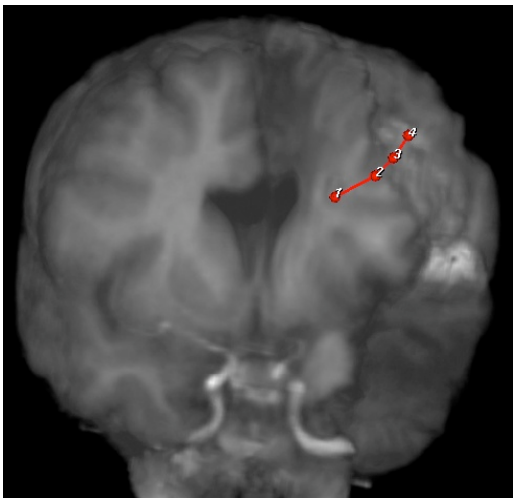

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Figure 1: Microdialysis probes and their implantation. A) Spencer depth electrode beside a modified CMA/20 microdialysis catheter (CMA, North Chelmsford, MA). **B)** Spencer depth electrode with the microdialysis catheter inserted as arranged prior to implantation. **C)** Intraoperative view with subdural grid, strip, and depth electrodes. The microdialysis catheter is inserted in the depth electrode marked by the black arrow. **D)** An MRI reconstruction showing bilateral hippocampal depth electrodes. The microdialysis membrane is located between the first two electrical contacts. Data from the two probes in the right hippocampus would be averaged. **E)** Cortical probes in the left orbitofrontal and cingulate cortices. F) Microdialysis probe within a lesion: here, in cortical dysplasia adjacent to a previous resection.

Zero-flow Microdialysis Method

All basal samples were obtained via the zero-flow quantitative microdialysis method, which was used to estimate the true basal neurotransmitter concentrations in the extracellular space (40, 42). To avoid any confounding effects due to blood-brain barrier disruption from implantation, anesthetics, circadian rhythm, and seizures, the first zero-flow collection was conducted 2-4 days after probe implantation in the afternoon while patients were quietly resting, at least 4-6 hours from any seizure activity. In many recent patients (since 2002), multiple basal collections have been performed during the same inpatient stay, often 2-3 days apart, under the same conditions. Sterile artificial cerebrospinal fluid (135 nM NaCl, 3 mM KCl, 1 mM $MgSO_4*7H_2O$, 1.2 mM $CaCl_2*2H_2O$ in 1 mM sodium phosphate buffer at pH 7.4) was infused using CMA107 syringe pumps (CMA, North Chelmsford, MA). After allowing for equilibration between flow rate changes, two samples were obtained each at 2.0, 1.0, 0.5, and 0.2 μL/min flow rates. The collected dialysate samples were stored over dry ice and then in a -80 ºC freezer for future high-performance liquid chromatography (HPLC) analysis. Basal levels were determined using regression analysis of the concentrations obtained at the different flow rates with a 2^{nd} order polynomial fit and extrapolation to a flow rate of zero. Data

from 53 out of a total of 89 included subjects were collected over a period of 6 years using the zero flow method. It has been demonstrated that microdialysis collection at a very low flow rate of 0.2 μL/min reliably allows for 80% recovery of the concentration of substrates estimated with the zero flow method (40, 42). Because the zero-flow method is very cumbersome, often taking 6 hours to complete, in more recent studies (since May 2004) involving 36 of 89 included subjects, this very low-flow microdialysis method was adopted. For this technique one 0.2 μL/min sample was collected over 1.5-2 hours, and, following HPLC analysis, the obtained value was corrected for 80% recovery to determine the basal concentration. Microdialysate was collected by M. Cassaday, D. Ocame, S. Forselius, W. Kassoff, G. Widi, and myself.

For the first analysis, which compared non-epileptic, epileptic, propagated, lesion, and non-localized probes within the hippocampus and cortex, only the first basal microdialysis collection (on the full AED dose) was used for each patient. The second analysis, which examined the effects of medication taper, was restricted to the subset of patients who had at least two basal collections. The first basal collection (B_1) was collected while on the full dose AED dose, while the second basal collection (B_{Tap}) was collected after the medications were tapered. For our analysis, we chose the basal collection closest to the point of maximum drug taper to be B_{Tap} and required that there had been a 50% reduction in at least one AED from the dose at B_1 (the full outpatient dose) within the 24 hours preceding collection. Medication status was verified by a chart review of daily inpatient medications and, if available, blood medication levels. While the actual AEDs and dose reductions involved in the taper differed between patients, clinically, the taper period was frequently related to increased seizure activity.

Neurochemical Concentration Determination

Glutamate and glutamine were analyzed by high-performance liquid chromatography (HPLC) (49). One μL of sample was mixed with 9 μL aminoadipic acid (AAA) internal standard and derivatized by adding 20 μL of O-phthaldialdehyde. Twenty μL of the derivatized sample was then injected into the HPLC column (3 m Phase II ODS column, 3.2 X 100 mm cartridge, Bioanalytic Systems, Inc., W. Lafayette, IN). The mobile phase was comprised of 0.1 M acetic acid (pH 6.0) with acetonitrile gradient from 12 to 20% flowing at 1 mL/min. The fluorescence detector (Shimadzu Scientific Instruments, Columbia, MD) was set with excitation wavelength 338 nm and emission wavelength 425 nm. Peaks occured at approximately 6.9 minutes for glutamate, 7.7 minutes for AAA, and 8.8 minutes for glutamine (Figure 2). Sensitivity limits were 0.1 M for glutamate and 10 M for glutamine, based on a signal to noise ratio of 10:1. The concentrations of amino acids were calculated by comparing their peaks to the external standard using EZCHROME elite software (Scientific Software, Inc., Pleasanton, OH).

GABA was analyzed on a separate system (Bioanalytic Systems, Inc., Indianapolis, IN) (50). 2 μL dialysate was mixed with 4 μL internal standard (2-amino-n-valeric acid) and derivatized with 13 μL O-phthaldialdehyde. This mixture was then injected into a column (BAS, ODS column, Phase II 100 X 3.2 mm 3 μm) with mobile phase of 0.1 M acetic acid (pH 4.85) in 37% acetonitrile solution flowing at 0.8 mL/min. Peaks were detected electrochemically (ESA, Chelmsford, MA, USA) at 43 mV with sensitivity limit 8 nM. The concentration was determined by comparing neurochemical peaks to the standard using EZCHROME elite software. HPLC analysis was performed by M. Cassaday, D. Ocame, G. Widi, and myself.

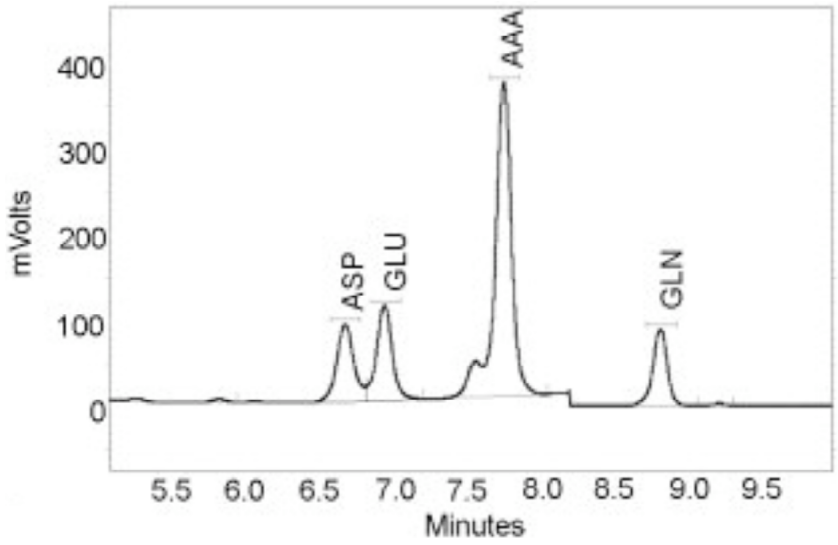


Figure 2: High performance liquid chromatogram showing peaks for aspartate, glutamate, α-aminoadipic acid (the internal standard) and glutamine (from Cavus et al., 2005).

Statistical Analysis

The concentrations for all three neurochemicals measured were positively skewed; therefore these values were-log-transformed to normalize them for statistical analysis. All statistics were calculated on the log-transformed data using SAS (SAS Institute, Cary, NC). Since many subjects had multiple microdialysis probes, it was necessary to determine whether there was a significant within-patient effect on the measured concentrations. For the purposes of this analysis, each intracranial study (n = 89), rather than individual patient (n = 81), was treated as a separate subject, because repeat intracranial studies were conducted months or years after the initial study under different sampling conditions (including time since last seizure, AED dose, background brain activity, etc.). The log-transformed data were modeled with a two-level nested data structure by probes within subjects. Between-subject and between-probe variations were

modeled using appropriate variance and covariance components. For the first analysis, the log-transformed concentrations of glutamate, glutamine, and GABA from the first basal collection were compared by group within the hippocampus and cortex using hierarchical linear modeling. Significance was set at $p < 0.05$. If there was a significant difference between groups, comparisons between groups were made by multiple t-tests with a Bonferroni correction for significance (3 comparisons in the hippocampus and 10 comparisons in the cortex). P-values are reported in their Bonferroni-adjusted forms (i.e. multiplied by the number of comparisons), where appropriate. Results are reported as arithmetic mean ± standard error of the mean (SEM).

The concentrations of glutamate, glutamine, and GABA were compared between the hippocampus and cortex within the three shared groups (non-epileptic, epileptic, propagated) by paired 2-tailed t-tests, and if there were no significant differences, the probes from the hippocampus and cortex were combined within each group for the medication taper analysis.

For the medication taper analysis, log-transformed data were modeled using the same two-level nested data structure by probes within subjects. This probes-within-subjects effect was found not to be significant. The log-transformed concentrations of glutamate, glutamine, and GABA were compared between B_1 and B_{Tap} within each probe category by a paired t-test with a Bonferroni correction for 5 comparisons to determine if there was a significant change in concentration with taper. Then, the difference in log-transformed concentration between B_{Tap} and B_1 was compared between probe categories by fixed-effects ANOVA, to determine if there was an interaction of category with the main effect of medication taper (i.e. if different probe categories responded differently to

taper). If the ANOVA indicated a significant interaction for given neurochemical, the log-transformed changes in concentration were compared between categories by a post-hoc Tukey's test.

Results

I. Subjects and Microdialysis Probes

Altogether, 91 patients implanted with 202 microdialysis probes were studied between October 1998 and December 2007. In accordance with our predefined exclusion criteria, 37 probes were excluded from the final analysis: 14 due to their location outside of the hippocampus or cortex (amygdala, n=5; thalamus, n=3; basal ganglia, n=2; ventricle, n=2; corpus callosum, n=1; and internal capsule, n=1), 8 due to their location in patients with tumors, 6 with missing neurochemical data, 4 with unclear probe epileptogenicity or location, and 3 with too few probes for a separate group analysis (all hippocampal in non-localized patients).

A total of 81 patients (38 males and 43 females) were included in the final analysis. These patients were 32.5 ± 9.91 (mean ± SD) years of age and had experienced recurrent seizures for an average of 18.9 ± 10.6 years prior to intracranial evaluation. Data were collected from 165 microdialysis probes implanted in 89 separate intracranial studies **(**Figure 3**)**. The number of intracranial studies is greater than the number of patients, because some patients with inadequate initial studies required repeat intracranial electrographic evaluations: six patients underwent two intracranial studies and one patient required three studies. The first basal collection was obtained interictally 2.7 ± 1.6 days (mean ± SD) after probe implantation while patients were on AED doses similar to their outpatient regimen.

