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GLUTAMATE REGULATION IN THE HIPPOCAMPAL TRISYNAPTIC PATHWAY IN AGING AND STATUS EPILEPTICUS

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ABSTRACT OF DISSERTATION

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The Graduate School

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GLUTAMATE REGULATION IN THE HIPPOCAMPAL TRISYNAPTIC
PATHWAY IN AGING AND STATUS EPILEPTICUS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Michelle Lee Stephens

Lexington, Kentucky

Director: Dr. Greg A. Gerhardt, Professor of Anatomy and Neurobiology

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A positive correlation exists between increasing age and the incidence of hippocampal-associated dysfunction and disease. Normal L-glutamate neurotransmission is absolutely critical for hippocampal function, while abnormal glutamate neurotransmission has been implicated in many neurodegenerative diseases. Previous studies, overwhelmingly utilizing *ex vivo* methods, have filled the literature with contradicting reports about hippocampal glutamate regulation during aging. For our studies, enzyme-based ceramic microelectrode arrays (MEA) were used for rapid (2 Hz) measurements of extracellular glutamate in the hippocampal trisynaptic pathway of young (3-6 months), late-middle aged (18 mo.) and aged (24 mo.) urethane-anesthetized Fischer 344 rats. Compared to young animals, glutamate terminals in cornu ammonis 3 (CA3) showed diminished potassium-evoked glutamate release in aged rats. In late-middle aged animals, terminals in the dentate gyrus (DG) showed increased evoked release compared to young rats. The aged DG demonstrated an increased glutamate clearance capacity, indicating a possible age-related compensation to deal with the increased glutamate release that occurred in late-middle age.

To investigate the impact of changes in glutamate regulation on the expression of a disease process, we modified the MEA technology to allow recordings in unanesthetized rats. These studies permitted us to measure glutamate regulation in the hippocampal formation without anesthetic effects, which showed a significant increase in basal glutamatergic tone during aging. Status epilepticus was induced by local application of 4-aminopyridine. Real-time glutamate measurements allowed us to capture never-before-seen spontaneous and highly rhythmic glutamate release events during status epilepticus. A significant correlation between pre-status tonic glutamate and the quantity of status epilepticus-associated convulsions and glutamate release events was determined. Taken together, this body of work identifies the DG and CA3 as the loci of age-associated glutamate dysregulation in the hippocampus,

and establishes elevated levels of glutamate as a key factor controlling severity of status epilepticus in aged animals.

Based upon the potential ability to discriminate brain areas experiencing seizure (i.e. synchronized spontaneous glutamate release) from areas not, we have initiated the development of a MEA for human use during temporal lobe resection surgery. The final studies presented here document the development and testing of a human microelectrode array prototype in non-human primates.

Michelle Lee Stephens

07/21/2009

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DISSERTATION

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Dedicated to Maw and Kate, no guts no glory!

ACKNOWLEDGEMENTS

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Chapter One: Introduction

The hippocampus, the brain area chiefly responsible for cognitive learning and memory, undergoes many biological changes during aging. Age-associated declines in learning and memory have been well established in humans and demonstrated in non-human primate and rodent models of human aging. Much is known about the morphological, biochemical and electrophysiological changes that occur during aging in the hippocampus, but an understanding about how these age-associated changes translate into deficits in the cognitive functions of the hippocampus is largely lacking. The hippocampus is also a primary substrate for the onset of age-associated pathology like Alzheimer's disease. Changes in cognitive function and on-set of disease, have led to the hypothesis that hippocampal aging and the associated biological changes may increase the susceptibility of aging populations to hippocampal dysfunction and neurodegenerative disease.

Anatomy and Neurophysiology of the Trisynaptic Pathway

The trisynaptic pathway or circuit is an ensemble of extrinsic and intrinsic pathways that adjoin cytoarchitectonically distinct regions in the hippocampal formation. All of the neuronal communication in the trisynaptic circuit is excitatory, using the neurotransmitter L-glutamate (Storm-Mathison, 1977). As described by Andersen and colleagues (2007), fast excitatory neurotransmission is mediated primarily by inward excitatory postsynaptic currents (EPSCs) flowing through ionotropic α-amino-3-hydroxyl-5-methyl-4-isoxazoleproprionate (AMPA) glutamate receptors, while slower hippocampal EPSCs are the result of activation of N-methyl-D-aspartate (NMDA) ionotropic glutamate receptors and certain subtypes of metabotropic glutamate receptors (mGluRs). The NMDA and AMPA ionotropic glutamate receptors are critical for long-term potentiation,

plasticity and hippocampal-dependent learning and memory (for review see Riedel et al., 2003).

Synapse 1 of the trisynaptic circuit is at the terminal end of projections from the entorhinal cortex (EC) to the dentate gyrus (DG); synapse 2 at the end of DG projections to cornu ammonis 3 (CA3) and synapse 3 at the end of CA3 projections to cornu ammonis 1 (CA1) (Anderson et al., 1971). Each set of projections in the trisynaptic circuit is named. The major input projections into the DG, termed the perforant path, arise from layer II of the EC (with minor layer V and VI contributions) (Steward and Scoville, 1976). Perforant path projections terminate in the molecular layer of the DG which contains the dendrites of the principal cell type in the DG, granule cells. The DG houses other cell types, most of which are inhibitory neurons, but only the granule cells provide axons that project outside of the DG. Granule cells provide efferents termed mossy fibers that only project to dendrites of CA3 principle cells, known as pyramidal cells (Claiborne et al., 1986). CA3 pyramidal cells project to dendrites of CA1 pyramidal cells via Schaffer collaterals (Ishizuka et al., 1990) (Figure 1.1).

Outside of the trisynaptic circuit, it is necessary to note that CA3 receives input from the perforant path in a similar manner to the DG which may actually be the major determinant of CA3 output (Yeckel and Berger, 1990; Witter, 1993). Also, CA3 is heavily innervated by 'associational connections/ collaterals' from other CA3 pyramidal neurons (Hasselmo et al., 1995). CA1 also receives perforant path afferents from the EC, which are unique because they originate in layer III (Andersen et al., 2007). A 'loop' of interconnectivity is created throughout the hippocampal formation when CA1 pyramidal cells project to the subiculum, which as a major source of hippocampal output projects back to all layers of the EC (Andersen et al., 2007). Other neurotransmitters in the hippocampus can modulate the flow of excitatory neurotransmission, namely acetylcholine (ACh). Ionotropic (nicotinic) ACh receptors can modulate glutamate release due to their positioning on presynaptic glutamatergic fibers and on inhibitory interneurons. Metabotropic (muscarinic) ACh receptors can be

found pre- and post-synaptically, and their effects are extremely excitatory (reviewed by Shepherd, 2004).

Though the DG, CA3 and CA1 subregions of the trisynaptic circuit are intricately connected, they each have unique characteristics distinguishing them from the other regions. This necessitates experimental protocols that investigate them individually, as opposed to studying the hippocampal formation as a homogeneous structure. Pyramidal cells in CA3 and CA1 differ from each other and from DG granule cells both anatomically and biophysically (Carnevale, et al. 1997). The most recent evidence of subregional differences has come from Greene and colleagues (2009). Using laser-capture microdissection and microarray hybridization, it was shown that greater than 45% of expressed gene transcripts were significantly different across the DG, CA3 and CA1 principal layers, and one third of those (>1000) showed at least a two-fold difference between layers. In the CA3 pyramidal layer, transcripts encoding proteins involved in glucose metabolism were highly expressed, and in the DG transcripts related to MAP kinase signaling and transcriptional regulator activity were most prominent. Differences between CA3 and CA1 included alterations in transcripts for calcium and potassium channels and glutamate receptors. Also, more synapse-related transcripts were expressed in CA3 compared to CA1. Greene et al. (2009) emphasized that functional differences across cell layers were likely secondary to wide-ranging expression differences of modest magnitude, rather than very large disparities in a few genes, but points out that these differences may be enough to account for subregion-specific susceptibility to insult including DG granule cell resistance to damage following ischemia and seizures, CA3 pyramidal cell vulnerability to damage from trauma and seizure and CA1 pyramidal cell vulnerability to ischemia and seizure (Ordy et al., 1993; Borges et al., 2007)

Overall, the trisynaptic circuit architecture and patterns of connectivity are similar between rats and humans. Humans do have a much expanded CA1 pyramidal cell layer (30 cells thick in humans, 5 cells thick in rats), and more developed entorhinal cortex (8 distinct subregions vs. 2 subregions) compared to

rats (Andersen et al., 2007). Rats have extensive commissural connections between the DG in each hemisphere (Gottlieb and Cowan, 1973), and though less numerous, humans also have these connections, which can allow contralateral seizure propagation (Gloor et al., 1993). Other minor differences exist, but overall the hippocampal formation is a phylogenetically conserved brain region in mammals (Andersen et al., 2007).

Anatomic and Neurophysiologic Changes in the Aged Hippocampus

Though some reports have shown a loss of hippocampal principal neurons during aging (for review see Geinisman et al., 1995), widespread use of unbiased stereological cell counting methods have contributed several studies to the literature that indicate no age-related loss of principal cells occurs in rats (Rapp and Gallagher, 1996; Rasmussen et al., 1996;), non-human primates (West et al., 1993; Peters et al., 1996), or humans (West, 1993; Morrison and Hof, 1997). Also, Rapp et al. (2002) found that aged rats with spatial learning deficits maintain neuron numbers at young levels in the primary input to the hippocampus, the EC. In addition to preserved number, several of the basic cellular characteristics of granule and pyramidal principal cells do not change with age (reviewed by Rosenzweig and Barnes, 2003) including but not limited to, resting membrane potential (Barnes, 1979; Barnes et al., 1987, 1992; Potier et al., 1992), and amplitude and duration of Na^+-mediated action potentials (Barnes and McNaughton, 1980; Segal, 1982; Barnes et al., 1987, 1992; Niesen et al., 1988; Potier et al., 1992). Alterations in hippocampal function do not appear to be caused by gross changes in cell numbers or basic cellular characteristics, therefore research efforts have shifted to focus on synapses as a substrate for hippocampal dysfunction.

Important changes in synaptic function are believed to occur in the aged trisynaptic circuit related to alterations in synapse number, efficiency and calcium regulation. Changes are subregion specific. A loss of perforant path axons

(Barnes and McNaughton, 1980) and synapses (Geinisman et al., 1992) in the DG has been demonstrated. Compensatory increases in the strength of the remaining synapses, shown by changes in the presynaptic fiber/EPSP ratio (Barnes and McNaughton, 1980, Foster et al., 1991) and increased quantal size (Foster et al., 1991), indicates that remaining perforant path synapses in aged rats may be stronger than in young rats. A loss of Schaffer collateral synapses are also believed to occur in CA1 based upon electrophysiological data (Landfield et al., 1986; Barnes et al., 1992; Deupree et al., 1993); however, different from the synapses in the DG, the CA1 synapse are not believed to increase in strength (reviewed by Rosenzweig and Barnes, 2003). Functional outcomes of age-associated synaptic changes are believed to correspond to changes in long-term potentiation (LTP), a mechanism underlying learning and memory processes in the laboratory setting (Moser et al., 1998). LTP is a long-lasting augmentation of synaptic responses following high frequency stimulation (Bliss and Gardner-Medwin, 1973).

Using high intensity stimulation protocols, synapses in the DG of young and aged animals have been shown to reach the same maximum LTP, though the process took longer in aged animals and aged animals lost the LTP faster (Barnes and McNaughton, 1985; Diana et al., 1994)). Similar to the DG, the maximum potentiation obtained in CA1 of young and aged animals is the same, though the time to reach peak potentiation is longer in aged rats (Landfield et al., 1978). The age-associated changes in the DG and CA1 LTP have been replicated many times. Interestingly, an opposite process of LTP, long-term depression (LTD) reduces synaptic strength, and is the experimental equivalent of erasing a memory (reviewed by Rosenzweig and Barnes, 2003). Aged animals have been shown to be more susceptible to LTD (Norris et al., 1996), so based upon LTP and LTD studies, aged animals not only have more difficultly learning or creating memories, they have an easier time forgetting too. CA1 pyramidal cells in aged animals have been shown to have an enhanced afterhyperpolarization (AHP) response (Landfield and Pitler, 1984; Moyer et al.,

1992) which usually diminishes in younger animals as a consequence of learning (for review see Wu et al., 2002).

Age-associated changes in CA3 have been less studied, but De Groot and Bierman (1987) did report a loss of synapses similar to that seen in the DG. Likewise, altered LTP characteristics comparable to those in the aged DG have been described in the aged CA3 following perforant path stimulation (Dieguez and Barea-Rodriguez, 2004).

Calcium regulation also significantly affects synaptic function. Based upon electrophysiological studies of Ca^{2+}-dependent neuronal processes, important biomarkers for aging in the hippocampus include increased L-type voltage gated Ca^{2+} channel (L-VGCC) activity (Thibault and Landfield, 1996), elevated Ca^{2+} transients (Thibault et al., 2001; Hemond and Jaffe, 2005) and the previously described enhanced AHP response and the corresponding impaired synaptic plasticity. Patrylo et al. (1994) has pointed out that elevated Ca^{2+} could create ion disturbances (i.e. K^+) leading to synchronized neuronal bursting, which may affect epileptogenic mechanisms in the aged hippocampus. It is also important to note that disrupted Ca^{2+} homeostasis has long been known to be centrally involved in NMDA receptor-mediated excitotoxicity (reviewed by Lipton, 2004).

Finally, neuroinflammation in the aged hippocampus must be discussed. The hippocampus is rich in glucocorticoid receptors (McEwen, 1996). Glucocorticoid exposure is linked to structural and functional changes in the hippocampus including decreased neuronal density, altered synaptic plasticity and deficits in spatial learning, all of which are ultimately associated with aging (for review see Landfield and Eldridge, 1994). However, the relationship between glucocorticoids and aging is not as straightforward as once believed (Landfield et al., 2007). Efficacy of glucocorticoid actions during aging are believed to be different for neurons and glia. For example, glia are thought to lose sensitivity to glucocorticoids. Therefore, normally suppressed inflammatory pathways are actually increased during aging (Landfield et al., 2007). Convincing evidence that neuroinflammatory changes (increases in

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proinflammatory cytokines and microglial activation) do occur in the hippocampus during aging, and are accompanied by deficits in LTP, has been reported by Griffin et al. (2006).

Glutamate Neurotransmission in the Young and Aged Hippocampus

Glutamate release and clearance are the key determiners of neurotransmitter availability for participation in receptor-dependent processes. Though release from depolarized nerve terminals in a Ca^{2+}-dependent manner is the predominant mechanism of glutamate release into the extracellular space, astroglia have been shown to release glutamate as well (Bezzi et al., 1998; Volterra and Steinhäuser, 2004). Astroglia also indirectly modulate glutamate release into the extracellular space via their most well known function, glutamate clearance with high affinity transporters, by controlling the ability of extrasynaptic glutamate to activate presynaptic mGluRs which feedback to adjust neuronal glutamate release (Huang and Bergles, 2004). As reviewed by Danbolt (2001); ionotropic and metabotropic glutamate receptors are widely distributed (dendrites, nerve terminals, neuronal cell bodies and glial cells), so to avoid improper activation of these receptors, extracellular glutamate levels must be highly regulated. The only way to remove glutamate from the extracellular space, regardless of the release mechanism, is through high affinity transporters located mainly on astroglia (Gegelashvili and Schousboe, 1998) (Figure 1.2).

As outlined earlier, much is known about age-associated changes in hippocampal synapse numbers, electrophysiological characteristics and plasticity. A reduction in hippocampal ionotropic glutamate receptors and their constituent subunits with age has been correlated with decline in memory function (Magnusson, 1998; Adams et al., 2001; Tang et al., 2001; Clayton et al., 2002). A similar correlation exists with memory impairment and reduced levels of the vesicle protein synaptophysin (Smith et al., 2000). Hippocampal aging is also associated with activated gliosis evidenced by increased GFAP staining,

increased astrocytic size and fibrosis (Vaughan and Peters, 1974; Landfield et al., 1977) and a complete loss of organization in astrocytic processes (Sykova, 1998). Changes in glia can possibly alter the expression and function of high affinity transporters and change glutamate levels in the extracellular space. Also, learning and memory deficits are associated with alterations in gamma-aminobutyric acid (GABA) neurotransmission associated with hippocampal interneurons that regulate excitatory transmission from principle cells (Shetty and Turner, 1998; Vela et al, 2003). Specifically, a loss of GABAergic neurons (but not synapses) has been reported in CA1, but not CA3 or the DG, with aging (Shi et al., 2004). Collectively, these data indicate that alterations in glutamatergic neurotransmission occur in the hippocampus during aging, but extensive investigations of glutamate release and clearance processes have only succeeded in filling the literature with contradicting reports (Segovia et al., 2001).

The majority of studies investigating regulation of glutamatergic neurotransmission during aging have used *ex vivo* experimental methodology (i.e. tissue homogenates, slices and synaptosomes). Total hippocampal content of glutamate has been shown to decrease (Banay-Schwartz et al., 1989) or not change (Wallace and Dawson, 1990) in aged compared to young animals. Basal extracellular glutamate levels in aged animals have been reported to decrease (Saransaari and Oja, 1995), increase (Freeman and Gibson, 1987) or not change (Meldrum et al., 1992) compared to young animals. Similarly, an age-associated increase (Meldrum et al., 1992; Saransaari and Oja, 1995) and decrease (Freeman and Gibson, 1987) have been reported for stimulus-evoked glutamate release, while no change has been reported in glutamate clearance when comparing young and aged animals (Gilad et al., 1990; Najerahim et al., 1990; Palmer et al., 1994). Isolating the hippocampus from its extrinsic neuronal connections and disrupting the intrinsic connections could account for the inconsistency in previous aging studies using *ex vivo* methods.

Only two prior studies have attempted to characterize *in vivo* glutamate regulation in the hippocampus of aging animals. Using microdialysis, Massieu and Tapia (1997) showed an increase in tonic hippocampal glutamate in aged

compared to young rats, and Zhang et al. (1991) reported a decrease. Though microdialysis does allow for *in vivo* measurements of tonic glutamate (i.e. steady-state glutamate concentration in the extracellular space), the slow temporal resolution limits the ability to investigate the rapid release and clearance dynamics associated with glutamate neurotransmission (Timmerman and Westerink, 1997; Danbolt, 2001). This is important because alterations in release and/or clearance may not manifest with changes in basal glutamate levels due to biological compensation, and therefore must be studied directly.

Animal Models of Status Epilepticus

The elderly population is the fastest growing age group in the United States (Collins et al., 1997). Studies over the last 20 years have established that the elderly population has an increased risk for status epilepticus (SE), status-related morbidity and mortality (DeLorenzo et al., 1992), and experiences the highest incidence of epilepsy in the population (Luhdorf et al., 1986; de la Court et al., 1996). SE is defined as continuous seizure activity persisting for more than 5-10 minutes, or 2 or more seizures without full recovery of neurologic function in between (Treiman et al., 1998; Lowenstein et al., 1999). Despite the obvious importance of understanding epilepsy in the elderly, especially SE, very few basic science studies have modeled epilepsy in aged animals (Leppik et al., 2006).

Chemically induced SE models typically use high doses of a convulsive agent (e.g. kainate, pilocarpine, 4-aminopyridine) administered systemically. These agents can also be applied intrahippocampally (Mathern et al., 1993; Fragoso-Veloz et al., 1990). Sustained electrical stimulation to the perforant path (Sloviter, 1987) and ventral hippocampus (Lothman et al., 1990) can also induce SE, though these models are less utilized. In all models animals display acute behavior and EEG activity consistent with clinical SE, and if allowed to continue for a sufficient duration will develop hippocampal neuronal damage, also

observed in humans (reviewed by Morimoto et al. 2004). After a variable lapse of time, animals will develop spontaneous seizures, and as pointed out by Morimoto et al. (2004), most researchers are interested in the development of this pathology and symptomatology similar to that seen in human temporal lobe epilepsy (TLE) following SE, and not the SE event itself.

Pilocarpine-induced SE is a commonly used animal model. Pilocarpine induces SE through M1 muscarinic receptor activation (Hamilton et al., 1997), but seizures are maintained by NMDAR activation (Nagao et al., 1996) and accompanied by increases in hippocampal glutamate (Smolders et al., 1997). Pilocarpine produces loss of hippocampal neurons and synaptic reorganization via mossy fiber sprouting (Mello et al., 1993). Historically, mortality rates have been high (at least 30-40%, Cavalheiro et al., 1991). Even though administering multiple low doses or using diazepam to limit the SE duration have improved mortality rates (for review see Curia et al., 2008), no examples of SE modeling in aged animals using pilocarpine exist in the literature.

Status epilepticus has been modeled in aged rats using a repeated low-dose (2.5 mg/kg per hour) kainic acid protocol (Darbin et al., 2004). This study showed that aged animals (22-25 months old) had a significantly shorter latency to seizure behavior compared to young rats (7 months) and significantly more wet-dog shakes, classified in this study as pre-seizure behavior. Likewise, aged rats also displayed altered electroencephalograms (reduction in higher frequencies) compared to young rats. As with pilocarpine, kainic acid treatments cause mossy fiber sprouting and also neuronal loss in CA3 (Leite et al., 1996).

Glutamate and Seizures

As previously mentioned, researchers have traditionally been more focused on the resultant TLE following SE modeling, rather than the SE event. An extensive relationship between glutamate dysregulation and TLE has been documented in the literature which can offer some insight into potential

mechanisms of glutamate dysregulation during and following SE that may ultimately lead to recurrent unprovoked seizures, especially in the elderly population.

From studies sing microdialysis in human TLE patients, extracellular glutamate has been shown to increase 30-fold above normal basal levels during seizure, and persist at levels approximately 12-fold higher than normal for at least 20 minutes after the cessation of seizure activity (During and Spencer et al., 1993). Glutamate levels are also elevated during interictal periods in epileptogenic hippocampi (Cavus et al., 2005). Indicating a universal involvement of glutamate in seizures, increases in glutamate have also been shown with microdialysis in rodent kainic acid models (Ueda et al., 2002) and pilocarpine models (Meurs et al., 2007) of epilepsy during the initial SE.

The very important involvement of astroglia in regulating extracellular glutamate concentrations has already been described. The predominant hippocampal excitatory amino acid transporter (EAAT2 or GLT-1; Rothstein et al., 1994) has been reported to be decreased in patients with TLE and accompanying hippocampal sclerosis (gliosis) (Mathern et al., 1999; Proper et al., 2002). This could alter clearance of extracellular glutamate and possibly contribute to the onset or propagation of epileptoform activity.

Astroglia are also responsible for metabolism of glutamate via the enzyme glutamine synthetase (GS), which converts glutamate recovered from the extracellular space to glutamine. Glutamine is then shuttled back to neurons, where it is converted back to glutamate, and packaged in synaptic vesicles for release (Figure 1.2). Patients with TLE have been shown to be severely deficient in GS (Eid et al., 2004). It is necessary to point out that GS activity has also been shown to be significantly decreased in the cortex during aging (Smith et al., 1991); though decreases in GS have not been shown in the aged hippocampus (Goss et al., 1991; Wu et al., 2005). Efficient glutamate clearance by astroglia may be compromised due to diminished metabolism from changes in GS, ultimately resulting in increased extracellular levels (Eid et al., 2008) which could lead to seizure or excitotoxicity.

Likewise, astroglia are important for the maintenance of extracellular K^+ levels which can also affect neuronal excitability. Buffering of extracellular K^+ via reuptake is thought to be primarily achieved with inward rectifying K^+ (Kir) channels in certain glial cell types (Newman, 1993). Several studies have shown a down regulation of Kir currents in patients with TLE (Bordey and Sontheimer, 1998; Hinterkeuser et al., 2000; Schroder et al., 2000). It is believed that diminished K^+ buffering from dysfunction of Kir channels may contribute to hyperexcitability in epileptic tissue (Steinhauser and Seifert, 2002). It should also be noted that pro-inflammatory cytokines are shown to transiently increase in experimental models of TLE. One cytokine, IL-1 β, has been shown to regulate astrocytic phenotype and stimulate Ca^{2+} -dependent glutamate release, which may trigger and maintain seizures (reviewed by de Lanerolle and Lee, 2005).

The common practice of temporal lobe resection as treatment for refractory TLE has allowed unique *in vitro* studies with human tissue. This has provided a large body of knowledge about potential mechanisms of excitatory neurotransmission dysregulation, including the changes in astrocytes outlined above. However, lacking in this cohort of patients, as is lacking in the basic science literature, is a representation of the elderly epileptic population (Leppik et al., 2006). Knowing that elderly patients are particularly affected by epilepsy and SE, but sometimes poor surgical candidates, makes the utilization of models especially important for understanding the pathology and unique treatment demands in the aged population. Though microdialysis has provided a correlation between increased extracellular glutamate and seizure, the temporal resolution of microdialysis limits the ability to measure alterations in the fast aspects of glutamate neurotransmission like release and clearance. A clear need exists for *in vivo* characterization of dynamic glutamatergic neurotransmission during status epilepticus, which may provide novel information about the role of glutamate in the aggressive clinical course of seizure disorders in the elderly (During and Spencer et al., 1993; Cavus et al., 2005).

Thesis Outline

For the work presented in this dissertation, it was especially important to consider the similarity in substrates for age-associated and seizure-associated changes in the hippocampus (i.e. glutamate neurotransmission, astrocytes). Chapter Two provides a detailed description of the materials, instrumentation, methodology and statistical analysis used for all dissertation experiments. In Chapter Three, ceramic enzyme-based microelectrode arrays (MEAs) were used for second-by-second measurements of glutamate neurotransmission in the discrete dentate gyrus (DG), cornu ammonis 3 (CA3) and cornu ammonis 1 (CA1) trisynaptic subregions. These experiments investigated the capacity of glutamate release and uptake in anesthetized young (3-6 months), late-middle aged (18 months) and aged (24 months) Fischer 344 (F344) rats. Potassium-evoked release of glutamate was greatest in the DG of late-middle aged rats as compared to young and aged animals. The most fascinating finding was that the CA3 subregion exhibited profound dysregulation during aging. CA3 had diminished evoked glutamate release in aged rats compared to young and late-middle aged animals. Also, alterations within the trisynaptic circuit during aging showed a significant loss of glutamate release capacity in CA3 and CA1 compared to the DG in both late-middle aged and aged rats. These findings suggest an altered function or competence of the glutamate fibers in aging animals. Finally, the aged DG showed a significantly increased glutamate uptake rate compared to the DG of young animals, suggesting that the increases in glutamate release in late-middle aged rats may result in compensatory increases in glutamate uptake capacity in aged rats. These data are the first to provide insight into age-related changes in functional glutamatergic neurotransmission in subregions of the rat hippocampus.

Chapter Four further explores glutamate regulation using MEAs adapted for use in unanesthetized animals. In summary, these studies were designed to investigate glutamate regulation in the hippocampus during normal aging, and to elucidate the potential relationship between glutamate dysregulation and rigor of

status epilepticus (SE) in aging populations. Tonic glutamate was shown to be significantly elevated in the hippocampal formation of late-middle aged and aged rats compared to young rats prior to SE modeling. The quantification and characterization of spontaneous and highly synchronized glutamatergic release events that co-manifested with the appearance of SE behavior provided a novel means to link glutamate dysregulation with seizure pathology. Pre-SE tonic glutamate significantly correlated with the severity of SE after low doses of 4-AP, as animals with the highest tonic levels experienced the most persistent and severe behavior, along with the largest amount of spontaneous glutamate release. Regardless of pre-SE tonic glutamate levels, after the high dose of 4-AP significantly longer SE durations and more spontaneous glutamate release activity was observed in aged animals. Taken together, these results indicate that a strong relationship exists between aberrant glutamate neurotransmission and SE, and support an etiological role for age-associated glutamate dysregulation in seizure disorders occurring with disproportionately high frequency and severity in elderly populations.

Chapter Five chronicles the development and testing of microelectrodes for future clinical uses. These studies demonstrated that we can obtain real-time measurements of extracellular glutamate in awake non-human primates using enzyme-based MEA technology coupled to amperometric recordings with a design that has been adapted for human use (Spencer-Gerhardt-2 MEA (SG-2)). The temporal resolution (2 Hz) of these measurements allowed us to record dynamic oscillations in extracellular glutamate, which to our knowledge has never been reported. We showed that sterilization did not significantly compromise the integrity and function of the enzyme/protein layers applied to SG-2 MEAs for the detection of glutamate. We also showed that the SG-2 MEAs have a shelf-life of close to a year.

