AGE-RELATED ALTERATIONS IN THE DYNAMICS OF L-GLUTAMATE REGULATION IN THE STRIATUM OF THE FISCHER 344 RAT

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ABSTRACT OF DISSERTATION

Justin Robert Nickell

The Graduate School
University of Kentucky
2006
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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
Justin Robert Nickell
Lexington, Kentucky

Director: Dr. Greg Gerhardt, Professor of Anatomy and Neurobiology
Lexington, Kentucky
2006

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L-glutamate is the predominant excitatory amino acid neurotransmitter in the mammalian central nervous system. Prior aging studies have focused primarily on dopaminergic circuitry of the striatum, and data obtained studying glutamate regulation in the striatum have been largely equivocal. These discrepancies are due in large part to the limitations of microdialysis; while it is extremely sensitive to minute concentrations of analyte, it is lacking in terms of the temporal resolution necessary to study a neurotransmitter with rapid release and clearance kinetics such as glutamate. In order to address this matter, our laboratory has designed a ceramic-based multisite microelectrode with the capability to detect and analyze fluctuations in extracellular glutamate concentrations on a sub-second basis. These microelectrodes were utilized to study the phasic release and uptake dynamics of potassium-evoked glutamate in the striatum of young (6 month), late-middle aged (18 month) and aged (24 month) Fischer 344 rats. Our results showed a reduced glutamate clearance rate and an attenuated response to potassium depolarization in the corticostriatal projections of aged animals in comparison to other age groups. In addition, average maximal glutamate release amplitudes were decreased in the striatum of aged animals. Pressure ejection of exogenous glutamate solution further confirmed the decreased glutamate clearance ability of the aged striatum. These potassium and exogenous glutamate data also highlighted a marked dorsoventral gradient in the striatum in terms of glutamate release and clearance ability. We further explored this phenomenon of age-related decreased glutamate uptake by coupling our in vivo technology with classical immunoblotting and biotinylation techniques in order to investigate glutamate transporter regulation. Decreased glutamate clearance in the aged rats cannot be attributed to a reduction in steady-state total transporter protein levels. Rather, our results indicate that reduced plasma membrane surface trafficking of GLAST in the aged striatum may be partially responsible for this effect. Finally, we modified our microelectrodes to study basal glutamate levels in the striatum of the aging, freely moving rat. This approach allowed us to study extracellular glutamate
regulation free from the potential confounding variable of anesthesia. Our results demonstrate that there is no significant alteration in basal glutamate levels in aging in the brain regions investigated. More importantly, this study validated the efficacy of the utilization of ceramic-based multisite microelectrodes for the study of alterations in glutamate neurotransmission in the aging, freely moving rat, and it lays the foundation for future work correlating such changes with age-associated impairments in motor function.

KEYWORDS: Glutamate, Voltammetry, Aging, GLAST, Freely moving

Justin Robert Nickell

06/26/06
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Dedicated to my wife Melissa, parents lynn and Robert, and precious daughter,
Rachel Morgan Nickell
ACKNOWLEDGMENTS

The pursuit of this degree has indeed led me down a long and arduous path, although I have not traveled in solitude. Many individuals have been instrumental in my personal and professional development. I would like to begin by expressing my gratitude for my mentor, Dr. Greg Gerhardt. It has been a privilege to receive my graduate training in a laboratory that encourages independence and strives to continually remain on the cutting edge of research. My writing, teaching and interpersonal skills have flourished in this environment, and I strongly believe that my growth in these areas will allow me to be very competitive in the ever challenging world of academic research. I would also like to thank my graduate committee, comprised of Drs. Joe Springer, Paul Glaser and Nada Porter, for their fostering of my ideas and intellectual contribution to the work contained in this dissertation, as well as Dr. Chris Norris for graciously agreeing to serve in the role of outside examiner on this committee. Dr. Michael Salvatore, Francois Pomerleau and Peter Huettl have also played a pivotal role in my professional development that is much appreciated.

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Chapter 1: Introduction

The term "aging" refers to a complex global series of events and processes that occur throughout the lifespan of an organism and ultimately result in a significant impairment in normal cellular functioning. The cardinal physical manifestations of aging related to locomotion have been well established, those being, but not limited to, reduced motor movement velocity (bradykinesia), stooped posture and shuffling gait (Mortimer and Webster, 1982; Teravainen and Calne, 1983; Bennett et al., 1996). Cognitive deficits have been observed as well, although the only consistent finding has been a marked decrease in the short-term memory of aged animals (Carey, 2002). While these features of aging are of a very concrete nature, the molecular and cellular changes underlying these deteriorations are not quite as evident. For instance, while the postural and motor impairments often observed with aging closely resemble those seen in individuals with Parkinson's disease, researchers have failed to implicate a substantial loss of dopaminergic neuron volume and transmitter content as being solely responsible for these deficits (for review, see Stanford et al., 2001b). Nonetheless, extensive aging research has been conducted in the basal ganglia, a neural network that operates in concert with the cerebellum and motor cortex for the initiation and progression of smooth, controlled locomotion.

Anatomy and Physiology of the Basal Ganglia

The basal ganglia are an interconnected circuit of subcortical nuclei that collectively act to inhibit the initiation of undesired motor movements and maintain proper posture (Fig. 1.1). An extensive review of the structures that comprise the basal ganglia is beyond the scope of this thesis, yet a fundamental understanding of the circuitry and its neurotransmitter components is crucial in the analysis and discussion of the alterations of glutamate neurotransmission in the aged striatum.
The human striatum is located in the forebrain and is composed of two functionally distinct regions: the caudate and putamen (dorsal striatum), which are delineated by the fibers of the internal capsule in primates and higher mammals, and the nucleus accumbens (ventral striatum), a pivotal structure in the reward/motivation pathway. Being a rich, heterogeneous environment composed of cholinergic, dopaminergic, GABAergic, and glutamatergic axons and cell bodies, the dorsal striatum serves as the primary input to the basal ganglia for a wide variety of glutamatergic signals generated throughout the cerebral cortex. These corticostriatal projections synapse on GABAergic medium spiny neurons in close proximity to cholinergic projections from large aspiny interneurons found in the area.

While the principle input to the striatum is glutamatergic in nature, another key component of the basal ganglia projects dopaminergic fibers to this region. The substantia nigra (SN) is located in the midbrain, and is also differentiated into two segments. The substantia nigra pars reticulata (SNpr) receives inhibitory input from the striatum, as well as excitatory information from the subthalamic nucleus (STN), and plays a prominent role in eye movements. The other subdivision of this region, the substantia nigra pars compacta (SNpc), is perhaps the most intensely researched component of the basal ganglia due to the finding that degeneration of this region is the prominent pathology in the brains of those afflicted with Parkinson's disease. This densely cellular region provides the primary dopaminergic input to the striatum and forms synapses in close proximity to corticostriatal projections on the medium spiny neuron. This juxtaposition of dopamine and glutamate allows for extensive interaction of these neurotransmitters and thus synaptic modulation at the level of the dendritic spines.

Signals processed in the striatum progress through the basal ganglia in one of two pathways depending on which variety of medium spiny neuron is stimulated. These neurons, while morphologically homogenous, are heterogeneous in terms of peptide neurotransmitters and the other molecules they contain, and therefore may be characterized into two distinct populations.
Medium spiny neurons with dendritic spines that house D1 class receptors contain dynorphin and substance P, and stimulate adenylate cyclase upon binding of dopamine. The peptide neurotransmitters found in the remaining neurons are enkephalin, and their D2 receptors serve an inhibitory role upon activation by dopamine.

Following stimulation of D1-containing neurons, GABAergic tone to the internal segment of the globus pallidus (GPi) and SNpr is increased, which leads to decreased GABAergic tone in the ventral lateral nucleus of the thalamus. The thalamus then increases glutamatergic tone to the motor cortex, thus influencing motor movement. Because of the non-circuitous nature of this route, it is termed the "direct", or striatonigral, pathway. Signals processed via the striatopallidal, or "indirect", pathway follow a much more complex route. Binding at D2 receptors inhibits GABA release in the external segment of the globus pallidus (GPe), which increases GABAergic tone at the subthalamic nucleus (STN). This inhibitory action on the STN reduces glutamate neurotransmission at the GPi/SNpr, reducing GABAergic tone in the ventral lateral thalamus leading to an overall increased glutamatergic tone in the motor cortex.

The Interaction of Glutamate and Dopamine in the Striatum

As previously mentioned, the dendrites of the medium spiny neuron are a juncture for the variety of neurotransmitters present in the striatum (Fig. 1.2). While the projections containing these transmitters may all synapse on a common dendrite, glutamate and dopamine projections often synapse in much closer proximity to one another; a large percentage of these projections have been discovered to share dendritic spines (Bouyer et al., 1984; Freund et al., 1984). As a result, glutamate/dopamine interplay is crucial in modulating information relayed from the cerebral cortex. Researchers have postulated for well over a decade that pharmacological intervention at this juncture could act as a feasible means of treatment of the motor abnormalities and deficiencies observed in patients with motor-related disorders such as Parkinson's disease.
Therefore, it should come as no surprise that many groups have extensively investigated corticostriatal glutamatergic control over dopamine release.

*In vitro* experiments conducted in rat striatal brain slices have demonstrated a concentration-dependent increase in extracellular dopamine levels following the administration of glutamate (Giorguieff *et al.*, 1977; Roberts and Sharif, 1978), a phenomenon that is blocked upon the application of the glutamate receptor antagonist glutamic acid diethylster (Roberts and Anderson, 1979). *In vivo* push-pull methodology has been used as well to study this phenomenon. Nieoullon *et al.* (1978) showed an increased concentration of dopamine in the striatum following electrical stimulation of the motor and visual areas of the feline cerebral cortex.

Following these discoveries, scientists strove to uncover the cellular mechanisms responsible for glutamate's facilitation of dopamine release in the striatum. The actions of synaptic glutamate are mediated by a number of receptors located both pre- and post-synaptically. Three classes of ionotropic glutamate receptors have been characterized based on their affinities for specific agonists: N-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), and kainic acid. Metabotropic glutamate receptors (mGluRs) are another family of receptors coupled to intracellular second messenger systems via G proteins, and consist of two classes. mGluR1 receptors (mGluR1, mGluR5) activate phospholipase C and adenylate cyclase, ultimately facilitating neurotransmitter release. The remaining mGluRs inhibit neurotransmitter release by inhibiting cAMP production, thus making this subclass of receptor a key member of the presynaptic mechanism responsible for maintaining low levels of extracellular transmitter in instances of excessive glutamate overflow.

An overwhelming majority of prior studies have focused on the NMDA receptor's role in stimulating dopamine release. Low concentration infusions of NMDA into the rat striatum strongly stimulate dopamine release, an effect that is tetrodotoxin (TTX)-dependent (Morari *et al.*, 1993). Likewise, this effect was also observed in the striatum of freely moving rats (Westerink *et al.*, 1992), and
electrophysiological studies also support the notion that NMDA receptors play a key role in facilitating dopamine release (Overton and Clark, 1991). Collectively, these data have led researchers to postulate that glutamate exerts its influence on dopamine release by binding to NMDA receptors present on the terminals of nigrostriatal afferents. Similar results have been obtained following applications of AMPA (Desce et al., 1992; Wang, 1991) and kainate (Jin and Fredholm, 1994) receptor agonists and antagonists, and the behavioral effects of mGluR manipulation suggest glutamate receptor-mediated dopamine release (Sacaan et al., 1992), but the data available for these receptor classes is relatively scarce in comparison to those collected in studies of the NMDA receptor.

The effects of dopamine on glutamate release have been intensively researched as well. While glutamate acts to stimulate the release of dopamine at the level of the medium spiny neuron dendrite, an elevation of extracellular dopamine corresponds with an inhibition of glutamate release. This has been demonstrated in many in vivo studies, as stimulation of the ventral tegmental area of the midbrain, dopamine iontophoresis, and the administration of dopaminergic drugs all resulted in a reduction of stimulus-evoked excitatory responses from corticostratial afferents (Pennartz et al., 1994; Floresco et al., 2001; Yang and Mogenson, 1984). In addition, the application of dopamine or D2 receptor agonists to the bath medium of striatal brain slices resulted in a reduction of the amplitude of excitatory postsynaptic potentials (EPSPs) evoked by the electrical stimulation of corticostratial pathways (O'Donnell and Grace, 1994; Hsu et al., 1995; Levine et al., 1996).

Substantial evidence has accumulated to identify the D2 receptor as being primarily responsible for mediating these effects. A neuroanatomical study localized these receptors to corticostratial afferents originating in the prefrontal cortex (Wang and Pickel, 2002), and the medium spiny neurons of mice with a deficiency of these receptors exhibit larger amplitude depolarizations and an increased frequency of spontaneous activity in comparison to wild type mice (Cepada et al., 2001). Collectively, these findings indicate that release of
dopamine from nigrostriatal afferents inhibits glutamate release in the striatum via a presynaptic D_{2}-receptor mediated mechanism on corticostriatal projections.