Of the included probes, 57 were located in the hippocampus and 108 were in the cortex. Ten hippocampi had 2 probes, which were averaged, resulting in 47 hippocampal

sites. Similarly, 2 probes located in the same cortical dysplasia lesion were averaged, resulting in 107 cortical sites. No probes located in the non-lesional cortex were averaged, as they were all located in separate cortical regions. Because of this probe averaging, all data are hereafter reported by site.

Cortical and hippocampal sites in patients with localizable seizure onsets were then further organized by seizure activity into: non-epileptic hippocampus (n = 17), epileptogenic hippocampus (n = 22), propagated hippocampus (n = 8), non-epileptic cortex (n = 29), epileptogenic cortex (n = 9), and propagated cortex (n = 33). Twenty probes were located in the cortex of subjects (n = 21) with non-localizing intracranial EEG studies, largely comprised of individuals with multifocal epilepsy, and were all classified as *non-localized*. Fifteen probes were located in non-neoplastic developmental or acquired lesions identified on imaging, representing 6 dysplasias (including the average of 2 probes), 4 heterotopias, 3 encephalomalacias, and 2 cortical tubers.

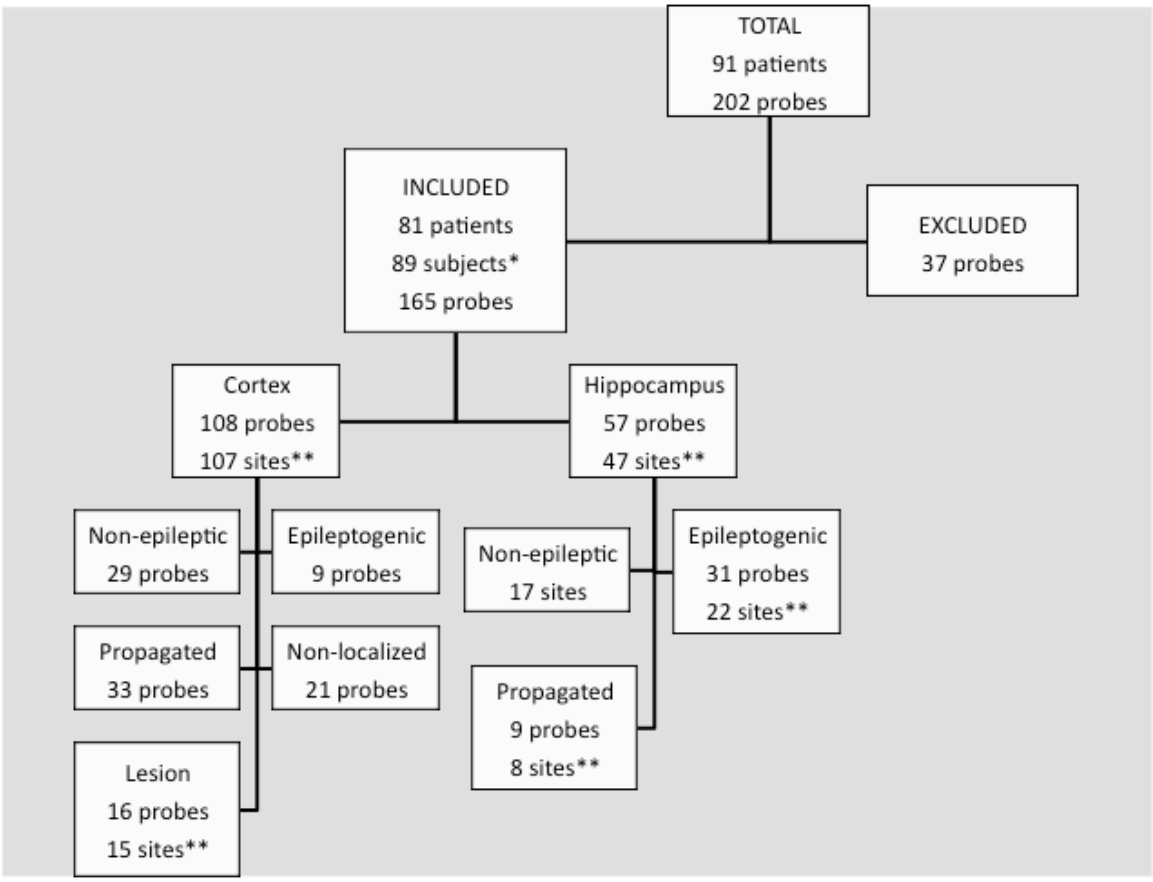


Figure 3: Flow-chart of microdialysis probe inclusion and classification within the hippocampus and cortex for the single basal analysis. *The number of subjects is greater than the number of patients, because several patients underwent multiple intracranial studies, which were treated separately. **Data from probes located in the same structure (hippocampus or lesion) were averaged to represent a single site.

II. Basal Glutamate, Glutamine, and GABA in the Cortex and Hippocampus

The mean levels of extracellular glutamate, glutamine, and GABA were compared within the cortex among non-epileptic, epileptogenic, propagated, non-localized, and lesion sites and within the hippocampus among non-epileptic, epileptogenic, and propagated sites (Table 3). The within-subject effects for glutamate, glutamine, and GABA were significant. Consequently, the within-subject effect was retained in the

statistical model for all three neurochemicals. All *p* values related to comparisons between sites are reported after Bonferroni-adjustment.

Probe Site	# Sites	Glutamate (μM)	Glutamine (μM)	GABA (nM)
Cortex	**107**			
Non-epileptic	29	2.6 ± 0.3	721 ± 124	265 ± 62
Epileptogenic	9	17.3 ± 5.1*	818 ± 152	921 ± 456
Propagated	33	25.8 ± 4.0*	669 ± 108	1503 ± 273*
Non-localized	21	43.9 ± 9.9*	801 ± 187	720 ± 172
Lesion	15	46.9 ± 9.0*	1181 ± 176	827 ± 183*
Hippocampus	**47**			
Non-epileptic	17	2.8 ± 0.5	482 ± 55	391 ± 169
Epileptogenic	22	10.3 ± 1.9*	557 ± 75	746 ± 182
Propagated	9	33.0 ± 13.8*	794 ± 215	1079 ± 395*

Table 3: Extracellular basal levels (mean ± SEM) of interictal extracellular glutamate, glutamine, and GABA in patients with medication-refractory epilepsy on full AED dosage in the hippocampus and cortex. * P < 0.05 compared to non-epileptic sites after Bonferroni correction.

A. Glutamate

A.1. Cortex

In the cortex basal extracellular glutamate levels were low in the non-epileptic sites (mean ± SEM, 2.6 ± 0.3 μM). Glutamate levels were higher in all of the other sites: 17.3 ± 5.1 μM in epileptogenic, 25.8 ± 4.0 μM in propagated, 43.9 ± 9.9 μM in non-localized, and 46.9 ± 9.0 μM in lesion sites. The effect of site on glutamate concentration was significant in the cortex ($F [4, 33] = 26.18$, $p < 0.0001$). Compared to non-epileptic sites, the elevation was significant in epileptogenic ($t [33] = 4.40$, $p = 0.0010$), propagated ($t [33] = 8.10$, $p < 0.0001$), non-localized ($t [33] = 7.58$, $p < 0.0001$), and lesion sites ($t [33] = 8.26$, $p < 0.0001$). No other differences among sites in the cortex were significant (adjusted $p > 0.05$) [FIGURE 4].

A.2. Hippocampus

In the hippocampus basal glutamate was similarly low in non-epileptic sites (2.8 ± 0.5 μM) and were comparable to the non-epileptic cortical sites (2.6 ± 0.3 μM). Glutamate concentrations were higher in the epileptogenic (10.3 ± 1.9 μM) and propagated (33.0 ± 13.8 μM) hippocampal sites. As in the cortex, the effect of site on glutamate concentration was significant in the hippocampus ($F [2, 3] = 21.70$, $p = 0.016$). Compared to non-epileptic sites, the elevation was significant in epileptogenic ($t [3] = 4.38$, $p = 0.044$) and propagated sites ($t [3] = 6.42$, $p = 0.016$). There was a non-significant trend toward higher glutamate in propagated compared to epileptogenic sites ($p = 0.081$). There were no significant differences between the cortex and hippocampus among sites of similar epileptogenicity [FIGURE 4].

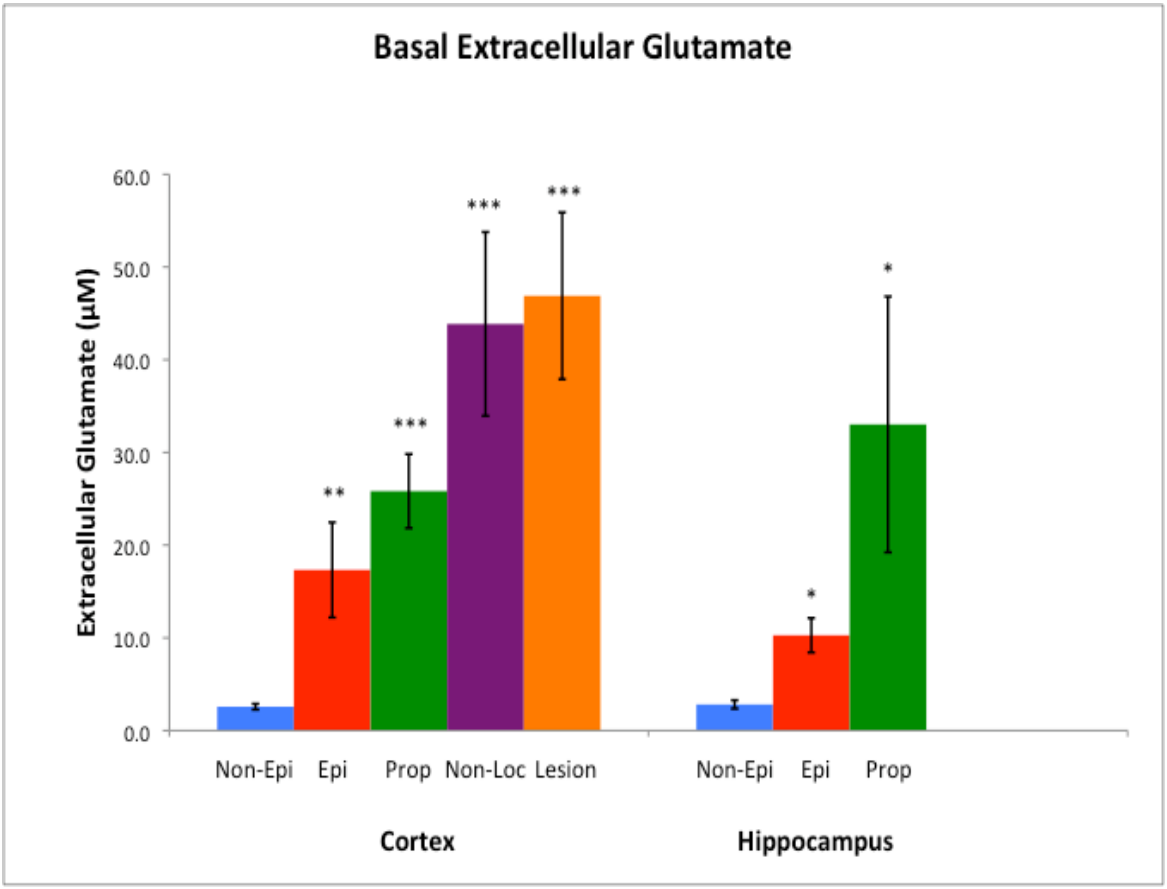


Figure 4: Basal extracellular glutamate in the cortex and hippocampus. Cortex: non-epileptic, n = 29 sites; epileptogenic, n = 9; propagated, n = 33; non-localized, n = 21; and lesion, n = 15. Hippocampus: non-epileptic, n = 17; epileptogenic, n = 21; and propagated, n = 8. In the cortex glutamate levels in the epileptogenic ($p = 0.0010$), propagated ($p < 0.0001$), non-localizable ($p < 0.0001$), and lesion ($p < 0.0001$) sites were significantly higher than in the non-epileptic sites. In the hippocampus glutamate was significantly elevated in the epileptogenic ($p = 0.044$) and propagated ($p = 0.016$) sites compared to non-epileptic sites. There were no significant differences between the cortex and hippocampus among sites of similar epileptogenicity. Abbreviations: non-epileptic (Non-Epi), epileptic (Epi), propagated (Prop), non-localized (Non-Loc), * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$.