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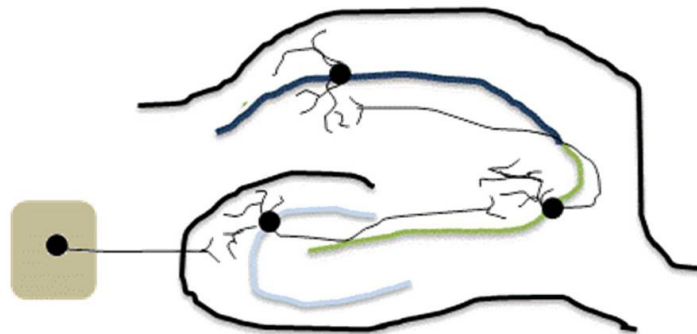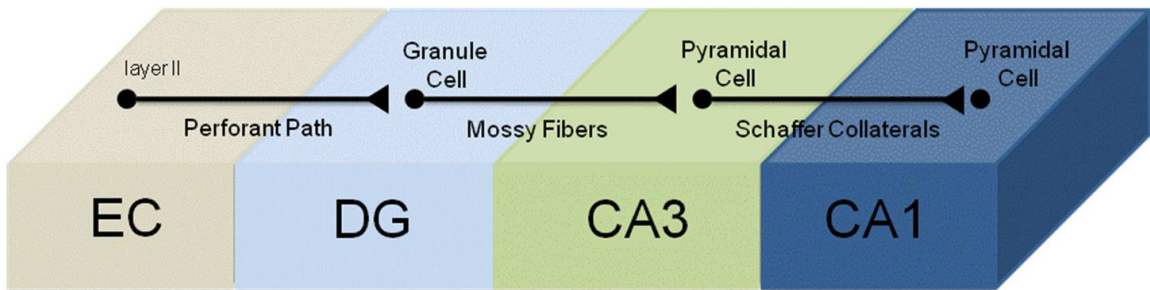
Figure 1.1 The Trisynaptic Pathway

The trisynaptic pathway or circuit is the unidirectional flow of excitatory glutamatergic neurotransmission through the hippocampal formation. Synapse 1 is at the terminal end of perforant path projections from the entorhinal cortex (EC) to the granule cells of the dentate gyrus (DG); synapse 2 is at the end of DG mossy fiber projections to pyramidal cells in cornu ammonis 3 (CA3) and synapse 3 is at the end of CA3 Schaffer collateral projections to pyramidal cells in cornu ammonis 1 (CA1).
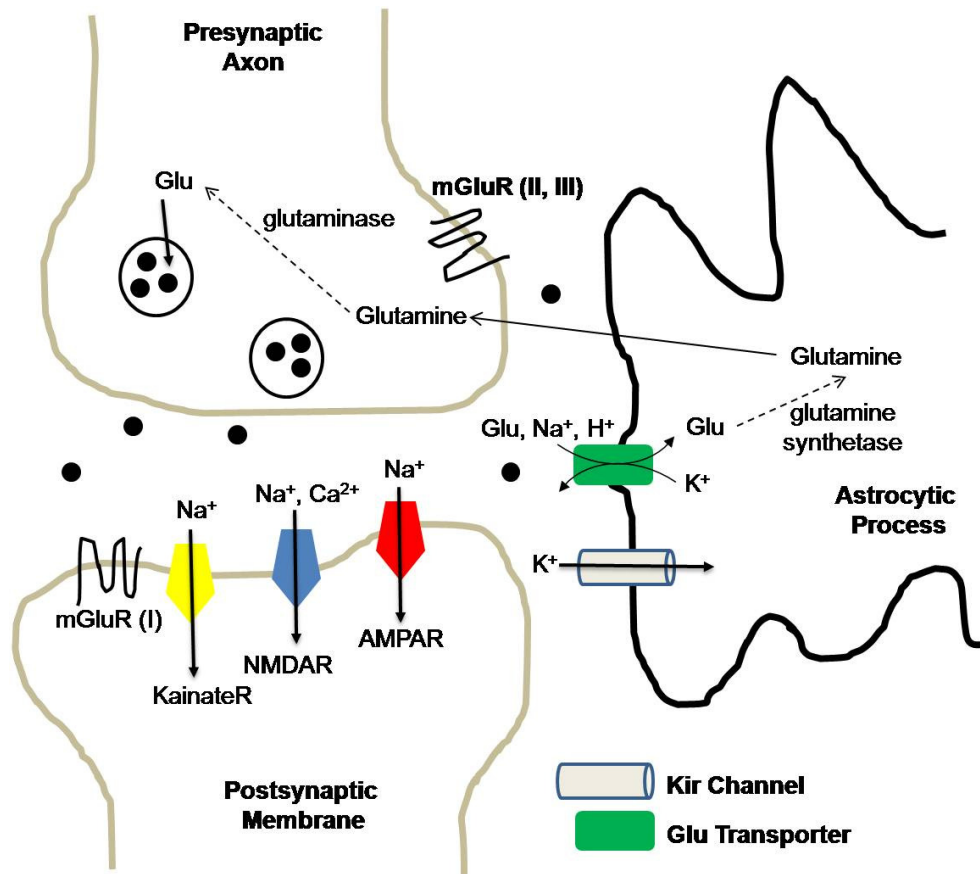
Figure 1.2 Typical Glutamate Synapse in the Trisynaptic Pathway

Excitatory post-synaptic potentials (EPSPs) are generated when glutamate is released from presynaptic vesicles and subsequently binds to receptors located on the postsynaptic membrane (mGluR, KainateR, NMDAR, AMPAR). Astrocytes express high-affinity transporters that function to remove glutamate from the extracellular space and terminate glutamatergic neurotransmission. Glutamate that is not recovered can diffuse to presynaptic mGluRs and auto-regulate glutamate release. Astrocytes are also important for buffering extracellular potassium via inward rectifying K^+ channels. Once recovered by glia, glutamate is converted to glutamine, shuttled across the extracellular space and reconverted to glutamate in presynaptic terminals where it is packaged for vesicular release.

Chapter Two: Materials and Methods

The Fischer-344 Rat as a Model of Human Aging

Rodent models have a long history of contributing to the scientific study of aging. Inbred strains have been particularly utilized because their genetic uniformity helps minimize sample sizes. Freedom from genetic variables also facilitates the isolation of aging effects on the data. Currently the National Institute on Aging maintains several inbred colonies for aging research including Fischer-344 (F344) and Brown-Norway (BN) rats and BALB/cBy, CBA, C57BL/6 and DBA/2 mice. Studies using F344 rats to model mammalian aging are the most numerous in the aging literature. Though the repeated use of F344 rats has brought criticism from some researchers with concerns that scientists are limiting the scope of our knowledge about aging (Weindruch and Masoro, 1991), the novelty of the studies and research techniques presented in this dissertation prompted us to choose the F344 strain because we felt the abundant body of prior work would assist in the interpretation of our data. Both male and female F344 rats are utilized for aging studies, but we chose to work with males to avoid the effects of hormonal fluctuations with age that can affect the hippocampal formation (reviewed by Foy et al., 2008).

A concern for researchers working with aged animals is the incidence of age-associated disease that may influence studies. Male F344 rats do commonly display glomerulonephropathy and testicular interstitial cell tumors at advanced ages, but a comprehensive examination showed that the male F344 rats displayed less of 10 other age-associated lesions than the other available inbred strain, BN rats (Holmes, 2003).

F344 rats have a typical life-span of approximately 27 months (Coleman et al., 1977). Though average life-span does vary across different strains, it has been agreed upon that a rat is considered aged at 22 months (Masoro, 1990).

For our studies characterizing the effects of aging on glutamate neurotransmission in the normal and actively seizing hippocampus, three age groups were chosen: young (3-6 months), late-middle aged (18 months) and aged (24 months). Using the conversion of '1 rat month = 3 human years', these age groups simulate humans of approximately 18, 54 and 72 years old. It is important to note that the youngest group is reproductively mature so these studies were not confounded by development. Approximately two-thirds of aged F344 rats are found to be impaired during memory tasks (reviewed by Geinisman et al., 1995), so we conclude that our aging model was a good choice for studying glutamatergic regulation in a functionally heterogeneous sample, representative of the aging human population.

The 4-aminopyridine Status Epilepticus Model

4-Aminopyridine (4-AP), a K^+ channel blocker, has been used extensively to model *in vitro* seizure activity (Perreault and Avoli, 1991 among many others), but much less *in vivo* research has been conducted. Interest in the 4-AP compound originated from its ability to prolong action potentials and increase neurotransmission, resulting in the use of 4-AP for the treatment of neurological disorders such as spinal cord injury (Cardenas et al., 2007). It was learned that over-exposure in humans caused convulsions and subsequently 4-AP became a compound of interest for epilepsy research (Spyker et. al., 1980).

4-AP produces limbic seizures when microinjected (Fragoso-Veloz, et al., 1990; Morales-Villagran et al., 2008) or applied with reverse microdialysis (Pena and Tapia, 1999; Media-Ceja et al., 2000; Pena and Tapia; 2000; Ayala and Tapia, 2005) into the hippocampal formation *in vivo*. Following a single dose of 4-AP, local seizure induction is rapid (< 1 minute for application into CA1, 4.2 nmol, Fragoso-Veloz, et al., 1990; ~10 min. for EC application, 10 nmol, Morales-Villagran et al., 2008), followed by propagation to other brain areas including the contralateral hippocampus and eventually the cortex. Animals display behaviors

characteristic of limbic seizures and analogous to those seen with kainic acid administration (Ben-Ari et al., 1981) including wet-dog shakes, oral automatisms and grooming. Microdialysis studies have shown that glutamate levels rise in the hippocampus during 4-AP-induced status epilepticus (SE), but slow sampling rates on the order of minutes have allowed for nothing more detailed than the basic conclusion that extracellular glutamate transiently increases to a maximum of 6-8 µM then returns to baseline (~2 µM) over the course of an hour or so (Pena and Tapia, 1999; Media-Ceja et al., 2000; Pena and Tapia; 2000; Ayala and Tapia, 2005). 4-AP also induces NMDAR-dependent neurotoxicity (Pena and Tapia; 2000; Ayala and Tapia, 2005) similar to that seen in kainic acid models (Brandt et al., 2003), so dysregulation of glutamate neurotransmission during SE needs to be more extensively studied.

We believe 4-AP-induced SE was the most appropriate and practical model for our research. In addition to inducing glutamate release which mimics numerous microdialysis recordings in seizure-prone humans and animal models of epilepsy, 4-AP blocks K^+ currents (neuronal and glial). A large body of work implicates 4-AP-sensitive K^+ currents in the modulation of high frequency neuronal discharges (reviewed by Bordey and Sontheimer, 1999), but most important for our studies is the ability of 4-AP to block glial Kir currents (Bordey and Sontheimer, 1999) in accordance with glial data collected from the hippocampi of TLE patients (see Chapter 1). The aged hippocampus experiences profound changes in glia, but the functional outcomes of these alterations are not known and may provide a critical link between age-associated hippocampal dysfunctional and the propensity for increased SE and negative outcomes. We feel other models of SE (i.e. kainic acid) do not adequately incorporate means to exploit potential alterations in glial function within the aging hippocampus as it relates to the manifestation of SE.

<center>**Animals**</center>

F344 Rat Studies

Young adult (3-6 months, n = 40), late-middle aged (18 months, n = 34) and aged (24 months, n = 42) male F344 rats were obtained from the National Institute on Aging colony (Harlan Sprague Dawley, Inc., Indianapolis, IN) and used for all experiments studying glutamate in the hippocampal trisynaptic circuit. Protocols for animal use were approved by the Institutional Animal Care and Use Committee. In accordance with approved guidelines, animals were housed in a 12 hour alternating light/dark cycle, with food and water available *ad libitum*. All glutamate measurements were obtained during the 'light phase' and on average were recorded at 1:00 PM (EST). These studies were focused on changes in glutamate regulation during 'normal aging', therefore animals were not enriched.

Non-human primates

Three adult female rhesus monkeys (*Macaca mulatta*) were obtained from a commercial supplier (Covance, Alice, TX) (NHP 1 = 11 years old, NHP 2 = 19 years old; NHP 3 = 21 years old). Protocols for animal use were approved by the Institutional Animal Care and Use Committee. Animals were housed in the Laboratory Animal Facilities at the University of Kentucky which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACI). Food and water were available *ad libitum*. All glutamate measurements were performed while the animals were awake during the 'light phase' of their 12 h light: 12 h dark maintenance schedule.

<center>**Principles of Electrochemistry**</center>

The recording surface or 'working-electrode' of the microelectrode array (MEA) is an inert metal (platinum) that can oxidize or reduce compounds of interest when a potential is applied versus a Ag/AgCl reference electrode in ionic

<center>20</center>

contact with the MEA. Computer-controlled low noise 'patch clamp-like' potentiostats with custom multiple-input recording software allow simultaneous measurements from several recording surfaces on a single MEA. The potentiostat, or 'headstage', creates the required difference in potential between the MEA and the Ag/AgCl reference electrode, and measures the currents created by electrochemical reactions. Applying a constant fixed potential to the microelectrode, known as amperometry, is one of the most straight-forward electrochemical techniques (Gerhardt and Burmeister, 2000). If the potential is sufficient, a molecule at the surface of the working-electrode may donate electrons (oxidize) or accept electrons (reduce), both Faradaic chemical reactions that generate current which is linear with respect to the concentration of electroactive molecules. Amperometry minimizes measurement of non-faradaic background current by the microelectrode, offering highly sensitive measurements of electrochemically active molecules. This technique also allows for rapid sampling rates (ms to sec), providing a fast means to monitor neurochemistry of the extracellular space in real-time (Gerhardt and Burmeister, 2000

Several neurochemicals of interest in the extracellular space are electrochemically active at a working electrode including dopamine (DA), norepinephrine, serotonin, nitric oxide, uric acid, and 3,4-dihyroxyphenylacetic acid (DOPAC). Most of these molecules can also be easily shielded from the recording surfaces of the MEA by application of selective coatings (Hascup et al., 2007). To expand the repertoire of detectable neurochemicals, the recording sites can be coated with a variety of oxidase enzymes which convert electrochemically inactive analytes of interest to 'reporter' molecules of hydrogen peroxide (Gerhardt and Burmeister, 2000). Applying a potential of +0.7 V vs. a Ag/AgCl reference is sufficient to oxidize hydrogen peroxide at the working sites. Current created by liberation of electrons during the oxidation of hydrogen peroxide is linear with respect to local concentration of the original enzymatic substrate.

Microelectrode Fabrication

MEAs used for the studies presented in this dissertation were constructed in conjunction with Thin Films Technology, Inc. (Buellton, CA). Details of the fabrication process are outlined in Burmeister et al. (2001). The multisite MEAs were mass fabricated using photolithographic methods. Photolithography helps decrease overall production costs as multiple MEAs can be fabricated with minimal waste of starting materials. Cost is an ever-present concern for research instrumentation, as well as for clinical diagnostic and treatment technologies. Arrays of platinum recording surfaces were patterned onto a ceramic substrate (Coors Superstrate 996 (alumina, Al_2O_3) Coors Ceramics, Golden, CO) which provides a strong non-flexible base for precise stereotactic placement, minimizes cross-talk noise between platinum recording sites, and can be polished to achieve excellent biocompatibility in chronic implants (Rutherford et al., 2007). The ceramic substrate was modified depending on the desired application (i.e. anesthetized or unanesthetized animal recordings; acute or chronic animal recordings). Extensive research and development of the MEA fabrication and cleaning processes have produced very deliberate protocols for the construction of durable and reusable MEAs (for review see Hascup et al., 2007).

For the *in vivo* studies presented here, the S2 MEA configuration was selected (Figure 2.1). The S2 has four platinum recording surfaces, each 15 x 333 μm. The platinum sites are arranged in two pairs, stacked in a dorsal-ventral orientation with 100 μm spacing between pairs and 30 μm spacing between sites within each pair. The intention of this design is to selectively configure one recording pair with glutamate oxidase (GluOX) for glutamate detection via production of the reporter molecule hydrogen peroxide and the other pair with non-active protein for self-referenced comparison (Burmeister and Gerhardt, 2001). For all calibrations and animal studies presented here, the Fast Analytical Sensing Technology system (FAST-16/mark II, Quanteon L.L.C, Nicholasville, KY) was used to perform constant voltage amperometry (+0.7 V vs. Ag/AgCl reference electrode).

Microelectrode Preparation for *In Vivo* Glutamate Measurements

Measurements in Anesthetized Rats

All S2 platinum recording surfaces were electroplated with meta-phenylenediamine (5 mM, Acros Organics, New Jersey, USA), which creates a molecular size exclusion layer blocking electrochemically active interferents such as DA, DOPAC and ascorbic acid based upon their size. The MEAs were configured for selective glutamate detection as per our previously published methods (Burmeister et al., 2002; Pomerleau et al., 2003, Day et al., 2006; Hascup et al., 2006). Briefly, the ventral pair of platinum recording surfaces were manually coated with approximately 1 μL of enzyme solution (1% bovine serum albumin, 0.125% glutaraldehyde, and 1% GluOX) which configured these sites for glutamate detection through the enzymatic conversion of glutamate to H_2O_2. The dorsal pair of platinum sites was coated with a similar BSA/glutaraldehyde solution lacking the glutamate oxidase enzyme. These sites are referred to as 'sentinel' (Figure 2.1).

A pre-pulled single-barrel micropipette (1 mm o.d., 0.58 mm i.d. glass, A-M Systems Inc., Everett, WA) was attached to each ceramic MEA substrate with Sticky Wax (Kerr Lab Corporation, Orange, CA). This allowed for intracranial application of a KCl solution to study stimulus-evoked glutamate release or an exogenous glutamate solution to study glutamate clearance. The tips of the micropipettes were pulled to an inner diameter of 10 μm, and positioned 70 ± 20 μm away from the microelectrode surface, centered in the 100 μm space between the dorsal and ventral platinum recording pairs (Figure 2.2).

Measurements in Unanesthetized Rats

All S2 platinum recording surfaces were coated with Nafion® and dried at 175°C for 4 minutes. Nafion® repels electrochemically active anionic interferents such as DOPAC and ascorbic acid (Gerhardt et al., 1984). Based upon empirical data, Nafion® is considered more stable than meta-phenylenediamine *in vivo*, warranting its use in all studies with unanesthetized animals. The MEAs were

configured for selective glutamate detection as per our previously published methods (Burmeister et al., 2002; Pomerleau et al., 2003, Day et al., 2006; Hascup et al., 2006) (Figure 2.1).

The ceramic substrate of the MEA used for studies in anesthetized rats, referred to as the paddle, was modified for chronic implantation which allowed glutamate measurements in unanesthetized rats. A detailed construction protocol has been previously published (Rutherford et al., 2007). Briefly, the ceramic MEA paddle was shortened and configured with an electrical connection for each platinum S2 recording site and attached to a miniature connector (Ginder Scientific, Ottawa, Ontario). A miniature a Ag/AgCl wire reference electrode was also attached to the connector. All electrical connections were covered with epoxy. For local delivery of solutions, a 26-gauge stainless steel guide cannula (Plastics One, Roanoke, VA, USA) was attached to the ceramic paddle with sticky wax (Kerr Corp., Orange, CA, USA) and positioned to allow solution application to the microenvironment between all four platinum recording sites through an inserted internal cannula. The distance between the internal cannula and the recordings sites was approximately 50 µm. A fixed stainless steel dummy cannula remained in the guide whenever the animal was not receiving solutions. The completed assembly containing the ceramic paddle, miniature connector and guide cannula is referred to as the rat pedestal (Rutherford et al., 2007) (Figure 2.2).

Measurements in Unanesthetized NHPs (Spencer-Gerhardt-2 MEA)

The Spencer-Gerhardt-2 microelectrode array (SG-2, Figure 2.3) was modeled after the clinically utilized SPENCER® Probe Depth Electrodes (ADTECH®, Racine, Wisconsin). The S2 configuration previously designed for basic science MEAs was conserved in the design of the SG-2 for comparisons of measures in rodents and non-human primates (Day et al., 2006; Nickell et al., 2005; Nickell et al., 2007; Pomerleau et al., 2003; Quintero et al., 2007) and eventually humans. Fabrication of the SG-2 was modified from the basic science MEA design to incorporate an extended flexible polyimide shaft for access to

ventral brain regions in primates. All S2 platinum recording surfaces were coated with Nafion® and dried at 175°C for 4 minutes. The recording sites were configured for selective glutamate detection as per our previously published methods (Burmeister et al., 2002; Pomerleau et al., 2003, Day et al., 2006; Hascup et al., 2006) (Figure 2.1).

In Vitro MEA Calibration

To correct for subtle inconsistencies in platinum deposition during photolithography and differences in thickness of manually applied coatings that may affect the efficiency of hydrogen peroxide oxidation, MEAs were calibrated prior to *in vivo* experimentation.

The platinum recording sites, and a glass Ag/AgCl reference electrode were submerged in phosphate-buffered saline (0.05 M, pH 7.4, 37°C) while serial aliquots of glutamate (20 mM) were added to yield final buffer glutamate concentrations of 20, 40 and 60 μM. The resulting increase in current (pA) from oxidation of hydrogen peroxide was measured by the FAST-16 electrochemistry instrument. Standard response curves were generated and used to equate a change in current from the oxidation of hydrogen peroxide with a proportional change in analyte concentration (Burmeister and Gerhardt, 2001). The recording sites measured typical linear increases in current following serial glutamate additions ($r^2 > 0.99$). The slope of calibration curve is referred to as the microelectrode sensitivity to glutamate (pA/μM). For each microelectrode the limit of detection (μM) was also determined (LOD = 3 times the baseline signal-to-noise ratio). Microelectrodes were also exposed to an ascorbic acid (250 μM) challenge to determine the selectivity ratio (glutamate:AA) as an indication of effective blockade of electrochemical interferents. Hydrogen peroxide was also added to the buffer as a positive control (Figure 2.4).

In vitro calibrations were also performed to determine the effects of sterilization on MEA function. Of note is that unlike the basic science calibration protocol utilizing a glass Ag/AgCl reference electrode, we submerged a Ag/AgCl skin reference electrode (4 mm, shielded; World Precision Instruments Inc.,

Sarasota, FL) directly into the calibration beaker with each SG-2. The Ag/AgCl skin reference electrodes can be sterilized, which will assist in development of a completely sterile SG-2 MEA calibration procedure for clinical use.

SG-2 Sterilization Studies

All four platinum recording sites of the SG-2 MEAs were configured for the detection of glutamate by the application of enzyme (GluOX) and protein (BSA) solution (< 1 µL, 1% GluOX/ 1% BSA/ 0.125% glutaraldehyde) (Burmeister et al., 2002). To assess shelf-life, one batch of coated SG-2 MEAs were stored for approximately 11 months (n = 16) and one batch for one month (n = 16) at room temperature prior to electron beam sterilization. Additional details of the sterilization procedure are proprietary.

Confirmation of Electrode Placement

Histological Evaluation (Rat Studies)

Upon completion of each anesthetized experiment, Fast Green dye (Sigma-Aldrich) was applied through the micropipette (500 nL) to mark the locations of the microelectrode. Dye was not needed to mark the location of MEAs implanted chronically for studies in unanesthetized rats. Animals were euthanized with isoflurane, the brains removed, frozen at -20C° and the positions of the microelectrode in the hippocampus verified by visual examination after slicing the brains (40 µm) on a cryostat (Figure 2.5). Data were excluded if there was clear indication that the MEA had missed the DG, CA3 or CA1 subregions of rat hippocampus (n = 5 for anesthetized studies; n = 4 for unanaesthetized).

Anatomical MRI (NHP Studies)

SG-2 MEAs were targeted to the ventral putamen through a surgically implanted access cannula (Figure 2.3). The brain coordinates for cannula implantation and subsequent MEA placement in the putamen were determined by

magnetic resonance imaging (MRI) before surgery, and confirmed post-implantation (Figure 2.6).

In Vivo Basal Glutamate Measurements

The most basic glutamate measurement obtained with MEAs is tonic, also called basal or resting, glutamate levels in the extracellular space. Tonic glutamate levels were a parameter of interest for all experiments described in this dissertation. Tonic glutamate levels are determined by the balance of glutamate release and clearance processes. For all measurements of tonic glutamate, the current produced by oxidation of background electroactive substances in the extracellular space and the charging current of the electrode surface was removed from the current measured with the glutamate-sensitive recording sites by subtraction. This is referred to as self-referencing and isolates the current generated by oxidation of hydrogen peroxide formed from the enzymatic breakdown of glutamate (Burmeister and Gerhardt, 2001; Day et al., 2006). For all studies presented here, current (pA) obtained by self-referenced *in vivo* MEA recordings was divided by the microelectrode sensitivity (pA/μM) obtained during calibration and reported as concentration of basal glutamate (μM) (Quintero, et al. 2007) (Figure 2.7).

In vivo Amperometric Recordings in Anesthetized Rats

In Vitro Microelectrode Calibration Specifications

Microelectrodes had a selectivity ratio of at least 20:1 (95% ascorbic acid blockade), and a very linear response to the serial glutamate aliquots ($r^2>0.99$). Average microelectrode calibration parameters for these experiments were as follows (n = 27, mean ± S.E.M.): glutamate sensitivity: 6 ± 0.3 pA/μM; limit of detection: 0.8 ± 0.2 μM.

Surgical Procedures

Male F344 rats were anesthetized with urethane (1.25 mg/kg, i.p.) and placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA). A bilateral craniotomy was performed removing a window of bone between bregma and lambda to allow access to the hippocampal formation. A miniature Ag/AgCl reference electrode (200 μm) was implanted into the right frontal cortex. The micropipette/microelectrode assemblies (Figure 2.2) were targeted to the dendritic trees of hippocampal principal cells in the DG, CA3 and CA1 sub-regions. Stereotactic coordinates were adapted from Paxinos and Watson (2004) to account for changes in animal size and skull thickness with age. Anterior-posterior and medial-lateral coordinates were taken from bregma, and dorsal-ventral coordinates were taken from the top of the skull. CA1 and CA3 recordings were performed at the same AP and ML coordinates with the microelectrode at two different depths. Placement coordinates were as follows: young (337 ± 50 g), CA1{CA3} AP: -(3.9-4.3), ML: ± 3.4-3.6, DV: -3.5 {-4.1}; DG AP: -4.1, ML ± 2.1, DV: -4.2; late-middle aged (452 ± 36 g), CA1{CA3} AP: -(4.1-4.5), ML: ± 3.7-3.9, DV: -3.9 {-4.5}; DG AP: -4.4, ML ± 2.2, DV: -4.5; aged (424 ± 32 g), CA1{CA3} AP: -(4.4-4.8), ML: ± 3.7-4.0, DV: -4.0 {-4.6}; DG AP: -4.6, ML ± 2.4, DV: -4.6.

Tonic and Phasic Glutamate Measurements

Basal glutamate measurements were obtained from each subregion following a baseline period of twenty minutes, prior to the application of any solutions. The order in which the subregions were targeted and in which solutions were applied was randomized during experimentation, so as to not bias the study by always targeting one particular area first. A Picospritzer III (Parker Hannifin Corp. NJ, USA) was used to precisely control the volumes of KCl (70

mM KCl, 79 mM NaCl, 2.5 mM CaCl$_2$, pH 7.4) or exogenous glutamate (100 µM in 0.9% physiological saline, pH 7.4) delivered during intracranial applications.

For KCl-evoked glutamate release, a dose response was performed in the hippocampal subregions to obtain the maximum glutamate release. Volumes delivered ranged from 12.5-75 nL and were quantified using a microscope fitted with a calibrated reticule which determined the volume of drug ejected from the micropipette (Day et al., 2006). Evoked glutamate release data were collected in young (n =11), late-middle aged (n = 8) and aged (n = 10) F344 rats.

For clearance studies, exogenous glutamate was locally applied in the hippocampal subregions (volumes ranging from 25-250 nL) to achieve a glutamate concentration in the microenvironment around the platinum recording sites within the range of concentrations most consistently obtained following KCl stimulation of glutamatergic terminals (10-20 µM, see Nickell et al., 2005). This allowed us to study clearance of glutamate at physiologically relevant concentrations and offered the benefit of isolating glutamate clearance mechanisms, in contrast to studying glutamate clearance following KCl-evoked release which causes a non-selective release of neurotransmitters such as GABA. This also allowed us to control for any effects of KCl on glia and glutamate transporters. Glutamate clearance data were collected in young (n =16), late-middle aged (n = 13) and aged (n = 14) F344 rats.

Data Analysis

Current (pA) obtained by self-referenced *in vivo* measurements in each hippocampal subregion was divided by the microelectrode sensitivity (pA/µM) obtained during calibration, and reported as concentration of tonic glutamate (µM) (Quintero, et al. 2007); young DG (n = 15), CA3 (n = 12), CA1 (n = 13); late-middle aged DG (n = 6), CA3 (n = 11), CA1 (n = 10); aged DG (n = 16), CA3 (n = 14), CA1 (n = 14).