**Previous Aging Research in the Striatum**

A thorough understanding of the interaction between these neurotransmitters in the striatum is critical in researching alterations in glutamate neurotransmission for several reasons, the foremost being that a preponderance of prior aging studies in the basal ganglia have been conducted in dopamine circuitry, in particular the nigrostriatal pathway. It has been widely known for nearly half of a century that the SNpc of those individuals afflicted with Parkinson's disease lacked dopamine (Ehringer and Hornykiewicz, 1960), and that the administration of the dopamine precursor L-dopa was effective at alleviating the motor abnormalities associated with this disease (Cotzias et al., 1967, 1969). Because many of the symptoms of Parkinson's disease (bradykinesia, shuffling gait) closely resemble the declines in motor function observed in aged individuals, the nigrostriatal circuitry became a popular target of research for those studying both Parkinson's disease and aging.

As a result, a literature search for alterations in dopamine transmission in aging yields a plethora of research relating to many aspects of cellular and molecular dopamine neuron functioning. A previous widespread tenet of aging has been that age-related motoric abnormalities are due in large part to a reduction in the number of dopamine neurons. While normal, non-pathological aging is accompanied with an approximate 50% reduction in tyrosine hydroxylase-immunoreactive neuron number, it has become apparent that cell loss alone cannot exclusively account for these deficiencies (West and Gundersen, 1990; West, 1993; West et al., 1994; Emborg et al., 1998). Rather, these motoric declines are most likely due to a combination of a number of age-related factors, including, but not limited to, reduced dopamine release and clearance, increased threshold for elicited electrophysiological responses, and
decreased dendritic branch volumes of medium spiny neurons (for review, see Stanford et al., 2001b).

**Glutamatergic Neurotransmission in the Young and Aged Striatum**

Due to the extensive interaction between dopamine and glutamate and the well-documented alterations that occur in the dopamine circuitry in aging, it is only natural to assume that similar changes occur in the glutamatergic corticostratal projections. Unfortunately, minimal data are available pertaining to this subject. The pathway of glutamate release, clearance, and metabolism in the young, healthy striatum, however, has been extensively studied (Fig. 1.3). Depolarization of corticostratal projections results in the influx of calcium ions in the axon terminals, which in turn stimulate the binding of vesicular glutamate transporters (VGLUTs) to the presynaptic terminal membrane and the subsequent release of glutamate into the extracellular space. Glutamate freely diffuses to the postsynaptic medium spiny neuron dendrite, at which point it binds to AMPA and NMDA receptors and induces the influx of sodium and calcium ions into the neuron.

The clearance of glutamate from the synapse is performed exclusively by a series of high-affinity, sodium-dependent excitatory amino acid transporters (EAATs). This process is robust and rapid, as glutamate poses a severe excitotoxic threat to surrounding neurons if conditions arise that facilitate accumulation of the neurotransmitter in the extracellular space (Olney, 1969). The two transporters responsible for >90% of glutamate clearance, glutamate transporter (GLT-1) and glutamate-aspartate transporter (GLAST), are localized to neuroglia, while the remaining ~10% of glutamate is removed by the excitatory amino acid transporter (EAAC) located on the postsynaptic membrane of the medium spiny neuron. The process of glutamate clearance takes advantage of the ion gradient established to drive neuron depolarization and so is extremely energetically favorable; co-transported with glutamate are three sodium ions and a proton, while a potassium ion is transported extracellularly (for review, see
For a more thorough discussion of these transporters and their regulation, please see below and Chapter Four. Once segregated in the glia, glutamate is metabolized to glutamine by the glial enzyme glutamine synthetase and returned to the corticostriatal neuron. The mitochondrial enzyme glutaminase then converts glutamine to glutamate which is to be packaged inside VGLUTs.

The aspects of this pathway have been studied in the context of aging, yet, as already mentioned, the data are scarce. Further complicating matters, the data that have been collected are equivocal to a large extent. Total glutamate content is a parameter that has been studied in a number of brain regions. Some researchers discovered a modest (10-20%) decrease in total glutamate levels (Price et al., 1981; Strolin-Benedetti et al., 1990, 1991), while others showed an apparent consistency of glutamate concentration in the striatum throughout aging (Saransaari and Oja, 1995; Wallace and Dawson, 1990). It must be noted that the striatum is a very heterogeneous structure (see Chapters Three and Four), suggesting that the obvious inconsistencies of these results may be due in large part to varying densities of corticostriatal projections in the different regions. In order to address this issue, Donzanti and Ung (1990) conducted a series of experiments in which the goal was to differentiate eight regions of the striatum based upon their total glutamate content. The results ranged from a significant increase in glutamate content of 40% in the aged anterior dorsolateral striatum to no alterations in regions such as the posterior dorsomedial striatum, yet in no regions was there a decrease in glutamate content as was indicated in studies by other groups.

The sensitivity of glutamate fibers is another area of interest for those studying aging in the striatum. Glutamate release can be investigated both in vivo as well as in vitro through the usage of two different models; push-pull microdialysis allows for sampling of extracellular neurotransmitters following chemical stimulation, while brain slice and synaptosome preparations afford the investigator ease of use to analyze the content of glutamate in the superfusion medium. Once again, the results are equivocal. Two groups found a large
decrease in the concentration of potassium-evoked glutamate in the aged striatum (Freeman and Gibson, 1987; Saransaari and Oja, 1995), while another found no change in the ability of the aged striatum to release glutamate despite the injection of a higher concentration of potassium (Corsi et al., 1997, 1999). In terms of basal glutamate release in aging, some researchers showed an astounding 77-190% increase in non-stimulated extracellular glutamate (Freeman and Gibson, 1987; Massieu and Tapia, 1997), others indicated a decrease in glutamate levels (Corsi et al., 1997), and yet others found no change in basal levels (Saransaari and Oja, 1995; Porras and Mora, 1995; Corsi et al., 1999).

The collective results that pertain to glutamate clearance in the aged striatum are inconclusive as well. Price et al. (1981) and Najerahim et al. (1990) both discovered a curious decrease in uptake rate in middle-aged rats that was restored in the striatum of the aged animals. A single investigator showed a significant increase in uptake rate in the aged rats, while others found no difference in glutamate clearance rates in aging (Palmer et al., 1994; Strong et al., 1984). Taken together, these data make it difficult to definitively determine the nature of glutamate regulation in the aged rat striatum (refer to Chapter Three for discussion).

One finding that is without such contention concerns alterations in glutamate receptor density in aged animals. NMDA receptor density in the striatum has been consistently reported to be moderately reduced (20-50%) in aged rats, regardless of the method used to assay for such a change (Cimino et al., 1993; Castorina et al., 1994; Wenk et al. 1991). In addition, a concurrent marked decrease in NMDA receptor mediated response has been observed (Cepeda et al., 1996). A single study found that no change in AMPA receptor density occurs in the aged rat striatum (Cimino et al., 1993), and no aging research has been conducted to determine if there are alterations of mGluR density in this region.
Glutamate Transporters: A Brief Overview

The process of termination of glutamate neurotransmission is quite different from the clearance and metabolic mechanisms observed in other neurotransmitter systems. There have been no synaptic enzymes identified to date that degrade extracellular glutamate to a significant degree, and while the presence of a presynaptic glutamate transporter remains plausible, all available evidence for such a transporter appear to have been collected using questionable experimental design (for review, see Danbolt, 2001). This has led researchers to formulate and accept the general conclusion that glutamate is cleared from the synapse almost exclusively via an efficient system of transporters localized to the glia and postsynaptic neuron membrane surfaces.

The common molecular structure of these transporters has historically been a topic of debate. The initial publications (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992) describing the discovery of these transporters in the last decade postulated that their structures consist of either six, eight or ten putative transmembrane spanning domains (TM). Hydropathy plots have confirmed that each transporter indeed possesses at least six TMs (Kanai et al., 1993) with an extended extracellular sequence of amino acids between TM3 and TM4 (Slotboom et al., 1996; Grunewald et al., 1998; Seal and Amara, 1998) that is a site for transporter glycosylation (for review see Seal and Amara, 1999). Also without contention is the finding that both the carboxy and amino termini are intracellular (Lehre et al., 1995; Danbolt et al., 1998; Dehnes et al., 1998). Further work in this area has since shifted the consensus opinion to these transporters having eight TMs (for reviews see Danbolt, 2001; Slotboom et al., 1999). This understanding of the conserved nature of these transporters is necessary when interpreting data pertaining to the regulation of glutamate clearance in the aged striatum (Chapter Four).
As evidenced by this discussion of alterations of glutamatergic neurotransmission in the aged rat striatum, there is much remaining to be done. The work presented herein serves to expand upon the foundation of the regulation of glutamate release and clearance in aging. In Chapter 3, we used a multisite ceramic-based microelectrode for rapid and low level measures of glutamate \textit{in vivo}. We measured the amplitude and clearance rate of phasic changes in glutamate release produced by local application of potassium by a micropipette placed adjacent to the recording sites in the striatum of anesthetized young (6 month), late-middle aged (18 month) and aged (24 month) Fischer 344 (F344) rats. Our results show that the amplitudes and clearance rates of potassium-evoked release of glutamate in the striatum are significantly decreased in aged rats as compared to the other age groups. In addition, the sensitivity of glutamate fibers to depolarization with potassium was significantly decreased in the aged rats. Taken together, these data are consistent with age-related alterations in glutamate release dynamics, which may involve a compensatory mechanism for maintaining static glutamate concentrations within the striatum.

Chapter 4 further explores the potential molecular underpinnings of this reduced glutamate clearance ability. Extracellular L-glutamate poses a severe excitotoxic threat to neurons and glia when unregulated, therefore low synaptic levels of this neurotransmitter must be maintained via a rapid and robust transport system. The included study utilized \textit{in vivo} electrochemical recordings, immunoblotting and biotinylation in young, late-middle aged and aged F344 rats to elucidate the potential role that glutamate transporters (GLT-1, GLAST, and EAAC1) may play in this mechanism. We found that the decreased glutamate clearance efficiency is not due to a decrease in the production of total transporter protein among these three transporters. Rather, a significant reduction in the amount of GLAST trafficked to the plasma membrane surface in the aged animals (~55% when compared to young rats) may be partially responsible for
this phenomenon. These age-related alterations in extracellular L-glutamate regulation may be key contributors to the increased susceptibility of the aged brain to excitotoxic insults such as stroke and hypoxia, and may also play a role in age-associated motoric decline.

Chapter 5 chronicles the development of in vivo, freely moving voltammetric recordings as a tool in studying the aging of glutamatergic neurotransmission in the rat striatum. This technology is a significant improvement over the anesthetized electrochemical recording preparations, as it affords us the opportunity to study glutamate release and uptake dynamics free from the influence of anesthetic, and also allows us to study the temporal dynamics of neurotransmitter regulation while an animal performs a behavioral task. Data obtained from these experiments indicate moderate but insignificant changes in basal glutamate levels throughout aging. This preliminary research will be pivotal in the design of future aging studies attempting to correlate age-related motor dysfunction with altered glutamate neurotransmission in the striatum.

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Figure 1.1 Circuitry of the Basal Ganglia
Schematic illustrating the principle projections to the striatum and their various neurotransmitter components. Checkerboard pattern represents dopamine projections, black arrows denote glutamate projections, and grey represents GABA projections; squares denote inhibition, while arrows represent neuron stimulation. Please see text for an extensive discussion of these pathways.

Adapted from Bar-Gad and Bergman (2001)
Figure 1.2 Diversity of Projections on the Medium Spiny Neuron

Cholinergic projections from interneurons synapse on dendritic shafts, while GABAergic projections from neighboring medium spiny neurons synapse directly on the neuron cell body. Corticostriatal glutamatergic and nigrostriatal dopaminergic afferents synapse on common dendritic spines. The close proximity of these terminals allows for direct modulation of presynaptic neurotransmitter release, a phenomenon which may play a prominent role in age-related motor dysfunction.
Following its release, glutamate binds to AMPA, NMDA and mGluR receptors located postsynaptically to influence ion influx and subsequent EPSP generation. Glutamate neurotransmission is terminated exclusively via the high-affinity sodium dependent transporters (GLAST, GLT-1 and EAAC1) present primarily on the glial cell membrane and to a lesser extent on the postsynaptic membrane. Presynaptic mGluR's act as autoreceptors to diminish glutamate release in the event of excessive spillover. At right is a magnified view of a standard transporter and the specific stoichiometry of the glutamate clearance process.