B. Glutamine

B.1. Cortex

In the cortex glutamine levels were 721 ± 124 μM in non-epileptic, 818 ± 152 μM in epileptogenic, 669 ± 108 μM in propagated, 801 ± 187 μM in non-localized, and 1181 ± 176 μM in lesion sites. There were no significant differences among any of the examined sites in the cortex ($F [4, 33] = 2.07$, $p = 0.11$) [FIGURE 5].

B.2. Hippocampus

In the hippocampus glutamine was 482 ± 55 μM in non-epileptic, 557 ± 75 μM in epileptogenic, and 794 ± 215 μM in propagated sites. Levels of glutamine in the hippocampus were also not significantly different between sites ($F [2, 2] = 3.99$, $p = 0.20$) [FIGURE 5]. In addition, there were also no significant differences between the cortex and hippocampus among sites of similar epileptogenicity ($p > 0.05$).

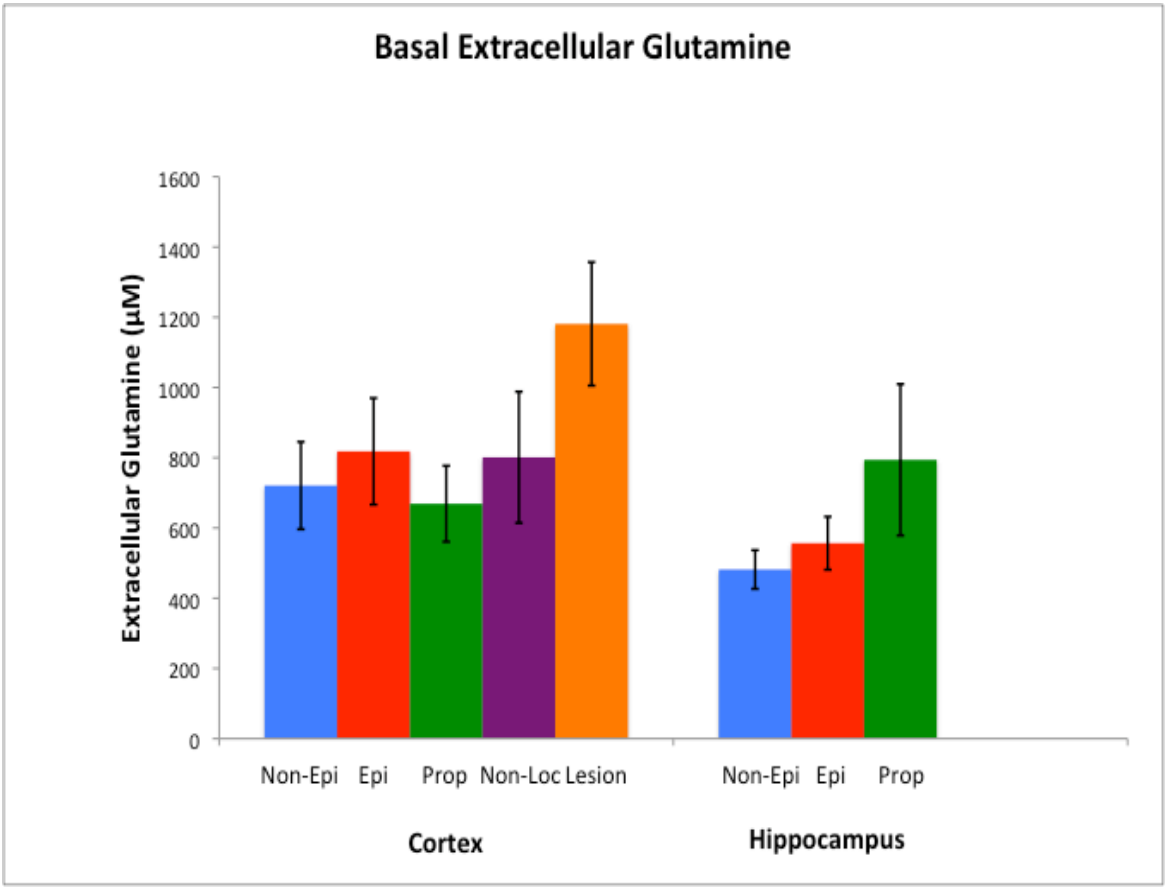


Figure 5: Basal extracellular glutamine in the cortex and hippocampus. Cortex: non-epileptic, n = 28 sites; epileptogenic, n = 9; propagated, n = 33; non-localized, n = 21; and lesion, n = 15. Hippocampus: non-epileptic, n = 14; epileptogenic, n = 19; and propagated, n = 8. There were no significant differences in glutamine among sites in the cortex or hippocampus. There were also no significant differences between the cortex and hippocampus among sites of similar epileptogenicity. Abbreviations: non-epileptic (Non-Epi), epileptic (Epi), propagated (Prop), and non-localized (Non-Loc).

C. GABA

C.1. Cortex

In the cortex GABA levels were lowest in the non-epileptic sites (265 ± 62 nM), compared to 921 ± 456 nM in epileptogenic, 1503 ± 273 nM in propagated, 720 ± 172 nM in non-localized, and 827 ± 183 nM in lesion sites. There were significant differences in GABA concentrations among cortical sites ($F [4, 31] = 5.03$, $p = 0.0030$). GABA was

significantly higher in propagated (t [31] = 4.13, p = 0.0022) and lesion sites (t [31] = 3.38, p = 0.016) than in non-epileptic sites. There was no significant difference between epileptogenic and non-epileptic sites (t [31] = 2.08, p = 0.25), nor were there any other significant differences in GABA concentrations between cortical sites [FIGURE 6].

C.2. Hippocampus

In the hippocampus GABA was also lowest in non-epileptic sites (391 ± 169 nM) compared to 746 ± 182 nM in epileptogenic and 1079 ± 395 nM in propagated sites. There were significant differences among hippocampal sites (F [2, 43] = 4.05, p = 0.025). GABA was significantly elevated in propagated compared to non-epileptic sites (t [43] = 2.73, p = 0.024). There were no significant differences between epileptogenic and non-epileptic sites (t [43] = 1.83, p = 0.17) or between epileptogenic and propagated sites (t [43] = 1.47, p =0.32) [FIGURE 6].

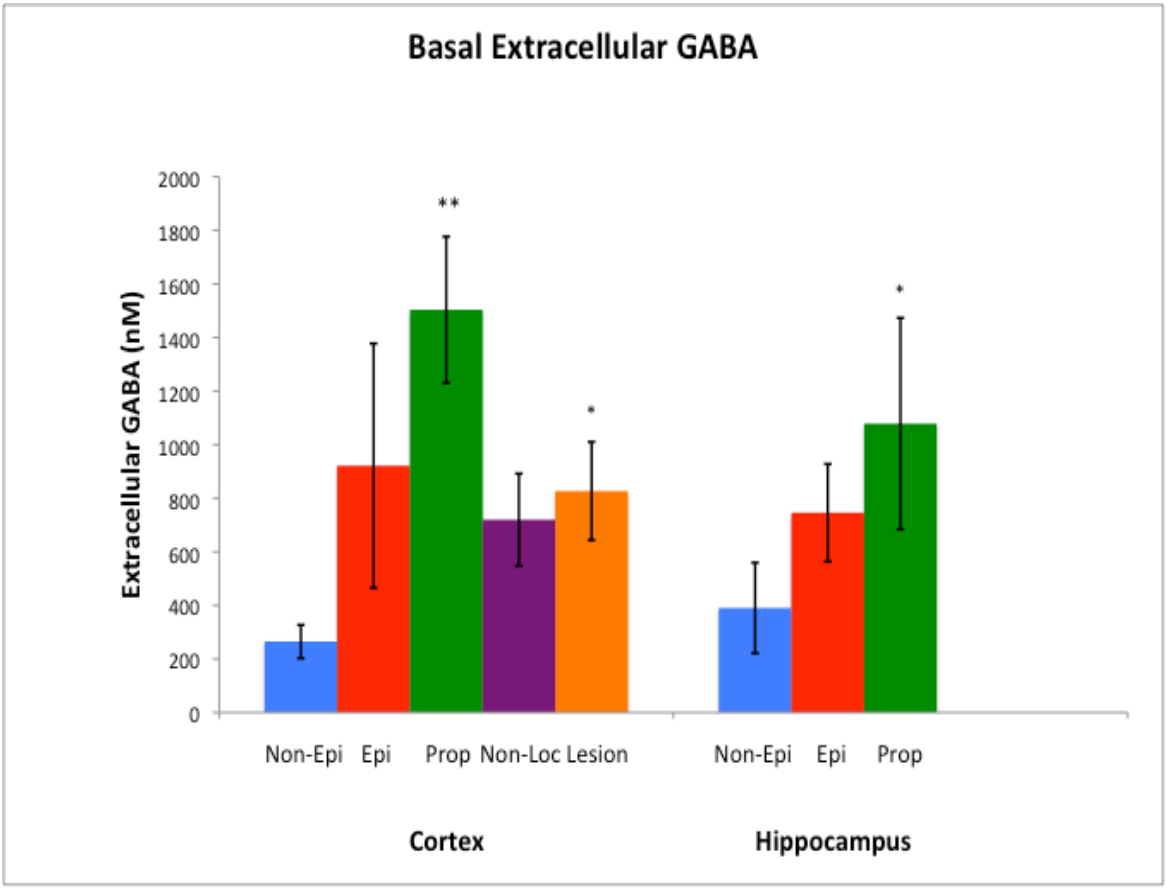


Figure 6: Basal extracellular GABA in the cortex and hippocampus. Cortex: non-epileptic, n = 27 sites; epileptogenic, n = 9; propagated, n = 31; non-localized, n = 21; and lesion, n = 14. Hippocampus: non-epileptic, n = 17; epileptogenic, n = 22; and propagated, n = 7. In the cortex GABA was significantly elevated in propagated ($p = 0.0022$) and lesion ($p = 0.016$) compared to non-epileptic sites. In the hippocampus GABA was significantly elevated in propagated compared to non-epileptic sites ($p = 0.024$) There were no significant differences among sites of similar epileptogenicity between the cortex and hippocampus. Abbreviations: non-epileptic (Non-Epi), epileptic (Epi), propagated (Prop), non-localized (Non-Loc), * $P < 0.05$, and ** $P < 0.005$.