Following local stimulation with 70 mM KCl in the DG, CA3, and CA1 hippocampal subregions, the maximum increase in the glutamate signal from

baseline due to evoked glutamate release was recorded by the FAST software (pA). Actual volumes delivered to obtain the maximum glutamate release were not significantly different across subregions or age groups (data not shown, two-way ANOVA, $p = 0.95$). Data were exported to a custom Excel™ spreadsheet for analysis. Maximum increase in glutamate signal from baseline (pA) was divided by the microelectrode sensitivity (pA/μM) and reported as the maximum amplitude of evoked-glutamate release (μM). The first order rate of decay (k^{-1} (sec^{-1})) was calculated for all evoked glutamate signals to examine the rate of return from maximum amplitude to baseline. Based upon the known reproducibility of KCl-evoked glutamate signals *in vivo* (Nickell et al. 2005, Day et al. 2006), data from an individual subregion were only analyzed if at least three similar signals could be evoked, upon which the data were averaged and reported as a single number for that subregion: young DG (n = 8), CA3 (n = 6), CA1 (n = 6); late-middle aged DG (n = 5), CA3 (n = 5), CA1 (n = 7); aged DG (n = 6), CA3 (n = 5), CA1 (n = 5).

Following local application of 100 μM glutamate in the DG, CA3 and CA1 hippocampal subregions, maximum increase in the glutamate signal from baseline was recorded by the FAST software. Data were exported to a custom Excel™ spreadsheet for analysis where the maximum increase in glutamate signal from baseline (pA) was divided by the microelectrode sensitivity (pA/μM) to determine the amplitude of glutamate signal following local application of exogenous glutamate (μM). Data from an individual subregion were only further analyzed if application of exogenous glutamate produced at least three signals in the range of 10-20 μM. For signals in the defined range, the concentration of glutamate removed from the extracellular space per second was calculated using an Excel™ spreadsheet and reported as uptake rate (k^{-1} x maximum amplitude (μM/sec)). Data from individual subregions were averaged and reported as a single number for that subregion: young DG (n = 9), CA3 (n = 7), CA1 (n = 11); late-middle aged DG (n = 7), CA3 (n =12), CA1 (n = 13); aged DG (n = 8), CA3 (n = 6), CA1 (n = 8).

A Grubb's test for outliers removed two young animals and one aged animal from analysis. Mean and SEM for all parameters of interest (tonic glutamate concentration, maximum amplitude of KCl-evoked glutamate signal and uptake rate following local exogenous glutamate application) were calculated for each subregion in each age group. Due to the defined guidelines for inclusion of data in the analysis, not every animal produced a complete set of tonic glutamate levels, KCl-evoked glutamate release and glutamate uptake rate data in all three subregions. Therefore, discrete comparisons were performed using a one-way analysis of variance (ANOVA, statistical significance defined as $p<0.05$) in GraphPad Prism 5 along with a Bartlett's comparison of data variance to look for differences in the trisynaptic circuit within each age group and for differences within each subregion across age groups. A Tukey-Kramer post-hoc test for unequal sample sizes was performed where necessary.

In vivo Amperometric Recordings in Unanesthetized Rats

MEA Calibration Specifications

Microelectrodes had a selectivity ratio of at least 20:1 (95% ascorbic acid blockade), and a linear response to the serial glutamate aliquots ($r^2>0.99$). The calibration parameters for the MEAs used in these experiments were (n = 55, mean ± S.E.M.): glutamate sensitivity: 5.4 ± 0.3 pA/μM; limit of detection: 0.42 ± 0.05 μM.

Surgical Procedures

One day before and the day of surgery, animals were given carprofen (15 mg/kg i.p., Pfizer) to minimize pain. Animals were placed in an induction chamber maintained with isoflurane (2% in O_2) for the onset of anesthesia and then transferred to a stereotactic frame outfitted with a isoflurane nose cone for maintenance of anesthesia (2% isoflurane). All procedures were performed in a Vertical Laminar Flow Workstation (Microzone Corp., Ottawa, ON, USA), with

sterile surgical instruments and dressings. Standard precautions for preparation of the incision site were observed including fur removal and Betadine scrubbing. A craniotomy was performed to remove skull (3 mm^2) and allow access of the S2 recording sites to the hippocampus. Stainless steel screws (Small Parts Inc., Miami Lakes, FL, USA) were anchored to the skull through three burr holes placed in areas away from the hippocampal access window (Rutherford et al., 2007). The Ag/AgCl reference electrode from the rat pedestal was placed into the left frontal cortex through a forth burr hole in the skull. All implants were targeted to a specific subregion in the right hippocampus. Anterior-posterior and medial-lateral coordinates for placement MEAs were taken from bregma, and dorsal-ventral coordinates were taken from the top of the skull: young (350 ± 11 g), DG AP: -4.1, ML -2.1, DV: -4.0; CA3 AP: -4.1, ML -3.4, DV: -4.0; CA1 AP: -4.1, ML: -2.4, DV: -3.3; late-middle aged (448 ± 5 g), DG AP: -4.3, ML -2.3, DV: -4.4; CA3 AP: -4.3, ML ± 3.6, DV: -4.4; CA1 AP: -4.3, ML: -2.5, DV: -3.4; aged (435 ± 6 g), DG AP: -4.3, ML -2.3, DV: -4.5; CA3 AP: -4.3, ML: -3.6, DV: -4.5; CA1 AP: -4.3, ML: -2.5, DV: -3.5. Dental acrylic (Lang Dental MFG, Wheeling, IL, USA) was applied to anchor the pedestal to the skull screws and fill the incision area. The entire surgical procedure required approximately 1.5 hours of animal anesthesia. Animals were allowed to recover two days before experimentation. Maintenance doses of carprofen were given as needed during recovery. The majority of animals tolerated the procedure, indicated by less than 10% loss of body weight after surgery. Only one animal (24 mo.) died from complications related to anesthesia. No cases of infection were reported.

During experimentation, implanted pedestals were connected to a miniature 4-channel low noise headstage amplifier ('rat hat', Quanteon, LLC, Nicholasville, KY). A locking AMS plug (Ginder Scientific) ensured secure attachment of the headstage amplifier to the rat pedestal, especially while animals were experiencing wet-dog-shakes characteristic of limbic seizures. The amplifier was connected to the FAST16 mk II electrochemistry instrument via a low-torque commutator, allowing animals minimally inhibited movement around an observation box (Rutherford et al., 2007).

4-aminopyridine-induced Status Epilepticus (Intrahippocampal Injections)

A 20 mM solution of 4-AP (Fischer Scientific) was prepared in physiological saline (0.9%, pH 7.4). On day 4 post-surgery, animals with CA1 MEA implants received a low dose of 4-AP (5.25 µmol, 250 nL) followed by a high dose (21 µmol, 1 µL) injected locally through an internal cannula placed into the guide cannula on the rat pedestal (young n = 6; late-middle aged n = 6; aged n = 5). All animals received the low dose of 4-AP first. A period of 20 minutes was allowed between the end of low dose 4-AP-induced status epilepticus (SE) and the administration of the high dose.

4-aminopyridine-induced Status Epilepticus (Behavioral Characterizations)

Intrahippocampal application of 4-AP produces behavior characteristic of limbic seizures including oral automatisms, grooming and paw tremor (Ben-Ari, 1981). The most evident limbic-associated behavior is wet-dog shaking (Fragoso-Veloz et al., 1990). Starting from the point of 4-AP injection, wet-dog shakes (WDS) were tallied in five minute epochs. For the purposes of these studies, the duration of SE was defined behaviorally. The start of SE was identified by the first WDS. Absence of WDS for an entire five minute epoch signaled the end of SE. SE behavior self-terminated in all animals.

Tonic and Phasic Glutamate Measurements

For tonic glutamate measurements, animals were acclimated to the observation box for 30 minutes and were then connected to the headstage amplifier. Animals required 1 – 3 hours to reach a stable baseline. Tonic glutamate measurements were obtained on days 3 and 4 post-surgery. After tonic glutamate levels were determined, animals with CA1 MEA implants were administered 4-AP on day 4. Dynamic fluctuations in extracellular glutamate were recorded on a second-by-second basis (1 Hz) during SE. After SE, animals

were allowed to recover for one day. On day 6, post-status basal glutamate levels were determined in a manner identical to days 3 and 4.

Data Analysis

GraphPad Prism 5 was used for all statistical analysis. For tonic glutamate measurements, current (pA) obtained by self-referenced *in vivo* measurements in each animal was divided by the microelectrode sensitivity (pA/μM) obtained during calibration (Quintero, et al. 2007) to determine concentration of basal glutamate (μM): young DG (n = 8), CA3 (n = 5), CA1 (n = 6); late-middle aged DG (n = 6), CA3 (n = 5), CA1 (n = 6); aged DG (n = 24), CA3 (n = 6), CA1 (n = 6). For all animals, tonic glutamate measurements obtained on days 3 and 4 were averaged and reported as a single value. A Grubb's test did not identify any significant outliers and all animals were included in the analysis. Tonic glutamate levels in the DG, CA3, and CA1 hippocampal subregions were discretely compared across age groups using one-way analysis of variance tests (ANOVA, statistical significance defined as $p < 0.05$). A Tukey-Kramer post-hoc test for unequal sample sizes was performed where necessary. The combined data from all three subregions representing tonic glutamate in the hippocampal formation were also compared across age groups using an ANOVA. A D-Agostino and Pearson omnibus normality test for Gaussian distribution was calculated for the combined hippocampal formation data. For animals receiving 4-AP, pre-status basal glutamate (days 3 and 4 combined) was compared to post-status basal glutamate (day 6) using a two-way ANOVA for repeated measures (statistical significance defined as $p < 0.05$), followed by a Bonferroni post hoc test when necessary.

Duration of SE, average WDS/ 5 minute epoch and maximum WDS/ 5 minute epoch was calculated for each animal following the low and high doses of 4-AP. All behavioral parameters were examined for dose and age-associated changes using a two-way ANOVA for repeated measures (statistical significance defined as $p < 0.05$) followed by a Bonferroni post-hoc test when necessary.

Custom MatLab-based software was used to identify and quantify spontaneous glutamate release events that occurred after 4-AP administration. A glutamate release event was defined as an increase in self-referenced current at least two-times the signal to noise ratio. To further isolate spontaneous glutamate release, any artifact producing change in current that was occurring faster than once every 15 seconds and was not already removed by self-referencing, was excluded. The total number of glutamate release events occurring during the periods of defined SE was calculated for each animal. Also, the latency to onset of spontaneous glutamate release events (min.), average magnitude of each release (peak size (μM)) and the average inter- event interval (release periodicity (sec.)) were determined for each animal. The spontaneous glutamate release characteristics were examined for dose and age-associated effects using a two-way ANOVA for repeated measures (statistical significance defined as $p < 0.05$) followed by a Bonferroni post-hoc test when necessary.

To examine the relationship between pre-SE tonic glutamate concentration and the progression of the behavioral and glutamate release activities during SE, we calculated the linear regression line (slope = r^2) for tonic glutamate (independent variable) verses 1) SE duration, 2) average WDS/ 5 min., 3) maximum WDS/5 min., 4) total glutamate release events, 5) average peak size and 6) release periodicity (dependent variables). We also calculated the linear regression lines for total glutamate release events verses total WDS and for average glutamate peak size verses average WDS/ 5 minutes to determine any correlations between behavior and spontaneous glutamate neurotransmission. Linear regression analysis determined if r^2 was significantly different than zero (statistical significance defined as $p < 0.05$)

In vivo Glutamate Studies in Unanesthetized Non-human Primates

SG-2 Calibration Specifications

To characterize the SG-2 MEAs on the day of experimentation, *in vitro* calibrations were performed. The same skin Ag/AgCl reference electrode used during calibration was later used during the animal experiments. SG-2 MEAs responded linearly to serial aliquots of glutamate ($r^2 > 0.99$), and demonstrated at least 95% AA blockade (glutamate:AA = 20:1). The calibration parameters for the SG-2 used in these experiments were (n = 6, mean ± S.E.M.): glutamate sensitivity: 3.5 ± 0.4 pA/μM; limit of detection: 2.7 ± 0.8 μM.

Surgical Procedures

Under sterile field conditions, a cannula was surgically implanted allowing chronic unilateral access to the ventral putamen (left hemisphere: NHP 1 and 2; right hemisphere: NHP 3). The cannula was a modified winged I.V. catheter (16G, JELCO™, Tampa, FL) fitted with a stainless steel cap (Figure 2.3). The brain coordinates for implantation were determined by magnetic resonance imaging (MRI) before surgery, and confirmed post-implantation (NHP 1, AP (from ear bars): +20 mm, ML: +11 mm, DV (from dura): -12 mm; NHP 2, AP: +18 mm, ML: +10.5 mm, DV: -15 mm; NHP 3, AP: +21.5 mm, ML: -11 mm, DV: -14.5 mm, Figure 2.6). The animals were anesthetized with isoflurane (1-3%) during these procedures and were allowed at least a 2 week recovery period before the first day of experimentation.

Tonic Glutamate Measurements

Animals were previously trained and extensively handled by the experimenters. Studies were performed in a non-human primate behavioral laboratory while the animals were seated in a MRI-compatible primate chair, which allowed free movement of the limbs and body but restrained the head (Figure 2.8) (Andersen et al., 2002). The SG-2 was lowered into the putamen

through the indwelling cannula. Similar to the basic science MEA technology developed in our laboratory for glutamate measurements in unanesthetized rodents (see *In vivo* Amperometric Recordings in Unanesthetized Rats), the SG-2 MEAs were connected to a miniature 4-channel low noise potentiostat and the FAST-16 electrochemistry instrument via a locking AMS plug (Figures 2.3 and 2.8). For these studies we used a non-invasive skin Ag/AgCl reference electrode which was modified to plug directly into the miniature potentiostat (Figure 2.8). Two depths in the putamen (2 mm apart) were targeted with the SG-2. The SG-2 was lowered manually utilizing millimeter increments marked on the SG-2 shaft. Glutamate was measured continuously (2 Hz) for around 40 minutes at each depth. Approximately four months after the first experiments, the studies were repeated to determine the reproducibility of the glutamate measurements.

Data Analysis

GraphPad Prism 5 was used for all statistical analysis. *In vitro* calibrations were performed to determine the effects of sterilization on SG-2 MEA function. Based upon empirical data, an MEA sensitivity to glutamate of 1 pA/μM or greater is considered functional. The percentage of SG-2 MEAs with at least one functional recording site was determined for each batch (1 and 11 month storage times). The average glutamate sensitivity and LOD for the functional sites was calculated. To determine if storage duration significantly affected MEA performance, a Student's t-test was used to compare the sensitivity and LOD between the batches.

Following a thirty minute stabilization period at each depth targeted by the SG-2, tonic glutamate was measured. Combining data from the first and the follow-up experiments, we compared the tonic glutamate levels in the dorsal putamen to levels in the ventral putamen with a Paired t-test (n = six each depth). We also compared the reproducibility of the basal glutamate levels between the first and follow-up recordings with a Paired t-test for repeated measures (n = 6).

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Portions of these methods have been previously published:

M. L. Stephens, D.D. Spencer, I. Cavus, M. Hsiao, D. Song, S.H. Courellis, S.A. Deadwyler, R.E. Hampson, D. Putz, J.E. Quintero, M.K. Bensalem-Owen, K.N. Hascup, E.C. Rutherford, B.K. Day, J.R. Nickell, F. Pomerleau, P. Huettl, J.J. Burmeister, P.M. Talauliker, V.Z. Marmarelis, J.J. Granacki, T. Berger, and G.A. Gerhardt. Microelectrode-based Epilepsy Therapy: A Hybrid Neural Prosthesis Incorporating Seizure Prediction and Intervention with Biomemetic Maintenance of Normal Hippocampal Function. Computational Neuroscience in Epilepsy (CH 33); Academic Press (2008)

M.L. Stephens, J.E. Quintero, F. Pomerleau, P. Huettl, and G. A. Gerhardt. Age-related changes in glutamate release in the CA3 and dentate gyrus of the rat hippocampus. *Neurobiology of Aging;* June 15 (2009) EPub ahead of print

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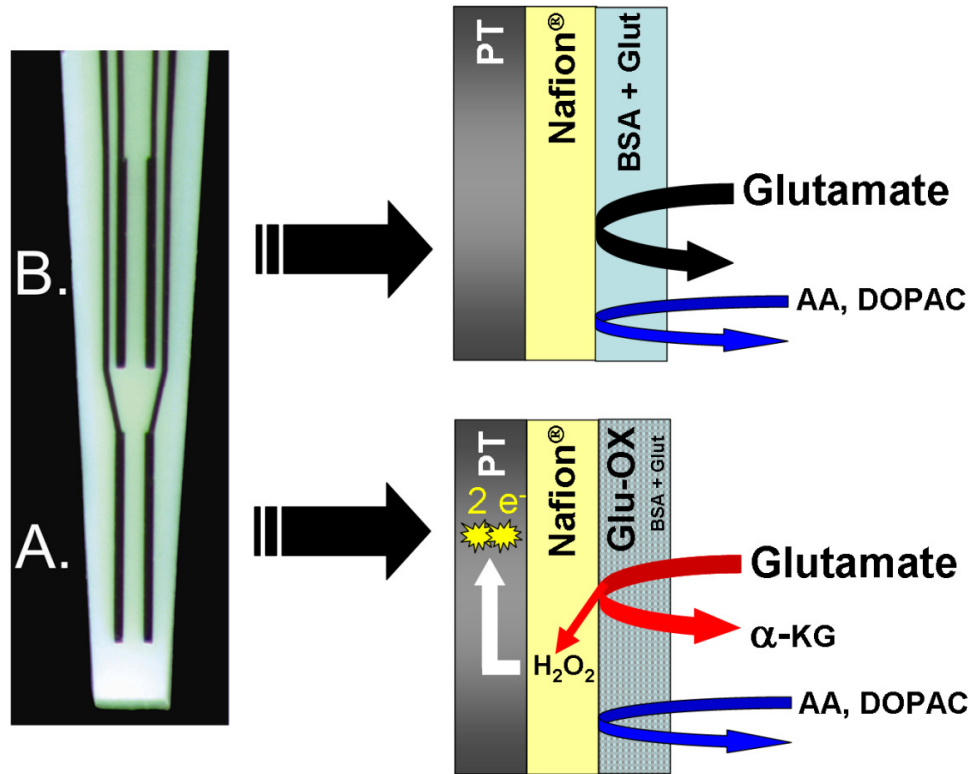
Figure 2.1 S2 MEA Configuration for Self-referenced
Glutamate Measurements

S2 MEA image with illustrations showing Nafion® exclusion layer, bovine serum albumin-glutaraldehyde inactive protein matrix (BSA-Glut) and glutamate-oxidase (GluOX) active enzyme coatings. S2 MEAs have four platinum recording sites (15 x 333 μm each).   A) Sites prepared for glutamate detection: current is generated when the hydrogen peroxide (H_2O_2) "reporter molecule" is oxidized (2 electrons, +0.7 V vs. Ag/AgCl reference electrode).   B) Sentinel sites (without GluOX): subtraction of current measured with 'B' sites from current measured with 'A 'sites serves to isolate current generated by enzymatic breakdown of glutamate. This is termed self-referencing. Note that Nafion® prevents oxidation of ascorbic acid and DOPAC. In lieu of Nafion, meta-phenylenediamine (mPD) can be electroplated to the recording sites which prevents oxidation of ascorbic acid, DOPAC and DA.
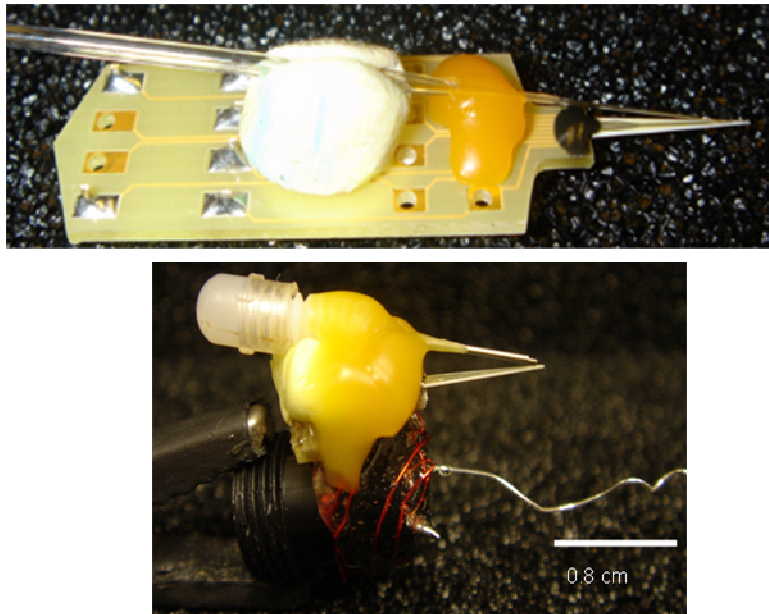
Figure 2.2 Various Electrodes with S2 MEA Configuration

Top panel: Ceramic paddle electrode used for glutamate measurements in anesthetized rats with attached glass pipette for local drug application (Stephens et al., 2008); *bottom panel*: modified ceramic paddle electrode ('rat pedestal') for chronic glutamate measurements in unanesthetized rats with attached stainless steel guide cannula for local drug application (Hascup et al., 2006).
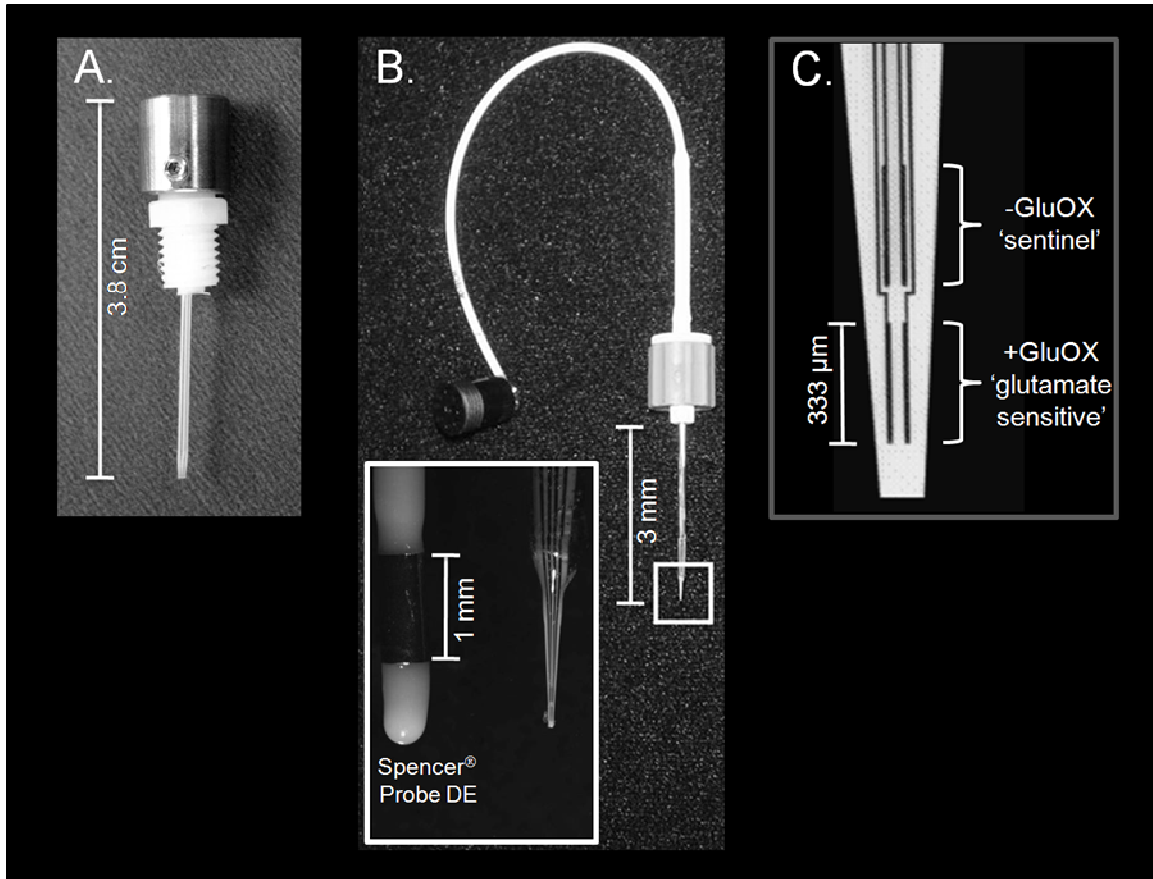
Figure 2.3 Spencer-Gerhardt-2 Microelectrode Array

A) Cannula and stainless steel cap for chronic access to the NHP putamen. B) SG-2 MEA, *inset:* side-by-side comparison of SPENCER® Probe Depth Electrode and SG-2 ceramic tip. C) Magnification of SG-2 ceramic tip (same as the S2 MEA), showing configuration of platinum recording surfaces for self-referenced glutamate measurements (Stephens et al., 2009[b]).
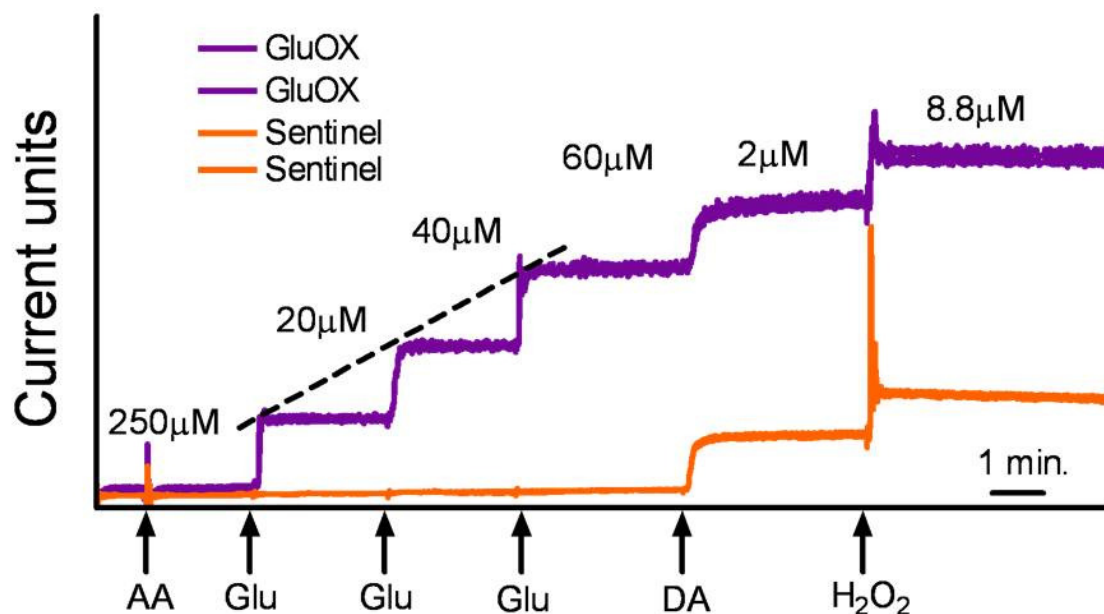
Figure 2.4 Calibration of S2 MEA