Figure 1.3 Typical Striatal Glutamatergic Synapse
Chapter Two: Materials and Methods

The F344 Rat as a Powerful Model of Aging

Aging is a multifaceted process that affects all aspects of the mammalian central nervous system at differing rates and to varying degrees. Therefore, an appropriate animal model must be selected that demonstrates many of the physical and cellular manifestations observed in aged humans. The rodent, specifically C57BL mice, Sprague-Dawley, Wistar, and the F344 rat, has traditionally been the model of choice for studying the aging of the nigrostriatal circuitry (Austad, 1997), as they exhibit marked age-related motor deficits. The F344 strain of rat has an inherent advantage over other rodent strains in that it is inbred. This high degree of genetic constraint limits the possibility that any of the alterations we observe in the aging of these animals are due to substantial genetic variation. In other words, any increases in life longevity and quality amongst animals will be limited, as genetic "superiority" will not be a confounding factor in these experiments. In addition, the body weight of these animals remains relatively consistent throughout their lifespan, limiting any maturation-dependent alterations in brain volume (Masoro, 1990).

Researchers agree that there is slight variability in lifespan amongst the various rat strains utilized for studying aging (Masoro, 1990; Sprott and Austad, 1996), yet a consensus has been reached as to when to classify a rat as "aged". All rat strains, including the F344 rat, are deemed aged upon reaching 22 months of age. With this in mind, three age groups were selected for the study of age-related alterations in glutamatergic transmission in the striatum: young (6 month), late-middle aged (18 months), and aged (24 months). These age groups approximately simulate humans of the ages of 18, 54, and 72 years, respectively. The addition of the late-middle aged F344 rats circumvents the confounding variable of differential maturation processes, as studies concerning aging should always be conducted in more than two age groups (Coleman and Flood, 1987).
**Animals**

Young adult (6 months, n = 31), late-middle aged (18 months, n = 28), and aged (24 months, n = 30) male F344 rats were obtained from the National Institute on Aging (Harlan Sprague Dawley, Inc., Indianapolis, IN) and used for all experiments. Please refer to Table 2.1 for distribution of animals across experiments. Protocols for animal use were approved by the Institutional Animal Care and Use Committee. Animals were housed according to approved guidelines, with food and water available *ad libitum*.

**Anesthetized, *In vivo* Amperometric Studies**

**Microelectrode Fabrication**

Ceramic-based multisite microelectrodes are mass produced in conjunction with Thin Films Technology, Inc. (Buellton, CA). The fabrication process consists of a series of photolithographic techniques that have several distinct advantages over the various other methods used to fabricate microelectrodes. Photolithography allows routine production of reproducible recording surfaces as small as 5-10 µm. In addition, multiple microelectrodes are patterned onto a single fabrication substrate (2.5 cm x 2.5 cm), allowing for increased fabrication number at a decreased cost. Finally, photolithographic methods are used to manufacture numerous microelectrode designs with multiple recording sites in well-defined, highly reproducible geometrical configurations (for review, see Hascup *et al.*, 2006).

The particular recording site geometrical configuration selected for the anesthetized electrochemical recordings was the R1 configuration (Fig. 2.2). This configuration consists of an in-line alignment of four recording sites with 50 µm spacing between sites. Individual recording sites possess the dimensions of 50 x 150 µm. This configuration provides a larger recording distance that is useful in the study of larger brain regions or layered structures, such as the striatum.
**Microelectrode Preparation**

The ceramic-based microelectrode arrays with platinum (Pt) recording surfaces were fabricated, assembled and selected for *in vivo* recordings using our previously published methods (Burmeister *et al.*, 2000, 2002) (Fig. 2.5). Microarrays were first coated with Nafion (Sigma-Aldrich, St. Louis, MI) to enhance their selectivity *in vivo* for glutamate versus ascorbic acid or other anionic interferents (Gerhardt *et al.*, 1984). Fresh stock Nafion solution was placed in a 1.5 ml Eppendorf tube and allowed to thicken to room temperature for 1 hour in a humidity-controlled dessicator. The microelectrode tip was then lowered into the Nafion and rotated gently for approximately five seconds prior to being oven baked at 175°C for four minutes to remove any traces of humidity and to dry the Nafion film. We have found that this procedure is optimal for attaining a desired selectivity of $\geq 100:1$ for ascorbate based on a calculated slope of at least 5 pA/micromolar glutamate. Additional Nafion coatings resulted in a reduced glutamate sensitivity due to H$_2$O$_2$'s inability to traverse a thicker layer of anion repellent, while the application of a thinner coat of Nafion led to a substantial increase in levels of background ascorbic acid in the striatum upon solution application (Burmeister *et al.*, 2002).

Microelectrodes were then coated with L-glutamate oxidase to make them sensitive to glutamate *in vivo*. A 10 µl solution consisting of 1% BSA, 0.125% glutaraldehyde, and 1% glutamate oxidase was prepared, and then applied to the Pt recording sites beneath a dissecting microscope. A small drop of the enzyme solution ($\sim$0.10 µl) was suspended from a pipette tip and placed on the sensor site for 1-2 seconds. If successfully applied, a thin, luminescent layer of protein was observed on the sensor sites when viewed under a dissecting microscope. To ensure that an adequate layer of enzyme was applied to the recording sites, and because we have observed that increasing layer thickness does not correspond to a decrease in glutamate sensitivity, two additional coats of enzyme were applied in the same manner. Microelectrodes were then stored in a low-humidity environment for at least two days to allow for optimal enzyme layer
curing time. Please refer to Chapter Three for further discussion of the necessity of these layers.

**Microelectrode Calibration**

Constant voltage amperometry was performed using a FAST-16 high-speed electrochemistry instrument (Quanteon, L.L.C.) using software (Fast Analytical Sensor Technology) designed for simultaneous four-channel recordings. An Ag/AgCl reference electrode was placed in a continuously stirred solution of 0.05 M PBS (37°C, pH 7.4) in conjunction with the microelectrode operated at an applied potential of +0.7 V vs. Ag/AgCl reference electrode. A recirculating water bath (Gaymar Co.) was utilized to maintain a constant buffer temperature of 37°C. Amplifiers were adjusted to obtain a final gain of 200 pA/V for all recordings. Calibrations were performed by achieving final buffer concentrations of 250 µM ascorbic acid and 10, 20, 30, and 40 µM L-glutamate through additions of aliquots of 20 mM ascorbic acid and 20 mM L-glutamate stock solutions. Selectivity ratios for glutamate over ascorbic acid were calculated as well as the slope (sensitivity), limit of detection (LOD), and linearity (R²) for glutamate.

**In Vivo Glutamate Measures in Anesthetized Rats**

Electrode/micropipette assemblies used for the *in vivo* recordings consisted of a ceramic-based working electrode and a single-barrel (1 mm o.d., 0.58 mm i.d. glass, A-M Systems, Inc., Everett, WA) micropipette. The tips of the micropipettes had inner diameters of 10-15 µm, and were positioned 100 ± µm between recording sites two and three of the microelectrode arrays. They were attached to the printed circuit board holder of the ceramic-based multisite microelectrode using Sticky Wax (Kerr Brand).

Male F344 rats were anesthetized with urethane (1.25g/kg, i.p.) and placed within a stereotaxic frame for the performance of a craniotomy. A three cm sagittal incision was made on the skin of the cranium, and underlying fascia was displaced with a cotton swab. Bulldog clips were applied to limit the
movement of the skin while performing this procedure. Using a common Dremel tool, a 2 mm x 2 mm square section of the skull cap was removed in the upper right quadrant relative to bregma and the sagittal suture. Underlying dura was carefully excised with the aid of forceps. A miniature (200 µm diameter) Ag/AgCl reference electrode was implanted into a site that was remote from the recording area to prevent drifting of the working electrode potential. A KCl (70 mM KCl, 79 mM NaCl, 2.5 mM CaCl₂, pH 7.4; Chapter Three) or glutamate solution (5 mM L-glutamate, pH 7.4; Chapter Four) was loaded into a single-barrel micropipette. The potassium solution was used to study phasic release of glutamate as described in Chapter 3, while the glutamate solution was used in Chapter 4 to determine the temporal dynamics of glutamate uptake. The microelectrode/micropipette array was inserted into the striatum (AP +1.0 mm, ML -2.5 mm, DV -3.0 to -7.0 mm) of the rat brain; coordinates were calculated from bregma based on the rat brain atlas of Paxinos and Watson (1986). Volumes of KCl and glutamate solution were pressure-ejected from the micropipettes using a Picospritzer II (Parker Hanninfin Corp., General valve operation) and monitored using a stereomicroscope fitted with a reticule (Friedemann and Gerhardt, 1992) (Fig. 2.3). The FAST-16 recording system (Quanteon, L.L.C.) was used to record the resulting L-glutamate signals at the multisite microelectrodes. Animals were euthanized at the conclusion of each recording session via an overdose of anesthetic.

**Glutamate Release and Clearance Parameters**

Chemical stimulation or the application of exogenous glutamate solution produces a glutamate signal with a very distinct graphical "fingerprint". Figure 2.4 illustrates the typical spike-shaped appearance of a glutamate signal that is representative of the fluctuation of extracellular glutamate concentration as a function of time. Amplitude (µM) is simply a measure of the maximal extracellular L-glutamate concentration achieved following its evoked-release or application, while rise time \( (T_R, \text{ sec}) \) describes the time necessary to achieve this amplitude from onset of the origin of the signal. \( T_{\text{Total}} \) indicates the time necessary for
glutamate levels to return to baseline and encompasses the entire duration of the
signal.

Several other parameters exist that are useful in describing the clearance
characteristics of the generated glutamate signals. \( T_{50}, T_{80}, \) and \( T_{100} \) (sec)
indicate the amount of time necessary to decay 50, 80, and 100 percent of the
signal from onset of maximum amplitude. \( K_1 \) (sec\(^{-1}\)) is a decay rate constant
(first exponential rate of fit) and is independent of signal amplitude. This value is
representative of the slope of the linear regression of the natural log
transformation of these data over time. Finally, uptake rate (\( \mu M/sec \)) is obtained
by multiplying the \( K_1 \) rate constant by the maximum amplitude of the signal.

**Potassium-Evoked L-Glutamate Release Data Analysis**

Dose-response studies were conducted in each striatal recording site to
determine the maximum potassium-evoked glutamate responses in the young,
late-middle aged, and aged rats (young rats, \( n = 182 \); late-middle aged rats, \( n =
304 \); old rats, \( n = 82 \)). Only signals ranging from 10-20 \( \mu M \) in amplitude were
selected for analysis of the temporal properties of the release signals, as
 glutamate signals within this range were the highest that were consistently
attainable in the aged animals. Approximately 20 signals were obtained from
each depth in the striatum (ventral positions -3.0 to -7.0 mm in 0.5 mm
increments). This resulted in a large total number of signals collected from the
young (\( n = 401 \)), late-middle aged (\( n = 318 \)) and aged (\( n = 279 \)) rats for the
temporal analyses of the glutamate signals. Data were analyzed using a one
way analysis of variance followed by Tukey's post hoc comparisons. Statistical
significance was defined as \( p<0.05 \).

**Exogenous L-Glutamate Clearance Data Analysis**

Only signals ranging from 10-20 and 20-40 \( \mu M \) in amplitude were selected
for analysis of the temporal properties of glutamate uptake, as uptake rate was
discovered to increase as amplitude does the same (see Chapter Four for further
discussion). Approximately 20 signals were obtained from each depth in the
striatum (ventral positions -3.0 to -7.0 mm in 0.5 mm increments). This resulted in a large total number of signals collected from the young (n = 1092), late-middle aged (n = 415) and aged (n = 838) rats for the temporal analyses of the L-glutamate signals. Because the number of data points obtained varies across animals, a nested between-groups analysis of variance (ANOVA), using SYSTAT’s Multivariate General Linear Model (SYSTAT Software, Richmond, CA, USA), was used to separate potential variance resulting from individual differences in sample number from the main effects for experimental groups. Making an assumption that each data point represents an individual animal would be pseudoreplication, which artificially inflates degrees of freedom (Hurlbert, 1984). The use of the nested between-groups ANOVA test does not assume that each data point was taken from an individual animal. A categorical variable was included that labeled each data point according to the individual animal from which it was obtained. By taking this approach, main effects for age groups were independent of variance due to differences in the numbers of samples per animal. Analyses were followed by Tukey’s post-hoc tests if statistical significance was evident from the omnibus F test. Statistical significance was defined as p< 0.05.