D. Cortex and Hippocampus Combined

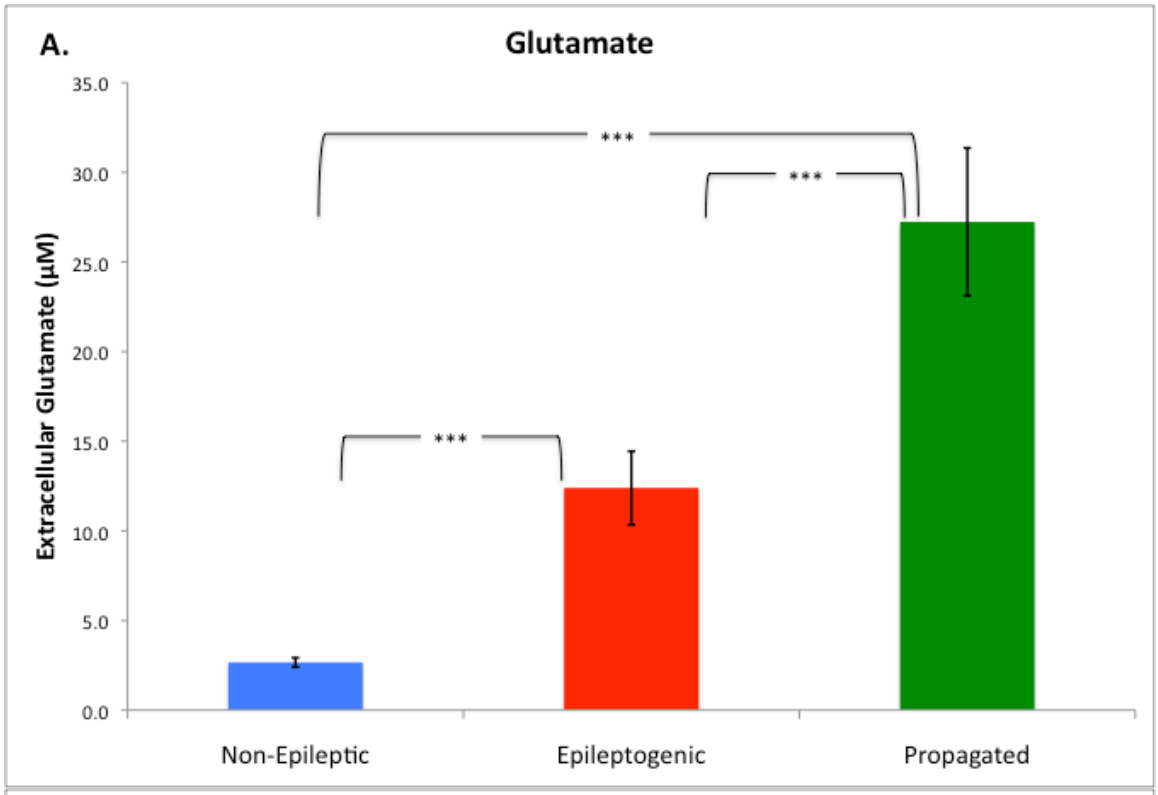
There were no significant differences in glutamate, glutamine, or GABA between the hippocampus and cortex within non-epileptic, epileptogenic, and propagated sites (all $p > 0.05$**).** Because of the similarity in neurochemical levels between the cortex and hippocampus, hippocampal and cortical sites were combined within non-epileptic,

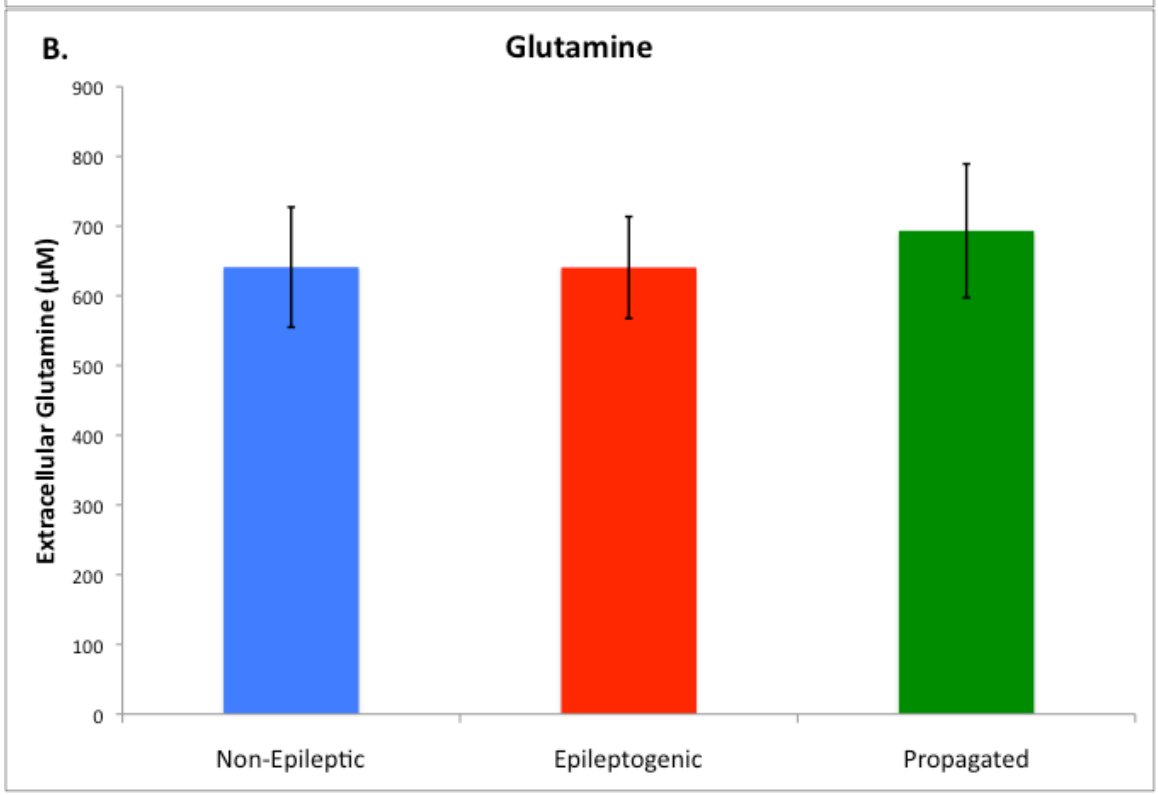
epileptogenic, and propagated groups to increase the power of this and the medication taper analysis. Concentrations in glutamate, glutamine, and GABA were then compared among these combined groups. For glutamate the combined means were 2.7 ± 0.3 μM in non-epileptic, 12.4 ± 2.0 μM in epileptogenic, and 27.2 ± 4.1 μM in propagated sites. Epileptogenic ($t [44] = 7.01$, $p < 0.0001$) and propagated sites ($t [44] = 11.82$, $p < 0.0001$) had significantly higher glutamate concentrations than non-epileptic sites. Propagated sites had significantly higher glutamate than epileptogenic sites ($t [44] = 4.50$, $p = 0.0001$). While no significant differences were detected in the earlier comparisons between epileptogenic and propagated probes in the cortex and hippocampus, the larger sample size afforded by combining hippocampal and cortical sites was now powered to detect this significant difference [FIGURE 7A].

For glutamine the combined means were 641 ± 86 μM in non-epileptic, 641 ± 73 μM in epileptogenic, and 693 ± 96 μM in propagated sites. There were no significant differences in glutamine between non-epileptic, epileptogenic, and propagated sites ($F [43, 2] = 1.39$, $p = 0.26$), reflecting the findings of the separate hippocampal and cortical analyses [FIGURE 7B].

For GABA the combined means were 313 ± 75 nM in non-epileptic, 797 ± 181 nM in epileptogenic, and 1425 ± 234 nM in propagated sites. Epileptogenic ($t [42] = 3.03$, $p = 0.011$) and propagated ($t [42] = 5.47$, $p < 0.0001$) sites had significantly higher GABA concentrations than non-epileptic sites. GABA in propagated sites was also significantly higher than in epileptogenic sites ($t [42] = 5.47$, $p = 0.028$). The increased power from combining hippocampal and cortical sites enabled detection of significant

differences between epileptogenic and non-epileptic sites and between propagated and epileptogenic sites [FIGURE 7C].





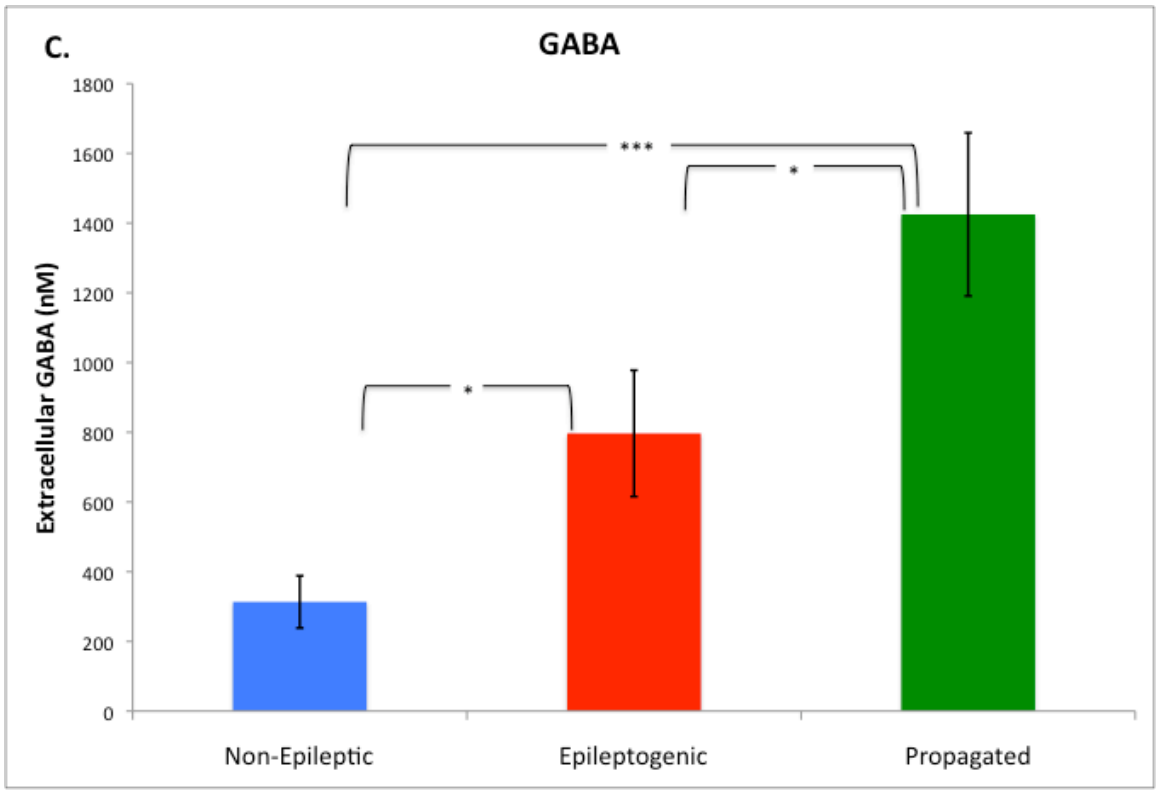


Figure 7: Basal extracellular glutamate, glutamine, and GABA after combining cortical and hippocampal sites. A) Glutamate in the epileptogenic (n = 30) and propagated (n = 41) sites was significantly higher than in non-epileptic (n = 46) sites (both $p < 0.0001$). Additionally, glutamate in the propagated sites was significantly higher than in epileptogenic sites ($p = 0.0001$). **B)** There were no significant differences in glutamine across sites (non-epileptic, n = 42; epileptogenic, n = 28; and propagated, n = 41; $p > 0.05$). **C)** GABA was significantly higher in epileptogenic (n = 31, $p = 0.011$) and propagated (n = 38, $p < 0.0001$) sites compared to non-epileptic sites (n = 44). GABA was also higher in propagated than epileptogenic sites ($p = 0.028$). * $p < 0.05$, *** $p < 0.0005$.