Platinum recording sites are submerged in phosphate-buffered saline (0.05 M, pH 7.4, 37°C) and serial aliquots of glutamate (20 mM) added to yield final buffer glutamate (Glu) concentrations of 20, 40 and 60 µM. Standard response curves (dashed line, r^2 > 0.99) are generated and used to equate a change in current from the oxidation of hydrogen peroxide with a proportional change in glutamate concentration (Burmeister and Gerhardt, 2001). The slope of the calibration curve is referred to as the microelectrode sensitivity to glutamate (pA/µM). Microelectrodes were also exposed to an ascorbic acid (AA, 250 µM) challenge to test effective blockade of electrochemical interferents, and to hydrogen peroxide (H_2O_2) as a positive control. Current produced from the oxidation of dopamine (DA) or other interferents can be removed by self-referencing.
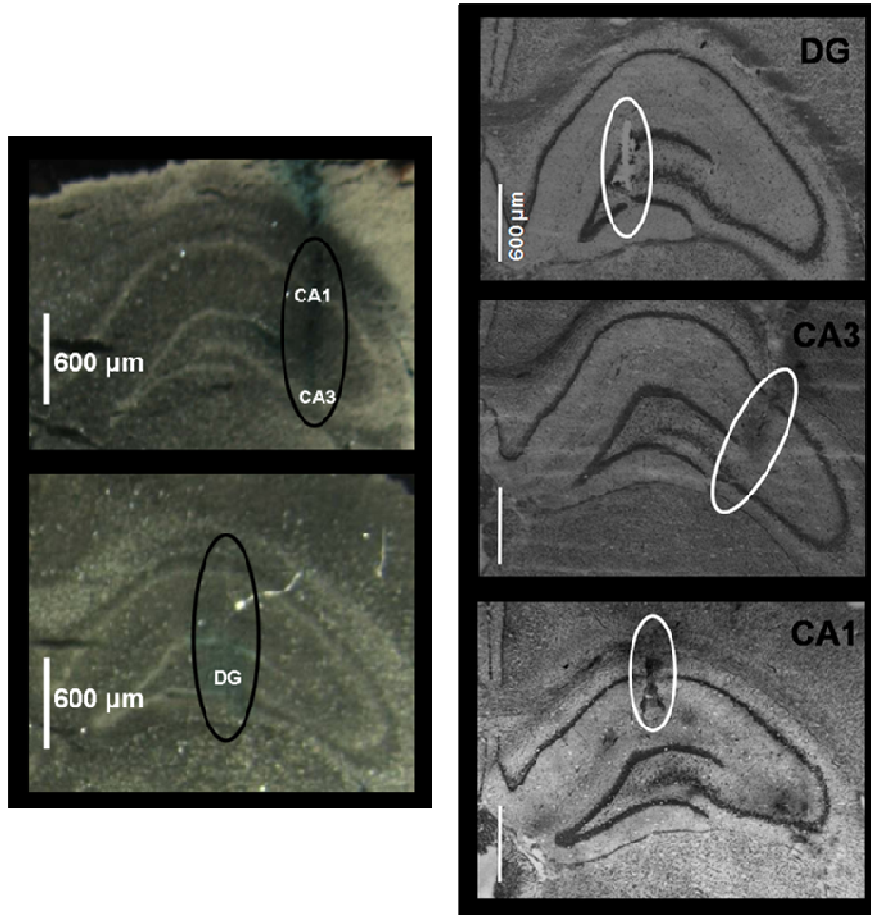
Figure 2.5 Microelectrode Placements in the Trisynaptic Pathway

Left: Fast Green (Sigma-Aldrich) staining in the hippocampal formation confirming placement of the MEA/micropipette assembly in the CA1 and CA3 subregions (upper panel) and the DG subregion (lower panel) during anesthetized rat studies. *Right:* MEA tracts in the hippocampal subregions targeted with chronic MEA implants confirming accurate placement in the DG, CA3 and CA1 during studies in unanesthetized rats. Note: During experiments in anesthetized rats, one MEA was used to measure glutamate in all the hippocampal subregions. In contrast, chronic MEA implants used for glutamate recordings in unanesthetized rats cannot be moved, therefore only one subregion could be studied in each animal.
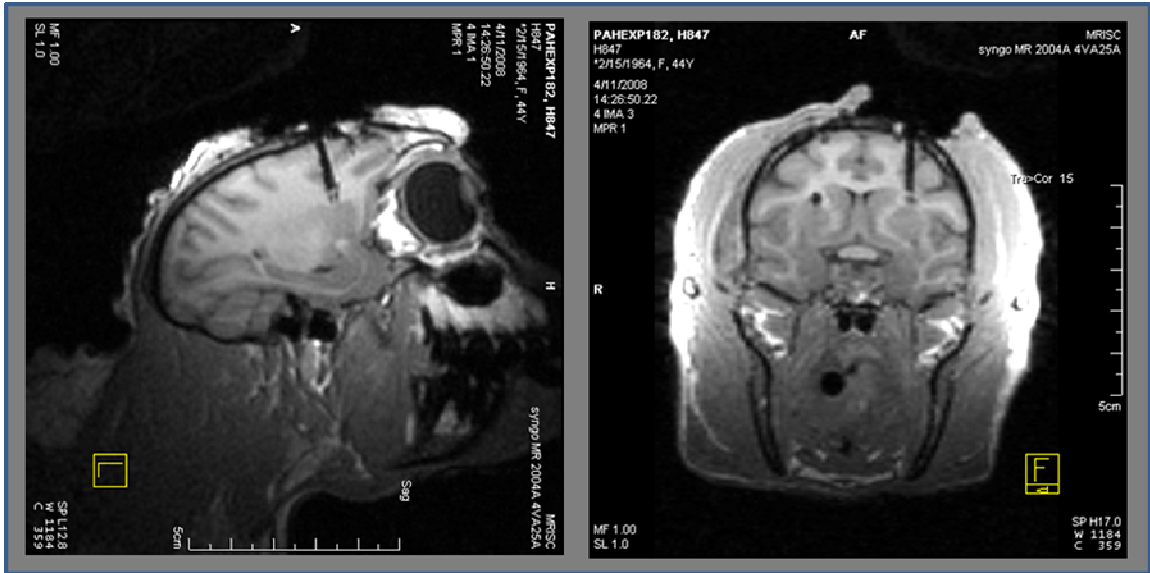
Figure 2.6 MRI Images of Chronic Cannula Placement in the NHP Putamen

Images confirm placement of the guide cannula allowing unilateral access of the SG-2 to the putamen of non-human primates.
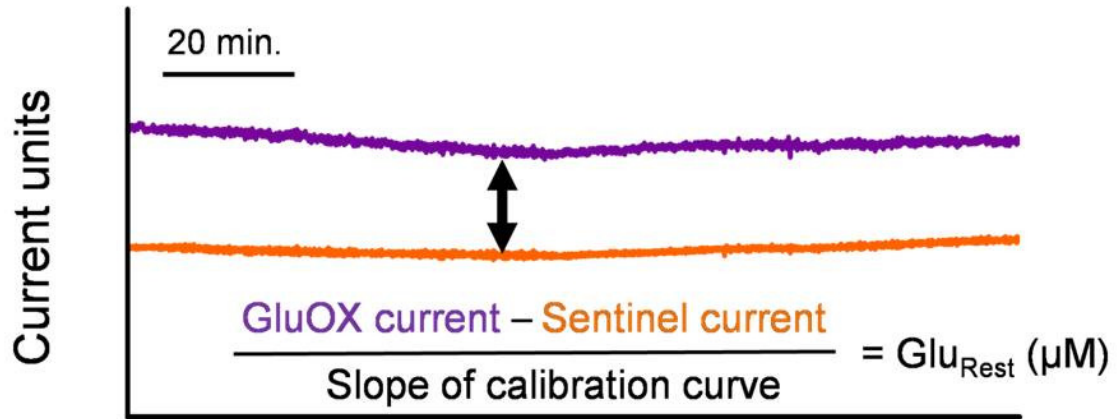
Figure 2.7 Measuring Basal Glutamate

Subtraction of current measured with sentinel sites from current measured with glutamate oxidase (GluOX) coated sites serves to isolate current generated by enzymatic breakdown of glutamate. This is termed self-referencing. Dividing the self-referenced current (pA) by the MEA sensitivity to glutamate (slope of the calibration line (pA/μM)) = Resting glutamate (Glu_{Rest} (μM)).
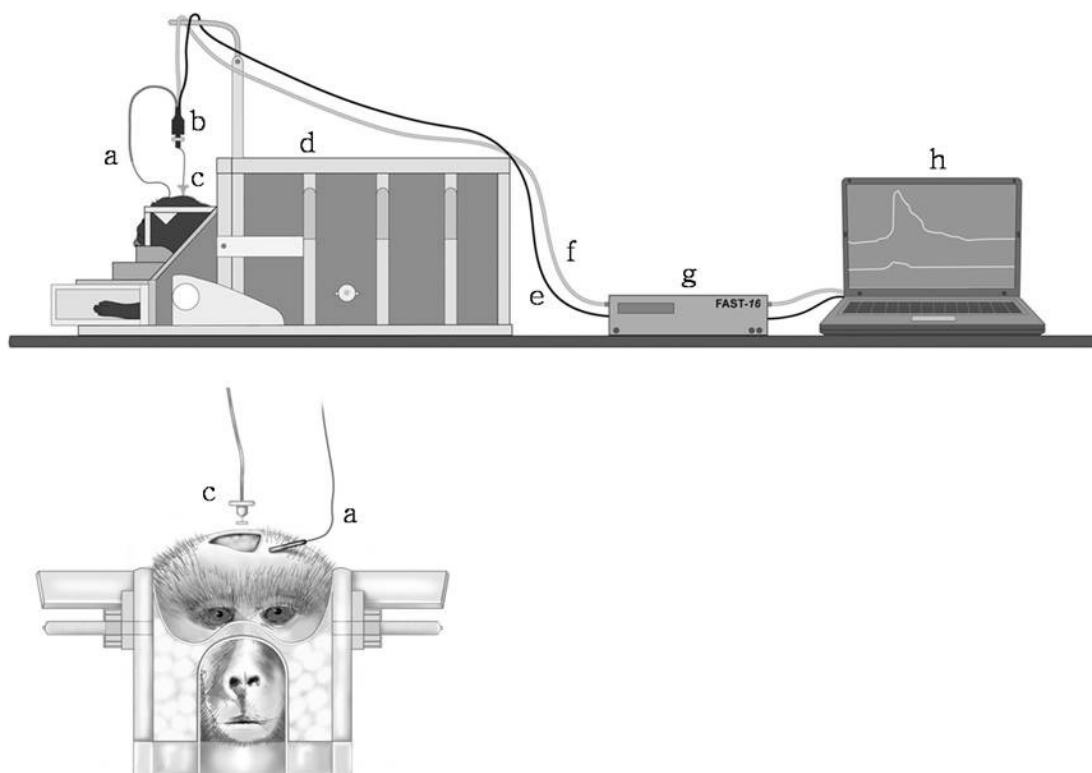
Figure 2.8 Experimental Set-up for Amperometric Studies in Awake-behaving NHPs

Illustrations provide a lateral view of the NHP chair and FAST-16 recording system, and an anterior view of the NHP during glutamate recordings. a) Ag/AgCl skin reference electrode, b) potentiostat (headstage), c) SG-2 MEA, d) NHP chair, e) ground wire, f) connector, g) FAST-16, h) laptop computer. (Courtesy Tom Dolan)

**Chapter Three: Age-related Changes in Glutamate Release and Clearance
in the Trisynaptic Pathway of Anesthetized F344 Rats**

Introduction

An understanding of how morphological and electrophysiological changes in brain regions critical for learning and memory translate into deficits in the cognitive functions of the hippocampus in aging is needed (for reviews see Rosenzweig and Barnes, 2003; Chawla and Barnes, 2007). In addition to preserved cell counts (West, 1993; West et al., 1993; Peters et al., 1996; Rapp and Gallagher, 1996; Rasmussen et al., 1996; Merrill et al., 2000; Merrill et al., 2001), most of the basic cellular characteristics of hippocampal principal cells do not change with advanced age including but not limited to resting membrane potential and amplitude and duration of Na^+-mediated action potentials (for review see Rosenzweig and Barnes, 2003). Therefore, changes in hippocampal cognitive functions during aging do not appear to be attributable to changes in neuron number or basic cellular physiology. It is believed that structural changes in synaptic connections during aging affect functional connectivity in the hippocampus (reviewed by Rosenzweig and Barnes, 2003; Chawla and Barnes, 2007); however, age-associated alterations in neurotransmission and subsequent effects on function of the hippocampal circuitry are far less defined.

In the hippocampus, the chief excitatory neurotransmitter is L-glutamate. The NMDA and AMPA ionotropic glutamate receptor subtypes are critical for long-term potentiation and hippocampal-dependent learning and memory (Riedel et al., 2003). A reduction in ionotropic receptors and their constituent subunits with age has been correlated with a decline in memory function (Magnusson, 1998[a,b]; Adams et al., 2001; Tang et al., 2001; Clayton et al., 2002) which can be modulated with pharmacological agents that facilitate activation of the glutamate receptors (Müller et al., 1994; Wu et al., 2002; Rosenzweig and Barnes, 2003). Thus, age-related changes in presynaptic and glial regulation of glutamate

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availability for participation in receptor-dependent processes may also be needed to help explain age-related changes in hippocampal neuron function during aging.

Regulation of glutamate release and clearance in the hippocampus has been extensively investigated using *ex vivo* methods like brain slices, tissue homogenates and synaptosomes (for review see Segovia et al., 2001). Due to the limitations of these methods the literature has been filled with contradicting reports about increases, decreases or no changes in glutamate regulation with aging. The dentate gyrus (DG), cornu ammonis 3 (CA3) and cornu ammonis 1 (CA1) hippocampal subregions are distinct on a cellular, molecular and functional basis, and are intricately connected via the trisynaptic circuit (for review Miller and O'Callaghan, 2005; Greene et al., 2008). Isolating the hippocampus from its extrinsic connections and disrupting the intrinsic connections could account for the inconsistency in previous aging studies. Only two prior microdialysis studies have reported on hippocampal glutamate regulation during aging using intact animals and the results are contradicting. Zhang et al. (1991) showed a decrease and Massieu and Tapia (1997) showed an increase in tonic extracellular glutamate levels in aged as compared to young rats. Though microdialysis does allow for *in vivo* measurements of tonic glutamate, controversy exists regarding the neuronal origin of the signals and its slow temporal resolution limits the ability to investigate the rapid release and clearance dynamics associated with glutamate neurotransmission (Timmerman and Westerink, 1997; Segovia et al., 2001). This is important because alterations in release and/or clearance may not manifest with changes in tonic resting glutamate levels due to biological compensation, and therefore must be studied directly. Furthermore, due to the size of microdialysis probes (mm), the previous studies are limited because they do not address discrete subregional variation within the hippocampus.

We have recently demonstrated altered regulation of glutamate neurotransmission during aging in the striatum of rodents (Nickell et al., 2005, 2007) and the cortex of nonhuman primates (Quintero et al., 2007) using enzyme-based microelectrode arrays coupled to amperometry for *in vivo*

glutamate recordings. In addition to a sub-second (2 Hz) temporal resolution that allows for rapid measurement of glutamate release and clearance in the extracellular space, our microelectrode technology measures from brain parenchyma with a spatial-resolution (µm) superior to that of microdialysis (Stephens et al., 2008) allowing accurate targeting of the DG, CA3 and CA1 subregions. The experiments presented here investigated the capacity of glutamate release and uptake in subregions of the hippocampus (DG, CA1 and CA3) in young, late-middle aged and aged Fischer 344 (F344) rats. First, we studied the effects of aging on tonic (resting) levels of glutamate using a self-referencing enzyme-based microelectrode array recording technology (Day et al., 2006). Second, we used local application of a high potassium solution to evoke reproducible synaptic overflow of glutamate with a micropipette attached to the microelectrode arrays. This was carried out to simulate phasic bursts of glutamate release and determine the effects of aging on depolarization-induced release of glutamate. Finally, using the attached micropipette for evoked release we locally applied finite amounts of glutamate to study the effects of aging on glutamate clearance. The present studies are the first to provide insight into age-related changes in functional glutamatergic neurotransmission in subregions of the rat hippocampus.

Materials and Methods

Please refer to Chapter Two for a detailed description of animal care, electrode preparation, electrode calibration, amperometric recording technique and methodology for measuring and comparing tonic and phasic glutamate neurotransmission across hippocampal subregions and age-groups.

Results

In these studies we utilized a novel microelectrode array recording technology to investigate the *in vivo* regulation of glutamate neurotransmission in the trisynaptic circuit of young, late-middle aged and aged F344 rats with spatial (µm) and temporal (2 Hz) resolutions unique to the scientific literature. Histological evaluation confirmed MEA placement in the dendritic trees of the hippocampal principal cells in the DG, CA3 and CA1 subregions. The co-localized granule cell dendrites and entorhinal cortex terminals were targeted in the infrapyramidal blade of the DG. In the stratum lucidum of CA3, the MEA was placed among mossy fiber terminals and their target CA3 pyramidal cell dendrites. In CA1, MEA placement was confirmed in the suprapyramidal stratum radiatum were CA1 pyramidal cell dendrites and Schaffer collateral terminals are co-localized.

Self-referenced tonic glutamate recordings in the DG, CA3 and CA1 hippocampal subregions were in the 3 µM range and showed no significant differences across the trisynaptic circuit within each age group (Figure 3.1, Table 3.1): young, DG: 3.4 ± 0.5 µM, CA3: 2.8 ± 0.6 µM, CA1: 2.5 ± 0.5 µM, $F_{(2,35)} = 2.72$, $p = 0.08$; late-middle aged, DG: 2.9 ± 1.1 µM, CA3: 3.3 ± 0.7 µM, CA1: 2.1 ± 0.5 µM, $F_{(2,24)} = 0.80$, $p = 0.46$; aged DG: 2.9 ± 0.5 µM, CA3: 3.0 ± 0.4 µM, CA1: 2.8 ± 0.4 µM, $F_{(2,41)} = 0.046$, $p = 0.96$. Average tonic glutamate levels did not change for any individual subregion when compared across age groups (DG, $F_{(2,34)} = 0.19$, $p = 0.83$; CA3, $F_{(2,34)} = 0.19$, $p = 0.83$; CA1, $F_{(2,34)} = 0.65$, $p = 0.53$). Thus, the resting or tonic levels of glutamate seem little affected with aging in the hippocampus, at least under these conditions and in anesthetized animals.

Application of KCl (70 mM, isotonic, pH 7.4, 12.5-75 nL) allowed us to produce discrete and well-defined stimulation of the local environment around the microelectrode, resulting in robust (5-60 µM) and reproducible (every 60 seconds) release of glutamate from terminals in the DG, CA3 and CA1

hippocampal subregions (Figure 3.2). Results are summarized in Table 3.2. The mean maximum amplitude of KCl-evoked glutamate signals was significantly increased in the DG of late-middle aged rats compared to young ($p < 0.05$) and aged ($p < 0.05$) rats (DG: young, 19.6 ± 3.9 µM; late-middle aged, 36.2 ± 7.7 µM; aged, 17.3 ± 1.8 µM, $F(2,16) = 4.3$, $p = 0.032$). The variances of these data were significantly different across age groups (Bartlett's test, $p = 0.038$). Though no significant difference existed in the maximum amplitude of glutamate signals recorded by the microelectrode in the young and aged DG, the first order rate of decay of the signals was significantly faster in the aged rats as compared to the young rats (0.29 ± 0.03 sec^{-1} vs. 0.21 ± 0.02 sec^{-1} respectively; t-test $p = 0.036$), indicating an increased clearance capacity of the aged DG following phasic release of glutamate (Figure 3.4). This was further investigated with exogenous glutamate clearance studies.

Perhaps of greatest interest was that the CA3 subregion showed significantly less glutamate release following KCl stimulation in aged rats when compared to young ($p < 0.05$) and late-middle aged ($p < 0.05$) animals (young, 14.6 ± 1.5 µM ; late-middle aged, 16.7 ± 2.6 µM; aged, 8.4 ± 2.4 µM, $F(2,13) = 3.82$, $p = 0.0495$). Diminished release in CA3 also contributed to significantly altered glutamate release capacities throughout the trisynaptic circuit within the late-middle aged and aged groups. Compared to the DG in each respective group, the CA3 and CA1 subregions had significantly less glutamate release following KCl stimulation (late-middle aged: DG, 36.2 ± 7.7 µM; CA3, 16.7 ± 2.6 µM, $p < 0.05$; CA1, 16.5 ± 5.4 µM, $p < 0.05$; $F(2,14) = 3.76$, $p = 0.0493$; aged: DG, 17.3 ± 1.8 µM; CA3, 8.4 ± 2.4 µM, $p < 0.05$; CA1, 10.9 ± 2.3 µM, $p < 0.05$; $F(2,13) = 4.89$, $p = 0.026$). In contrast to the DG and CA3, no age-associated changes in maximum amplitude of evoked-glutamate signals were seen within the CA1 subregion across age groups (young, 12.7 ± 2.7 µM; late-middle aged, 16.5 ± 5.4 µM; aged, 10.9 ± 2.3 µM, $F(2,15) = 0.47$, $p = 0.63$). Thus, the major findings of this study were increased glutamate release from the entorhinal cortex terminals in the DG of late-middle aged rats, decreased glutamate release from mossy fiber terminals in the CA3 of aged rats and diminished glutamate release

capacity during aging from the mossy fibers and Schaffer collaterals comprising the trisynaptic circuit downstream of the DG.

To better determine potential age-related changes in glutamate uptake we locally applied an exogenous glutamate solution (100 µM in 0.9% saline, pH 7.4) into the DG, CA3 and CA1 hippocampal subregions of all three age groups to study clearance of glutamate from the extracellular space by glia and neuronal elements. Volumes ranging from 25-250 nL were delivered intracranially to obtain signals with maximum amplitudes in the range most frequently obtained during the KCl-evoked glutamate release studies (10-20 µM). Because the high-affinity glutamate transporters operate under Michaelis-Menton kinetics, it is important to compare clearance rates where experimental conditions supply approximately the same amount of substrate to transporters as physiological conditions. Actual maximum amplitudes analyzed across subregions and age groups were not statistically different (data not shown, ANOVA, $p = 0.17$); therefore, variability in the size of peaks analyzed did not contribute to differences in uptake rate.

Results of the exogenous glutamate clearance experiments are summarized in Table 3.3. In the DG of aged rats, the glutamate uptake rate was significantly faster compared to the uptake rate in the DG of young rats (5.5 ± 0.7 µM/sec vs. 3.3 ± 0.4 µM/sec, $p < 0.05$), with the late-middle aged rats falling in between (4.1 ± 0.6 µM/sec; $F_{(2,22)} = 3.88$, $p = 0.036$) (Figures 3.3 and 3.4). Also, within the aged trisynaptic circuit uptake rates were significantly different ($F_{(2,19)} = 5.38$, $p = 0.014$), showing a faster clearance rate in the DG (5.5 ± 0.7 µM/sec) when compared to the other hippocampal subregions (CA3: 3.7 ± 0.5, p < 0.05; CA1: 3.9 ± 0.4, $p < 0.05$) (Figure 3.4). No significant differences in uptake rate across the trisynaptic circuit were seen in the young and late-middle aged groups: (young, DG: 3.3 ± 0.4 µM/sec, CA3: 4.2 ± 0.4 µM/sec, CA1: 4.0 ± 0.3 µM/sec, $F_{(2,24)} = 1.68$, $p = 0.21$; late-middle aged, DG: 4.1 ± 0.6 µM/sec, CA3: 4.4 ± 0.4 µM/sec, CA1: 4.5 ± 0.5 µM/sec, $F_{(2,29)} = 0.18$, $p = 0.84$). The uptake rate within the CA3 and CA1 subregions was not different across age

groups ($F(2,22) = 0.86$, $p = 0.44$ and $F(2,29) = 0.61$, $p = 0.55$, respectively) (Figure 3.3).

Discussion

In this study, we have shown that tonic glutamate levels in the discrete DG, CA3 and CA1 subregions of the hippocampus do not change significantly during aging in anesthetized F344 rats. However, the trisynaptic pathway did exhibit changes in glutamate release and uptake in specific subregions. Interestingly, evoked release of glutamate was greatest from perforant path terminals in the DG of late-middle aged rats as compared to young and aged animals. The most fascinating finding was that the CA3 subregion exhibited profound dysregulation during aging. Mossy fiber terminals in CA3 had diminished evoked glutamate release in aged rats compared to young and late-middle aged animals. Also, alterations within the trisynaptic circuit during aging showed a significant loss of glutamate release capacity in CA3 (mossy fibers) and CA1 (Schaffer collaterals) compared to the DG (perforant path) in both late-middle aged and aged rats. These findings suggest a diminished function or competence of the glutamate fibers in aging animals. Finally, the aged DG showed a significantly increased glutamate uptake capacity compared to the DG of young animals, suggesting that the increased glutamate release in late-middle aged rats may result in increased glutamate uptake in aged rats.

Aging Does Not Alter Tonic Glutamate Levels in the Trisynaptic Circuit

Only two *in vivo* studies of resting glutamate in the hippocampus during aging have been reported. Their results are contradicting, showing an increase in aged compared to young Wistar rats (Massieu and Tapia, 1997) and a decrease in aged compared to young F344 rats (Zhang et al. 1991). A

shortcoming of these studies is the lack of subregional resolution attained due to the large size of the microdialysis probes utilized.

In contrast to the previous *in vivo* studies, our data show that resting glutamate levels were approximately equal throughout the individual subregions of the trisynaptic circuit across age groups. We caution that these results too could be misleading. Our laboratory has recently begun to appreciate that urethane anesthesia can lower resting glutamate levels in the striatum and pre-frontal cortex (Rutherford et al., 2007). Tonic glutamate concentrations across the hippocampus could be affected by anesthesia. However, the microelectrode technology used in these studies is the first *in vivo* technology that allows for measures of resting glutamate in discrete subregions of the trisynaptic circuit. Additional studies are needed in unanesthetized rats to determine the effects of aging on resting levels of glutamate.

Aging Alters Stimulus-evoked Glutamate Release in the Trisynaptic Circuit

In these studies we showed that significantly less glutamate release was evoked by local KCl application in the CA3 subregion of aged rats compared to the CA3 of young and late-middle aged rats. In the late-middle aged and aged groups, glutamate release was impaired from terminals in both CA3 and CA1 when compared to terminals in the DG. A decrease in stimulus-evoked glutamate release in the hippocampus with aging has been previously demonstrated in brain slices (Freeman and Gibson, 1987). Diminished glutamate release indicates that less glutamate is available for receptor activation, potentially altering the relay of glutamatergic neurotransmission through the trisynaptic circuit which is critical for hippocampal-dependent learning and memory.

The CA3 subregion has been repeatedly identified as having age-associated neurochemical alterations (Le Jeune et al., 1996; Kadar et al., 1998; Adams et al., 2001). Mossy fiber projections to CA3 have shown age-associated changes in dynorphin, an opioid-like peptide believed to be intimately involved

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with glutamatergic neurotransmission. Zhang et al. (1991) showed that an increase in dynorphin was localized to the DG granule cells and their MF projections in F344 rats, which correlated with an impaired memory performance and decreased glutamate release *in viv*o; however, their microdialysis study lacked anatomical resolution, localizing the diminished glutamate response only to the ventral hippocampus. Loss of glutamate release capacity in the CA3, both in aged compared to younger animals and within the trisynaptic circuitry of late-middle aged and aged rats, is perhaps our most important finding. These experiments have potentially exposed the functional outcomes of the age-associated molecular changes in mossy fiber inputs previously described in the literature.

Our *in vivo* studies also showed that the DG subregion was significantly altered in late-middle aged rats as compared to younger and older animals because glutamatergic terminals in the late-middle aged DG released more neurotransmitter following local KCl stimulation. The augmented evoked-release of glutamate from perforant path fibers in the DG indicates that changes in input to the hippocampal formation occur by late-middle age. An age-related increase in glutamate release has also been reported in brain slices (Meldrum et al., 1992; Saransaari and Oja, 1995). Prior work has shown that synapses from afferent projections to the DG decrease with age, but the remaining synapses are electrophysiologically stronger (reviewed by Geinisman et al., 1996). Until now, the effects of these age-associated changes on glutamatergic neurotransmission *in vivo* have never been specifically investigated in the DG subregion.

A compensatory increase in synaptic strength is supported by our data because we showed an enhanced evoked glutamate release in late-middle aged rats. From this we can conclude that more glutamate is available from perforant path terminals in the late-middle aged DG to participate in glutamate-dependent processes. This is important because age-associated alterations in hippocampal connectivity due to loss of synapses may not immediately result in functional changes due to the ability of the late-middle aged glutamatergic afferents to the DG to compensate. The return in the aged DG to evoked glutamate release

levels seen in young animals led us to two interesting, yet very different conclusions about the effects of aging on glutamate release in the DG. First, assuming the augmented release of glutamate in late-middle aged animals is a compensatory response to synaptic loss, the inability of the aged DG to maintain the increase may explain the loss of cognitive function in these animals. Alternatively, the aged DG may not be exhibiting a loss of compensation, but rather a different mechanism of compensation. The first order rate of glutamate clearance following evoked release was significantly faster in the aged DG compared to the young, even though the maximum release of glutamate measured by the microelectrode in both age groups was not significantly different. We believe the aged DG develops a compensatory increase in clearance capacity in response to the increased glutamate release in late-middle age. This is further supported by our exogenous glutamate clearance data. Increased clearance ability in the aged DG may be masking an increase in perforant path evoked-glutamate release similar to that seen in late-middle aged animals, such that higher release of glutamate in the aged DG is not measured by the electrode because a significant portion is rapidly cleared following release. If the increase in glutamate release during aging exceeds compensation and becomes pathologic, the faster glutamate clearance in aged animals may be a neuroprotective mechanism against excitotoxicity.