**Total Transporter Protein and Biotinylation Assays**

**Tissue Dissection and Preparation for Immunoblotting**

All animals were decapitated under urethane (1.25 g/kg, i.p.) anesthetic, and their brains rapidly removed and dissected in 1 mm coronal slices using a chilled rodent brain matrix. The dorsal striatum was extracted from three or four slices using a 14-gauge needle, and the tissue was frozen at -70 °C. Frozen tissue samples were sonicated in 300 µl 1% sodium dodecyl sulfate solution (pH ~8) using a Fisher 60 sonic Dismembrator. Protein concentrations of the tissue samples were determined using the bichinchoninic acid method (BCA), and appropriate volumes of sample buffer were administered to achieve a final protein concentration of 2 µg/µl in each sample. Following electrophoresis,
sample proteins were transferred overnight in Tris/glycine/methanol buffer onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The blots were then placed in Ponceau stain to visualize and verify approximate protein loads, followed by a 1 hr. immersion in quenching buffer (a buffer that reduces non-specific binding of the antibodies to protein) containing 1% polyvinylpyrrolidone and 0.05% Tween 20 for a minimum of 1 hr. After a 1 hr. incubation in primary antibody, blots were incubated in appropriate secondary antibody (swine anti-rabbit IgG (0.8 µg/ml) or rabbit anti-mouse IgG (1 µg/ml; Dako, Glostrup, Denmark) for 1 hr., followed by a 1 hr. incubation with [125I] protein A (specific activity 1.3 GBq/mg; Amersham, Piscataway, NJ).

**Antibodies and Blot Immunolabeling**

A guinea pig polyclonal antibody targeting the carboxy-terminus of GLT-1 was purchased from Chemicon International (cat. # AB1783) and used at a dilution of 1 µg/ml. A rabbit polyclonal antibody raised against the carboxy-terminus of GLAST was purchased from Novus Biologicals (cat. # ab416) and used at a dilution of 1 µg/ml. A monoclonal antibody targeting the carboxy-terminus of EAAC1 was obtained from Chemicon International (cat. # MAB1587) and used at a dilution of 2 µg/ml. These antibodies were used to assay both total transporter protein levels as well as plasma membrane-associated protein levels, and the amount of protein loaded to assay total levels of transporter (30 µg for GLT-1, 15 µg for GLAST, and 60 µg for EAAC1) was well within the linear working range of each particular antibody.

Quantification of total and plasma membrane-associated EAAT protein levels was carried out via autoradiography. Immunoreactive portions corresponding to the protein of interest on the blots were detected, and these areas were excised and gamma radioactivity measured for each sample. Please see Results in Chapter Four for an extensive discussion of biotinylation data and analysis.

In the EAAT plasma membrane expression study, plasma membrane-associated EAAT was defined as EAAT-immunoreactivity (EAAT-IR) from Lamelli
Buffer eluant collected from avidin beads and normalized to EAAT-IR in the lysate (which represents total EAAT-IR present prior to avidin bead addition) in each sample.

**Preparation of Crude Striatal Synaptosomes/Glial Plasmalemmal Vesicles and Determination of Plasma Membrane-Associated EAAT**

We assessed the plasma membrane content of EAAT in crude striatal synaptosomes/glial plasmalemmal vesicle preparations using the membrane impermeant biotinylating reagent, sulfosuccinimidobiotin (NHS-biotin). This method has been previously established for studies of protein plasma membrane expression of DAT in these preparations (Salvatore et al., 2003; Zhu et al. 2005). Striatal tissue was homogenized in ice-cold 0.32 M sucrose and spun at 1000 x g for 10 min. The resulting supernatant was spun at 12, 500 x g for 15 min. and the pellet containing the crude synaptosomes was suspended in Krebs bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, Na₂EDTA, 1.7 mM CaCl₂, 10 mM glucose, 100 µM paraglyine, 100 µM ascorbic acid; bubbled with 95:5 O₂:CO₂). 500 µg total protein aliquoted from these preparations for each age group was incubated for 1 hr. at 4 ºC (a temperature that arrests endocytosis) in 500 µl of 1.5 mg/ml NHS-biotin in PBS/Ca/Mg buffer (138 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 9.6 mM Na₂HPO₄, 1 mM MgCl₂, 0.1 mM CaCl₂, pH 7.3). Biotinylating agents were removed by twice rinsing the pellet formed, followed by a 30-min. incubation with ice-cold 100 mM glycine in PBS/Ca/Mg buffer. Preparations were further washed three times with PBS/Ca/Mg buffer and then lysed in 300 µl Triton X-100 buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) containing protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µM pepstatin, 250 µM PMSF). Aliquots of these Triton-sonicated lysates were taken for subsequent normalization of total EAAT against the biotinylated fraction and the remaining lysate was centrifuged for 20 min. at 20,000 x g at 4 ºC to remove nuclear components. The supernatants were then incubated with monomeric avidin beads (100 µl/tube) in the same buffer for 1 hr. at room temperature. The beads
were then washed in Triton X-100 buffer and the adsorbed biotinylated proteins were eluted by incubating in Lamelli buffer (62.5 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, and 5% bromophenol blue) for 20 min. at room temperature. Two 20-min. elutions with Lamelli buffer were typically required for maximal elution of EAAT immunoreactivity. The extracts were then processed for blot immunolabeling for EAAT as described above.

**Total and Membrane-Associated Protein Data Analysis**

All data were generated from individual rats. Mean and SEM were determined from each result obtained from the total number of rats used for each study. One-way analysis of variance (ANOVA) was used to determine any age-related changes in EAAT total and membrane-associated protein levels. We used Tukey’s post hoc tests to determine significance among groups if the ANOVA results indicated a significant difference among the three age groups (p < 0.05).

**Drugs and Reagents**

All chemicals were used as received unless otherwise stated.

**Electrode Preparation and Calibration**

Nafion (5% in a mixture of aliphatic alcohols and water), ascorbate, L-glutamate monosodium salt, and glutaraldehyde were obtained from Sigma-Aldrich. L-glutamate oxidase was purchased from Seikagaku America, Inc. 0.05 M phosphate buffered saline solution (PBS) was created using sodium chloride, sodium phosphate monobasic, and sodium phosphate dibasic obtained from Fisher Scientific (Fair Lawn, NJ). Glutaraldehyde was stored at -20°C. All solutions were prepared using distilled water, which was de-ionized using a Millipore water treatment system.
**Intracranial Pressure Ejection**

All solutions administered intracranially were prepared in 0.09% saline solution, adjusted to pH 7.4, and filtered prior to use through a sterile syringe filter (0.22 µm pore size, Costar, Corning, NY). All components of the 70 mM potassium (sodium chloride, potassium chloride, calcium chloride dihydrate) and 5 mM glutamate were purchased from Fisher Scientific.

**Immunoblotting and Biotinylation**

NHS-biotin and monomeric avidin beads were purchased from Pierce Biotechnology (Rockford, IL). Protease inhibitors were obtained from Sigma. All other solutions pertaining to biotinylation were purchased from Fisher Scientific and Sigma unless otherwise stated.

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Table 2.1 Distribution of Fischer 344 Rats Across Experimental Conditions

<table>
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<th>Study</th>
<th>6 Month</th>
<th>18 Month</th>
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<tr>
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Figure 2.2 Ceramic-Based Pt Microelectrode (R1 Configuration)

This geometrical configuration consists of four in-line recording sites, each measuring 50 x 150 µm with 50 µm spacing between sites.
Figure 2.3 Anesthetized In Vivo Glutamate Recording Apparatus
Figure 2.4 Extracellular L-Glutamate Signal Parameters

Peak representative of a typical signal generated via application of 70 mM KCl and 5 mM L-Glutamate solution. Please refer to text for detailed discussion of these parameters.
Extracellular L-glutamate diffuses to the microelectrode surface, at which point it contacts the glutamate oxidase layer. Glutamate is metabolized to $\alpha$-ketoglutarate, a non-factor in the determination of synaptic glutamate, and H$_2$O$_2$, which traverses the Nafion layer and is oxidized at the Pt recording site surface at +0.7V vs. Ag/AgCl reference electrode. This process generates two electrons per molecule of peroxide, thus the current produced is an indirect indication of extracellular glutamate concentration. Note that the Nafion layer excludes potential negatively charged interferents via electrostatic repulsion.
Chapter Three: Age-Related Changes in the Dynamics of Potassium-Evoked L-Glutamate Release in the Striatum of Fischer 344 Rats

Introduction

L-Glutamate is the primary excitatory neurotransmitter in the central nervous system, and plays a primary role in a number of activities related to brain plasticity including motor behavior, cognition, and memory. It is believed that alterations in glutamate release and uptake dynamics are partially responsible for the deficits in these functions that often occur with aging. Prior studies of glutamate release, however, are equivocal (Segovia et al., 2001b). Some studies have indicated that basal extracellular glutamate concentrations in the hippocampus of aged (24 months) rodents may rise to levels 94% greater than those found in young (6 months) animals (Freeman and Gibson, 1987). By contrast, more recent studies have attempted to show that these levels in aged rodents may actually be 60% lower than in young animals (Saransaari and Oja, 1995). Similarly, researchers have observed increases, decreases, or no changes in basal extracellular glutamate levels in the aged striatum (Freeman and Gibson, 1987; Massieu and Tapia, 1997; Corsi et al., 1997). Thus, it is unclear why there are major discrepancies in the literature regarding the regulation of glutamate release in aging.

Taken together, it may be that basal extracellular glutamate levels remain constant throughout aging in rodents. However, these constant glutamate levels are not necessarily indicative of static glutamate release and uptake processes. Extracellular glutamate levels reflect a delicate balance between release and uptake, meaning that age-related decreases in release may be compensated by a regional decrease in uptake, and vice-versa (Segovia et al., 2001b). It is difficult, therefore, to accurately postulate mechanisms for the possible alterations in glutamate dynamics as a result of aging, as prior studies using brain slice methods and microdialysis techniques provide only a "snapshot" of glutamate concentration at a particular time, and cannot differentiate between
neuronal and glial sources of glutamate (Timmerman and Westerink, 1997). Thus, they are limited in their capacity to provide us with the high temporal and spatial resolution necessary for studying release of fast neurotransmitters such as L-glutamate in the brain.

Contrasting data also exist for the phenomenon of glutamate clearance from the synapse in the cerebral cortex and striatum. While most studies have demonstrated a reduction in clearance of glutamate by astrocytes in aged rodents (Wheeler and Ondo, 1986; Najerahim et al., 1990; Saransaari and Oja, 1995; Vatassery et al., 1998), other have reported no change (Dawson et al., 1989; Palmer et al., 1994) or an increase in astrocyte clearance ability (Strong et al., 1984). Once again, despite the discrepancies in data, there is a general belief that aged rats have a lower uptake capacity for extracellular glutamate and a decreased number of high affinity glutamate transporters in the striatum and prefrontal cortex (Price et al., 1981; Wheeler and Ondo, 1986; Najerahim et al., 1990; Vatassery et al., 1998; Saransaari and Oja, 1995). However, it is difficult to interpret prior data, as researchers were limited in their ability to obtain a high degree of temporal and spatial resolution regarding glutamate uptake.

In order to more rapidly measure L-glutamate release dynamics in vivo, our laboratory has developed ceramic-based multisite microelectrodes for rapid measures in the intact brain (Chapter Two). The enzyme-based microelectrode has been developed and characterized for second-by-second measures of L-glutamate in the intact rat brain (Burmeister et al., 2000, 2002). Phasic glutamate release is produced in vivo by pressure ejection of a potassium solution from a micropipette placed adjacent to the recording sites; the glutamate released from nearby presynaptic terminals forms hydrogen peroxide by its interaction with the glutamate oxidase coating on the microelectrode. This hydrogen peroxide is directly proportional to the L-glutamate concentration and can be converted to extracellular L-glutamate levels by calibration. This technology allows us to accurately measure phasic changes of glutamate concentrations within various brain regions on a second-by-second basis, while maintaining a high degree of spatial resolution (microns) (Burmeister et al.,
The aim of this study was to utilize this technology to evaluate the amplitudes and temporal dynamics of potassium-evoked L-glutamate release and uptake in the young, late-middle aged, and aged F344 rat striatum. In addition, the heterogeneity of this brain region was characterized according to the nature of its release and clearance properties throughout aging.

**Materials and Methods**

Please refer to Chapter Two for an in-depth discussion of all matters pertaining to animal care, electrode preparation, generation of potassium-evoked glutamate signals, and data analysis.

**Results**

Local applications of potassium for 1-2 seconds produced robust, transient, and reproducible changes in extracellular levels of L-glutamate in the striatum of the young, late-middle aged, and aged rats. The average L-glutamate amplitudes were lower in both the late-middle aged (36.0 ± 0.8 µM) and aged rats (18.9 ± 0.8 µM) when compared to the young animals (41.7 ± 1.2 µM; both p<0.001). Thus, aging was seen to cause a robust decline in the maximal amplitude of L-glutamate signals recorded in the striatum of the aged rats as compared to young animals. In addition, a small but significant decline in the release amplitudes was seen in the late-middle aged rats as compared to the young rats (Fig. 3.2).