III. Antiepileptic Medication Taper

For this analysis, only patients who had two basal microdialysis studies (on initial dose of AED meds, "AED on" and on tapered dose, "AED tapered", as defined in the methods) were included. Data from 38 patients (18 males and 20 females) of age 32.2 ± 9.6 years (mean ± SD) studied between 2002 and 2008 were analyzed. This represented data from 40 intracranial studies (subjects) involving 76 microdialysis probes in 73

microdialysis sites **(**Figure 8**)**. Two patients were studied twice, and these repeated intracranial studies were treated as separate subjects (n = 40), as in the above basal comparison. Two hippocampi contained two probes each and one dysplasia contained two probes; their values were averaged for each structure. Seventeen probes were excluded due to: missing microdialysis data (n = 4), too few probes for group analysis (n = 3, all hippocampi in non-localized patients), unclear probe location (n = 2), excluded location (n = 7: basal ganglia, thalamus, tumor, ventricle, internal capsule, and corpus callosum), and seizure activity during collection (n = 1). The basal collections after AED taper were obtained 4.4 ± 1.8 days (mean ± SD) after the basal collection on initial dose of AEDs (AED On). The AED condition was defined as tapered (AED Tap) if at least one medication was reduced by 50% from the full medication dose within 24 hours of sample collection. AEDs administered included acetazolamide (ACTZ) carbamazepine (CBZ), felbamate (FBM), gabapentin (GBP), lamotrigine (LTG), levetiracetam (LEV), oxcarbazepine (OXC), phenobarbital (PB), phenytoin (PHT), topiramate (TPM), valproate (VPA), and zonisamide (ZNS). Six subjects were on AED monotherapy, 21 were on 2 AEDs, 12 were on 3 AEDs, and 1 was on 4 AEDs.

In an earlier analysis (see Results II.D) we found no significant differences in glutamate, glutamine or GABA between the cortex and hippocampus within the non-epileptic, epileptogenic or propagated sites. Therefore, to increase the statistical power, data from the cortical and hippocampal sites for each category (i.e. non-epileptic, epileptogenic, or propagated) were combined for glutamate, glutamine and GABA. Thus, there were 13 non-epileptic, 9 epileptogenic and 27 propagated combined sites. Non-localized and lesion sites were exclusively located in the cortex: 15 non-localized

sites located in 8 subjects and 9 lesion sites (10 probes) were included. The lesion sites comprised 2 cortical tubers, 3 dysplasias (containing 2 averaged probes), 1 CSF cleft, 1 encephalomalacia, and 2 heterotopias.

The mean pre-taper level, post-taper level, and differences in glutamate, glutamine, and GABA are reported below (Table 4). In contrast to the previous single basal analysis, the within-subject effect on neurochemical concentration was non-significant and, therefore, the nested statistical model was abandoned for the medication taper analysis.

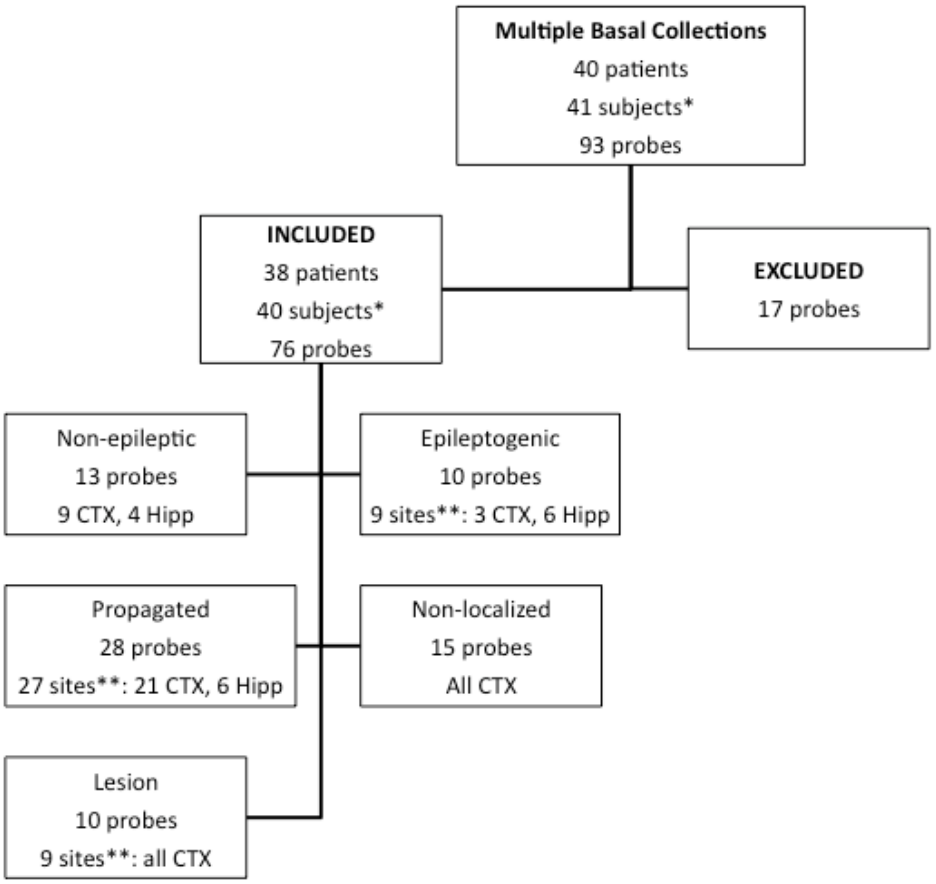


Figure 8: Flow-chart of microdialysis probe inclusion and classification within the hippocampus and cortex for the medication taper analysis.* The number of subjects is greater than the number of patients, because several patients underwent multiple intracranial studies. ** Data from probes located in the same structure (hippocampus or lesion) were averaged to represent a single site.

Table 4A.

Probe Sites	# Sites	GLU On (μM)	GLU Tap (μM)	Δ GLU (μM)
Non-epileptic	13	3.6 ± 0.7	3.6 ± 1.4	0 ± 1.4
Epileptogenic	9	16.1 ± 3.2	28.3 ± 9.7	+12.2 ± 10.3
Propagated	27	30.4 ± 4.7	32.1 ± 6.3	+1.6 ± 6.8
Non-localized	15	57.5 ± 12.1	35.4 ± 6.9	-22.1 ± 10.5
Lesion	9	63.0 ± 11.1	68.8 ± 19.7	+5.8 ± 22.1

Table 4B.

Probe Sites	# Sites	GLN On (μM)	GLN Tap (μM)	Δ GLN (μM)
Non-epileptic	13	753 ± 214	513 ± 129	-240 ± 107
Epileptogenic	9	582 ± 122	543 ± 71	-39 ± 130
Propagated	27	730 ± 132	668 ± 129	-62 ± 43
Non-localized	15	980 ± 248	743 ± 98	-237 ± 207
Lesion	9	1299 ± 202	522 ± 99	-776 ± 208*

Table 4C.

Probe Sites	# Sites	GABA On (nM)	GABA Tap (nM)	Δ GABA (nM)
Non-epileptic	11	221 ± 70	133 ± 43	-88 ± 63
Epileptogenic	8	1229 ± 445	780 ± 334	-450 ± 544
Propagated	21	1438 ± 296	1506 ± 482	+68 ± 326
Non-localized	15	894 ± 224	330 ± 59	-563 ± 226*
Lesion	7	1170 ± 249	845 ± 310	-325 ± 307

Table 4: Basal levels of interictal (A) glutamate, (B) glutamine, and (C) GABA on full AED dose (On) and after the medications are tapered (Tap) with the change in concentration after taper (Δ). * $P < 0.05$, Bonferroni-adjusted.

A. Glutamate

The effect of AED taper on glutamate levels was examined across all sites (non-epileptic, epileptogenic, propagated, non-localized, and lesion). The overall taper effect on glutamate across all sites showed a non-significant trend toward glutamate decrease ($t[72] = -1.66$, $p = 0.1017$). There were no significant changes in glutamate with taper (adjusted $p > 0.05$) in any of the five sites (all Bonferroni adjusted $p > 0.05$). There was a non-significant trend toward glutamate decrease in non-localized sites (-22.1 ± 10.5 μM, $t[14] = -2.68$, $p = 0.090$). There were no significant differences in the magnitude of glutamate change between sites ($F[4, 72] = 1.43$, $p = 0.2317$).

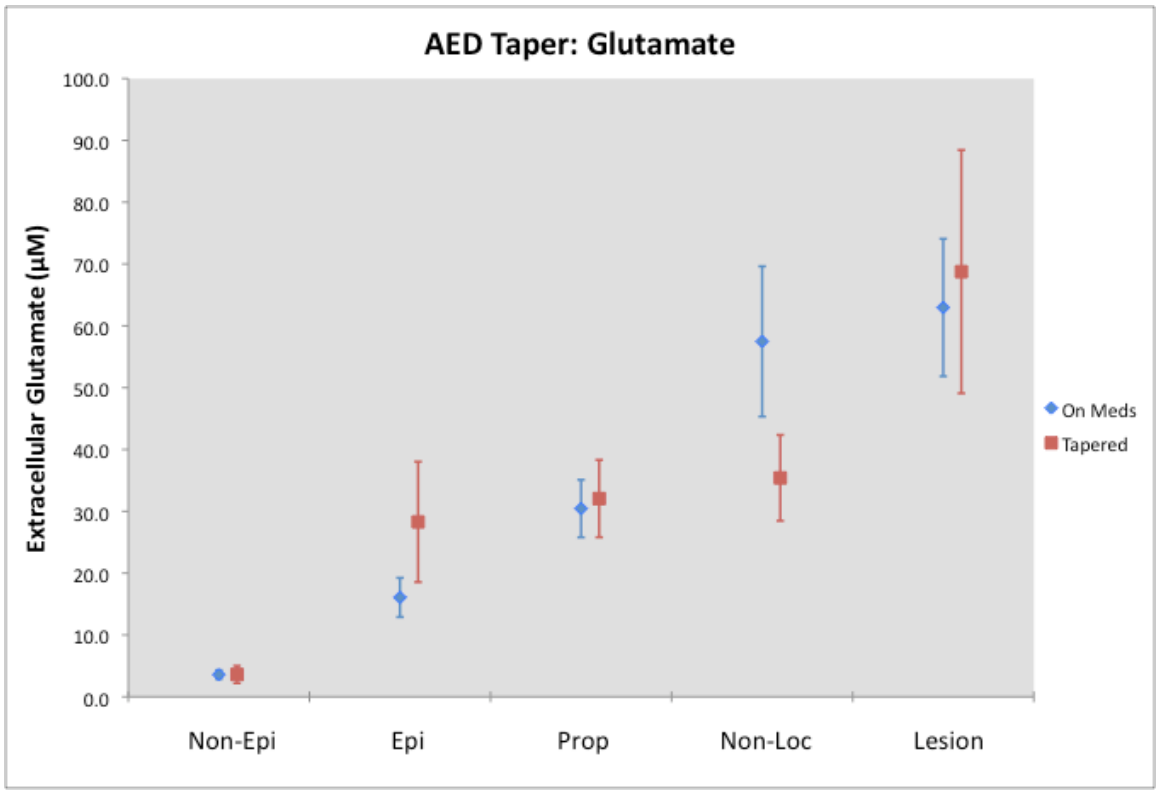


Figure 9: Effect of AED taper on basal extracellular glutamate levels by site. Non-epileptic, n = 13 sites; epileptogenic, n = 9; propagated, n = 27; non-localized, n = 15; and lesion, n = 9. There were no significant changes ($p > 0.05$) in glutamate with taper in any of the 5 sites (all $p>0.05$). There was a non-significant trend toward decreased glutamate in non-localized sites ($p = 0.09$). Data are presented as mean ± SEM.