Aging Alters Glutamate Clearance in the Trisynaptic Circuit

Our data indicate that the DG acquires significant glutamate clearance capacity with age, showing approximately a 66% increase in the uptake rate of aged rats as compared to young rats. A significant increase is seen when the DG is compared to other trisynaptic subregions within the aged hippocampus as well. These data are in contrast to prior work that has shown no consistent changes in glutamate clearance with aging (Najerahim et al, 1990; Gilad et al., 1990; Palmer et al., 1994). Glutamate clearance from the synaptic cleft is controlled by uptake and diffusion (Sykova, 2005). Approximately ninety percent

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of glutamate uptake is performed by high affinity transporters (GLAST, GLT-1) located predominantly on astroglia (for review see Danbolt, 2001). Sykova et al. (2002) showed that a decrease in extracellular space (ECS) and loss of glial organization, especially in the DG, were evident in aged rats that were impaired learners. The aged F344 hippocampus maintains numbers of GFAP[+] astrocytes at young levels (Hattingly and Shetty, 2008), so it is possible that age-associated gliosis may be due to an increase in the fibrous character of astrocytes rather than an increase in astrocyte number (Wu et al., 2005). Sykova et al. (2002) proposed that the compacted ECS could predispose the aging population to excitotoxicity due to synaptic 'trapping' of glutamate.

We believe the increased clearance capacity of the aged DG could be a very effective neuroprotective mechanism against over-activation of glutamate receptors resulting from age-associated increases in release capacity of glutamate terminals in the DG and excitotoxic trapping due to gliosis. The significant increase in uptake rate is likely related to increased surface expression of transporters on glia and neurons. We have recently reported that decreased surface expression of GLAST may contribute to slower regulation of glutamate uptake in the dorsal rat striatum (Nickell et al., 2007). How aging affects the function of astrocytic glutamate transporters in the hippocampus is a critical piece of information not yet fully understood. Additional studies are needed to investigate the mechanism(s) involved with enhanced clearance of glutamate in the aged DG observed in the present study.

To our knowledge, this is the first study describing age-associated subregional heterogeneity of hippocampal glutamatergic neurotransmission *in vivo*. Though tonic levels of glutamate in the extracellular space were unchanged with aging, we were able to show subregion-specific alterations in stimulus-evoked glutamate release and clearance capacity in the trisynaptic circuit during aging. Taken with our previous aging studies in the rodent striatum and non-human primate cortex, we can begin to outline the vast variability of CNS susceptibility to changes in glutamate regulation during aging.

Portions of this work have been previously published:

M.L. Stephens, J.E. Quintero, F. Pomerleau, P. Huettl, and G. A. Gerhardt. Age-related changes in glutamate release in the CA3 and dentate gyrus of the rat hippocampus. *Neurobiology of Aging;* June 15 (2009) EPub ahead of print
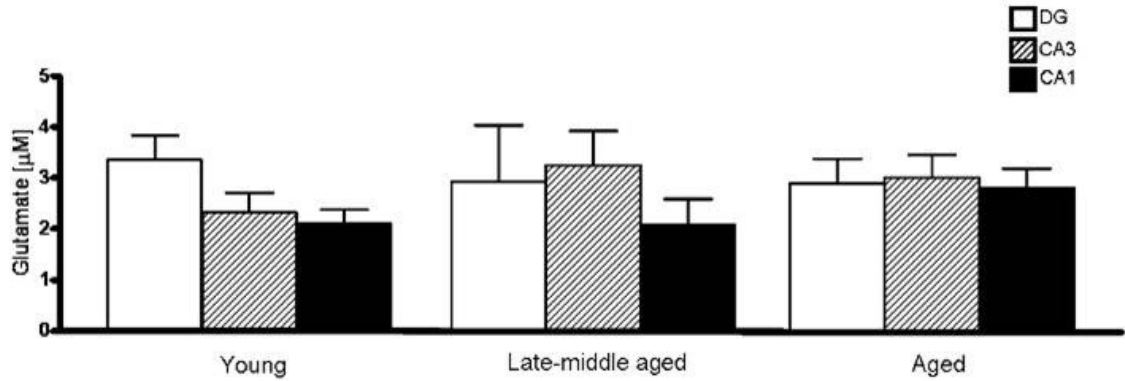
Figure 3.1 Mean Tonic Glutamate Concentrations in the Trisynaptic Circuit During Aging

Aging did not significantly alter tonic glutamate levels in the DG ($F_{(2,34)} = 0.19$, $p = 0.83$), CA3 ($F_{(2,34)} = 0.19$, $p = 0.83$) or CA1 ($F_{(2,34)} = 0.65$, $p = 0.53$).
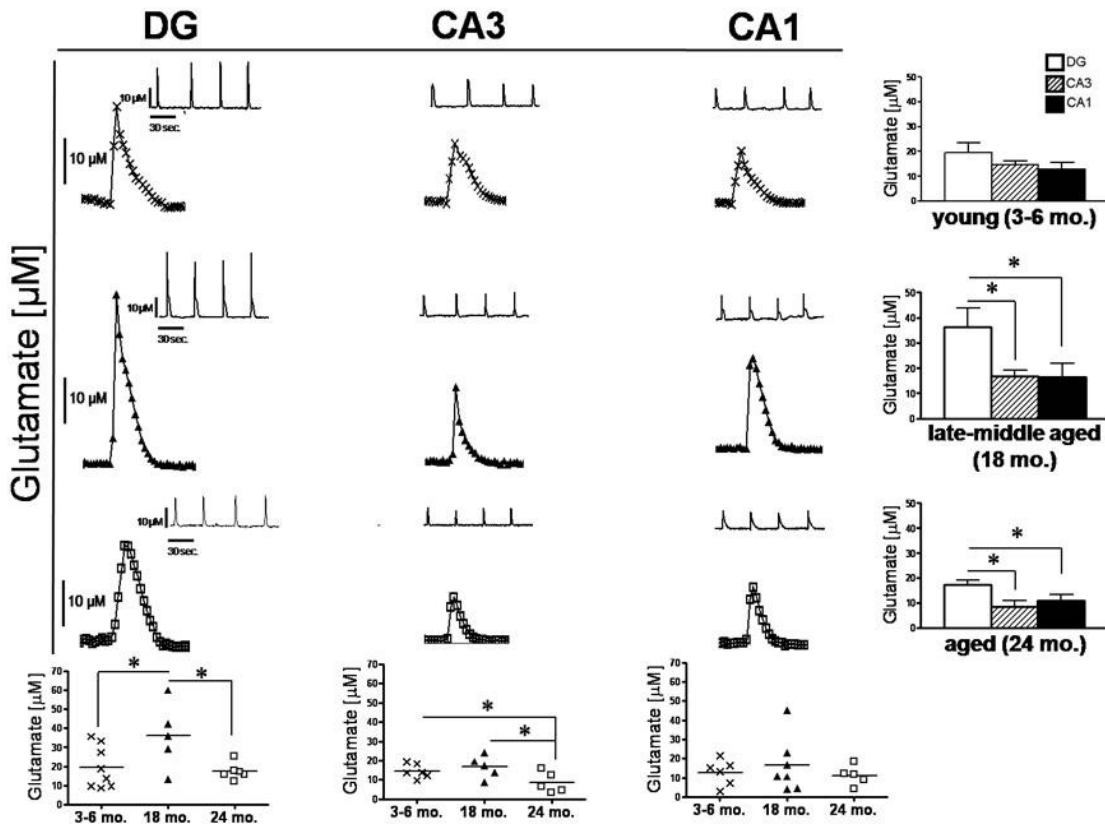
Figure 3.2 Representative Glutamate Release Signals Following Local Application of KCl in the Trisynaptic Circuit of F344 Rats During Aging

Each symbol in the traces is an individual glutamate measurement (2 Hz). Inset signals show reproducibility of evoked-glutamate release signals. *Right:* Bar graphs represent the mean maximum amplitude of glutamate signals across the trisynaptic circuit. Terminals in CA3 and CA1 had significantly less glutamate release compared to terminals in the DG in the 18 mo. and 24 mo. groups. *Bottom:* Graphs represent the mean max. amplitude of glutamate signals across age groups. Each symbol is an individual animal. The perforant path terminals in the DG of late-middle aged rats released more glutamate compared to young and aged rats. The mossy fiber terminals in the CA3 of aged rats released less glutamate compared to young and late-middle aged animals.
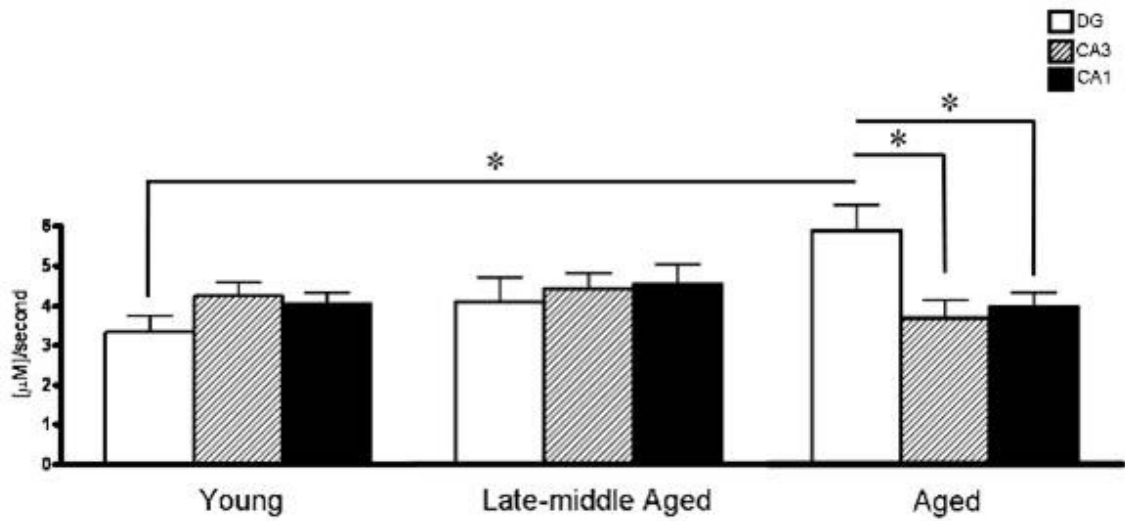
Figure 3.3 Mean Glutamate Uptake Rate Following Local Application of Exogenous Glutamate in the Trisynaptic Circuit of F344 Rats During Aging

Following local application of exogenous glutamate (100 µM), uptake rate was significantly faster in the aged DG compared to young rats. Within the aged trisynaptic circuit, uptake rates were significantly different showing faster glutamate uptake rate in the DG when compared to CA3 and CA1.
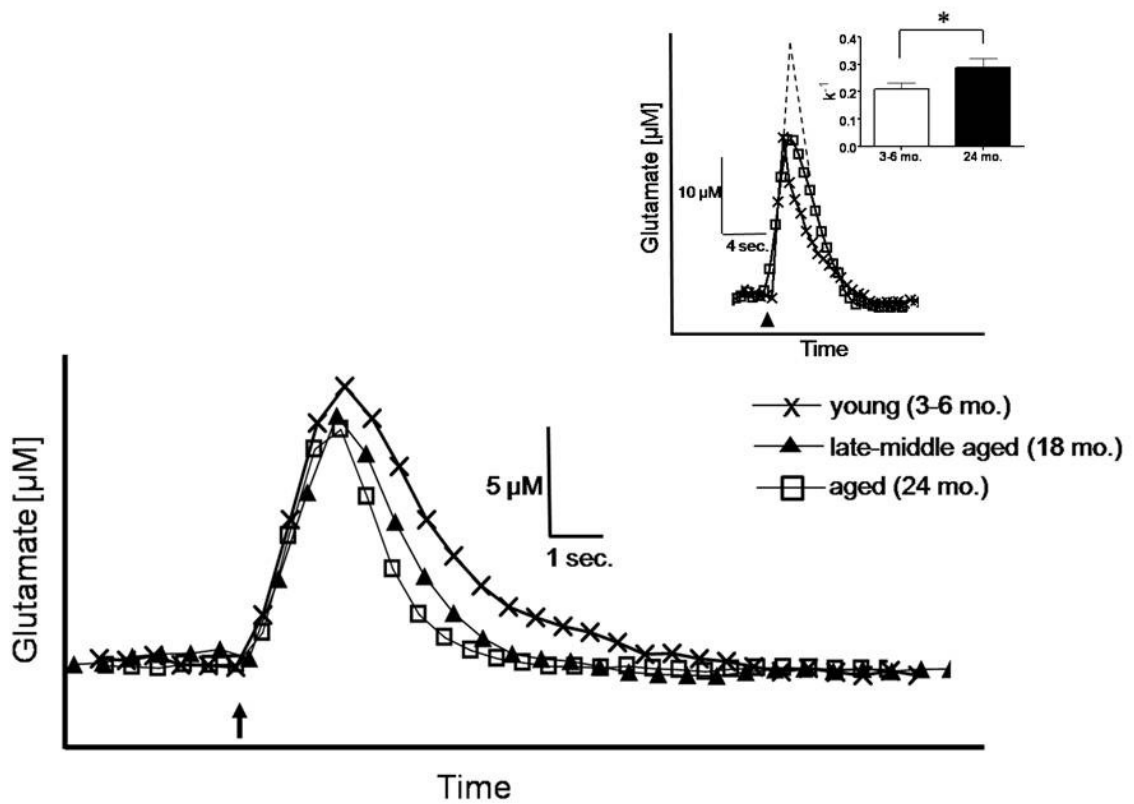
Figure 3.4 Comparison of Amplitude-matched Signals in the DG Showing Faster Clearance of Glutamate in Aged Animals

Each symbol is an individual glutamate measurement (2 Hz). Arrow indicates local application of exogenous glutamate (100 µM in 0.9% saline, pH 7.4). *Inset:* KCl-evoked glutamate signals in DG of young and aged rats. Aged rats have a faster first order rate of glutamate clearance. Dashed line indicates predicted maximum amplitude of glutamate signal in an aged animal without increased glutamate clearance capacity.

Table 3.1 Tonic Glutamate in the Hippocampus of Anesthetized Rats [µM]

Age	DG	CA3	CA1	Comparison Across Tri-synaptic Circuit (One-way ANOVA)
Young [a]	3.4 ± 0.5	2.8 ± 0.6	2.5 ± 0.5	p = 0.08
Late-middle aged [b]	2.9 ± 1.1	3.3 ± 0.7	2.1 ± 0.5	p = 0.46
Aged [c]	2.9 ± 0.5	3.0 ± 0.4	2.8 ± 0.4	p = 0.96
Comparison Across Age Groups Within Each Subregion (One-way ANOVA)	p = 0.83	p = 0.83	p = 0.53	

Values expressed as Mean ± SEM
[a] 3-6 months; DG n = 15, CA3 n = 12; CA1 n = 13
[b] 18 months; DG n = 6, CA3 n = 11; CA1 n = 10
[c] 24 months; DG n = 16, CA3 n = 14; CA1 n = 14

Table 3.2 Potassium-evoked Glutamate Release in the Tri-Synaptic Circuit During Aging [μM]

Age	DG	CA3	CA1	Comparison Across Tri-synaptic Circuit (One-way ANOVA)
Young [a]	19.6 ± 3.9	14.6 ± 1.5	12.7 ± 2.7	$p = 0.06$
Late-middle aged [b]	[‡,α] 36.2 ± 7.7	[#] 16.7 ± 2.6	[◊] 16.5 ± 5.4	*$p = 0.049$ [#]$CA3 < DG, p < 0.05$ [◊]$CA1 < DG, p < 0.05$
Aged [c]	17.3 ± 1.8	[¥, €,†] 8.4 ± 2.4	[Ω] 10.9 ± 2.3	*$p = 0.026$ [†]$CA3 < DG, p < 0.05$ [Ω]$CA1 < DG, p < 0.05$
Comparison Across Age Groups Within Each Subregion (One-way ANOVA)	*$p = 0.032$ [‡] Late-middle aged > Aged, $p < 0.05$ [α]Late-middle aged > Young, $p < 0.05$	*$p = 0.049$ [¥] Aged < Late-middle aged, $p < 0.05$ [€]Aged < Young, $p < 0.05$	$p = 0.86$	

All values represent mean $\pm$ S.E.M. of glutamate release following local application of 50 nL KCl (70mM, isotonic, pH 7.4)

[a] 3-6 months; DG n = 8, CA3 n =6; CA1 n = 6

[b] 18 months; DG n = 5, CA3 n = 5; CA1 n = 7

[c] 24 months; DG n = 6, CA3 n = 5; CA1 n = 5

Table 3.3 Clearance of Glutamate in the Tri-Synaptic Circuit during Aging

µM/sec

Age	DG	CA3	CA1	Comparison Across Tri-synaptic Circuit (One-way ANOVA)
Young [a]	3.3 ± 0.4	4.2 ± 0.4	4.0 ± 0.3	p = 0.21
Late-middle aged [b]	4.1 ± 0.6	4.4 ± 0.4	4.5 ± 0.5	p = 0.84
Aged [c]	†,#,◊5.5 ± 0.7	3.7 ± 0.5	3.9 ± 0.4	*p = 0.014 #DG > CA3, p < 0.05 ◊DG > CA1, p < 0.05
Comparison Across Age Groups Within Each Subregion (One-way ANOVA)	*p = 0.036 †Aged > Young, p < 0.05	p = 0.61	p = 0.55	

All values represent mean $\pm$ S.E.M. of uptake rate following local application of exogenous glutamate (100µM, pH 7.4)

[a] 3-6 months; DG n = 9, CA3 n = 7; CA1 n = 11

[b] 18 months; DG n = 7, CA3 n = 12; CA1 n = 13

[c] 24 months; DG n = 8, CA3 n = 6; CA1 n = 8

Chapter Four: Age-associated Changes in Hippocampal Glutamate Regulation Significantly Correlate With Status Epilepticus Behavior and Spontaneous Glutamatergic Neurotransmission

Introduction

The elderly population is the fastest growing age group in the United States (Collins et al., 1997). Studies have established that the elderly population has an increased risk for status epilepticus (SE) and status-related morbidity and mortality (DeLorenzo et. al., 1992). During aging, numerous alterations occur in the brain, including anatomical and physiological changes in the hippocampus (see Chapter Three) which is the most common brain structure involved in seizure disorders (Leppik et al., 2006). It can be hypothesized that aging of the CNS may directly affect seizure susceptibility. Age-associated alterations in the hippocampus that might predispose older individuals for SE need to be identified and studied. In addition, an understanding of how aging contributes to the increased severity of SE observed in elderly patients is also needed.

It has been widely suggested that dysregulation of excitatory neurotransmission involving glutamate is a predominant mechanism of age-associated increases in hippocampal vulnerability to neurodegenerative disease (for review see Danbolt, 2001; Segovia et. al., 2001). Availability of neurotransmitter for participation in glutamate receptor-dependent processes (normal and excitotoxic) is a dynamic balance of release and uptake (Segovia et al., 2001). One approach for investigating glutamate regulation involves looking for changes in tonic glutamate levels as an indication of disruption in glutamate release and/or uptake. To investigate *in vivo* neurotransmission, microdialysis coupled with high-performance liquid chromatography has traditionally been the gold standard though this technique has some significant limitations including slow sampling rates (minutes), poor spatial resolution (mm) and significant CNS glial activation and cellular inflammation around the sampling area (Clapp-Lilly et.

al., 1999). Only two studies using microdialysis have reported on age-associated changes in hippocampal tonic glutamate, and the results were contradictory (glutamate increased in aged animals, Massieu and Tapia, 1997; decreased in aged animals, Zhang et al., 1991). Our laboratory uses enzyme-based ceramic microelectrode arrays (MEA) coupled to real-time amperometry to study *in vivo* glutamate neurotransmission without the limitations of microdialysis. We can reliably measure tonic glutamate, as well as dynamic glutamate release and clearance events, in the mammalian CNS with sub-second temporal resolution (Burmeister et al. 2001, 2002; Pomerleau et al., 2003; Nickell et. al. 2005, 2007; Day et al., 2006; Quintero et al., 2007). The small size of the MEA recording sites (μm) allows targeting of discrete hippocampal trisynaptic subregions (e.g. dentate gyrus (DG), cornu ammonis 3 (CA3) and cornu ammonis 1 (CA1), Stephens et al., 2009[a]). Also, the MEAs have been shown to cause minimal disruption of the biological microenvironment evidenced by nominal glial activation, even after chronic implantation (Rutherford et al., 2007).

We recently showed that evoked-glutamate release and glutamate clearance capacity was significantly altered in the trisynaptic circuit of aged F344 rats, but no changes were evident in tonic extracellular glutamate (Chapter Three). Our tonic glutamate measurements were most likely affected by the presence of urethane anesthesia (Rutherford et al., 2007) warranting the current studies of tonic glutamate in awake-behaving animals, free of anesthetic effects. A clear benefit of conducting experiments in unanesthetized animals is the ability to incorporate behavioral experimental paradigms. For the studies presented here, we were able to measure tonic glutamate levels in the hippocampus of young and aging animals and then examine the manifestation of SE as it related to age and pre-SE glutamate levels.

Convulsive SE animal models using 4-aminopyridine (4-AP) administration (systemic and intrahippocampal) have been an important experimental method for understanding the causal role of excitatory synaptic neurotransmission in seizure-related neuronal death (Pena and Tapia, 1999; Pena and Tapia, 2000; Ayala and Tapia, 2005). Intrahippocampal 4-AP exposure results in robust EEG

activity in rodents (Fragoso-Veloz et al., 1990; Medina-Ceja et al., 2000; Pena and Tapia, 1999; Pena and Tapia, 2000; Ayala and Tapia, 2005) which is accompanied by increased hippocampal extracellular glutamate (Pena and Tapia, 1999; Pena and Tapia, 2000; Morales-Villagran et al., 2008). The need to monitor dynamic glutamate neurotransmission during SE has been recognized (Morales-Villagran et al., 2008) but never achieved on the second-by-second time scale of rapid glutamate release and clearance events (Danbolt, 2001). The data presented here highlight the use of real-time amperometric measurements of glutamate as a novel assessment of glutamate regulation before, during and after seizure in a rodent model of human aging.

To our knowledge, no previous studies have reported on the use of the 4-AP in aged animals, but we believe it is an attractive model because it can be applied intrahippocampally to cause limbic seizures and induces fast onset of SE with a single injection (Fragoso-Veloz et al., 1990). Further supporting the utility of the 4-AP SE model, especially in aged animals, we determined through the course of this study that our experimental paradigm had zero mortality and produced self-terminating SE which allowed us to compare the SE duration as a function of age In summary, these studies were designed to investigate glutamate regulation in the hippocampus during normal aging, and to elucidate the potential relationship between glutamate dysregulation and rigor of SE in aging populations. First, we studied the effects of aging on tonic glutamate levels in the DG, CA3 and CA1 subregions of the trisynaptic circuit of unanaesthetized animals using a self-referencing enzyme-based recording technology (Day et al., 2006). Second, we measured dynamic alterations in extracellular glutamate occurring in real-time during SE. We accomplished this by coupling behavioral characterizations with amperometric recording techniques to monitor *in vivo* extracellular glutamate on a second-by-second basis. We then compared SE-associated behavioral events and dynamic glutamatergic neurotransmission across age groups. Finally, we looked for lasting changes in glutamate regulation after SE by comparing pre- and post-SE tonic glutamate measurements.

Materials and Methods

Please refer to Chapter Two for a detailed description of animal care, electrode preparation, electrode calibration, amperometric recording technique, the 4-AP status epilepticus model and methodology for measuring and comparing tonic and phasic glutamate neurotransmission across hippocampal subregions and age-groups before, during and after SE.

Results

Measurements obtained in unanesthetized F344 rats showed that tonic glutamate in the collective hippocampal formation in both late-middle aged (16.3 ± 3.0 μM) and aged rats (16.7 ± 3.2 μM) was significantly higher than young rats (8.4 ± 1.1 μM), $F(2,52) = 3.479$, $p < 0.05$ (Table 4.1). The D'Agostino and Pearson omnibus normality test confirmed that the data were normally distributed (young: $p = 0.232$; late-middle aged: $p = 0.414$; aged: $p = 0.190$). In all trisynaptic subregions there were late-middle aged and aged rats with tonic glutamate levels higher than young rats (mean ± S.E.M.): DG: young 7.9 ± 1.4 μM, late-middle aged 17.5 ± 5.6 μM, aged 17.3 ± 12.6 μM; CA3: young 11.7 ± 2.9 μM, late-middle aged 23.6 ± 5.3 μM, aged 22.2 ± 7.1 μM; CA1: young 6.2 ± 1.0 μM, late-middle aged 9.1 ± 3.1 μM, aged 10.7 ± 4.3 μM, but comparisons of the individual subregions across age groups did not produce a particular subregion that was primarily responsible for the age-associated increase in hippocampal glutamate (DG $F(2,18) = 1.990$, $p > 0.05$; CA3 $F(2,13) = 1.240$, $p > 0.05$: CA1 $F(2,15) = 0.5208$, $p > 0.05$) (Figure 4.1).

Self-referenced recordings showed spontaneous glutamatergic release events in CA1 after local application of 4-AP which persisted throughout SE, and was not due to movement artifact from SE-associated convulsions (i.e. wet-dog

shakes (WDS)) (Figure 4.2). Spontaneous glutamate neurotransmission, characterized by a 'peak' in current recorded by the MEA as rapid release and clearance of glutamate in CA1 caused an increase in extracellular concentration followed by a quick return to baseline, was TTX (100 µM, 1 µL applied locally) sensitive (Figure 4.3). Except for one young rat, the low dose 4-AP produced WDS behavior and spontaneous glutamate release events in all animals (Figure 4.4). All animals displayed WDS behavior and glutamate release events following the high dose (Figure 4.5). A highly significantly dose effect was observed as all age groups showed increases in duration of SE ($F(1, 14) = 71.70$, $p < 0.001$), average WDS/ 5 minutes ($F(1, 14) = 31.89$, $p < 0.001$) and maximum WDS/ 5 minutes ($F(1, 14) = 29.54$, $p < 0.001$) after the high dose of 4-AP compared to after the low-dose. All age groups also showed a significant increase in total glutamate release events during SE from high dose 4-AP ($F(1, 14) = 102.33$. $p < 0.001$) compared to SE from low dose 4-AP. The latency to onset of spontaneous glutamate release events ($F(1, 14) = 0.49$, $p > 0.05$), average peak size ($F(1, 14) = 1.8$, $p > 0.05$) and release periodicity ($F(1, 14) = 1.37$, $p > 0.05$) did not change as a function of 4-AP dose. More severe behavior (clonic spasms, rearing) was not seen in any of the animals, though use of 4-AP has been reported to cause these behaviors (Medina-Ceja et al. 2000). Results are summarized in Tables 4.2 and 4.3.

Regression analyses showed that the status duration ($r^2 = 0.3667$, $F(1,15) = 8.685$, $p = 0.01$), average WDS/ 5 minutes ($r^2 = 0.6136$, $F(1,15) = 23.82$, $p < 0.001$), maximum WDS/ 5 minutes ($r^2 = 0.6059$, $F(1,15) = 23.06$, $p < 0.001$), and average glutamate peak size ($r^2 = 0.2375$, $F(1,15) = 4.67$, $p < 0.05$) increased linearly with respect to pre-SE tonic glutamate levels obtained before experimentation with low dose 4-AP (Figure 4.6). The total number of glutamate release events ($r^2 = 0.1819$, $F(1,15) = 3.33$, $p > 0.05$) and the release periodicity ($r^2 = 0.006$, $F(1,15) = 0.081$, $p > 0.05$) during SE did not significantly change due to pre-SE tonic glutamate concentration. The behavioral and glutamate release characteristics of SE induced by high dose 4-AP did not correlate with pre-SE tonic glutamate: status duration ($r^2 = 0.0576$, $F(1,15) = 3.33$, $p > 0.05$), average

WDS/ 5 minutes ($r^2 = 0.0673$, $F(1,15) = 1.082$, $p > 0.05$), maximum WDS/ 5 minutes ($r^2 = 0.0472$, $F(1,15) = 0.7428$, $p > 0.05$), total glutamate release events ($r^2 = 0.0344$, $F(1,15) = 0.5346$, $p > 0.05$), average glutamate peak size ($r^2 = 0.0515$, $F(1,15) = 0.8140$, $p > 0.05$), release periodicity ($r^2 = 0.040$, $F(1,15) = 0.6315$, $p > 0.05$).