Due to the age-related changes in potassium-evoked L-glutamate signal amplitudes, the L-glutamate release data were sub-divided into groups composed of 10-20 µM signals from all age groups to study the temporal dynamics of the signals in aging. When examining glutamate release dynamics, the average volume of potassium necessary to evoke glutamate signals in the range of 10-20 µM was greater (*p<0.05) in the 24 month rats (204 ± 12 nl) than in the young rats (169 ± 8 nl). Likewise, the average amplitude of extracellular...
glutamate concentration achieved per nanoliter of potassium ejected was attenuated in the 24 month rats (0.09 ± 0.006 µM/nl) as compared to young animals (0.15 ± 0.007 µM/nl). Interestingly, signals from the striatum of late-middle aged rats showed an increased response to potassium (0.21 ± 0.01 µM/nl) in comparison to young rats (Figs. 3.1, 3.3).

Upon further examination of the data, L-glutamate uptake dynamics were also compromised in the aged rats. When compared to young rats (3.9 ± 0.08 µM/sec), the L-glutamate uptake rate in 24 month rats (3.1 ± 0.10 µM/sec) was significantly slower (***p<0.001) (Fig. 3.3). Figure 3.2 illustrates this phenomenon in much greater detail, as it compares the time dynamics of individual signals representative of each of the three age groups. While the release rate and total amplitude achieved were similar amongst the three age groups, the time necessary for complete removal of glutamate from the extracellular space in the 24 month rats was longer than in the 6 and 18 month old animals. Thus, the time dynamics of potassium-evoked release of L-glutamate were seen to be altered in the aged rat striatum as compared to the young and late-middle aged animals.

Depth-related alterations in potassium-evoked release and uptake in aging were examined as well. The average uptake rate and amp/nl were calculated for each depth (DV -3.0 to -7.0 mm, 0.5 mm increments) and compared amongst age groups. This analysis yielded an abundance of data and highlighted many age-related significant differences in glutamate release and clearance parameters. In consideration of space and clarity, please refer to Figures 3.4 and 3.5 for these results. In terms of glutamate uptake rate, the significant reduction in global clearance rate in the aged animals mirrored in the dorsal regions of the striatum (DV -3.0 to -4.5 mm) was not present in the more ventral depths of this structure (DV -5.0 to -6.5 mm). This disparity between age-related local and global alterations of glutamate regulation became more apparent upon analysis of corticostriatal projection excitability, as the global attenuation of apparent fiber excitability in the aged animals was only observed in the dorsal striatum (DV -3.0 to -4.5 mm), and the overall increase in excitability in the late-
middle aged striatum was only found in the more ventral regions (DV -5.0 to -6.5 mm). Thus, global alterations in glutamate release and clearance in the striatum are a reflection of a compilation of these parameter averages at all depths of the structure.

**Discussion**

L-glutamate is a neurotransmitter of extreme value to normal CNS functioning, and it possesses the capability to produce severe excitotoxic damage. Therefore, it must be tightly regulated within the brain and in brain areas such as the striatum. Our study indicates that while phasic release of L-glutamate is detectable in the aged rat, significant decreases in release amplitudes and uptake rate occur in the aged rat brain. In the young animals, potassium-evoked glutamate signals were robust and required smaller volumes of potassium to achieve glutamate concentrations equal to those recorded in aged rats. Late-middle aged rats had L-glutamate signals with larger amplitude in response to the ejection of equivalent volumes of potassium when compared to young rats. In addition, the potassium effects on glutamate fibers were diminished in aged rats, as there was a decrease in the apparent excitability in comparison to young rats. Glutamate clearance was significantly compromised in the aged striatum as well. This technology also permitted us to study depth-related alterations in glutamate clearance and release in the layered striatum, which highlighted that the majority of changes found at individual depths were representative of the global alterations in these parameters. Thus, the present studies employing the new ceramic-based microelectrodes for rapid measures of L-glutamate support that there are significant age-related changes in phasic glutamate release produced by local applications of potassium.

Previous studies of glutamate release and uptake in the aged rat striatum are inconclusive. While there are reports of reduced potassium-evoked glutamate release that are in agreement with the results of our study (Freeman and Gibson, 1987; Saransaari and Oja, 1995), there is an overwhelming number
of studies supporting the idea that there is no net change in basal (Porras and Mora, 1995; Saransaari and Oja, 1995; Corsi et al., 1999; Segovia et al., 1999) or chemically induced (Donzanti et al., 1993; Sanchez-Prieto et al., 1994; Corsi et al., 1997) extracellular glutamate concentrations. The data remain equivocal on the issue of increased clearance rate (Strong et al., 1984), decreased clearance rate (Wheeler and Ondo, 1986; Najerahim et al., 1990) or no change in clearance rate of glutamate uptake in aging (Dawson et al., 1989).

We believe that this discrepancy in prior studies is due in part to the limitations of the technique employed. Microdialysis is lacking in terms of the high degree of temporal resolution necessary for studying neurotransmitters with rapid release and uptake dynamics such as L-glutamate. In addition, this low degree of temporal resolution increases the likelihood of detecting non-neuronal glutamate; neuron-mediated glutamate release is rapid and is likely cleared from the extracellular space in a matter of seconds. Over a time frame of several minutes, the glutamate obtained in microdialysis experiments reflects glutamate not only from neuronal stores, but from other sources as well. In fact, much of this glutamate is not obtained via the fulfillment of classical release criteria, and many researchers prefer to explain this phenomenon as being a result of reversed-uptake by carrier-mediated processes. This has been shown to occur, however, only in situations of excessive stimulation (Timmerman and Westerink, 1997). Our ceramic-based microelectrodes provide the sub-second temporal resolution necessary to give a more accurate representation of the dynamics of phasic changes in extracellular L-glutamate. This allows us to better elucidate the possible compensatory mechanisms responsible for maintaining static extracellular glutamate concentrations in the rat striatum in young and aged rats.

Compensatory mechanisms are known to exist throughout the mammalian CNS, and these may very well explain the striatum's ability to maintain relatively stable basal glutamate concentrations during the aging process (Segovia et al., 2001a). As demonstrated by Cobo et al. (1993), neurons in the prefrontal cortex display a marked decrease in excitability in order to compensate for the loss of glutamatergic neurons in the frontal cortex. In all, this results in no alteration in
net basal glutamate levels. Our studies are in accordance with this notion of decreased excitability, as corticostriatal glutamatergic nerve endings in aged rats require a greater amount of potassium stimulation in order to reach the threshold necessary for neurotransmitter release. Further studies are needed to determine the mechanisms which may underlie this apparent change in excitability of L-glutamate synapses in the aged rat brain.

This decrease in apparent excitability of glutamate fibers may be guarding striatal neurons from potential excitotoxic damage, as glutamate clearance rates are significantly attenuated during aging. This carries with it several possible interpretations. The first and most obvious hypothesis that could be drawn from these data would be that there is either a down regulation of EAAT's or a reduction in their efficiency. Prior studies have confirmed that there is a decrease in the maximal velocity of glutamate uptake in synaptosomes from aged animals (Price et al., 1981; Wheeler and Ondo, 1986). This would, however, only account for the neurons' contribution to glutamate clearance, which is relatively small in comparison to glial glutamate clearance (Danbolt, 2001), and no data, to the best of our knowledge, currently exist on glial clearance rates in aged animals.

Regarding potential compensatory changes in aging, astrocytes have been shown to increase in number and activity in the aged brain (Brizzee et al., 1983; David et al., 1997). This could very well offset a loss of neuronal transporter efficiency. An increase in the number of transporters, however, does not necessarily correlate with an increase in affinity (Segovia et al., 2001b), and there have been few studies conducted on the glutamate affinity of these transporters. Recent studies in our laboratory indicate a reduction in glutamate clearance rates in aged animals (Chapter Four), which were associated with an indication of increased numbers of transporters in the plasma membrane of glia and/or neuronal synapses. Clearly, additional studies are warranted to determine the effect of aging on the functional dynamics of glutamate release and uptake in the striatum and many other brain regions.
The development of the microelectrode technology utilized in this study has allowed us to obtain new insights into the temporal dynamics of potassium-evoked glutamate release and uptake in young and aged rat striatum. A particular strength of this technology is its capability to investigate neurotransmitter temporal dynamics in several depths of a layered structure such as the striatum. The performance of such an assay is especially critical when analyzing release and clearance in larger brain regions, as variance in the density and release characteristics of neuronal projections then becomes a potential confounding factor. The heterogeneity of the striatum has been well characterized in a number of recent studies. Segovia et al. (1999) showed that glutamate's facilitation of dopamine release was diminished in the ventral striatum of middle-aged and aged rats, yet there was no significant loss of this stimulatory effect during aging in the dorsal striatum. Marked changes were also observed in neuronal activity in the ventral striatum in aged animals following the administration of amphetamine or a D2 receptor antagonist, an effect that was not present in the dorsal striatum (Crawford and Levine, 1997). Moreover, Friedemann and Gerhardt (1992) highlighted a dorsoventral gradient with respect to the phenomenon of the amount of potassium stimulation necessary to evoke dopamine release in the striatum. Collectively, these data suggest that dopaminergic and glutamatergic projections to the striatum are not uniform in terms of distribution and/or excitability. In addition, our uptake assay indicates that the clearance properties of glial and postsynaptic glutamate transporters may change as a function of depth in aging animals as well. Therefore, when attempting to investigate the temporal dynamics of transmitter release and uptake in the striatum, it is necessary to analyze these parameters at many depths of the structure and not simply infer that the data obtained from a single layer are representative of the structure as a whole.
Portions of this work have been published in the Journal of Neural Transmission:


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Figure 3.1 Dose Dependency of Potassium-Evoked Glutamate Release

Representative tracings depicting the volume of potassium solution necessary to elicit glutamate release of increasing amplitudes in both young (upper tracings) and aged (lower tracings) F344 rats. Note the significant attenuation in response in the aged animals upon application of similar volumes of potassium solution. Potassium solution was applied at the time points indicated by arrows.
Figure 3.2 Potassium-Evoked Glutamate Uptake Rate: Representative Tracings

Series of recordings showing differences in amplitude-matched, potassium-evoked L-glutamate release dynamics in 6, 18 and 24 month old F344 rats. Note the significantly slower uptake rate in the 24 month rats as compared to the 6 and 18 month old animals. Potassium solution was applied at the time points indicated by the arrows. The figure inset shows the decrease in average maximum amplitude of potassium-evoked L-glutamate release that occurs in the rat striatum in aging. ***p<0.001 vs. young animal group based on one-way ANOVA followed by Tukey's post-hoc test.
Figure 3.3 Average Potassium-Evoked Glutamate Signal Parameters

Average values in the different age groups were for 1) comparison of L-glutamate signal amplitudes, 2) volumes of potassium ejected, 3) L-Glutamate signal uptake rates, and 4) maximal glutamate signal amplitudes obtained per nanoliter of potassium ejected amongst the three age groups. Each bar represents mean ± SEM from seven animals in each age group. *p<0.05 and ***p<0.001 vs. young animal group based on one-way ANOVA followed by Tukey’s post-hoc tests.
Figure 3.4 Depth-Related Alterations in Glutamate Uptake Rate in Aging
Glutamate uptake rates were calculated at several depths within each animal (DV -3.0 to -7.0 mm) and compared amongst age groups. Note the that while the dorsal striatum (DV -3.0 to -4.5 mm) exhibits a marked reduction in glutamate clearance ability in the aged animals in comparison to young rats, no such difference exists in the more ventral regions (DV -5.0 to -6.5 mm) of this structure. Each bar represents mean ± SEM from seven animals in each age group. ***p<0.001 and **p<0.01 vs. young animal group based on one-way ANOVA followed by Tukey’s post-hoc tests.
Excitability of corticostriatal projections (amp/nl) was calculated at several depths within each animal (DV -3.0 to -7.0 mm) and compared across age groups. A reduction in the apparent excitability of corticostriatal fibers in aged animals was only observed in the more dorsal striatum (DV -3.0 to -4.5 mm), while an increase in excitability of these fibers in the late-middle aged rats was only found in the ventral striatum (DV -5.0 to -6.5 mm). Above data are reported as mean ± SEM from seven animals in each age group. ***p<0.001, **p<0.01 and *p<0.05 vs. young animal group based on one-way ANOVA followed by Tukey’s post-hoc tests.
Chapter Four: Reduced Plasma Membrane Surface Trafficking of GLAST Mediates Decreased Glutamate Regulation in the Aged Striatum

Introduction

L-glutamate is the predominant excitatory amino acid neurotransmitter in the mammalian CNS and plays a primary role in neural processes underlying cognition, behavior, and motor functioning (Collindridge and Bliss, 1987; Cotman et al., 1988; Mora and Cobo, 1990; Cobo and Mora, 1991; Schmidt et al., 1992). While important for normal CNS functioning, deficits in extracellular glutamate regulation pose a serious threat to the viability of surrounding neurons, as elevated synaptic glutamate concentrations greatly increase the risk of excitotoxic loss of neurons (Olney, 1969). Glutamate dysregulation is also believed to be a major player in aging, as the aged CNS is very susceptible to excitotoxic insults from stroke or hypoxia (Liu et al., 1996; Brewer, 1998). Thus, it is crucial that low extracellular glutamate levels be maintained via an efficient, rapid, and robust transport mechanism (Balcar and Johnston, 1972; Logan and Snyder, 1972; Johnston, 1981). To date, five sodium-dependent EAAT subtypes have been cloned, and they act in concert as the exclusive means of terminating glutamate neurotransmission (Pines et al., 1992; Storck et al., 1992). GLAST and GLT-1 are located primarily on astrocytes, and account for approximately ninety percent of all glutamate transport. Postsynaptically, EAAC1 is responsible for removal of the remaining ten percent. EAAT4 and EAAT5 have been characterized in humans and have very specific localizations, the cerebellum and retina, respectively, and so they are excluded from all discussions pertaining to the cerebral cortex and deep structures of the rat brain (for review, see Danbolt, 2001).