B. Glutamine

When site was not considered, glutamine demonstrated a significant decrease with AED taper (t [72] = -3.40, p = 0.0011). When examined by site, there was a significant decrease in glutamine in the lesion sites only (-776 ± 208 μM, t [8] = -4.54, p = 0.0095). No other sites exhibited significant changes in glutamine (p > 0.05).

There were significant differences in the magnitude of glutamine change between sites (F [4, 72] = 4.50, p = 0.0028). Post-hoc analysis on the magnitude of glutamine change demonstrated that the glutamine decrease in lesions was significantly greater than in the other four sites, but no distinction could be made between non-epileptic, epileptogenic, propagated, or non-localized sites.

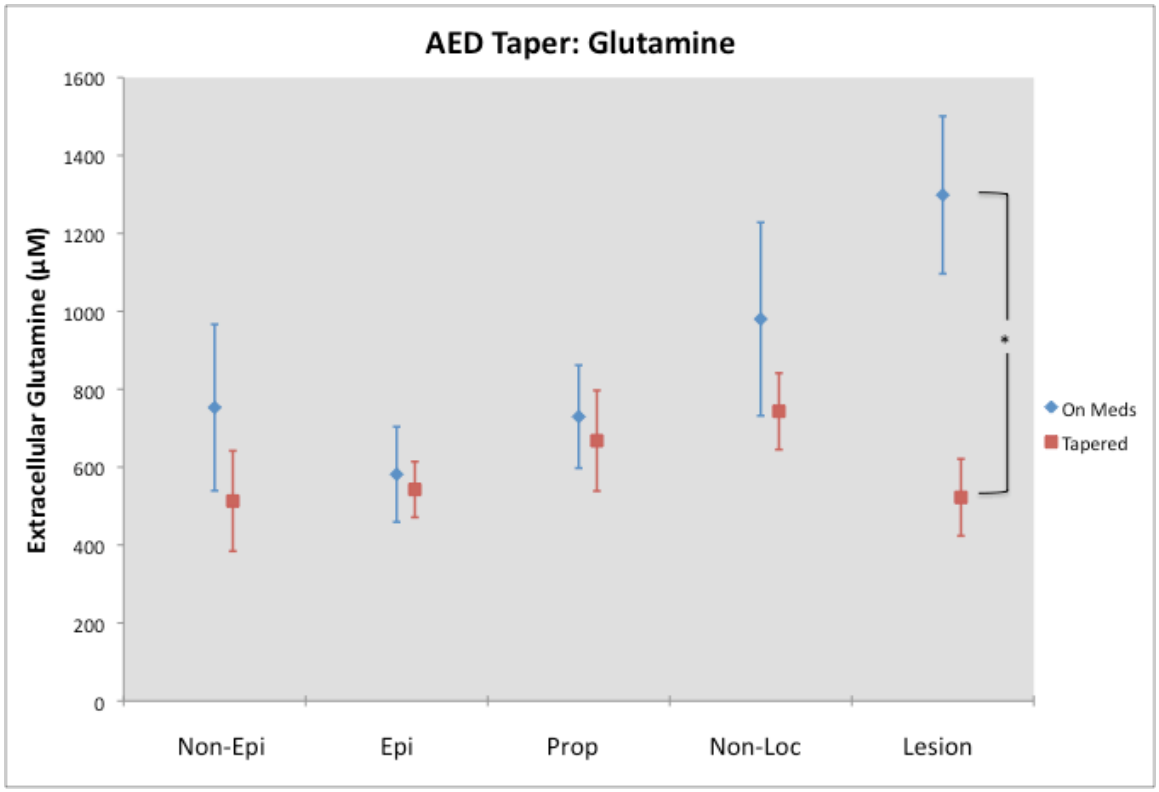


Figure 10: Effect of AED taper on basal extracellular glutamine levels by site. Non-epileptic, n = 13 sites; epileptogenic, n = 9; propagated, n = 27; non-localized, n = 15; and lesion, n = 9. AED taper resulted in significant glutamine decrease only in the lesions sites (*p = 0.0095). Data are presented as mean ± SEM.

C. GABA

When site was not considered, GABA decreased significantly overall with AED taper ($t\ [61] = -4.65$, $p < 0.0001$). Examined by site, AED taper was associated with a significant decrease in GABA in the non-localized sites only (-563 ± 226 nM, $t\ [14] = -3.61$, $p = 0.014$). There were no significant changes in GABA in non-epileptic, epileptogenic, propagated and lesion sites (adjusted $p > 0.05$).

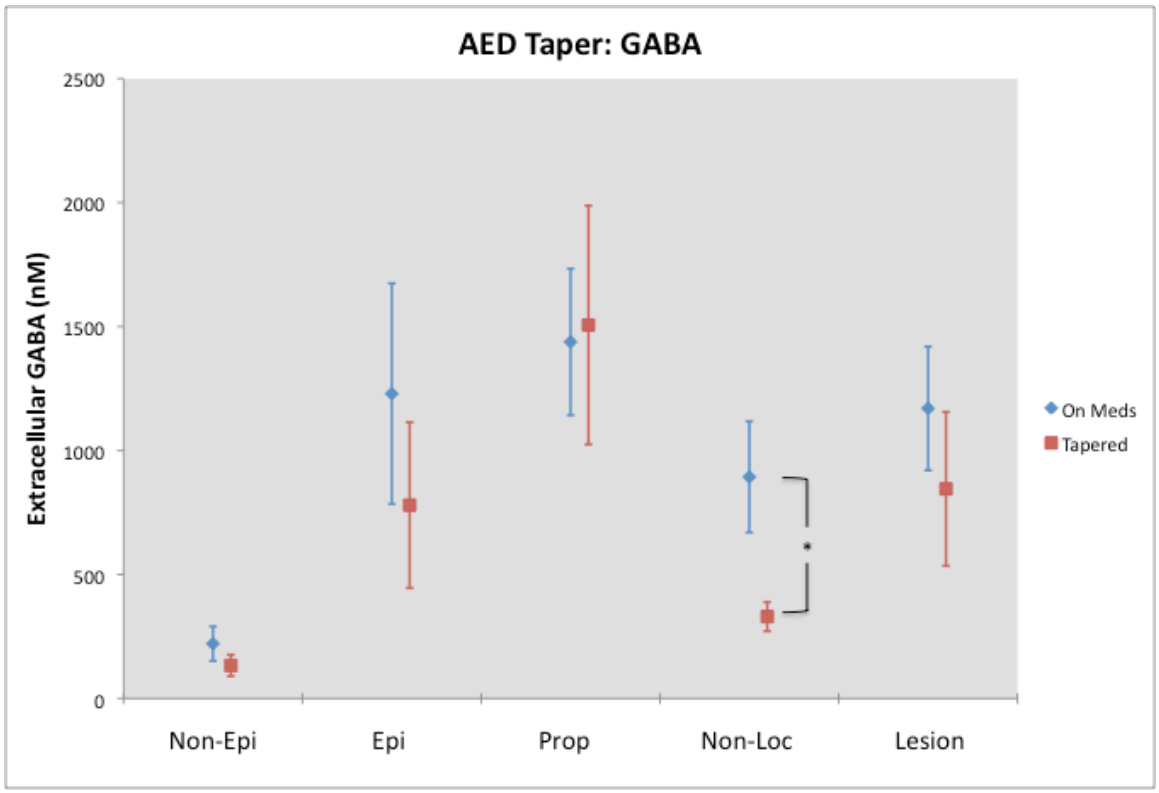


Figure 11: Effect of AED taper on basal extracellular GABA levels by site. Non-epileptic, n = 12 sites; epileptogenic, n = 9; propagated, n = 24; non-localized, n = 15; and lesion, n = 8. AED taper was associated with a significant decrease in GABA only in the non-localized sites (*p = 0.014). Data are presented as mean ± SEM.

Discussion

This study has built upon the previous work of Cavus and colleagues (49) that examined the basal interictal extracellular concentrations of glutamate and glutamine in the epileptogenic and non-epileptic hippocampus and cortex of patients with medically-refractory epilepsy. Here, in a much larger sample size involving a total of 89 subjects, including the original 38 patients from Cavus et al.'s 2005 publication (49), we report the interictal basal levels of glutamate, glutamine and GABA not only in the epileptogenic and non-epileptic human hippocampus and cortex, but also in seizure propagation sites, MRI-identified lesions, and patients in whom the seizure onset site could not be localized (non-localized). In addition, we examined the effect of AED taper on the interictal glutamate, glutamine and GABA levels in a subset of these subjects. To our knowledge, this is the first study reporting on such work in such an extensive number of subjects with refractory epilepsy.

I. Basal Glutamate, Glutamine, and GABA in the Cortex and Hippocampus

Glutamate

The interictal basal glutamate levels in the non-epileptic hippocampus and cortex were low and remarkably similar (hippocampus, 2.8 ± 0.5 μM vs. cortex, 2.6 ± 0.3 μM). These values were also similar to those previously reported in the non-epileptogenic cortex and hippocampus of a subset of our subjects by Cavus et al. (49, 50) and in the striatum of normal freely-moving rats (3.0 ± 0.6 μM) (206). Although the invasiveness of

the microdialysis studies precludes obtaining data from control human subjects, the findings in non-epileptic brain sites presumably closely approximate normal brain tissue. The consistency of our findings in non-epileptic sites with previously reported results in both humans and animals demonstrates that extracellular glutamate is very tightly regulated under normal conditions (86, 88, 207). These findings further confirm the consistency of microdialysis measurements under chronic conditions in human subjects.

In a previous study in 38 of our subjects, Cavus and colleagues found that the basal concentration of glutamate was significantly elevated in the epileptogenic hippocampus compared to the non-epileptic hippocampus, while the elevation in the epileptogenic cortex did not reach significance (49). Here, we confirm higher interictal glutamate levels in the epileptogenic hippocampus (10.3 ± 1.9 μM) compared to the non-epileptogenic hippocampus ($p = 0.044$). Furthermore, with the advantage of adding additional subjects (since 2004) to the original 38, we find that the glutamate in the epileptogenic cortex (17.3 ± 5.1 μM) is now *significantly* higher than in the non-epileptic cortex ($p = 0.0010$). There were no regional differences (i.e. hippocampus vs. cortex) in glutamate levels in the non-epileptic, epileptogenic, or propagated sites. Although hippocampal and neocortical epilepsies have different pathologies (208) and biogenic amine profiles (209), the levels of glutamate, glutamine, and GABA in these regions were comparable, which suggests that metabolic abnormalities associated with epilepsy have a similar effect on extracellular neurochemicals.

Remarkably, we found that extracellular glutamate was significantly elevated in all other examined sites: in both the propagated cortex (25.8 ± 4.0 μM) and hippocampus (33.0 ± 13.8) and in non-localized (43.9 ± 9.9 μM) and lesion (46.9 ± 9.0 μM) sites in the

cortex. In fact, the interictal elevation of glutamate in the propagated hippocampal and cortical sites was even significantly higher than in the epileptogenic sites ($p = 0.0001$). This was surprising because, although propagated sites are exposed to greater seizure activity than non-epileptic sites, they are located in comparably normal brain tissue not known to be affected by the synaptic reorganization and gliosis seen in epileptogenic and lesion sites. However, evidence from neuroimaging studies in epilepsy patients also indicates that abnormalities extend beyond the sites of seizure origin (210). In patients with MTS, bilateral cortical thinning is present on MRI (211, 212), and PET abnormalities project beyond hippocampal (213) and neocortical (214) sites of seizure origin along established intra-cortical connections. Our findings of elevated interictal glutamate in sites outside of the seizure origin are consistent with these findings, and suggest that, in medication-refractory epilepsy patients, there is a widespread impairment in glutamate homeostasis. This elevation in the extracellular glutamate could be a result of increased neuronal and glial release due to impaired metabolism, inadequate glutamate reuptake following repeated seizures, or a combination of both (70, 73, 74, 79, 215). It is possible that this glutamate elevation may enhance seizure propagation, as increased neuronal activity can amplify astrocytic glutamate release (82), and the release of astrocytic glutamate could initiate neuronal depolarization and glutamate release distant from the seizure focus (83).