Though pre-SE tonic glutamate did not correlate with behavior and spontaneous glutamate release during the more severe SE induced by high dose 4-AP, age did significantly affect the high dose SE duration ($F(2,14) = 5.96$, $p < 0.05$) and total glutamate release events ($F(2,14) = 5.74$, $p < 0.05$). Aged rats experienced a longer duration of SE (105 ± 15 min.) than young rats (55 ± 10 min., $p < 0.01$) and late-middle aged rats (66 ± 9 min., $p < 0.05$). Related to the SE duration, total glutamate release events were also higher in aged rats (105.0 ± 14.8) than young rats (55.0 ± 10.3, $p < 0.05$) (Figure 4.5). Average WDS/ 5 minutes ($F(2,14) = 0.92$, $p > 0.05$), maximum WDS/ 5 min. ($F(2,14) = 1.02$, $p > 0.05$), latency to onset of spontaneous glutamate release ($F(1, 14) = 0.44$, $p > 0.05$), average peak size ($F(2,14) = 0.48$, $p > 0.05$) and release periodicity ($F(2,14) = 2.46$, $p > 0.05$) after high dose 4-AP were not affected by age. Age did not significantly affect any of the behavioral or spontaneous glutamate release characteristics during SE induced by low dose 4-AP ($p > 0.05$ for all parameters) (Figure 4.4). Results are summarized in Tables 4.2 and 4.3.

WDS behavior and glutamate release events were significantly correlated after both the low and high doses of 4-AP (Figure 4.7). The quantity of WDS was linear with respect to the quantity of glutamate release events (low dose, $r^2 = 0.7391$; high dose, $r^2 = 0.5034$). During the low dose 4-AP experimental paradigm, the average number of WDS/ 5 minutes increased as the average size of the glutamate peaks increased ($r^2 = 0.3668$). This relationship was not seen in the high dose experimental paradigm.

Comparing tonic glutamate concentrations obtained before and after SE showed that exposure to 4-AP and the subsequent induction of SE did not significantly change tonic glutamate in any age group ($F(1, 14) = 0.50$, $p > 0.05$).

Pre-SE vs. post-SE: young, 6.2 ± 1.0 µM vs. 7.8 ± 1.5 µM; late-middle aged, 9.1 ± 3.1 µM vs. 7.7 ± 2.7 µM; aged, 7.6 ± 3.8 µM vs. 9.1± 5.9 µM (Figure 4.8).

Discussion

The studies in Chapter Three demonstrated age-associated alterations in glutamate release and clearance in the DG and CA3 hippocampal subregions of F344 rats. One goal of the current study was to determine if previously demonstrated alterations in glutamate and release and clearance manifested with an age-associated change in tonic glutamate levels. It was important to perform these experiments in unanesthetized animals to avoid influence of urethane anesthesia on tonic glutamate which confounded prior measurements.

A second goal of this study was to determine the influence of age on the expression of SE. The quantification and characterization of rapid, spontaneous and highly synchronized glutamatergic release events that co-manifested with the appearance of SE behavior provided a novel means to connect glutamate dysregulation and seizure pathology. Tonic glutamate was shown to be significantly elevated in the hippocampal formation of late-middle aged and aged rats compared to young rats. Pre-SE tonic glutamate significantly correlated with the rigor of SE after the low dose of 4-AP, as animals with the highest tonic levels experienced the most persistent and severe WDS behavior along with the largest glutamate peaks. Regardless of pre-SE tonic glutamate levels, after the high dose of 4-AP significantly longer SE duration and more spontaneous glutamate release events were observed in aged animals. Taken together these results indicate that a strong relationship exists between aberrant glutamate neurotransmission and SE, and also support an etiological role for age-associated glutamate dysregulation in epilepsy and SE which occur with disproportionately high frequency and severity in elderly populations.

Basal Glutamate Is Significantly Elevated in the Hippocampal Formation During Aging

Though no particular subregion showed a statistically significant increase in tonic glutamate during aging, when data from all subregions were combined within each age group, a significant elevation in tonic glutamate was seen in the collective hippocampal formation of late-middle aged and aged animals compared to young animals. Mean tonic glutamate levels were approximately the same in the late-middle aged and aged groups indicating that onset of glutamate dysregulation occurs earlier than late-middle age, and persists throughout the remainder of the life span. Animals with age-associated increases in DG and CA3 tonic glutamate seemed to contribute most to the significantly elevated levels in the collective hippocampal formation. This is interesting because our prior work has shown that age-associated alterations in evoked- glutamate release and clearance are specific to the DG and CA3 subregions, with no significant alterations occurring in CA1 (see Chapter Three).

The question remains, are age-associated changes in glutamate regulation cause, effect or compensation? The answer probably depends on the animal. Significant increases in evoked-glutamate release in the DG of late-middle animals could be an initial substrate for increasing tonic glutamate levels during aging which cues the DG of aged animals to increase clearance capacity. A diminished CA3 evoked glutamate release in aged animals could certainly be interpreted as a mechanism contributing to loss of NMDAR-dependent learning and memory functions, but alternatively might be a compensatory response to elevated basal glutamate in the aging CA3, possibly mediated by mGluR auto-regulation of presynaptic glutamate release. It is important to note that even though some older animals did have elevated tonic glutamate, several animals in the late-middle aged and aged groups had tonic glutamate levels similar to young animals. This could be interpreted to mean that effective compensatory mechanisms are in place in these 'successfully' aging animals, or that dysregulation never began in the first place. A very interesting alternative interpretation is that these animals are not successfully aging. According to

Geinisman and colleagues (1995), approximately two-thirds of aged animals are impaired on learning and memory tasks, and so it must be considered that the one-third of the our aging experimental populations with elevated tonic glutamate levels may function comparably to young animals in a scenario where increased extracellular glutamate compensates for synaptic loss, yielding aged animals that have maintained hippocampal function. Definitive conclusions about the origin of increased tonic glutamate in the hippocampal formation, specifically in DG and CA3, cannot be made at this point, but it is important to reflect upon the fact that CA1 appears to be robust in the face of age-associated glutamate dysregulation, evidenced by prior studies and the tonic glutamate measurements presented here. In the future, combining studies of glutamate regulation with memory testing could help discriminate the role of glutamate neurotransmission in maintenance of hippocampal function and successful aging of the trisynaptic circuit.

Intrahippocampal 4-AP Causes Dose-dependent Status Epilepticus Characterized by Glutamate Bursting Activity and Limbic Behavior

Local application of 4-AP produced repetitive glutamate release events (~2 µM) that displayed a very rhythmic periodicity (approximately 1 peak per minute). On average, spontaneous release activity began about 2 minutes after the administration of 4-AP. Glutamate release events occurred throughout the duration of WDS behavior. Regression analysis confirmed a significant correlation between total numbers of WDS and total glutamate release events during SE induced after both low and high doses of 4-AP. The average peak size, release periodicity and latency to onset were not dose-dependent, but were sensitive to TTX which immediately halted spontaneous glutamate release and WDS behavior. The total number of glutamate release events occurring during SE was dose dependent, as was the SE duration. Together these data indicate that SE coincides with homogeneous, periodic neuronal glutamate release

events that persist for a given duration of time, dependent upon the severity of the seizure (i.e. low verses high dose 4-AP-induce SE).

All of the SE behavioral parameters (duration, average WDS/ 5 minutes, maximum WDS/ 5 minutes) were highly dose dependent. This is especially interesting in light of the very consistent spontaneous glutamate release activity regardless of dose. It is important to emphasize that the MEA measures glutamate through direct contact to the analyte, and is therefore measuring neurotransmission in a discrete area. One interpretation of dose-dependent increases in behavioral severity and dose-independent spontaneous glutamate release, is that high dose 4-AP facilitated the recruitment of brain areas distal to the seizure focus (injection site). Through the subsequent spread of synchronized neuronal firing, behavior became more severe while the spontaneous glutamatergic neurotransmission at the MEA remained constant in size and periodicity.

We now know that spontaneous glutamate release events initiate within five minutes after intrahippocampal (CA1) 4-AP injection, but this study was not designed to determine the latency of behavioral onset as behavior was tallied over five minute epochs and not on a second-by-second basis like the glutamate measurements. A few animals did not display WDS until after the first five minute epoch, so we can conclude that spontaneous glutamate release activity preceded WDS behavior in these animals, although other seizure behaviors like oral automatisms may have had a shorter latency, but were more difficult to monitor and therefore not quantified. Future studies should be designed to determine which comes first, bursting or behavior. If it is determined that aberrant glutamate neurotransmission does in fact begin before behavior, spontaneous release could become a powerful biomarker of imminent seizure useful to therapeutic interventions aimed at halting synchronized neuronal firing before recruitment of adjacent brain areas and transition to overt seizure occurs.

These studies established that a key feature of the SE-associated glutamate release events was the uniformity of the peak size and periodicity after both low and high doses of 4-AP. Average glutamate release events were

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approximately 2 µM in all age groups, but some animals had 3 - 4 µM peaks while others had 0.5 µM peaks. This small variation about the mean had significant effects on the manifestation of WDS behavior after low-dose 4-AP. Regression analysis confirmed that animals with larger glutamate peaks averaged more WDS per 5 minute epoch. Smaller glutamate bursts resulted in less WDS per 5 minute epoch. These data fundamentally implicate dysregulation of dynamic glutamate neurotransmission in the progression of SE.

Severity of Status Epilepticus is Predicted by Pre-Status Basal Glutamate

Perhaps the most important finding in this study for aging research is the significant correlation between pre-SE tonic glutamate levels in CA1 and the severity of SE following low-dose 4-AP. Though tonic glutamate was not significantly increased in CA1 with age, some late-middle aged and aged rats had elevated levels compared to the rest of the animals in the study. Animals with increased tonic glutamate experienced a longer SE duration, increased average and maximum WDS per 5 minute epoch and also larger spontaneous glutamate peaks. Increased tonic levels indicate an underlying dysregulation in glutamate neurotransmission, though the mechanism cannot be identified with these studies. Prophylactic steps to regulate tonic glutamate in high risk populations like the elderly could dramatically reduce SE related morbidity and mortality.

Age Affects Status Epilepticus Behavior and Glutamate Bursting After High Dose 4-AP

Duration of SE behavior and the corresponding number of glutamate release events was significantly increased after high-dose 4-AP in the aged rats compared to younger animals. This is intriguing because during severe SE, pre-SE tonic glutamate no longer mattered, but rather age controlled the manifestation of WDS behavior and spontaneous glutamate release events. This

indicates that properties of the aged hippocampus other than CA1 tonic glutamate levels predispose this population for severe seizures. As mentioned earlier, we believe high-dose 4-AP may potentiate the spread of synchronous neuronal firing. Recruitment of the other hippocampal subregions with known age-associated glutamate dysregulation, in addition to extrahippocampal brain areas that display altered glutamate regulation during aging (i.e. the striatum (Nickell et al., 2005, 2007), which is susceptible to 4-AP-induced status epilepticus, (Morales-Villagran and Tapis, 1996) may contribute to the increase in WDS behavior and spontaneous glutamatergic neurotransmission during severe SE in aged animals.

Do 4-AP Mechanisms of Action Tell Us Anything About Aged Hippocampus?

At least acutely, the aged hippocampus appeared to recover from SE similarly to the young hippocampus, evidenced by consistent pre- and post-SE tonic glutamate levels. It has been shown that 4-AP induced neurodegeneration can take up to five days, so chronic studies of post-SE tonic glutamate are needed to assess differences in glutamate neurotransmission as a result of neurotoxicity in each age group (Pena and Tapia, 1999).

The choice of 4-AP for our SE model was based upon the hypothesis that age-associated alterations in glial function could be exploited by 4-AP. Consider the scenario where aged astrocytes have impaired transporter function. This has been previously shown in the aged striatum (Nickell et al., 2006). Because recovery of glutamate form the extracellular space occurs with reverse transport of a K^+ ion, diminished glutamate recovery could result in increased intraglial K^+ and potentially an attenuation of the inward rectifying current (K_{ir}) which helps to buffer extracellular K^+. Elevated extracellular glutamate and K^+ could enhance glutamate receptor activation and neuronal excitability, possibly pre-disposing the aged hippocampus to synchronous neuronal firing and excitotoxicity.

What if the aged Kir was inherently disrupted like in TLE patients? Inappropriate buffering and the subsequent increase in extracellular K^+ can reverse transporter function, and actually result in the release of glutamate from astrocytes. Again, elevated extracellular glutamate and K^+ could enhance neuronal excitability while increased glutamate could result in over-activation of ionotropic receptors leading to excitotoxicity. Due to the ability of high levels of extracellular K^+ to depolarize neurons in both scenarios, auto-regulation of pre-synaptic glutamate release via mGluRs would potentially fail to attenuate glutamate release even though extracellular levels would be increasing.

In theory, 4-AP could facilitate dysregulation already present in the aged hippocampus by potentiating the inappropriate buffering of K+ (first scenario), or paradoxically fail to produce activity in aged rats with inherently disrupted K^+ regulation which is the result of loss of Kir channels (second scenario). Our data indirectly support that Kir channels are intact in the aged hippocampus; however this is not an indication of proper function, especially when extracellular glutamate levels are elevated. In addition to therapeutic strategies aimed at regulating glutamate levels, the pharmacological facilitation of proper K^+ buffering may reduce the occurrence and severity of SE in elderly. These studies do not allow for conclusions to be drawn about transporter function in the aged hippocampus. Prior studies actually demonstrated that transporter function may be increased in the aged DG. Based on the present study, we can conclude that glutamate neurotransmission is severely disrupted during SE and that age-associated increases in tonic glutamate levels in the extracellular space, regardless of origin, correlate with increased severity of SE.
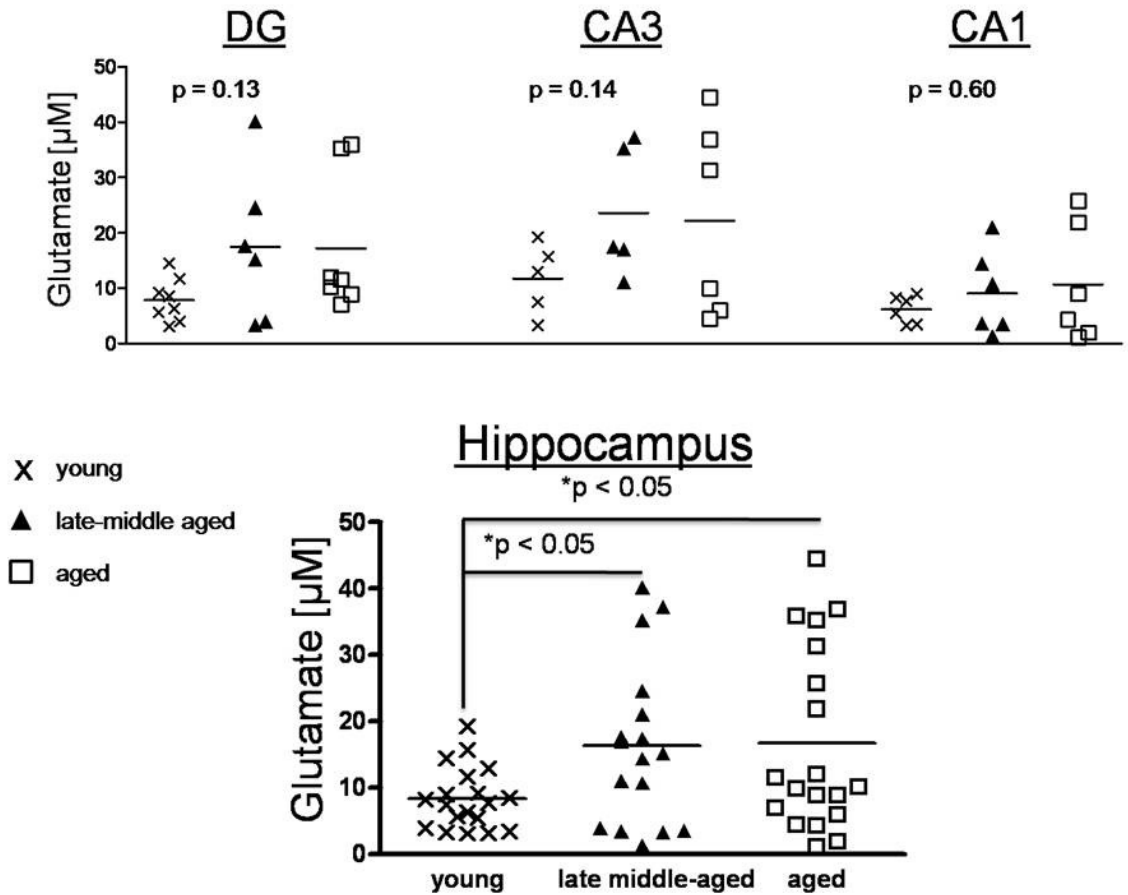
Figure 4.1 Tonic Glutamate in the Trisynaptic Circuit of Awake Rats During Aging

Compared to young animals (8.4 ± 1.1 µM), tonic glutamate levels were significantly elevated in the late-middle aged (16.3 ± 3.0 µM, $p < 0.05$) and aged (16.7 ± 3.2 µM, $p < 0.05$) hippocampal formation. Each symbol represents an individual animal. As is typical in aging studies, data variability increases with age, but the data are normally distributed (young, p = 0.23; late middle-aged, p = 0.41; aged, p = 0.19, D'Agostino and Pearson omnibus normality test).
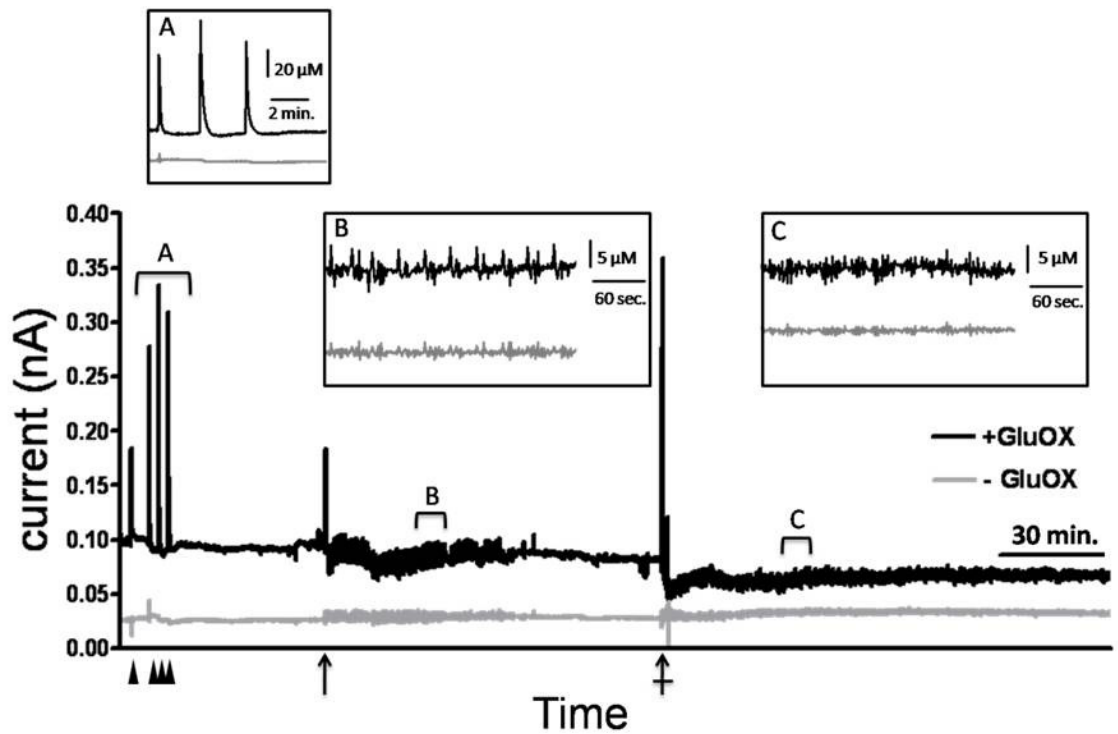
Figure 4.2 Traces from 'Glutamate-sensitive' and Sentinel MEA Recording Sites During Status Epilepticus in the Awake Rat

Representative traces for the entire duration of Day 4 recordings in CA1. Local application of glutamate (5 mM, triangles) produces a robust response on the glutamate-sensitive recording site (black trace) and no response on the sentinel recording site (grey trace). High levels of glutamate following exogenous application are rapidly cleared and do not result in spontaneous release events (A). Local 4-AP (arrow = low dose; hashed arrow = high dose) produces a large transient increase in glutamate, followed by spontaneous synchronized glutamate release events that only appear on the glutamate-sensitive sites (B and C). Any movement artifact from WDS can be removed by self-referencing.
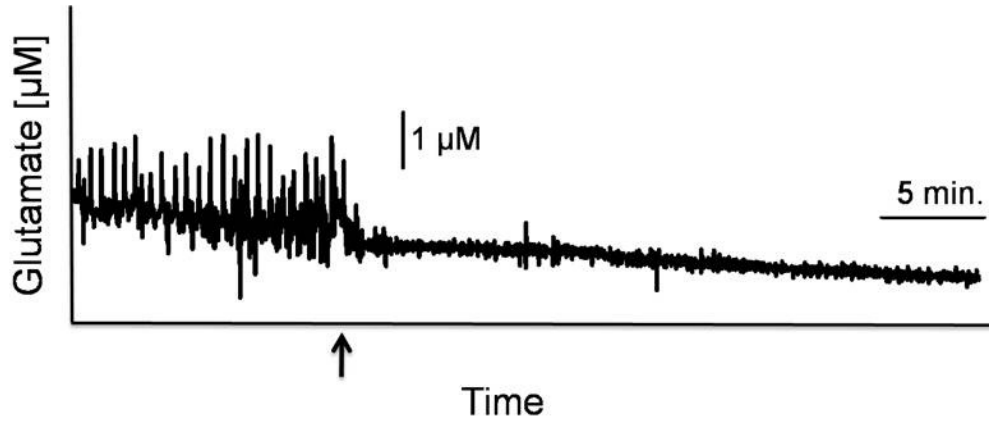
Figure 4.3 Spontaneous Glutamate Release During Status Epilepticus Is TTX-sensitive

Self-referenced recording showing that glutamate release events during 4-AP-induced SE are halted following local application of TTX (100 μM, 1 μL, arrow). TTX also halted the expression of WDS.
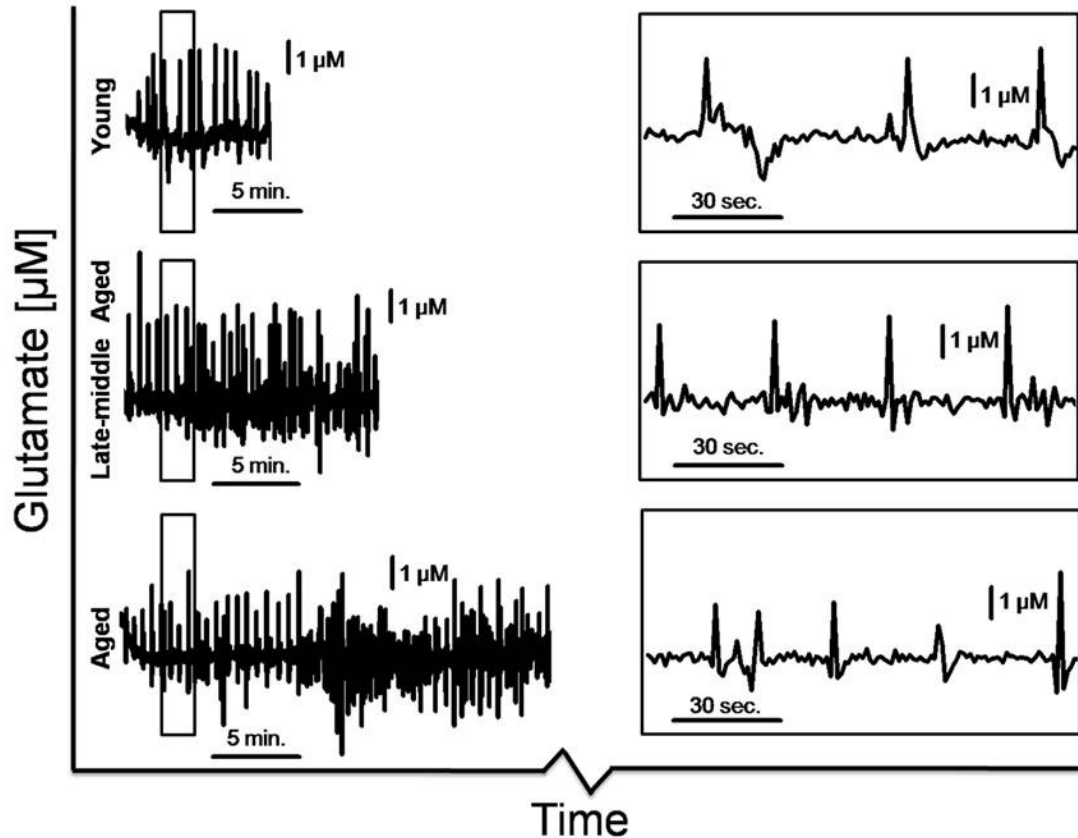
Figure 4.4 Spontaneous Glutamate Release After Low-dose 4-AP

Self-referenced recordings showing highly synchronous glutamate release events in CA1 following intrahippocampal injection of 4-AP (5.25 nmol). *Left:* Spontaneous glutamate activity during WDS behavior. *Insets:* Magnification of traces showing amplitude and period of glutamate peaks across age groups. Age did not significantly affect any of the behavioral or glutamate release characteristics during SE induced by low dose 4-AP ($p > 0.05$ for all parameters).
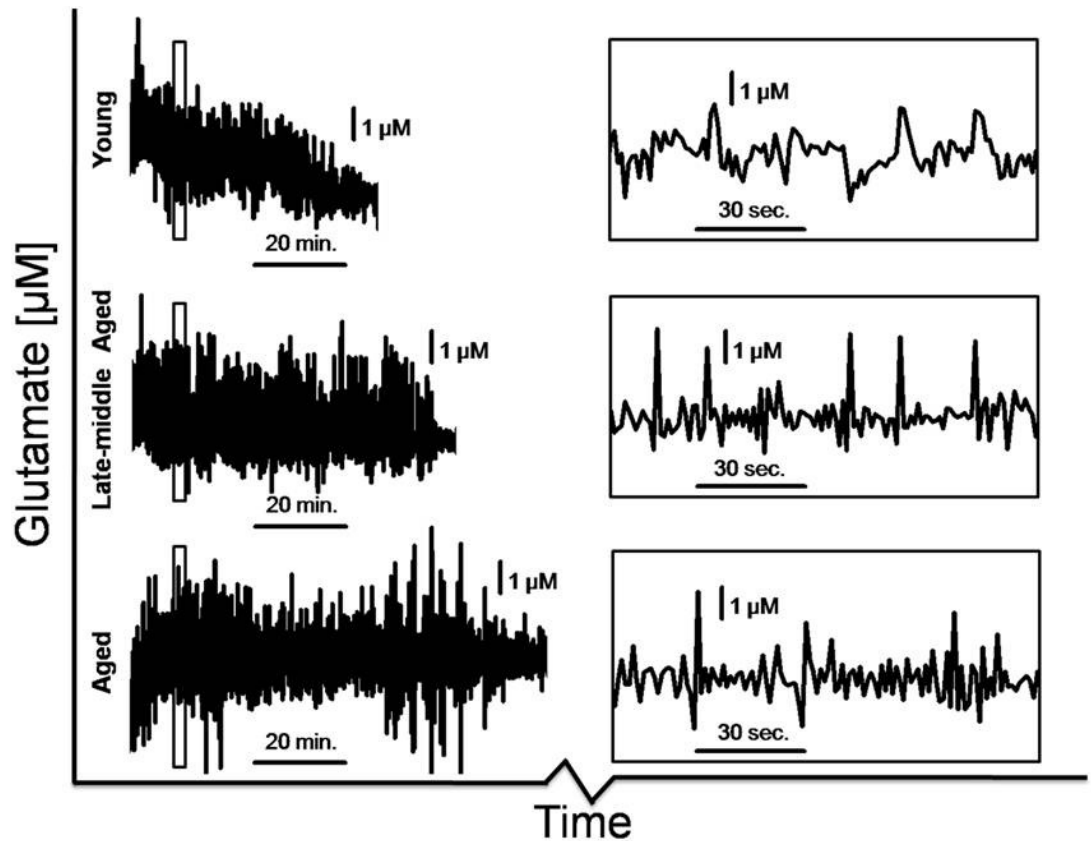
Figure 4.5 Spontaneous Glutamate Release After High-dose 4-AP

Self-referenced recordings showing glutamate release events in CA1 following intrahippocampal injection of 4-AP (21 nmol). *Left:* Spontaneous glutamate activity during wet-dog shaking behavior. *Insets:* Magnification of traces showing amplitude and period of glutamate peaks across age groups. Aged rats experienced a longer duration of status epilepticus (105 ± 15 min.) than young rats (55 ± 10 min., $p < 0.01$) and late-middle aged rats (66 ± 9 min., $p < 0.05$). Related to the SE duration, total glutamate release events were also higher in aged rats (105.0 ± 14.8) than young rats (55.0 ± 10.3), $p < 0.05$.
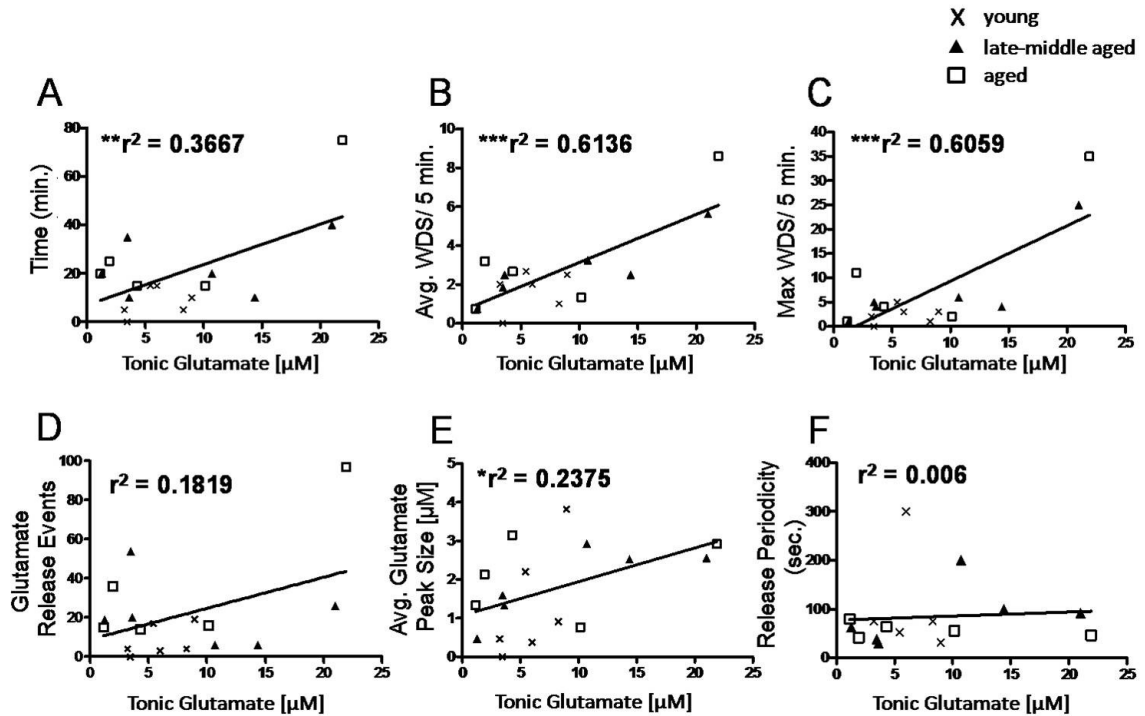
Figure 4.6 Tonic Glutamate Predicts Status Epilepticus Outcomes

Regression analysis showed that higher levels of pre-status basal glutamate (seen in late-middle aged and aged animals) resulted in significant increases in A) status duration ($p < 0.01$), B) average WDS/ 5 min. ($p < 0.001$), C) maximum WDS/ 5 min. ($p < 0.001$), and E) average glutamate peak size after low-dose 4-AP ($p < 0.05$). Total numbers of glutamate release events (D) and release periodicity (F) were not affected by pre-SE basal glutamate levels ($p > 0.05$).