Prior studies of aging in the striatum have not implicated these transporters as having a significant contribution to alterations in glutamate neurotransmission believed to play a role in age-related motor deficits and increased secondary damage susceptibility. Further complicating matters, a
large portion of previous data pertaining to glutamate uptake are equivocal. For example, Price et al. (1981) noted a decrease in glutamate uptake rate of 41%, with a concurrent 32% increase in transporter affinity in aged rat synaptosome preparations. In contrast, Strong et al. (1984) reported attenuation in transporter affinity of 116%, with an accompanying increase in uptake rate of 84% in aged animals. Furthermore, another study indicated no significant alteration in either glutamate uptake rate or affinity in the aged rat model (Palmer et al., 1994).

We believe that these inconsistencies are due in large part to limitations of the techniques employed; the analysis of glutamate release and clearance dynamics requires a technique that provides a high degree of temporal resolution (for review, see Segovia et al., 2001b; Timmerman and Westerink, 1997). We recently used novel methodology to record glutamate release on a second-by-second basis, which determined a reduction in glutamate uptake rate and a concurrent decrease in the apparent excitability of glutamate fibers (Nickell et al., 2005). However, our prior study did not address the potential mechanisms underlying the decrease in glutamate uptake rate. The present study aimed to further explore this phenomenon by coupling in vivo electrochemical recordings of glutamate uptake with classical immunoblotting and biotinylation in order to determine the potential roles of total transporter protein levels and membrane surface trafficking of these transporters in this apparent age-related decrease in glutamate uptake rate. Collectively, the data support that changes in the surface expression of GLAST may contribute to decreased regulation of extracellular glutamate in the aged brain.

Materials and Methods

Please refer to Chapter Two for an extensive discussion of all methods relating to the following research, including animal care, voltammetric recordings, immunoblotting, and biotinylation.
Results

A new recording approach was used to more directly investigate the regulation of extracellular L-glutamate in vivo. Local applications of exogenous L-glutamate were used to directly investigate glutamate uptake and clearance time in the striatum of young, late-middle aged and aged F344 rats. Rapid (1-2 sec.), local ejections of nanoliter volumes of glutamate solution into regions of the striatum produced robust, transient, and well-defined elevations in extracellular glutamate concentrations, followed by a time period of rapid glutamate clearance (see Figs. 4.1, 4.2). We investigated two ranges of signal amplitudes for our temporal analyses of glutamate clearance, as our prior study (Nickell et al., 2005) showed that the average maximal potassium-evoked release of glutamate in young F344 animals was approximately 40 µM, while the average maximal amplitude of potassium-evoked L-glutamate release in the aged rat striatum was ~20 µM. Therefore, glutamate signal amplitude ranges of 10-20 and 20-40 µM were selected to simulate these two extracellular levels of L-glutamate clearance. It must be noted that it is impractical to compare glutamate signals with vastly differing amplitudes, as there is a direct correlation between synaptic glutamate concentrations and clearance rates. Thus, the rate of glutamate uptake increases as extracellular levels of glutamate increase as seen in Figure 4.1.

The average extracellular glutamate concentration achieved with each application among the three age groups in the 20-40 µM range was similar, as there was no significant difference when comparing the young (30.10 ± 0.17 µM), late-middle aged (30.07 ± 0.23 µM), and aged (29.57 ± 0.20 µM) signal amplitudes. Likewise, no differences existed among the three age groups when comparing average signal amplitudes in the 10-20 µM range as well, as the signals obtained in the young (15.88 ± 0.15 µM), late-middle aged (15.88 ± 0.20 µM) and aged (14.97 ± 0.13 µM) rats did not vary much in amplitude (Fig. 4.3).

Our primary finding is that both the late-middle aged and the aged rat brain display a compromised ability to remove extracellular L-glutamate, as evidenced by clearance times using a parameter called $T_{80}$. This measure is a
calculation of the time necessary to clear 80% of the maximum signal amplitude, and has proven to be an extremely reliable means to characterize clearance efficiency in prior studies of both the dopamine (David et al., 1998) and serotonin (Daws et al., 1998) systems of the brain. Within the extracellular glutamate amplitude range of 10-20 µM, T80 values in both the 18 (7.41 ± 0.15 sec.; p<0.01) and 24 month (8.85 ± 0.29 sec.; p<0.001) rats were significantly prolonged in comparison to those in the 6 month (6.00 ± 0.11 sec.) animals. Likewise, T80 values for 20-40 µM signals obtained in young rats (6.10 ± 0.07 sec.) were significantly lower than those in the late-middle aged (7.80 ± 0.10 sec.; p<0.001) and aged (8.70 ± 0.18 sec.; p<0.001) animals (see Fig. 4.3).

Upon further examination of these signals, the data also showed a reduction in the rate at which the aged brain is capable of removing L-glutamate from the extracellular space when examining both signal amplitude ranges. The glutamate transporters in the young animals were significantly (p < 0.001) more efficient (10.47 ± 0.13 µM/sec.) in clearing glutamate in the 20-40 µM range than those in the aged animals (8.25 ± 0.20 µM/sec.). The clearance of glutamate signals in the 10-20 µM range was also significantly (p<0.001) more rapid in the young rats (4.62 ± 0.10 µM/sec) than in the aged animals (2.70 ± 0.10 µM/sec). Interestingly, while there was no significant difference in glutamate uptake in the late-middle aged striatum in comparison to the young animals in the 20-40 µM amplitude range, glutamate clearance in these animals (5.08 ± 0.16 µM/sec) in the 10-20 µM range was significantly (p<0.01) faster than in the young or aged groups.

Depth-related alterations in clearance time and uptake rate in aging were examined as well. The average T80 and uptake rate were calculated at each depth (DV -3.0 to -7.0 mm; 0.5 mm increments) and compared amongst age groups. This analysis yielded an abundance of data and highlighted many age-related significant differences in glutamate clearance ability. In consideration of space and clarity, please refer to Figure 4.4 and Table 4.5 for these results. Generally speaking, the relationship amongst age groups observed at dorsal depths (DV -3.0 to -4.5 mm) paralleled the above reported global effects in terms
of both uptake rate and clearance time. However, when examining clearance times in more ventral regions of the striatum (DV -5.0 to -6.5 mm), the large discrepancy in T80 amongst the age groups is greatly diminished. Thus, the aged rat striatum displays a marked dorsoventral gradient in its ability to clear glutamate from the extracellular space.

This age-related reduction in glutamate clearance time and uptake rate led us to investigate the possibility that perhaps this phenomenon is due, in part, to an attenuation in the production of total transporter protein. When comparing the transporter responsible for the majority of glutamate clearance among the three age groups, GLT-1, no significant difference was found (Fig. 4.6A). Likewise, there was no difference in either GLAST or postsynaptic EAAC1 total protein levels (Fig. 4.6B, C). Thus, we can state with confidence that the decreased glutamate uptake rate we observe in aging is not due to reduced steady-state levels of transporter proteins.

We continued to explore the potential mechanism underlying this decreased clearance time and uptake rate by investigating relative plasma membrane surface expression of the glutamate transporters via a biotinylation technique. In order to process striatal homogenates for the assessment of all three glutamate transporters from each animal, we determined expected signal (as cpm from using I\(^{125}\)-protein A for detection) from each transporter based upon immunoreactivities given from each antibody and the aliquots used from each step of preparation: P2 fraction, lysate (representing all protein), supernatant from avidin bead spin (representing cytosolic protein), and two eluants from treatment of avidin beads with Lamelli buffer (representing plasma membrane protein). The relative immunoreactivities of the three transporter antibodies were such that GLT-1 gave the strongest immunoreactivity per µg protein, followed by GLAST, and then EAAC1. Aliquot volumes taken from each step were guided by these immunoreactivities and in accordance with general handling as described in previous studies (Salvatore et al., 2003; Gates et al., 2004; Zhu et al., 2005). Each assayed amount was calculated for total amount based upon aliquot volume, total volume of prepared sample, and total amount processed. In Figure
5, the relative yields for each step in the processing of striatal tissue for assessment of GLT-1 is shown. Briefly, a band of ~65-70 kDa was excised from the blot and counted for gamma radioactivity. Total recovery of GLT-1 immunoreactivities in the supernatant and eluant fractions was 73% of that obtained from the lysate. Furthermore, assuming equal recovery of the GLT-1 from both fractions, the percent of GLT-1 associated with the eluant fraction was ~90% and that from the supernatant ~10%. Therefore, it appears that the majority of this glutamate transporter is expressed at the plasma membrane (Fig. 4.7).

No difference existed among the age groups when comparing GLT-1 and EAAC1 in terms of membrane surface-association (Fig. 4.8B). However, when comparing the surface expression of GLAST among the three age groups, there was a significant reduction in surface expression in both the late-middle aged (42 ± 11 %; p < 0.05) and aged (55 ± 13 %; p < 0.01) striatum in comparison to the young animals (Fig. 4.8A). Thus, a strong age-related decline in GLAST membrane surface-association was observed that is in accordance with our clearance time data.

**Discussion**

Regulation of L-glutamate signaling presents the mammalian CNS with an interesting paradox. While a great majority of all excitatory neurotransmission in the brain and spinal cord is mediated by glutamate (Fonnum, 1984; Orrego and Villanueva, 1993), slightly elevated extracellular concentrations of this neurotransmitter over a period of time establish a toxic environment for surrounding neurons (Olney, 1969). As a result, maintenance of relatively low synaptic glutamate levels is critical for cell survival and proper neuron functioning. The current study investigated glutamate clearance alterations in aging, as well as several potential mechanisms responsible for those changes. When examining the clearance times of amplitude-matched applications of exogenous glutamate, the data showed a significant decrease in clearance ability
in both the late-middle aged and aged rat striatum. This age-related decrease in clearance ability was shown to demonstrate a marked dorsoventral gradient, as clearance times were prolonged in the dorsal striatum in comparison to the ventral striatum of the aged animals. We also provided evidence to conclusively show that a potential alteration in total transporter protein levels in aging is not a feasible mechanism to explain this phenomenon. Rather, it appears that an age-related decrease in the membrane surface trafficking of GLAST is at least partially responsible for this slowing of glutamate clearance in the aged brain.

Interestingly, the late-middle aged rat striatum exhibits a slightly increased glutamate uptake rate in the 10-20 µM range despite a reduction in the glia membrane surface expression of GLAST in the animals of this age group. A recent study conducted by Salvatore et al. (2003) discovered a more rapid dopamine clearance rate coupled with a reduction in the surface trafficking of the dopamine transporter of nigrostriatal projections in late-middle aged rats in comparison to young animals. This finding validates the point that several factors are responsible for determining the net uptake rate of glutamate; while glutamate transporter steady-state protein levels and membrane expression are critical components of this process, affinity of these transporters must also be taken into consideration. Therefore, the determination of the affinity of each EAAT in the striatum of all age groups is necessary in the future in order to more fully characterize the mechanism underlying impaired glutamate clearance, but the lack of selective transporter inhibitors makes this difficult at this time. In addition, clearance curves of injected substances are comprised of both diffusion and transporter-mediated uptake processes (Cass et al., 1993), which further complicate the interpretation of the rate data. We have previously determined that the 80% decline times, or T80's, of clearance signals are extremely reliable to characterize clearance of both the dopamine (David et al., 1998) and serotonin (Daws et al., 1998) systems of the brain. In the present studies, the T80 values were found to be highly reproducible and correlated with changes in the surface expression of the glutamate transporters, analogous to the prior work of
Salvatore et al. (2003). Therefore, we believe that $T_{80}$ is a more reliable parameter for the study of the rapid temporal dynamics of glutamate clearance.