These findings of widespread glutamate elevation may have implications for the outcomes of surgical treatment of these patients. It is unknown if high extracellular glutamate in propagated sites, which are usually not resected with the focus (20), predicts poor surgical outcome. However, the presence of extensive PET abnormalities before

surgery has been associated with decreased seizure freedom following resection (203). Alternatively, PET abnormalities in seizure projection sites may improve after resective surgery (216, 217), and it is possible that the elevated glutamate in propagated sites may decrease following resection of the epileptogenic focus.

Patients are classified as non-localized if their intracranial EEG study fails to consistently identify a seizure focus. This often occurs because seizures originate from multiple foci, although, in some cases, there may be a single seizure focus that was inadequately covered by the electrode array. The presence of elevated glutamate in multiple sites in non-localized patients could be consistent with the presence of global dysfunction in the regulation of glutamate and multifocal seizure onset. Although not completely analogous to microdialysis conducted in non-localized patients, MRS studies on patients with generalized epilepsy demonstrate elevation of glutamate-glutamine (GLX) in the occipital (218) and frontal lobes (219), suggesting a global dysfunction.

Glutamate was elevated in lesions, which included heterotopias, dysplasias, tuberous sclerosis, and encephalomalacias. Because of the limited sample sizes, lesion sites have been previously excluded from other microdialysis reports (35, 49, 50). However, our findings are consistent with the limited human and animal data on glutamate changes in tumors and lesions. While non-neoplastic lesions have not previously been studied by microdialysis in humans, microdialysis conducted in patients with high-grade astrocytoma has identified elevated glutamate both within tumors and in the adjacent (radiographically normal) brain (220). Although not examined here, we have observed similar glutamate elevations both within neoplasms and in the surrounding brain (I. Cavus, unpublished observations). MRS studies of malformations of cortical

development in patients with refractory epilepsy have identified elevated GLX within the lesion sites (221). Microdialysis in a mouse tuberous sclerosis model identified elevated extracellular hippocampal glutamate, which was associated with neuronal death (222), suggesting that some developmental abnormalities may be associated with widespread dysfunction in glutamate homeostasis and that this elevation in glutamate may be neurotoxic. Rats in a cortical dysplasia injury model are susceptible to excitability when glutamate transport is blocked (223), which may elucidate the mechanisms underlying the epileptogenic properties of lesions in humans. This widespread elevation of glutamate in the propagated, non-localized, and lesion sites suggests that the dysregulation of glutamate homeostasis in the epileptic brain is much more widespread then originally anticipated.

Multiple microdialysis studies have identified elevations in extracellular glutamate in medically-refractory epilepsy patients both during seizures (43, 47, 48, 113) and in the interictal period (35, 49, 50, 52). Although the origin and significance of this glutamate in epileptic patients is not completely known (73), elevated glutamate has been shown to be neurotoxic in cell culture (95), and chronic exposure is associated with neurodegeneration in rats (98) and hippocampal atrophy in humans (50). Although a rise in glutamate alone does not appear to induce seizures, it lowers the seizure threshold and enhances epileptogenesis (87). Consequently, extracellular glutamate is a promising target for future epilepsy pharmacotherapy (204).

GABA

The interictal extracellular levels of GABA in the non-epileptic hippocampus and cortex were comparable (hippocampus, 391 ± 169 nM vs. cortex, 265 ± 62 nM, $p > 0.05$)

and were remarkably similar to those obtained in the striatum of normal rats (270 ± 40 nM) (224). Surprisingly, we found that compared to the non-epileptic sites, GABA was significantly elevated in the propagated sites in both the hippocampus (1079 ± 395 nM; p = 0.024) and in the cortex (1503 ± 273 nM; p = 0.0022), as well as in the cortical lesions (827 ± 183 nM; P = 0.016). Although the metabolic substrates underlying extracellular GABA elevation in the hippocampus and cortex may differ, (51), there were no differences in extracellular GABA concentration between the hippocampus and cortex within non-epileptic, epileptogenic, and propagated sites. When the hippocampal and cortical sites were combined, it became apparent that GABA was also significantly elevated in epileptogenic sites compared to non-epileptic sites (p = 0.011) and, furthermore, that the GABA in propagated sites was higher than in epileptogenic sites (p = 0.028). These significant and novel differences were only appreciated after combining the hippocampal and cortical probes, illustrating the necessity of larger sample sizes to appreciate extracellular neurochemical variations.

The elevation of extracellular GABA within lesions is a novel finding. Although the specific mechanism of this elevation is unclear, there is evidence from tissue studies of impaired regulation of GABA in lesions. In resected dysplasias GABAergic neuron and GABA transporter organization have been reported to be abnormal (225), and decreased inhibitory postsynaptic potentials, GABA clearance and transporter expression have been identified (226).

The similar distributions of elevated extracellular GABA and glutamate suggest that metabolic abnormalities extend beyond seizure onset sites and lead to increased concentrations of both neurotransmitters. It is unknown if increased GABA is due to

chronic seizure activity, follows seizure propagation tracts, reflects interictal impairment in GABA regulation, or is an effect of medications. The medication taper analysis (see below), demonstrated an overall GABA reduction in response to AED withdrawal, which suggests that elevated GABA may be partially related to AED administration and that, if present, this AED-related amplification of GABA may be greater in abnormal brain regions (i.e. outside of non-epileptic sites). Extracellular GABA increases with seizures in patients with refractory epilepsy and may serve to limit seizure activity (47, 48). Indeed, there is evidence of increased interictal inhibitory activity in epileptogenic regions in humans (227) that may be related to the elevated GABA we find in those sites. There is also substantial evidence from sclerotic human hippocampi and animal models of epilepsy of impaired GABA transport in epileptogenic states (113, 114, 228).

Alternately, the elevated extracellular GABA may enhance rather than mitigate epileptogenesis. Chronic seizure activity has been reported to increase the reversal potential of GABA, rendering GABA paradoxically excitatory (116). This excitatory property of GABA is hypothesized to contribute to interictal electrical activity (229) and the development of epileptogenic foci in human MTLE (230). It is possible that excitatory or inhibitory properties of GABA may predominate in different brain regions within the same patient. For example, GABA elevation is associated with improved mitochondrial energy metabolism in non-epileptic hippocampi but with worsened energy metabolism in epileptogenic hippocampi (51), so increased GABA in epileptogenic hippocampi may be pathologic and excitatory, while GABA in non-epileptic or propagated sites may be a normal response to chronic excitation that counteracts seizure spread. Nevertheless, the inhibitory properties of GABA may be attenuated in patients

with epilepsy, as there is evidence that GABA receptors are maximally activated but functionally impaired in the presence of chronic seizures (231). Also, extracellular basal GABA concentrations may not accurately reflect the GABAergic response to seizure initiation, which is diminished in epileptogenic compared to non-epileptic hippocampi (47).

Glutamine

Glutamine levels in the non-epileptic hippocampus and cortex were similar (hippocampus, 482 ± 55 μM vs. cortex 721 ± 124 μM, $p > 0.05$). These levels were higher than levels found in the normal rat hippocampus (193 ± 10.9 μM) (232). There were no significant differences in basal extracellular glutamine concentrations between any of the sites and regions examined, regardless of their involvement in seizure activity.

Although glutamine does not, itself, function as a neurotransmitter, it is a critical component in the metabolism of both glutamate and GABA (88, 100). A low ratio of glutamine to glutamate (i.e. high glutamate and low glutamine), as measured by microdialysis, has been associated with poor outcome in children with traumatic brain injury (233), although its prognostic significance in epilepsy is unknown. These microdialysis findings of low glutamine in the setting of high glutamate support evidence of impaired glutamate-glutamine cycling, which could result from a combination of impaired glutamate uptake and decreased glutamine synthetase function in glia. Evidence of decreased glial glutamate transport (89, 234), glutamine synthetase expression (102, 235), and impaired glutamate-glutamine cycling (49, 101) have been identified in the human epileptogenic hippocampus Reduced glutamine availability or glutaminase function could also impair inhibitory GABAergic neurotransmission (103, 236). Our

results suggest that despite the reported decrease in glutamine synthetase in the epileptogenic hippocampus, extracellular glutamine levels are regulated at a constant level across all regions regardless of their degree of involvement in seizure activity.

II. Antiepileptic Medication Taper

Although they have been examined in animal models, the effects of AEDs on extracellular glutamate, glutamine, and GABA have not previously been studied by microdialysis in humans. Several microdialysis studies have been conducted on basal extracellular neurochemical levels at a single point in time (35, 49-52), often when patients are on their full dose of AEDs, but the extent to which AEDs may contribute to these levels has not been specifically examined. Since most AEDs exert their effects by decreasing neuronal excitation, which is primarily glutamatergic, or enhancing inhibition, which is primarily GABAergic, medication withdrawal might be expected to influence these extracellular neurotransmitter levels. Many AEDs have multiple mechanisms of action, and their effects may be astrocytic as well as neuronal. For example, valproate, gabapentin, and phenytoin have been shown to decrease calcium-mediated signaling between astrocytes, which may enhance their antiepileptic properties in neurons by decreasing the astrocytic contribution to extracellular glutamate (83). Due to these complex interactions, the effect of AEDs on glutamate, glutamine, and GABA in the human brain may be more complicated than that predicted by individual drug mechanisms or their effects in animal models. Because the nature of this study renders correlation of neurochemical concentrations with individual drugs and their doses impossible, the taper period should be viewed as a state of increased susceptibility to seizures rather than the result of specific medication changes, as has been done in several

studies on the electrographic effects of AED taper (33, 34, 36), rather than the result of specific medication changes.

Glutamate:

Following AED taper, glutamate did not change significantly in any of the examined sites (non-epileptic, epileptogenic, propagated, or lesions). There was a trend to decrease only in the non-localized sites (-22.1 ± 10.5 μM, $p = 0.09$). The failure to detect any significant changes in the extracellular glutamate with AED taper is consistent with animal studies on many AEDs, including CBZ (135), LEV (146), LTG (64), PHT (67, 135), TPM (148), VPA (S. Ahmad et al., 2005b; (67), and ZNS (137), in which no change in basal extracellular glutamate was observed after AED administration. MRS data in humans, though predominantly measuring intracellular concentrations, also suggest that AEDs may not significantly reduce glutamate in patients with medication-refractory epilepsy (121), the patient subtype studied by microdialysis. Many of the current AEDs may not target mechanisms that regulate the basal extracellular glutamate. Because some AEDs, especially sodium-channel blockers, are known to be use-dependent (130), their effects on basal glutamate levels, obtained during periods of relative inactivity, may be minimal. This property may also explain why the same medication may affect basal (Table 2) and evoked (Table 1) neurotransmitters differently in animal microdialysis studies.