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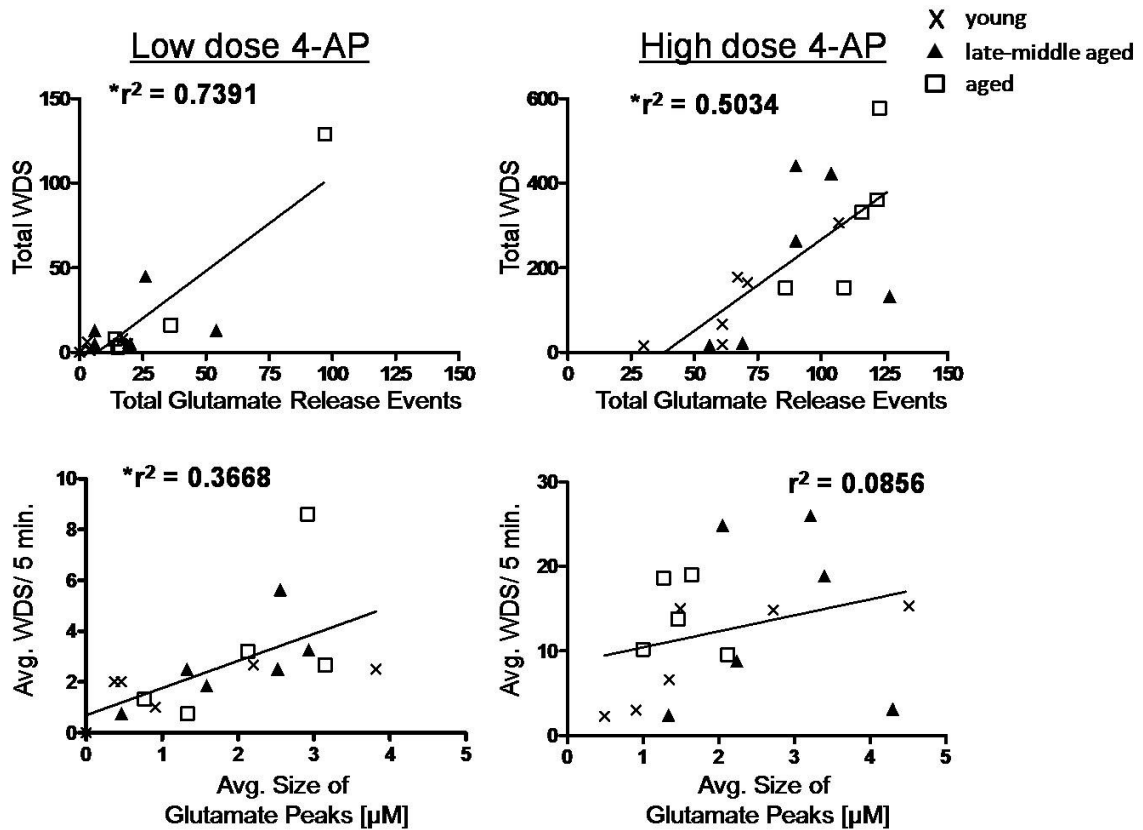
Figure 4.7 Quantity and Size of Glutamate Release Events Correlates with Behavior During Status Epilepticus

Regression analysis showed after both the low and high doses of 4-AP, the total number of glutamate release events significantly correlated with the total number of WDS (p <0.05). The average size of the glutamate peaks predicted the average WDS/ 5 min. after the low dose (p < 0.05), but this relationship was not significant after the high dose (p > 0.05).
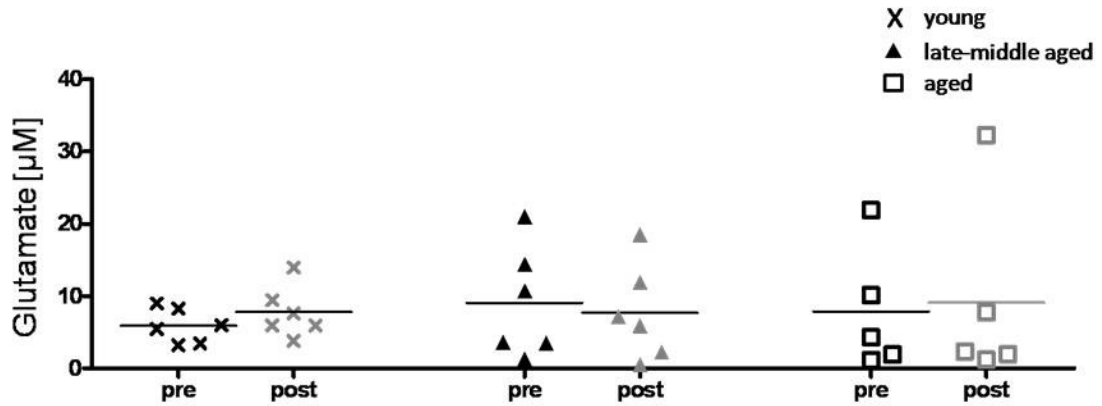
Figure 4.8 Pre- and Post-status Epilepticus Tonic Glutamate Levels in CA1

Basal glutamate concentrations obtained before (black) and after (grey) SE showed that exposure to 4-AP and the subsequent induction of SE did not significantly change tonic glutamate in any age group ($p > 0.05$, two-way ANOVA). Pre-SE vs. post-SE: young, 6.2 ± 1.0 µM vs. 7.8 ± 1.5 µM; late-middle aged, 9.1 ± 3.1 µM vs. 7.7 ± 2.7 µM; aged, 7.6 ± 3.8 µM vs. 9.1 ± 5.9 µM.
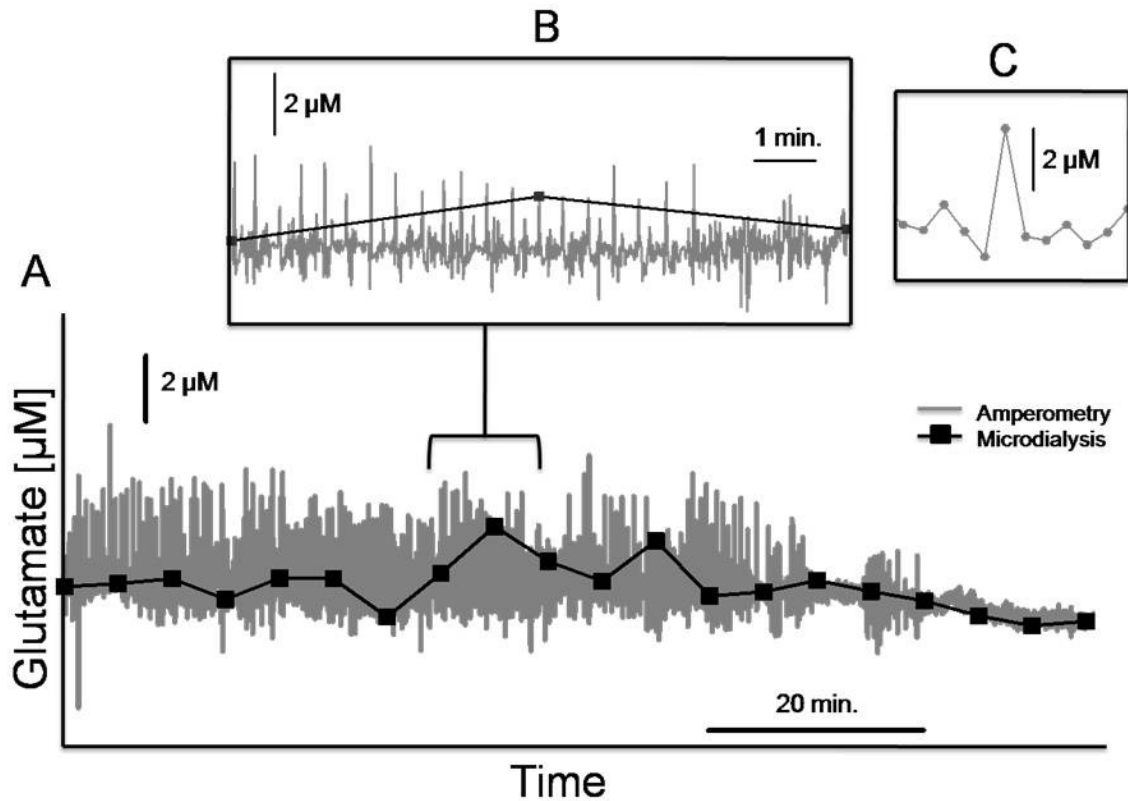
Figure 4.9 Real-time Amperometry vs. Microdialysis

A) Comparisons of a self-referenced amperometric glutamate recording during 4-AP-induced SE (grey trace) to a hypothetical microdialysis recording (black trace) with a dialysate fraction collection period of 5 minutes (squares). B) Due to the temporal resolution, traditional microdialysis is not capable of measuring spontaneous glutamate release events during SE. C) Second-by-second glutamate measurements (circles) capture fast dynamic glutamate release and clearance processes.

Table 4.1 Tonic Glutamate in the Hippocampus of Unanesthetized Rats
[μM]

	DG	CA3	CA1	Combined Hippocampal Glutamate
Young	7.9 ± 1.4	11.7 ± 2.9	6.2 ± 1.0	8.4 ± 1.1
Late-middle Aged	17.5 ± 5.6	23.6 ± 5.3	9.1 ± 3.1	16.3 ± 3.0
Aged	17.3 ± 12.6	22.2 ± 7.1	10.7 ± 4.3	16.2 ± 3.3
Comparison Across Age Groups (One-way ANOVA)	$p = 0.13$	$p = 0.14$	$p = 0.60$	$*p = 0.038$ Y < LMA, $p < 0.05$ Y < A, $p < 0.05$

All values represent the mean ± S.E.M.

Young (Y, 3-6 months), DG: n = 8; CA3: n = 5; CA1: n = 6

Late-middle Aged (LMA, 18 mo.), DG: n = 6; CA3: n = 5; CA1: n = 6

Aged (A, 24 mo.), DG: n = 7; CA3: n = 6; CA1: n = 6

Table 4.2 Behavioral Characteristics During 4-AP-Induced Status Epilepticus

	Young	Late-middle Aged	Aged	Dose-Effect	Age-Effect
SE Duration (minutes)				***p < 0.0001	*p < 0.05
low dose	8.3 ± 2.5	22.5 ± 5.1	30.0 ± 11.4		
high dose	55.0 ± 10.3	65.8 ± 8.6	105.0 ± 14.8		A > Y, **p < 0.01 A > LMA, *p < 0.05
Average WDS/ 5 min.				***p < 0.0001	p > 0.05
low dose	1.7 ± 0.4	2.7 ± 0.7	3.3 ± 1.4		
high dose	9.5 ± 2.6	14.0 ± 4.3	14.2 ± 2.0		
Max. WDS/ 5 min.				***p < 0.0001	p > 0.05
low dose	2.3 ± 0.7	7.5 ± 3.6	10.6 ± 6.3		
high dose	22.8 ± 6.8	30.5 ± 8.2	31.2 ± 2.9		

All values represent the mean ± S.E.M.

Young (3-6 months), n = 6

Late-middle Aged (18 mo.), n = 6

Aged (24 mo.), n = 5

Table 4.3 Glutamatergic Activity During 4-AP Induced Status Epilepticus

	Young	Late-middle Aged	Aged	Dose-Effect	Age-Effect
Latency to Spontaneous Release (minutes)				$p > 0.05$	$p > 0.05$
low dose	1.4 ± 0.8	2.3 ± 0.5	2.3 ± 0.6		
high dose	1.3 ± 0.4	2.0 ± 0.8	2.5 ± 0.7		
Glutamate Release Events				***$p < 0.0001$	*$p < 0.05$
low dose	7.8 ± 3.3	21.8 ± 7.2	35.6 ± 15.9		
high dose	66.2 ± 10.1	89.3 ± 10.3	111.1 ± 6.8		A > Y, **$p < 0.01$
Ave. Peak Size (µM)				$p > 0.05$	$p > 0.05$
low dose	1.3 ± 0.6	1.9 ± 0.4	2.1 ± 0.5		
high dose	1.9 ± 0.6	2.8 ± 0.4	1.5 ± 0.2		
Release Periodicity (seconds)				$p > 0.05$	$p > 0.05$
low dose	109 ± 49	87 ± 25	58 ± 7		
high dose	49 ± 2	44 ± 3	56 ± 6		

All values represent the mean ± S.E.M.

Young (3-6 months), n = 6

Late-middle Aged (18 mo.), n = 6

Aged (24 mo.), n = 5

Chapter Five: Real-time Glutamate Measurements in the Putamen of Awake Rhesus Monkeys Using an Enzyme-based Human Microelectrode Array Prototype

Introduction

Chapter Four highlighted the potential to use tonic hippocampal glutamate levels as a biomarker for the identification of populations at high-risk for status epilepticus-associated morbidity. Also, dysregulation of glutamatergic neurotransmission during seizure, manifesting as repeated spontaneous synchronized glutamate release events, was recognized as a novel marker of ongoing seizure. Based upon the potential to translate these findings into clinically-oriented initiatives, we began the development of microelectrode array (MEA) technology for use in humans.

The gold standard for *in vivo* monitoring of neurochemistry has been microdialysis. In addition to the complexity of the procedure, the most limiting characteristic of microdialysis has been the time resolution of the method, which acts like a low-pass filter when sampling chemicals in the extracellular space. This is especially important when measuring glutamate due to its rapid release and clearance processes. Researchers utilizing microdialysis techniques have explored many ways of improving the sampling of dialysates. One of the most popular techniques has been the coupling of microdialysis with on-line enzymatic conversion of analytes such as glutamate, followed by amperometric or fluorescence detection (Obrenovitch and Zilkha, 2001; Galvan et al., 2003; Zhang and Mao, 2005; Jin et al., 2008). Using similar principles, we have developed and extensively characterized a more streamlined approach using ceramic-based MEAs with glutamate oxidase (GluOX) cross-linked to the platinum recording surfaces coupled with real-time amperometric detection for

sub-second (2 Hz) *in vivo* measurements of glutamate (Burmeister et al., 2002; Pomerleau et al., 2003).

Monitoring of extracellular glutamate has been an area of interest for neurosurgeons and neuroscientists trying to understand a wide variety of pathological processes that are believed to have an excitotoxic component like epilepsy and traumatic brain injury (Hillered et al., 2005). The goal of intracerebral microdialysis monitoring is to detect impending pathological events before they are clinically evident; therefore, time resolution is absolutely critical. The commercially available ISCUS*flex* clinical microdialysis analyzer (CMA Microdialysis, Solna, Sweden) has a 60 second sampling rate, but again a faster time resolution is needed for certain applications (Valadka et al., 1998; Reinert et al., 2000; Schulz et al., 2000). Despite several studies over the last 15 years reporting on the use of microdialysis in patients, no examples of clinical management guidelines based upon glutamate exist (Hillered et al., 2005) which may be related to the limitations of the microdialysis method.

The size, structure and function of non-human primate (NHP) brains have made them very desirable for neuroscientists aiming to closely approximate the human CNS in their investigations (for review see Bradberry, 2000). Despite the advantages over rodents, few studies report on glutamate measurements in the NHP brain (Graham et al., 1989; Kling et al., 1993; Yin et al., 1997; Enblad et al., 2001; Kodama et al., 2002; Galvan et al., 2003; Quintero et al., 2007; Zhao et al., 2007). Using the enzyme-based MEA recording technology, we have extensively investigated glutamate regulation in the mammalian CNS (Pomerleau et al., 2003; Nickell et al., 2005; Day et al., 2006; Nickell et al., 2007; Quintero et al., 2007; Stephens et al., 2009). Recently, we have begun adapting the basic-science MEA for potential clinical use. Our recording technology has a distinct advantage in temporal resolution compared to currently utilized cerebral microdialysis techniques. Our ability to monitor glutamate in real-time may advance the development of clinical decision algorithms based upon glutamate fluctuations in the extracellular space to assist care-providers in determining the clinical course of patients with traumatic brain injury or sub-arachnoid

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hemorrhage, and may assist neurosurgeons during epilepsy surgery (see Stephens et al., 2008).

In these studies we used our human MEA prototype (Spencer-Gerhardt-2 (SG-2), ADTECH® Medical Instrument Corp., Racine, WI) in awake NHPs to determine glutamate levels in the putamen. These studies contribute information to the literature about the NHP basal ganglia, which is known to be involved in a variety of pathological processes including Parkinson's disease, Huntington's diseases and psychiatric disorders (Kreitzer and Malenka, 2008), and evaluate the abilities of our SG-2 MEAs to target sub-cortical structures and measure glutamate in a clinical setting. We also performed functional assessments of SG-2 MEAs post-sterilization, setting the stage for future studies in humans.

Methods

Please refer to Chapter Two for a detailed description of animal care, electrode preparation, sterilization procedure, electrode calibration, amperometric recording technique, and methodology for measuring and comparing tonic glutamate neurotransmission in the NHP putamen.

Results

In the batch of SG-2 MEAs that had been coated with the GluOX solution 11 months prior to sterilization, 10 out of 16 (63%) had at least one recording site that retained 1 pA/μM glutamate sensitivity or greater. Of these 10 functional SG-2 MEAs, 8 (80%) actually had 3 or 4 recordings sites that remained sensitive to glutamate post-sterilization. The average sensitivity of the functional recording sites was 5.6 ± 0.7 pA/μM (n = 29), well above the threshold for *in vivo* use. The average LOD was 0.37 ± 0.09 μM. In the batch of SG-2 MEAs that had been coated with the GluOX solution one month prior to sterilization, 14 out of 16

(88%) had at least one recording site with 1 pA/μM glutamate sensitivity or greater. Again, almost all 14 of the functional SG-2 MEAs actually had multiple sites that retained the ability to measure glutamate (12/14 (86%) had 3 or 4 functional sites). The average glutamate sensitivity of the functional recording sites was 7.5 ± 0.7 pA/μM (n = 47). The average LOD was 0.47 ± 0.14 μM. Though the mean sensitivity of the functional recording sites was greater in the batch prepared one month prior to sterilization, the difference was not significant (sensitivity $p = 0.06$, ($t = 1.9$, df = 74)). Likewise, there was no significant difference in the LOD of the two batches ($p = 0.58$, $t = 0.57$, df = 74), indicating that enzyme coated SG-2 MEAs have a good shelf life, and can withstand sterilization (Table 5.1).

The primary objective of these studies was to measure extracellular glutamate concentrations in the putamen of unanesthetized NHPs using the newly developed SG-2 MEA human prototype (Table 5.2). With self-referencing recording techniques (Day et al., 2006; Quintero et al., 2007) we were first able to determine tonic glutamate levels at a dorsal position in the putamen (NHP 1 = 25.4 μM; NHP 2 = 6.9 μM; NHP 3 = 9.8 μM) (Figure 5.1). As the SG-2 MEA was progressed 2 mm to a more ventral position in the putamen, we measured a robust increase in extracellular glutamate followed by clearance and stabilization of glutamate levels after approximately 15 min. This increase is probably due to ruptured glutamate terminals. Tonic glutamate levels at the ventral depth were: NHP 1 = 34.2 μM; NHP 2 = 10.6 μM; NHP 3 = 11.5 μM. In the follow-up experiments conducted four months later, glutamate levels were as follows: *dorsal*: NHP 1 = 15.1 μM; NHP 2 = 4.9 μM; NHP 3 = 7.7 μM; *ventral*: NHP 1 = 12.6 μM; NHP 2 = 5.3 μM; NHP 3 = 5.6 μM). There was not a significant difference between tonic glutamate measurements obtained in the dorsal recording position compared to the ventral position ($p = 0.38$ ($t = 0.97$, df = 5). The glutamate measurements recorded on the follow-up day of experimentation were significantly lower than the first measurements ($p = 0.047$ ($t = 2.6$, df = 5). No medical complications were reported throughout the duration of the study, which gave a preliminary indication of SG-2 MEA safety.

Perhaps the most interesting findings from these studies were the rapid fluctuations of glutamate in the extracellular space of the putamen. We were able to capture dynamic fluctuations because of the fast temporal resolution (2 Hz) of the glutamate measurements obtained with the FAST16 instrument. We arbitrarily chose a 60 second window 10 minutes after the lowering of the SG-2 MEA to the ventral depth in the putamen to compare recordings from each animal (Figure 5.2). A qualitative assessment showed the youngest NHP had the most spontaneous activity with some of the rapid release events resulting in changes in extracellular glutamate of 2 µM or greater. This is remarkable because the youngest animal also had the highest tonic glutamate levels. It is important to note that these events were not synchronized and showed no rhythmic periodicity. We can be sure these dynamic changes in extracellular glutamate are not artifacts because of our self-referenced recording technique. Self-referencing removes background noise and artifact whether electrical or due to movement of the animal. Though we do not have enough subjects in this study to make a statistical comparison across animals, we are very encouraged about the potential of this high-temporal resolution recording technique for use in monitoring glutamate in clinical applications where rapid and dynamic changes in extracellular glutamate may predict outcome or guide intervention, especially in light of the spontaneous release activity recorded during status epilepticus in rats (Chapter Four).

Discussion

These studies demonstrated that we can obtain real-time measurements of extracellular glutamate in awake non-human primates using enzyme-based MEA technology coupled to amperometric recordings with a design that has been adapted for humans (SG-2). Through a chronically implanted access cannula, we obtained tonic and phasic glutamate measurements at two different depths in the putamen and repeated the study four months later. The temporal resolution

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(2 Hz) of these measurements allowed us to record dynamic oscillations in extracellular glutamate, which to our knowledge has never been reported. We showed that sterilization did not significantly compromise the integrity and function of the enzyme/protein layers applied to SG-2 MEAs for the detection of glutamate. We also showed that the SG-2 MEAs have a shelf-life of at least 11 months.

SG-2 MEAs Can Reliably Measure Glutamate in a Clinical Setting

The tonic glutamate measurements in our youngest NHP animal agreed with a prior study from Galvan et al. (2003) that reported ~29 µM glutamate in the putamen of awake juvenile (3-4 kg) Rhesus monkeys. Glutamate levels appeared lower in the older animals. We have previously shown age-associated alterations in glutamate regulation in the striatum of rodents (Nickell et al., 2005; Nickell et al., 2007), but the current study was not designed to determine if a statistical difference in tonic glutamate concentrations existed across age groups. Based upon this study, future studies of glutamate regulation in the striatum should also closely examine dynamic transients in the glutamate measurements. The youngest NHP appeared to have larger magnitude peaks and dips in glutamate concentration compared to the older animals. This type of activity simply cannot be measured with microdialysis techniques, and potentially could provide a completely novel parameter to assess glutamatergic tone *in vivo*.

Due to the small size of the recording sites, the SG-2 MEAs can discriminate differences in extracellular glutamate in NHP brain areas separated by as little as 500 µm (Quintero et al., 2007). We measured tonic glutamate in the putamen at two depths separated by a 2 mm dorsal-ventral progression of the SG-2 MEA. The measurements obtained at the two depths were not statistically different, but did highlight the ability of the SG-2 MEAs to target different brain layers and obtain a stable basal glutamate measurement much faster than the most recent report in the literature using microdialysis to measure glutamate in the NHP striatum (SG-2 MEA = 20-30 min. baseline vs.

microdialysis = 60 min. baseline (Galvan et al., 2003)). Shortening the delay-to-stabilization of the glutamate measurements indicates that our technology could potentially sample from at least twice as many brain areas compared to microdialysis in a fixed amount of time.

The NHPs used in our studies were maintained in our animal care facility for four months after the first experiment day, and no adverse events were reported. This is a preliminary indication that the SG-2 MEAs can be safely used *in vivo*. Compared to the first day, tonic glutamate measurements in the putamen were significantly lower after the four month maintenance period. We believe this is most likely due to presence of the chronic access cannula, which may have caused the formation of scarring in the areas targeted by the SG-2 MEA. We have previously shown that our MEAs cause minimal damage and scarring in the surrounding tissue, even in chronic implants (Rutherford et al., 2007). In the future, we would like to utilize the methodology proposed by Kolachana et al. (1994) which via attachment of a 'guide holder' to the skull of rhesus monkeys, repeated studies can be performed in 'fresh' or 'experienced' brain parenchyma without the need of indwelling cannulae or multiple surgeries.

SG-2 MEAs Tolerate Sterilization

The commercially available ISCUS*flex* clinical microdialysis analyzer (CMA Microdialysis, Solna, Sweden) uses kinetic enzymatic conversion followed by colorimetry to measure glutamate in dialysate samples obtained once every 60 seconds. Our recording technology bypasses the collection of samples and detects glutamate via enzymatic conversion at GluOX-coated recording sites on an indwelling MEA. Coupled to high speed amperometry, our MEAs are able to measure glutamate *in vivo* on a second-by-second time scale. Adapting our basic science MEA technology for potential clinical use necessitated the development of a sterilization protocol. With these studies we have determined that the performance of the SG-2 MEA remains well above the functional threshold for detection of glutamate following electron beam sterilization.