The marked dorsoventral gradient in clearance ability observed in the aged striatum holds important implications for investigations of the motor dysfunction that arises in senescence. Several studies conducted by Hebert and Gerhardt (1998, 1999) discovered a compromised dopamine release capacity in aged animals that was met with a concurrent reduction in dopamine clearance in both the dorsal and ventral striatum. This phenomenon is believed to be a crucial compensatory mechanism for maintaining the normal synaptic lifespan of dopamine in the aged brain. While these alterations were observed throughout the striatum, our data indicate that glutamate uptake is compromised solely in the dorsal striatum. Collectively, these data suggest that the primary motor circuitry of the striatum located in the dorsal depths of the region incur a reduction in glutamate clearance ability in order to sustain this transmitter's facilitation of dopamine release in the aged basal ganglia. Additional studies are warranted to further characterize the chronology and molecular underpinnings of such age-related changes.

Emerging data have identified a number of molecules as potential factors in the process of glutamate transporter regulation. These glial and postsynaptic transporters share common putative protein phosphorylation sites in their primary amino acid sequences (Kanai et al., 1993), leading many researchers (Pisano et al., 1996; Casado et al., 1991; Sims and Robinson, 1999) to investigate the possibility that EAAT regulation is mediated by protein kinases, notably protein kinase C (PKC). Data have shown that activation of PKC in C6 glioma cells rapidly increased cell surface expression of EAAC1 (Dowd and Robinson, 1996; Davis et al., 1998) and similar results have been obtained in primary cultures of neurons and astrocytes (Munir et al., 1997). However, these data are in stark contrast with results obtained in alternate cell line models of other studies. For example, EAAC1 activity and expression were decreased in *Xenopus* oocytes (Trotti et al., 1998, 2001) cell lines, while GLT-1 membrane expression was decreased in C6 glioma and primary cultures upon PKC activation (Kalandadze
et al., 2000; Robinson, 2002). The equivocal nature of these studies continues when examining the influence of PKC activation on GLAST membrane trafficking, as researchers have noted increases, decreases, or no change in transporter surface association following an increase in activated intracellular PKC levels (Casado et al., 1991; Gonzalez and Ortega, 1997; Daniels and Vickroy, 1999; Gonzalez et al., 1999). Taken together, these data show that glutamate transporter phosphorylation has differing effects that are dependent upon the particular cell line utilized for study. Despite these inconsistencies, however, these studies clearly demonstrate that alterations in glutamate transporter membrane surface trafficking are a viable means whereby to regulate extracellular glutamate levels in vivo as well as in vitro.

While the focus on glutamate transporter regulation continues to intensify, scarce data, if any, are available on alterations of these transporters in aging. In fact, a reduction in the density of NMDA receptors on the medium spiny neuron postsynaptic membrane (Petersen and Cotman, 1989; Mitchell and Anderson, 1998) is the only finding that is without contention among researchers studying aging in the striatum. Many prior studies have investigated the rate of glutamate clearance in the striatum, and the results have led to the general conclusion that glutamate uptake is compromised in the aged brain (for review, see Segovia et al., 2001). However, one must use caution when interpreting these results, as the technique used in these studies (microdialysis) does not possess the necessary time resolution to completely study neurotransmitters with rapid release and uptake dynamics such as glutamate (Timmerman and Westerink, 1997). In addition, these data provide only indirect evidence of glutamate transporter regulation (Segovia et al., 2001). To the best of our knowledge, our study is the first to demonstrate that the decrease in glutamate clearance efficiency observed in aging is due in part to a selective reduction in GLAST membrane surface trafficking and not a reduction in total EAAT protein levels.

The results obtained in this study hold promising implications for the field of aging research in the striatum. It has been well established that the aged brain has an increased susceptibility to neurotoxic damage (Liu et al., 1996;
Brewer, 1998), yet a reduction in the concentration of antioxidants has been the only explanation offered for this phenomenon (Vatassery et al., 1998; Lynch, 1998). Our data provide further insight to this increase in susceptibility by examining the role of impaired glutamate transport and its potential contribution to the establishment of a neurotoxic environment in aging (see Fig. 4.9). As we have shown, the glia in the aged striatum exhibit a 55% reduction in the trafficking of GLAST to the membrane surface, limiting its access to the extracellular environment and subsequently reducing the rate at which glutamate is cleared from the synapse. Over time, this gradual elevation of tonic and phasic glutamate concentration may result in increased NMDA receptor stimulation, calcium and sodium influx, and reactive oxygen species (ROS) production (Bondy and Lee, 1993; Lafon-Cazal et al., 1993; Reynolds and Hastings, 1995; Savolainen et al., 1995). These elevated ROS levels could lead to increased lipid peroxidation and glutamate transporter impairment via peroxidation byproducts such as 4-hydroxynonenal (HNE) (Springer et al., 1997; Pedersen et al., 1999; Keller et al., 1997; Blanc et al., 1998). In addition, energy consumption of these medium spiny neurons may increase, as the ATP demand placed on the pumps responsible for restoring ionic gradients will be greater (Pellerin and Magistretti, 1994, 1997; Sibson et al., 1998; Novelli et al., 1988; Kohmura et al., 1990). These are conditions that may be taxing to neurons over a prolonged period of time and may contribute to increased age-related damage of the CNS.

We conclude that the age-associated reduction in L-glutamate clearance efficiency in the striatum of the aged F344 rat is partially due to decreased membrane surface trafficking of GLAST. Altered glutamate transport has long been implicated in the etiology of a number of neurodegenerative conditions in humans, including amyotrophic lateral sclerosis (Rothstein et al., 1992, 1995; Lin et al., 1998), episodic ataxia, seizures (Jen et al., 2005), and the age-related Alzheimer’s disease (AD) (Cross et al., 1987; Cowburn et al., 1988). Researchers have more recently illustrated a strong relationship between AD and GLAST expression. In transgenic mice with the London mutation (a mutation of
familial AD), a reduction in GLAST expression in the neocortex was observed (Masliah et al., 2000). Likewise, biochemical studies have discovered reduced high-affinity L-glutamate uptake sites in cortex extracted from human brain tissue (Cowburn et al., 1988; Cross et al., 1987; Scott et al., 1995). While a number of these studies attributed the link between disease and transporter to decreased GLAST production, more recent studies acknowledged that internal sequestration of transporters due to increased oxidative stress or amyloid beta-peptide deposition could mask detection of a portion of the transporter pool from specific antibodies (Zoia et al., 2005; Butterfield and Lauderback, 2002). Thus, there is a strong possibility that decreased GLAST membrane-association may play a role in AD etiology. Another potential causative factor in AD pertains to the structure of the transporters themselves. According to Scott et al. (1995), variant forms of GLAST are present in the cortex of Alzheimer's disease-afflicted brains. Taken together, these alterations in GLAST may very well account for much of the neurotoxic pathology observed in this age-related disease, as well as the increased susceptibility to damage observed in ischemia and hypoxia. Even so, these data were collected in brain regions other than the striatum, and to the best of our knowledge no studies have been conducted in this region concerning GLAST expression and its correlation with neurotoxic damage. Clearly, additional studies are warranted to determine the role of GLAST in age-related diseases and conditions in both animals and humans.

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Figure 4.1 Glutamate Uptake Rate/Amplitude Correlation

Glutamate clearance signals in the rat striatum. Shown are representative recordings illustrating the positive correlation between glutamate signal amplitude and uptake rate. Tracings are comprised of signal amplitudes representative of potassium-evoked glutamate release in the old (Amplitude: 16 µM, Uptake Rate: 4.38 µM/sec) and young (Amplitude: 35 µM, Uptake Rate: 13.5 µM/sec) F344 rat striatum, as well as a signal representing glutamate release dynamics well beyond the physiological range (Amplitude: 59 µM, Uptake Rate: 27.6 µM/sec). Indicated on the left and right tracings are the portions of the decay curve representing 80% glutamate clearance (T80), while the center tracing denotes the portions of the signal utilized for calculation of uptake rate (maximum amplitude x first order rate of the decay signal). The figure inset shows glutamate signal amplitude versus uptake rate from signals recorded in a young animal (Pearson correlation coefficient = 0.7186, ***p<0.001).
Figure 4.2 Exogenous L-Glutamate Clearance: Representative Tracings
Series of L-glutamate voltammetric recordings showing differences in amplitude matched L-glutamate clearance dynamics in 6, 18 and 24 month old F344 rats. Note the significantly prolonged glutamate clearance time in the late-middle aged and aged striatum in the left tracing, as well as the reduced glutamate uptake rate in the signal on the right tracing corresponding to an aged rat. L-glutamate solution was applied at the time points indicated by the arrows.
Figure 4.3 Average Exogenous Glutamate Clearance Parameters

Average signal parameters from glutamate signals (10-20 and 20-40 µM) recorded in the striatum of 6, 18 and 24 month old F344 rats. Average values in the different age groups were for 1) comparison of L-glutamate signal amplitudes and 2) the time necessary to clear 80% of exogenous glutamate in the striatum of young, late-middle aged and aged rats. Each bar represents mean ± SEM from six, four and seven animals in 6, 18 and 24 month old rats, respectively. ***p<0.001 and **p<0.01 vs. young animal group based on nested between-groups ANOVA followed by Tukey's post-hoc tests.
Figure 4.4 Depth-Related Alterations in Glutamate Clearance Time ($T_{80}$) in the Striatum as a Function of Age

The average $T_{80}$ for each depth (DV -3.0 to -7.0 mm; 0.5 mm increments) was calculated and then compared amongst age groups. Note that while the average $T_{80}$ values of the young and late-middle aged animals display little variance...
amongst depths, the prolonged clearance time found in the dorsal striatum of the aged rats is diminished as placement of the microelectrode proceeds in a ventral direction. Each bar represents mean ± SEM from six, four and seven animals in 6, 18 and 24 month old rats, respectively. ***p<0.001 and *p<0.05 vs. young animal group based on nested between-groups ANOVA followed by Tukey's post-hoc tests.
Table 4.5 Depth-Related Alterations in Uptake Rate as a Function of Aging

The average uptake rates for each depth (DV -3.0 to -7.0 mm; 0.5 mm increments) were calculated and then compared amongst age groups. Glutamate uptake dynamics at most depths are representative of the structure as a whole, the notable exception for both amplitude ranges being the most dorsal depth studied (DV -6.0 to -6.5 mm). Data represent mean ± SEM from six, four and seven animals in 6, 18 and 24 month old rats, respectively. ***p<0.001 and *p<0.05 vs. young animal group based on nested between-groups ANOVA followed by Tukey's post-hoc tests.