Another possibility, especially in a patient population that is defined by resistance to multiple AEDs, is that changes in the drug targets or reduced drug accumulation in the extracellular space due to transporter-mediated efflux by P-gp or MRPs (237) prevent

these medications from exerting their effects on extracellular glutamate. Increased efflux transporter expression is seen in epileptogenic tissue (178, 179, 181) and is associated with chronic seizures in humans and animal models (187-189). Still, the effect of efflux transporters on AED function in humans is unknown. If efflux transporters are clinically significant in humans, they would be expected to be associated with resistance to more than one AED (202). The association of elevated extracellular glutamate with increased efflux transporter expression (190, 191) may directly link the microdialysis glutamate data with medication response. If high glutamate at baseline were associated with poor drug influx, then the medication effect should be attenuated in areas where glutamate is already high. In addition, cortical dysplasias and tubers are associated with increased drug efflux transporter expression at baseline (179-181).

Nonetheless, glutamate did decrease, albeit non-significantly, in non-localized patient sites. The decrease of glutamate in these sites likely heavily influences the trend in glutamate decrease ($p = 0.102$) observed when all sites are considered together. Since these patients are defined by the inability to localize their seizure onset to a single site, often due to multifocality, this may represent a pathology that is distinct from that of single-onset epilepsy. Since medication taper is associated with decreased Teager energy (36) and Teager energy is positively correlated with extracellular glutamate levels (51), it would follow that medication taper is associated with decreased extracellular glutamate. One plausible hypothesis is that this is related to increased feedback inhibition on extracellular glutamate release, a process that might be modulated by presynaptic metabotropic glutamate receptors (238). However, since these non-localized sites represent a heterogeneous group, ranging from localization-related epilepsy with

undetectable foci to true generalized seizure onset, any conclusions from these results should be approached with caution. Also, the failure to detect significant changes in other sites could be a function of the relatively small sample sizes examined.

Glutamine:

Following AED taper glutamine did not significantly change in non-epileptic, epileptogenic, propagated, or non-localized sites but decreased significantly in lesion sites (-776 ± 208 μM, $p = 0.0095$). These results may be due to differences in glutamate-glutamine cycling within lesions, although there was no accompanying significant change in glutamate within lesions. The glutamine decrease in lesions may indicate a different metabolic pattern from non-lesional cortex. In a C-13 labeling study comparing human tissue from MTS and dysplasias, both tissues exhibited evidence of decreased glutamate uptake, although glutamine synthetase was intact in dysplasias and impaired in MTS, while neuronal mitochondrial function was impaired in dysplasias but relatively unaffected in MTS (239). It is important to emphasize that the lesions considered in our microdialysis study were comprised of multiple pathologies, including heterotopias, tubers, and encephalomalacias in addition to dysplasias. A possible explanation for decreased extracellular glutamine in the setting of unchanged extracellular glutamate is that more glutamate is produced during the pro-epileptogenic state of AED taper, but it is located intracellularly or in synapses and is consequently not detectable by microdialysis (73). This would be consistent with the theory that pro-epileptogenic conditions favor the conversion of excess glutamine to glutamate rather than GABA (105). Another consideration is that glutamine levels are in balance with ammonia and related to nitrogen metabolism. For example, valproate has been shown to increase brain glutamine by

inducing hyperammonemia (165). This is a less likely explanation in the present study, because only one of nine lesion sites underwent a taper in valproate (although, this was accompanied by a reduction of glutamine from 1850 to 525 μM). Interestingly, since several AEDs incidentally inhibit glutamine synthetase, glutamine might actually be expected to increase after drug withdrawal (166).

GABA:

Following AED taper GABA decreased significantly in non-localized sites (-563 ± 226 nM, $p = 0.014$), similar to glutamine, but it did not significantly change in non-epileptic, epileptogenic, or propagated sites. There was an overall significant decrease in GABA when all sites were examined together ($p < 0.00001$), and the degree of decrease in GABA concentration was not significantly different between sites when they were examined separately. Although it was not possible to examine the change in GABA by individual drugs or drug classes due to the small number of patients in whom only one AED was tapered, these results suggest that many of these AEDs enhanced extracellular GABA. This effect has been demonstrated on whole brain GABA for some AEDs in patients with epilepsy (123-125) and in healthy subjects (126). Increased basal GABA has been also demonstrated in animal microdialysis studies after administration of AEDs, such as valproate (67, 131, 139), vigabatrin (149), and tiagabine (147). In this study, no patients in the current microdialysis study were on vigabatrin or tiagabine, but valproate was a constituent of many therapeutic regimens. The prominence of this effect in non-localized patients, as for glutamate, may be due to the larger sample size of this group

(n=15 sites) or may indicate a different metabolic profile from true localization-related epilepsy.

Medication taper is clinically associated with increased seizure frequency, which may be related to decreased neuronal inhibition. Increased whole-brain GABA identified on MRS is associated with improved seizure control (107), although patients with medication-refractory epilepsy are more likely to have subnormal GABA levels after AED administration (121). Based on our microdialysis results, it is possible that extracellular, as well as neuronal, GABA is involved in mitigating epileptogenesis. GABA may have effects in regions of different epileptic activity. For example, an AED-induced increase in extracellular GABA may attenuate seizure spread in propagated regions. Although increasing GABAergic tone may enhance seizure control, this effect is broadly distributed and contributes to the cognitive impairments seen with GABAergic AEDs (240, 241). It is also important to consider that, as discussed previously, increased GABA may promote inhibition as well as excitation, depending on its reversal potential (115, 116, 229, 230).

III. Limitations and Future Directions

While *in vivo* microdialysis in conscious humans has provided many valuable insights into the neurochemical substrates of medication-refractory epilepsy, this technique has several inherent limitations. Although these studies involve the largest dataset of epilepsy patients studied by microdialysis, the sample sizes involved, especially in the medication taper analysis, are still small relative to the number of comparisons made. Unfortunately, as intracranial electrographic studies are a significant

undertaking and only eight to twelve patients are studied annually at our center, it would take many years to accrue a larger number of subjects. All data were collected in subjects with medication-refractory epilepsy, since normal controls and patients with drug-responsive epilepsy would not ethically be candidates for the invasive catheter implantation procedure. Consequently, the findings may not be applicable to patients with drug-responsive epilepsy, and comparisons can only be made to non-epileptic sites in patients with epilepsy rather than normal controls, with the assumption that non-epileptic sites approximate the conditions in normal brain. Microdialysis probe locations are chosen in advance based on clinical suspicion of epileptic activity; therefore, the number and types of sites to sample with microdialysis cannot be predetermined or controlled. In addition, not all microdialysis probes function after insertion. Consequently, some patients have multiple probes and some probe groups are over-represented, such as cortical seizure propagation sites, while others, such as cortical seizure onset sites, are under-represented. These limitations have been considered by classifying probe activities according to a consensus of independent epileptologists and by adjusting for the within-subject effect on probes in patients with multiple probes.

Every attempt possible has been in the microdialysis methodology to standardize collection conditions between patients. To decrease the variability in neurochemical levels based on diurnal variations, sleep, food intake etc, all basal samples were collected in the afternoon, at least 4-6 hours after any seizure activity. However, differences in clinical conditions, behavioral activity, administered AEDs and other medications (such as antibiotics, corticosteroids, and pain relievers), seizure frequency, and other variables may also affect neurotransmitter concentration and cannot presently be controlled for.

Following several initial measurements and continual random sampling and quality control, all probes have been assumed to have the same fractional recovery of 0.8, although it is possible that slight differences may exist between probes and could change over the sampling period. Although samples have been obtained over a period of ten years under different investigators and staff, the procedures for microdialysate collection, storage, and analysis have been standardized, and any changes to the protocol have been followed by quality analysis to ensure that the results are consistent with those obtained by previous methods.

As the medication taper analysis was conducted on a subset of patients included in the single basal analysis, the sample sizes were even smaller, and many patients were on multiple medications. The medication taper was individualized to fit the clinical needs of each patient, guided by the need to record an adequate number of seizures for electrographic localization. Consequently, it was impossible to discern effects of single medications on glutamate, glutamine, and GABA. This is unfortunate, because, while these effects have been studied in animal models, these data are not available in humans, in whom the drugs may have different effects. Ideally, if data from more patients were available, it may become possible to explore the effects of medication classes, if not individual AEDs.

The neurochemicals of interest may also have been subject to a number of confounding factors. Non-AED medications known to affect glutamate were also adjusted over the taper period. Most patients received prophylactic antibiotics, usually cephalosporins, and corticosteroids during the implantation surgery, which were discontinued over the same time period that the AEDs were tapered. β-lactam antibiotics,

which include cephalosporins, have been reported to upregulate glutamate transporters, protect neurons from glutamatergic excitotoxicity, and prevent seizures (Rothstein, Patel et al. 2005; Tanaka 2005). Glucocorticoids acutely raise ECF glutamate concentrations in the rat hippocampus (242, 243) and have longer-term effects that involve regulation of glutamate transporters (244) and glutamine synthetase (245). It is unlikely that these non-AED medications would have affected the neurochemical comparisons between different sites in the single basal analysis, because many patients were on the same antibiotics and steroids, but they may have confounded the taper results. Glutamine levels are related to blood levels of ammonia, which can be influenced by dietary protein and medications such as valproate (165), and this was not examined. Finally, magnetic spectroscopy studies (246) and unpublished microdialysis data from our lab indicate that GABA levels differ between men and women and fluctuate throughout the menstrual cycle. The gender effect on GABA and other neurotransmitters will be examined in a subsequent work.

IV. Conclusions

This is a two-part study on the extracellular basal levels of glutamate, glutamine, and GABA in subjects with medically-refractory epilepsy initially on their full AED doses and then, within a smaller subset, after AED taper. Consisting of 89 subjects, this report constitutes the largest microdialysis study, to our knowledge, on basal levels of these neurochemicals in patients with medication-refractory epilepsy. This is also the first microdialysis study to examine basal GABA levels in both the hippocampus and cortex and to examine the neurochemical substrates of seizure propagation sites, lesions, and patients with non-localizable epilepsy.

Basal glutamate was elevated in epileptogenic and seizure propagation sites compared to non-epileptic sites in both the hippocampus and cortex. Glutamate was also elevated within lesions and in the cortex of patients with non-localized seizure onset. In the hippocampus and cortex, GABA was significantly elevated in epileptogenic and propagated compared to non-epileptic sites, as well as in lesions. There were no significant differences in glutamine within the cortex or hippocampus. Since glutamine was unchanged, the glutamine/glutamate and glutamine/GABA ratios were lower outside of the non-epileptic sites, which could suggest differences in glutamine-glutamate and glutamine-GABA cycling. There was no difference between the hippocampus and cortex with regard to concentrations of glutamate, glutamine, and GABA. When data from the cortex and hippocampus were combined, propagated sites were shown to have higher glutamate and GABA than sites of seizure onset, which suggests that neurochemical abnormalities may extend beyond and be more prominent in brain regions outside of seizure onset sites.

The second part of this study examined the effects of antiepileptic drug withdrawal on levels of glutamate, glutamine, and GABA to ascertain if these concentrations were influenced by AEDs. There was a trend toward glutamate decrease in non-localized sites. Glutamine decreased significantly in lesion sites following taper, and GABA decreased significantly in non-localized sites. When sites were considered altogether, there were significant decreases in glutamine and GABA with taper.

The findings in the single basal component of this study suggest that increased extracellular glutamate and GABA extend far beyond the epileptogenic zone, which is consistent with findings on structural and metabolic imaging. Glutamate and GABA are

also elevated in lesions. The medication taper component of this study suggests that antiepileptic drugs may reduce seizure frequency by increasing extracellular GABA throughout the brain, although their effect on extracellular glutamate is minimal. The inability of AEDs to suppress extracellular glutamate to normal levels may be related to the medication resistance of epilepsy in these subjects. These results suggest that modulation of extracellular glutamate, both in seizure onset and propagation regions, should be examined for future antiepileptic pharmacotherapy.

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