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Additionally, the SG-2 MEAs have a shelf-life of at least the duration of the study, 11 months, which is very important to the logistics of distributing MEAs for clinical use. We have initiated development of a clinical calibration protocol using sterile reagents. Preliminary results indicate that there is no difference in the functional parameters (e.g. glutamate sensitivity) of MEAs when calibrated with sterile verses the non-sterile reagents (see Appendix). We are currently performing a functional characterization of enzyme/protein coated SG-2 MEAs that have also been coated with Nafion® to determine the effects of sterilization on the anion repelling properties of Nafion®.

Advantages of Amperometric Recordings Using Enzyme-based SG-2 MEAs

Using enzyme-based MEAs for *in vivo* amperometric detection of glutamate has several advantages over the current gold standard technique, microdialysis. To our knowledge, the fastest sampling rate for glutamate measurements obtained with microdialysis in a laboratory setting is 1 second, but the diffusional exchange of analytes across the microdialysis membrane results in a delay of several seconds (for review see Kennedy et al. 2002). Also, Rossell *et al.* (2003) reported that based on their data in awake-behaving rats, glutamate release and clearance events were not adequately measured with 1 Hz resolution because these events seemed to occur faster. We routinely measure glutamate at 2 Hz. As demonstrated by our measurements of dynamic fluctuations in extracellular glutamate in unanesthetized NHPs, our temporal resolution is superior for monitoring the rapid events associated with glutamatergic neurotransmission.

Microdialysis procedures can be laborious and time consuming, especially when incorporating adaptations to improve temporal resolution (Rossell et al., 2003). Major contributors to this are procedures for calibrating microdialysis probes and determining probe recovery rates (Hillered et al., 2005). Calibration of SG-2 MEAs is very straight-forward, and requires only 20 minutes to complete (Burmeister et al., 2002; Pomerleau et al., 2003). Microdialysis experimental

protocols require tubing and pumps to flow artificial cerebral spinal fluid (aCSF) through the microdialysis probe which can introduce mechanical interference to measurements made by on-line detection methods that improve the temporal resolution (e.g. fluorometry). Even though this may be believed to be negligible (Galvan et al., 2003), our self-referencing technique allows for the removal of background noise which in a clinical scenario could arise from a variety of sources including patient movement and the myriad electrical devices. Also the microdialysis procedure may have additional confounders like the use of room-temperature aCSF and air-saturated solutions (with markedly different O_2 and CO_2 saturations than the interstitial fluid) that can affect neurotransmission (Hillered et al., 2005). We avoid this by directly measuring glutamate in the extracellular space, without the exchange of fluid, which minimizes disruption of the extracellular space and leaves the microenvironment around the MEA intact.

Attached microelectrodes on microdialysis probes for dual electrophysiological and electrochemical recordings have been used to study a number of brain processes including epilepsy and TBI (Obrenovitch and Zilkha, 1995; During and Spencer, 1993; Fried et al., 1999; Alves et al., 2005). It has recently been reported that our enzyme-coated MEAs for electrochemical measurements can simultaneously measure electrophysiological currents *in vivo* (Zhang et al., 2009). Combining information about the electrical activity of neurons and the resulting chemical environment in the extracellular space can provide a more comprehensive understanding of pathological processes and response to injury.

The spatial resolution of our MEAs (microns) allows us to obtain information closer to the synapse than microdialysis probes which are millimeters in length. The size of microdialysis probes not only lowers the spatial resolution of the measurements, but is also a substrate for damage to the surrounding tissue. It has been reported that microdialysis probes extensively injure brain parenchyma with damage extending up to 1.4 mm from the implant site (Clapp-Lilly et al. 1999). In contrast, chronic studies with our MEAs showed minimal

damage around the implant site, ranging 50 to 100 microns after eight weeks *in vivo* (Rutherford et al., 2007).

Currently, a clear limitation of the SG-2 MEA is the ability to measure only one analyte at a time, whereas microdialysis allows for detection of several analytes. Though our standard MEAs have four platinum sites, arrays with up to 16 recording sites have been fabricated. To date, enzyme coatings have been developed to measure glutamate, choline, acetylcholine, lactate, and glucose (Burmeister et al., 2004; Burmeister et al., 2008). Electroactive compounds such as dopamine, norepinephrine, and O_2 can also be measured (Burmeister *et al.*, 2004). In the future we hope to be able to measure multiple analytes *in vivo* with a single MEA, and provide a real-time compositional analysis of the extracellular fluid.

Clinical Applications for Enzyme-based MEAs

Many studies using intracerebral microdialysis have shown that glutamate may be an important indicator of clinical course following brain injury. In acute focal ischemia imposed during the resection of brain tissue, patients have shown increased glutamate levels (Hillered et al., 1990; Kanthan et al., 1995) that are proportional to the extent of tissue resected, i.e. the duration of the ischemia (Kanthan et al., 1996). This has indicated a potential use for monitoring glutamate levels to predict intra-operative hypoxia (Mendelowitsch et al., 1998), but study outcomes have not been consistently positive (Hutchinson et al., 2000; Kett-White et al., 2002). The prospects for glutamate as a diagnostic tool have been better in subarachnoid hemorrhage (SAH). Increased glutamate levels have preceded and predicted the deterioration of SAH patients (Unterberg et al., 2001; Sarrafzadeh et al., 2002). Monitoring glutamate levels, known to be very important to secondary injury cascades, for management of traumatic brain injury patients is also promising (for review see Hillered et al., 2005). Though glutamate, and other analytes such as lactate, glucose and O_2, show potential as markers of injury and clinical course, no medical guidelines are based upon

cerebral monitoring with microdialysis (Hillered et al., 2005). For the reasons explained above, MEAs could monitor relevant compounds in the CNS faster than microdialysis while causing less damage than microdialysis probes to already injured tissue. We believe this may give care-providers a more accurate analysis of the brain's chemical profile, and facilitate the development of clinical algorithms based upon neurochemistry.

An especially novel application of MEAs may be in epilepsy surgery. Resection of diseased brain tissue has provided favorable outcomes for some patients with seizures refractory to medication; however, patients without a discrete focus of seizure activity or with foci in eloquent brain regions have remained difficult to treat.   Inadequate delineation of the normal brain-epileptogenic zone interface has been the major contributor to poor surgical results in these patients, despite advances in multimodality image-guided surgery and brain function testing (Murphy et al., 2004). We believe MEAs could be used as a supplemental diagnostic to provide neurosurgeons with a real-time measurement of extracellular glutamate, which is known to be elevated in seizure foci (reviewed by Eid et al., 2008). Also, the unique synchronized glutamate release events shown to occur during status epilepticus in rats could certainly identify seizing neurons from adjacent dormant brain tissue (Chapter Four). The spatial resolution of the MEA glutamate measurements could improve accuracy of the margin of excision for seizure foci from millimeters to micrometers, which is absolutely critical when performing excision surgery (Murphy et al., 2004). In addition, providing neurosurgeons and neuroscientists with a novel tool to investigate the role of glutamate neurotransmission in human seizures, SAH, ischemia and TBI could possibly lead to the development of new medications and interventions for difficult to treat neurologic patient populations.

Portions of this work have been previously published:

M.L. Stephens, F. Pomerleau, P. Huettl, G. A. Gerhardt and Z. Zhang. Real-time glutamate measurements in the putamen of awake rhesus monkeys using an enzyme-based human microelectrode array prototype. *Journal Neurosci. Methods* (submitted, 2009)
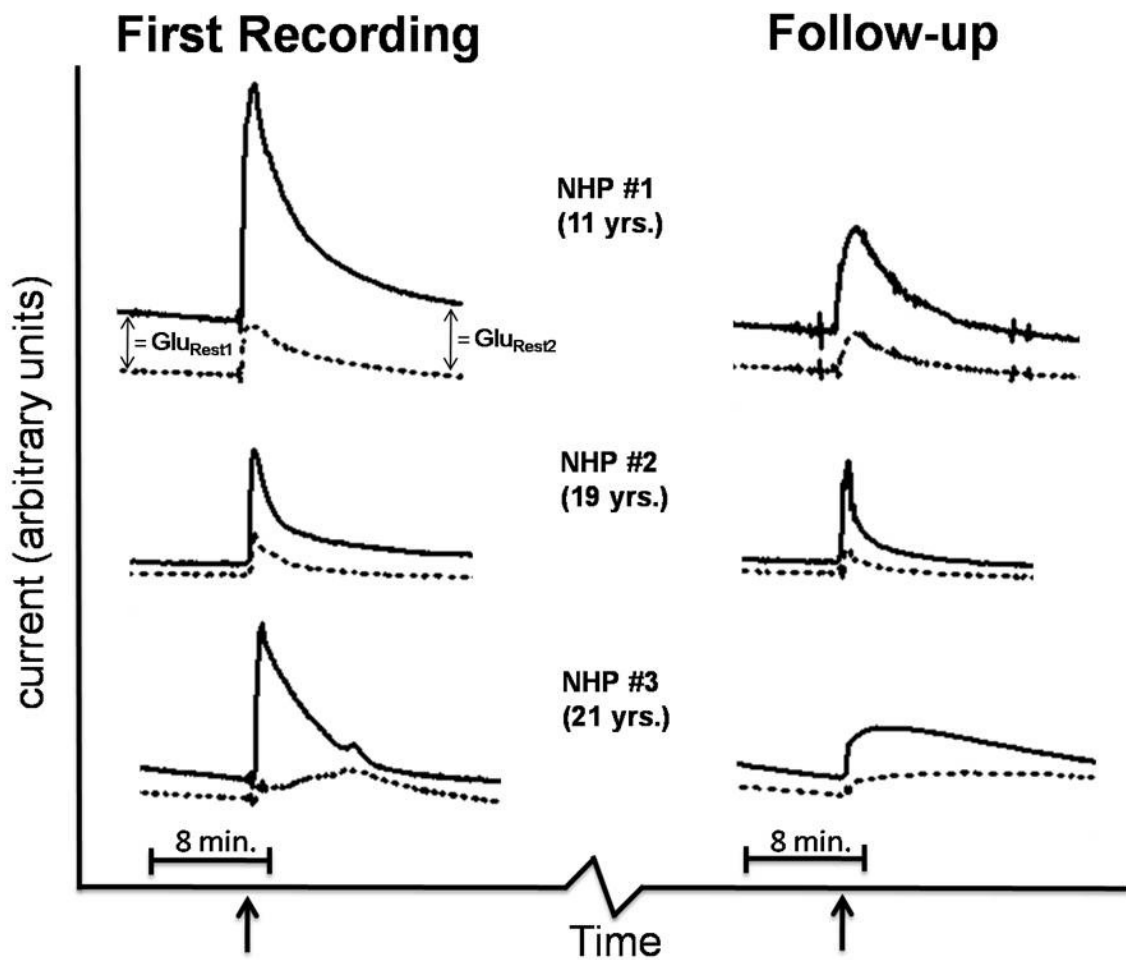
Figure 5.1 Basal Glutamate Measurements in the NHP Putamen

Traces show current measured on 'glutamate-sensitive' recording site (solid line) and sentinel site (dashed line). Arrow indicates 2 mm ventral progression of the SG-2 MEA in the putamen. Note the robust increase in glutamate, most likely due to disruption of terminals during MEA movement. Self-referencing yields basal glutamate measurements at depth 1 (Glu_{Rest1}) and depth 2 (Glu_{Rest2}). Recordings are four months apart. The youngest NHP appears to have the highest basal glutamate levels.
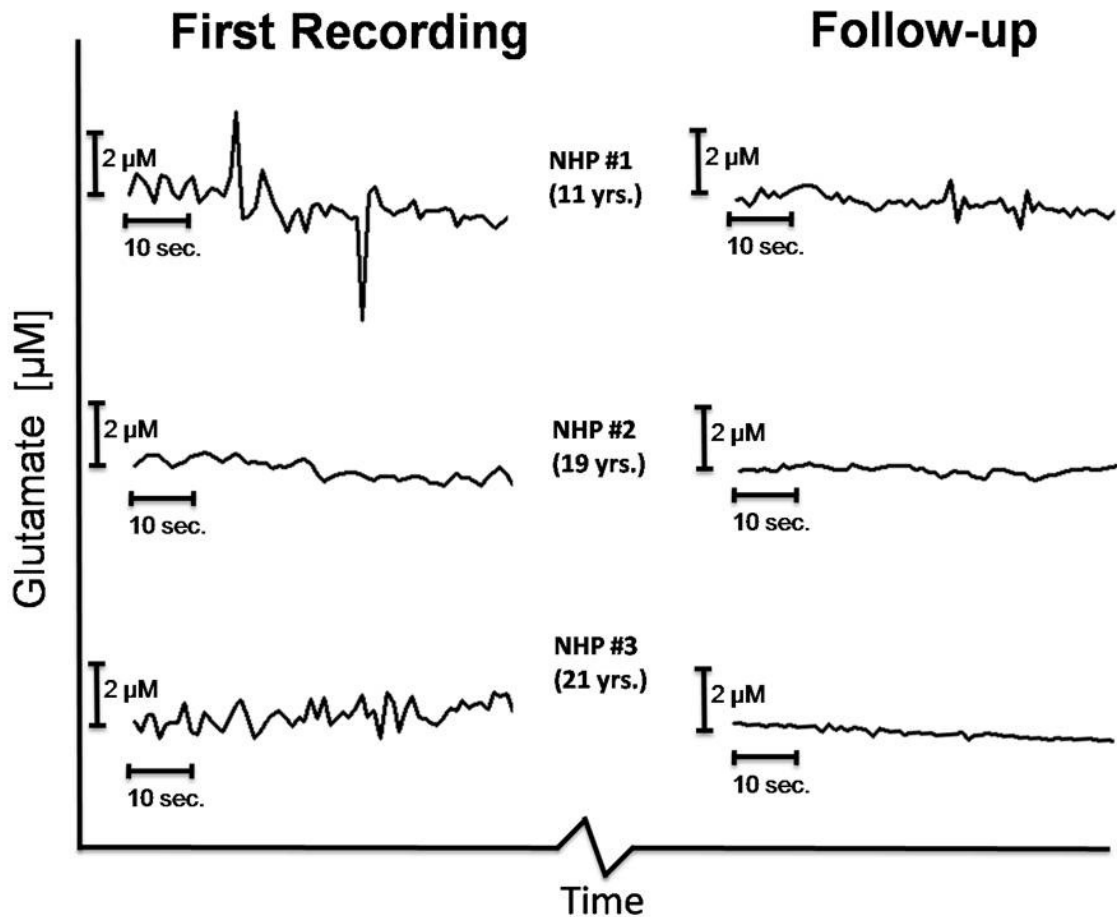
Figure 5.2 Dynamic Glutamate Oscillations in the NHP Putamen

Self-referenced recordings show dynamic glutamate oscillations at the second recording depth. Recordings are four months apart. The youngest NHP appears to have the most oscillatory behavior. The attenuation of oscillations in the follow-up recordings could be due to could be damage from the chronically implanted guide cannula, or might indicate positioning of the SG-2 MEA in a slightly different location than the first recordings.

Table 5.1 Functional Analysis of SG-2 MEAs Post-sterilization

Batch	Functional / Total	%	Average Sensitivity (mean ± SEM)	Average LOD (mean ± SEM)
Coated with GluOX 1 month prior to sterilization	10 / 16	63	5.6 ± 0.7 pA/μM	0.37 ± 0.09 μM
Coated with GluOX 11 months prior to sterilization	14 / 16	88	7.5 ± 0.7 pA/μM	0.37 ± 0.09 μM
t-test (1 mo. vs. 11 mo.)			**p = 0.06**	**p = 0.57**

Table 5.2 Tonic Glutamate Measurements in the NHP Putamen

Animal	Depth	First Recording (µM)	Follow – up Recording [†] (µM)
NHP 1 (11 y.o.)	1	25.4	15.1
	2	34.2	12.6
NHP 2 (19 y.o.)	1	6.9	4.9
	2	10.6	5.3
NHP 3 (21 y.o.)	1	9.8	7.7
	2	11.5	5.6

[†] Follow–up recordings were significantly lower compared to the first recordings
(Paired t-test for repeated measures, p = 0.047)

Conclusions

Several interesting things happened in the news media while I was finishing this dissertation. Just a few days before I distributed this document to my committee members for review, Newsweek's cover story was 'The Mystery of Epilepsy, Why We Must Find A Cure'. I also heard a report on National Public Radio about the rapid rate at which the population of the world is aging, and how this is going to affect every aspect of life, as we as a society collectively try to care and provide for elderly people. To me, this is additional validation that the questions addressed in this dissertation are extremely timely and relevant.

I believe the work I completed as a graduate student has really contributed something of worth to the aging and epilepsy research communities. I have potentially revealed a terrible irony during aging. My studies in the rodent hippocampus revealed that approximately one-third of late-middle aged and aged rats have elevations in their tonic extracellular glutamate levels. This is the same proportion of the population that has been shown to perform as well as younger rats in learning and memory tasks. Functional testing of the rodents used for glutamate regulation studies in the hippocampus is a critical piece of information I was not able to collect at the time of studies; however, I feel comfortable hypothesizing that animals with higher levels of extracellular glutamate have developed a mechanism to compensate for synaptic loss in the hippocampus during aging, rendering them functionally 'young'. The irony I mentioned is that these same animals are predisposed to have longer and more severe seizures. At this point, I cannot be sure at what age tonic hippocampal glutamate levels start to rise. Future studies should incorporate a middle-aged experimental group (12 months old) to help address this question.

My work focused on understanding glutamate dysregulation during prolonged seizures, termed status epilepticus, has the potential to really change the way researchers and clinicians have traditionally viewed glutamate's role in seizure. It is now clear that seizing hippocampal neurons display very rhythmic

glutamate neurotransmission, resulting in dynamic rapid glutamate release and synaptic spillover approximately once every thirty seconds. In my studies, the overall tonic glutamate levels did not change during status epilepticus, but rather were punctuated by spontaneous phasic glutamate release events. This is in stark contrast to previous beliefs that altered glutamate neurotransmission during seizure led to slow, steady and very large increases in extrasynaptic glutamate levels (several hundred times the tonic baseline). The spontaneous glutamate release events were extremely sensitive to the Na^+ channel blocker, TTX. This could explain why anti-epileptic drugs with mechanisms of action at Na^+ channels work. Based upon my studies, I believe development of novel therapeutics should continue to focus on ways to inhibit seizure-associated spontaneous glutamate release. Also, the magnitude of the release events significantly correlated with the severity of the seizure-associated convulsive behavior. Perhaps when larger boluses of glutamate are released during the spontaneous neurotransmission, inappropriate glutamate receptor activation potentiates the severity of behavior. Drug developers should also explore ways to minimize excessive receptor activation. Potentially the most important question remains 'how do we minimize risk for seizure-related morbidity (i.e. lower the tonic glutamate levels), while not compromising hippocampal function in elderly populations?'.

Though rodents are an excellent tool for studying the central nervous system, the fact remains that to really understand human biology, we must study glutamate in the human brain. Patients with refractory epilepsy that have been identified as candidates for surgical foci resection are the ideal patient population in which to pioneer human glutamate recordings with microelectrode arrays (MEA). A large part of my efforts as a graduate student were directed toward the development and testing human MEA technology. At the completion of this dissertation, the human MEA prototype (Spencer-Gerhardt-2 MEA (SG-2)) was in the 'final post-sterilization validation studies' phase of development, with hopeful imminent progression to obtainment of a FDA Investigational Device Exemption and IRB approval. As an MD/PhD student, I have two more years of clinical

training at the University of Kentucky. I hope the first human glutamate recordings occur before I graduate. If a unique glutamatergic neurotransmission profile exists in seizure foci of humans, like what I have shown in rodents, MEAs could provide neurosurgeons with a novel supplemental diagnostic for the identification of diseased neurons during surgery. Initially, I believe information gained from human glutamate recordings in seizure foci will be of most value for validating animal models of epilepsy. Eventually, MEAs could be expanded for second-by-second monitoring of extracellular glutamate in stroke, subarachnoid hemorrhage and traumatic brain injury patients which could push the integration of glutamate measurements into clinical decision-making algorithms.

Appendix: Additional Considerations for Clinical Glutamate Recordings

The tonic glutamate data presented in Chapters Three and Four emphasize the need to further characterize the affects of anesthesia on basal glutamate. This is especially important for future human studies. The first human recordings are currently being planned, and will proceed in patients referred for temporal lobe resection for the treatment of persistent epilepsy. Though patients are sometimes awake during certain portions of a resection surgery when brain areas need to be evaluated for essential-function (i.e. speech, motor movement) prior to removal, measurements with glutamate-sensitive MEAs will most likely occur while patients are under general anesthesia.

Isoflurane is a halogenated hydrocarbon inhalational anesthetic commonly used for neurosurgical procedures. A preliminary study evaluating the effects of isoflurane anesthesia on tonic glutamate levels in the rat hippocampus was conducted. The subjects were animals already used for the experiments in Chapter Four upon completion of their use in that study. Tonic glutamate levels were measured in each animal (unanesthetized), and then compared within animal to tonic glutamate levels obtained while under isoflurane anesthesia (2% in O_2). Animals represented all age groups and hippocampal subregions (n = 15). Isoflurane anesthesia did not significantly alter tonic glutamate levels (Paired t-test, p = 0.93, Figure A.1). To confirm these results, glutamate was continuously measured as an animal awoke from isoflurane anesthesia. No change in tonic glutamate was observed (Figure A.2).

An additional concern for future human glutamate recordings is MEA sterility. We know that SG-2 MEAs are functionally robust following electron beam sterilization, but a sterile calibration protocol is needed to maintain MEA sterility during evaluation prior to *in vivo* use. Pilot studies indicate that MEAs can be calibrated using sterile buffer and analyte (glutamate) (Table A.1), but additional studies are needed to determine if calibration parameters obtained with non-sterile and sterile buffer and analyte solutions are significantly different.

**Figure A.1 Comparison of Tonic Glutamate Before and During
Isoflurane Anesthesia**

Within animal comparison shows that measurements of tonic glutamate when animals were unanesthetized were not significantly different than when animals were anesthetized with isoflurane. Mean unanesthetized basal glutamate = 16.5 μM ± 4.0; Mean isoflurane basal glutamate = 16.3 μM ± 4.1. Paired t-test, $p > 0.05$.
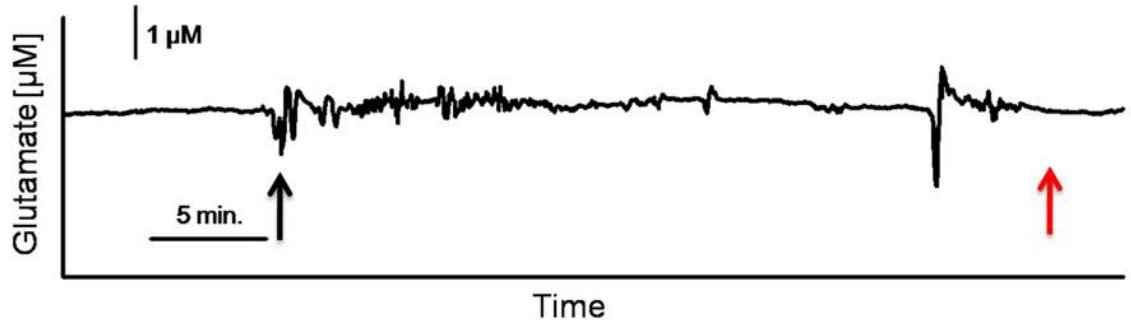
Figure A.2 Tonic Glutamate Unchanged as Animal Recovers From Isoflurane Anesthesia

Self-referenced recording of tonic glutamate showing no change after removal of isoflurane anesthesia (black arrow). Some transient glutamate activity is seen, but overall basal glutamate remains stable until the animals is fully recovered and grooming (red arrow).

Table A.1 Comparisons of Non-sterile and Sterile MEA Calibrations

Calibration Solution	Slope (nA/µM)	LOD (µM)
Non-sterile PBS / Glutamate	**2.1**	**0.3**
Non-sterile PBS/ Sterile Glutamate	**2.3**	**0.2**
Sterile PBS/ Sterile Glutamate	**1.6**	**0.2**

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08/08   Invited Speaker
Development of Intraoperative Diagnostics for
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Monitoring Molecules in Neuroscience
12th International Conference on *in vivo* Methods
Vancouver, British Columbia

05/07   Invited Speaker
Subregional Comparisons of Second-by-second
Glutamate Clearance in the Rat Hippocampus During
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Graduate Student Interdisciplinary Conference
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04/07   Invited Speaker
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Bluegrass Society for Neuroscience
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	Neural Therapy and Repair
	Clearwater Beach, Florida
04/06	Graduate Student Poster Presentation Award
	RNA Therapy for Neurodegenerative Disorders
	University of Kentucky
01/06-01/09	Department of Health and Human Services
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	Don Gash, Ph.D., Supervisor
05/04, 05/05	Promotion 'With Distinction'
	University of Kentucky College of Medicine
08/03-05/11	MD/PhD Scholarship
	University of Kentucky College of Medicine
05/01, 05/02	Chemistry Major of the Year (Capital University)
	American Chemical Society
03/01	Invited Speaker
	Use of Muscarinic Receptor Agonists in Medication
	Development for Cocaine Addiction
	National Conference on Undergraduate Research
	University of Kentucky
08/98-05/02	Battelle Industries Scholar
	Capital University

Publications

Manuscripts:

Jorge E. Quintero, B. K. Day, Z. Zhang, R. Grondin, M.L. Stephens, P. Huettl, F. Pomerleau, D. M. Gash, and G.A. Gerhardt. (2007). Amperometric measures of age-related changes in glutamate regulation in cortex of rhesus monkeys. *Experimental Neurology*; 208(2):238-46. (2007)

M.L. Stephens, J.E. Quintero, F. Pomerleau, P. Huettl, and G. A. Gerhardt. Age-related changes in glutamate release in the CA3 and dentate gyrus of the rat hippocampus. *Neurobiology of Aging;* June 15 (2009) EPub ahead of print

M.L. Stephens, F. Pomerleau, P. Huettl, G. A. Gerhardt and Z. Zhang. Real-time glutamate measurements in the putamen of awake rhesus monkeys using an enzyme-based human microelectrode array prototype. *Journal Neurosci. Methods* (submitted, 2009)

Book Chapters:

M.L. Stephens, D.D. Spencer, I. Cavus, M. Hsiao, D.Song, S.H. Courellis, S. A. Deadwyler, R.E. Hampson, D. Putz, J.E. Quintero, M.K. Bensalem-Owen, K.N. Hascup, E.C. Rutherford, B.K. Day, J. R. Nickell, F. Pomerleau, P. Huettl, J. J. Burmeister, P.M. Talauliker, V.Z. Marmarelis, J.J. Granacki, T. Berger, and G. A. Gerhardt. Microelectrode-based Epilepsy Therapy: A Hybrid Neural Prosthesis Incorporating Seizure Prediction and Intervention with Biomemetic Maintenance of Normal Hippocampal Function. In: Computational Neuroscience in Epilepsy, Editors: I. Soltesz and K. Staley, (p. 540-563). Academic Press (2008).

Abstracts:

Stephens M.L., Berger S.P. Use of Muscarinic Receptor Agonists In Medication Development For Cocaine Addiction. Honors Research Symposium, Capital Univ., Columbus, OH; Central Ohio Undergraduate Research Symposium, Denison Univ., Granville, OH; National Conference on Undergraduate Research (NCUR), University of Kentucky, Lexington, KY. (2000)

Stephens M.L., Spencer M., Bhangale H. Nanofabrication and Selective Filtration Properties of Collagen. Honors Research Symposium, Capital Univ. Columbus, OH; Central Ohio Undergraduate Research Symposium, Kenyon College, Gambier, OH. (2001)

Day B.K., Stephens M.L., Quintero J.E., Huettl P., Grondin R., Zhang Z., Luan L., Gash D.M., Gerhardt G.A. Second-By-Second L-Glutamate Measurements In Cortical Areas of Young and Aged Rhesus Monkeys. Society for Neuroscience Annual Conference, Washington D.C., USA (966.15) (2005)

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Day B.K., Stephens M.L., Quintero J.E., Huettl P., Grondin R., Zhang Z., Luan L., Gash D.M., Gerhardt G.A. Second-By-Second L-Glutamate Measurements In Cortical Areas of Young and Aged Rhesus Monkeys. RNA Therapy for Neurodegenerative Disorders Conference, University of Kentucky, Lexington, KY, USA (2006)

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Stephens M.L., Quintero J.E., Talauliker P.M., Huettl P., Pomerleau F., Gerhardt G.A. Second-by-second L-Glutamate Recordings in the CA1 and CA3 Regions in the Young Rat Hippocampus. *In Vivo* Methods, Monitoring Molecules in Neuroscience Sardegna, Italy (2006)

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