**Uptake Rate (µM/sec)**

### 20-40 µM

<table>
<thead>
<tr>
<th>Depth</th>
<th>6 Months</th>
<th>18 Months</th>
<th>24 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0-3.5</td>
<td>10.15 ± 0.22</td>
<td>10.73 ± 0.29</td>
<td>6.56 ± 0.47</td>
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<tr>
<td></td>
<td>***p&lt;0.001</td>
<td>***p&lt;0.001</td>
<td>***p&lt;0.001</td>
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<tr>
<td>4.0-4.5</td>
<td>11.11 ± 0.23</td>
<td>12.87 ± 0.32</td>
<td>7.95 ± 0.34</td>
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<td></td>
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<td>***p&lt;0.001</td>
<td>***p&lt;0.001</td>
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<tr>
<td>5.0-5.5</td>
<td>11.05 ± 0.37</td>
<td>13.21 ± 0.33</td>
<td>8.99 ± 0.40</td>
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<td></td>
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<td>***p&lt;0.001</td>
<td>***p&lt;0.001</td>
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<tr>
<td>6.0-6.5</td>
<td>9.57 ± 0.24</td>
<td>11.20 ± 0.67</td>
<td>9.19 ± 0.42</td>
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</tbody>
</table>

### 10-20 µM

<table>
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<th>6 Months</th>
<th>18 Months</th>
<th>24 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0-3.5</td>
<td>3.52 ± 0.18</td>
<td>4.77 ± 0.27</td>
<td>1.81 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>*p&lt;0.05</td>
<td>***p&lt;0.001</td>
<td>***p&lt;0.001</td>
</tr>
<tr>
<td>4.0-4.5</td>
<td>5.02 ± 0.17</td>
<td>6.24 ± 0.35</td>
<td>2.63 ± 0.18</td>
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<tr>
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<td>***p&lt;0.001</td>
<td>***p&lt;0.001</td>
<td>***p&lt;0.001</td>
</tr>
<tr>
<td>5.0-5.5</td>
<td>5.08 ± 0.25</td>
<td>5.77 ± 0.30</td>
<td>3.73 ± 0.31</td>
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<tr>
<td></td>
<td>***p&lt;0.001</td>
<td>***p&lt;0.001</td>
<td>***p&lt;0.001</td>
</tr>
<tr>
<td>6.0-6.5</td>
<td>4.81 ± 0.25</td>
<td>4.08 ± 0.31</td>
<td>2.46 ± 0.15</td>
</tr>
</tbody>
</table>
Figure 4.6 Striatal L-Glutamate Transporter Protein Levels as a Function of Age

Western blots denoting striatal L-glutamate transporter total protein levels as a function of aging. Numbers located below lanes for each transporter denote age of animals in months.  (A) Total GLT-1 levels per protein (cpm/µg; 30 µg per lane) in the striatum across the three age groups analyzed.  Data represent the mean ± SEM of values (n = 6).  (B) Total GLAST levels per protein (cpm/µg; 15 µg per lane) in the striatum across the three age groups analyzed.  Data represent the mean ± SEM of values (n = 6).  (C) Total EAAC1 levels per protein
(cpm/µg; 60 µg per lane) in the striatum across the three age groups analyzed. Data represent the mean ± SEM of values (n = 6).
Figure 4.7 Representative Biotinylation Assay

Western blot representing the relative immunoreactivities obtained from each step of processing striatal homogenate for determining GLT-1 levels (~65-70 kDa band) in each cellular fraction. For the P2 fraction, 15 µl of sample was loaded from 225 µl of sample volume; for lysate, 15 µl of 225 µl; for supernatant (representing cytosolic protein), 80 µl of 450 µl; for both eluants, 15 µl of 105 µl. During processing of striatal homogenates, 100 µl of 300 µl of sample (representing 500 µg of total protein) was set aside for lysate analysis. Therefore the remaining 200 µl (representing ~330 µg protein) was processed for assessment of cytosolic and plasma membrane levels. Sample volumes stated above represent volumes from sample handling steps plus the addition of sample buffer for electrophoresis. The remaining volume of sample from each step was processed for analysis of GLAST and EAAC1. Numbers above bands represent age group in months.
Figure 4.8 Striatal Synaptosome Protein Recoveries in Plasma Membrane (Bead Eluants) Fractions as a Function of Age

(A) Western blot illustrating GLAST immunoreactivities in avidin bead eluant (representing plasma membrane fraction) from striatal synaptosomes from 6-, 18- and 24-month-old rats. Relative GLAST-IR in the eluted fractions was normalized to relative GLAST-IR for each age group in the lysates (representing total GLAST prior to the addition of the avidin beads). For each experiment, the GLAST-IR in the 18- and 24-month-old fractions was compared against that in the operationally matched 6-month-old fraction (n = 5 animals per age group). Numbers located above lanes denote age group of animals in months. Data presented in bar graphs represent mean ± SEM from five animals in each age group. Asterisks indicate a significant difference as compared to 6 month fractions (*p<0.05 and **p<0.01 vs. young animal group; Tukey’s post-hoc test).

(B) Relative GLT-1 and EAAC1 immunoreactivity in the avidin bead eluant. The GLT-1 and EAAC1 immunoreactivity in the 18- and 24-month-old fractions was compared against that in the operationally matched 6-month-old fraction (n = 5
animals per age group). Data represent mean ± SEM for all age groups.
Figure 4.9 The Role of GLAST in Glutamate Dysregulation
Schematic of a young and aged striatal synapse illustrating the danger of a dysregulation of extracellular L-glutamate. Note that an identical number of glutamate molecules are included in each diagram, and that an approximate 55% reduction in plasma membrane-associated GLAST has occurred in the aged schematic. As this decrease in GLAST surface expression becomes prevalent in the aged striatum, extracellular L-glutamate concentrations become elevated, as is evidenced by the concurrent reduction in clearance efficiency. This may lead to increased ionotropic NMDA receptor stimulation and elevated intracellular levels of Na⁺ and Ca²⁺ ions. This is potentially detrimental to neuron viability, as this environment is conducive to the formation of increased levels of reactive oxygen species and ATP consumption (see Chapter Four Discussion).
Chapter Five: Investigations of Basal L-Glutamate Levels in the Aging Striatum: An *In vivo*, Freely Moving Amperometric Study

Introduction

The strengths of the *in vivo* voltammetric technology employed in the studies of the previous chapters are great in number; it provides us with the high degree of spatial and temporal resolution necessary to study the rapid kinetics of glutamate, while also providing us a means to characterize temporal release and uptake dynamics as a function of regional depth. It must be noted, however, that all previous applications of this technology were conducted in anesthetized preparations, and the potential effects of anesthesia on glutamatergic neurotransmission in the striatum have yet to be determined. The anesthetic of choice for prior experiments, urethane, has been extensively studied in the dopamine circuitry of the brain. The central nervous system depression imparted by urethane is mediated by the interaction of this drug with a wide variety of receptor classes, including glycine, GABA, NMDA, and AMPA receptors (Hara and Harris, 2002), and while urethane may depress neuronal activity in dopamine neuronal terminal fields, electrophysiological responsiveness to the indirect dopamine agonist amphetamine remains unaltered (Warenycia and McKenzie, 1988). This, along with the finding that dopamine uptake remains consistent following administration of urethane in the striatum of unrestrained rats (Sabeti *et al.*, 2003), suggests that urethane is an optimal anesthetic for non-survival surgeries based on its minimal influence on dopaminergic motor circuitry. No such studies, however, have been conducted to determine the potential effects of this drug’s interaction with glutamate receptors and transporters.

The inability to study age-related deficits in motor functioning in anesthetized animals is another concern. *In vivo* electrophysiological studies have provided great insight to dopaminergic motor neuron activity in aging. Stanford and Gerhardt (2001a) demonstrated an age-related decrease in amphetamine-induced firing rates of medium spiny neurons, as well as an
increased pre-drug intraburst firing rate in the amphetamine-excited neurons in the aged animals. This increased basal firing rate of the medium spiny neurons has also been observed in other studies following the eradication of inhibitory dopaminergic afferents via 6-hydroxydopamine lesions, and is believed to be due in large part to the unchecked influence of glutamatergic corticostriatal projections (Centonze et al., 1999; Herrling and Hull, 1980). While these experiments suggest that alterations in striatal glutamate dynamics may play a substantial role in the motor dysfunction of aged animals, there has yet to be any studies conducted to observe age-related differences in glutamate neurotransmission in awake young vs. aged rats. More recently, the multisite microelectrodes utilized in the studies described in previous chapters have been configured for in vivo recordings of extracellular glutamate, and they have reliably detected alterations in glutamate concentrations upon repeated ejections of exogenous L-glutamate in the behaving animal. Whisker stimulation-induced glutamate response in the striatum has been observed as well (Pomerleau et al., 2003). While the current state of the technology lacks the capability to accurately quantify the amount of drug ejected (< 1 µl) and characterize the heterogeneity of glutamatergic terminals within the striatum, it is important to consider the possible ramifications that anesthesia may have on glutamate signaling. Therefore, this technology is extremely beneficial in examining the dynamics of corticostriatal glutamate in the awake, behaving animal. This chapter chronicles the continual development of the chronic implantation of ceramic-based microelectrode arrays and their involvement in laying the foundation for future work correlating alterations in glutamate neurotransmission in aging with behavior.

Materials and Methods

Microelectrode Design

The microelectrode recording site geometrical configuration selected for these studies was the S2 electrode (Fig. 5.1). This configuration consists of four 15 x 333 µm recording sites arranged in a rectangular formation, 30 µm spacing
between sites in an individual pair and a distance of 100 µm between pairs. This particular recording site arrangement allows the investigator to employ a technique known as "self-referencing", a method valuable for further validating the purity and origin of all signals generated, that will now be discussed.

**Microelectrode Selectivity**

One issue that must be addressed in all experiments relying on the specific detection and analysis of a single neurotransmitter of interest is that of selectivity. Currently, there are several techniques available to enhance the selectivity of the microelectrode arrays for L-glutamate. The first method, as discussed in Chapter Two, is the application of a layer of Nafion film which serves to electrostatically repel negatively charged potential interferents such as ascorbic acid. Varying the voltage applied to the Pt recording sites is another useful tool in eliminating signal components which may be attributed to interferents. By reducing the applied voltage from +0.7V to +0.2V, most peroxide may no longer be oxidized at the surface of the electrode recording site. Dopamine and other molecules, however, may still be detected at this voltage. The discrepancy in signal between the two voltages can then be interpreted as glutamate’s contribution to the signal generated at +0.7V vs. Ag/AgCl reference electrode.

A third method that was utilized for this study is self-referencing (Burmeister and Gerhardt, 2001; Day *et al.*, 2006). Figure 5.3 illustrates the concept of this technique. Briefly, the microelectrode recording site pair in closest proximity to the ceramic tip was rendered sensitive to glutamate via the normal addition of the glutamate oxidase/glutaraldehyde/BSA matrix as described in Chapter Two. The remaining recording site pair was coated with an inactive matrix of simply glutaraldehyde and BSA, resulting in sites nearly identical to the other pair with the exception that these lack the capability to detect glutamate. Following the conclusion of each recording session, basal glutamate levels were calculated as described below.
Microelectrode Preparation

Microelectrodes utilized in freely moving recordings must undergo extensive modification prior to implantation and the determination of basal glutamate levels (Fig. 5.2). The initial step in converting the electrode to a stable device for implantation is the addition of four 30 AWG varnished copper wires, each corresponding to a single Pt recording site. Approximately 0.5 mm sections of both ends of each wire were stripped prior to one end being soldered to a pin-hole on the row nearest the microelectrode tip as depicted in Figure 5.2B. The other end was soldered to a gold-plated socket amphenol (Ginder Scientific Part #220-S02). The microelectrode was then coated with five minute epoxy to protect the connections from moisture damage for the remainder of the procedure. All microelectrodes were then coated with Nafion and the glutamate oxidase/BSA/glutaraldehyde matrix as previously described.

The final steps of the conversion process were conducted one day prior to implantation. All amphenols were firmly inserted into a miniature connector (Ginder Scientific, 9-pin ABS Plug, Part #GS09PLG-220) with the aid of pliers so that all amphenols were completely encased. A miniature Ag/AgCl reference electrode was prepared and inserted in a similar fashion (Fig. 5.2C). All copper wires were wrapped around the connector and the microelectrode positioned parallel to the longitudinal axis of the connector. Five minute epoxy was administered to the microelectrode/connector apparatus to insulate all connections from moisture damage and to secure the microelectrode in position.

Microelectrode Calibration/Recording Apparatus

The recording of glutamate signaling in the awake, freely moving rat dictates that the equipment utilized for anesthetized in vivo recordings be modified in such a way as to allow for greater stability in the mobile animal. Therefore, the recording setup for these experiments varies greatly from that used to record in anesthetized rats. The headstage attaches directly to the rat microelectrode assembly, connecting the chronically implanted microelectrode to the FAST-16 recording system. This headstage is comprised of a miniature
connector with five connector pins designated for each of the four recording sites and the Ag/AgCl reference electrode. The connector pins lead to a four channel mini-amplifier positioned in close proximity to the animal in order to minimize noise artifacts. Shielded connecting wire leads to an electrical swivel (commutator) at the ceiling of the recording chamber that contains twelve electrical contacts. This configuration is optimal for allowing free rotation of the headstage as the animal navigates the recording chamber.

*In vitro* calibrations were conducted as per the methods outlined in Chapter Two (Fig. 5.4).

**Microelectrode Implantation**

Surgical implantation was conducted in its entirety in a Vertical Laminar Flow Workstation (Microzone Corp., VLF-2-4). Animals were anesthetized with sodium pentobarbital solution (50 mg/ml) administered in a single injection prior to being placed in a stereotaxic frame. All hair immediately surrounding the surgical area was shaved, and an iodine solution was applied to the shaven area in order to prevent potential infection. A 2 mm midline sagittal incision was made and the skin reflected with bulldog clamps. All underlying fascia was gently displaced with a cotton swab, and the skull thoroughly cleaned with saline solution to enhance the viewing of the skull sutures. A craniotomy was performed as described in Chapter Two. Four additional holes (< 0.5 mm diameter) were created for placement of the Ag/AgCl reference electrode and three stainless steel skull screws (Small Parts Inc., Part #MPX-080-02-M). The microelectrode/connector apparatus was implanted into the dorsal striatum (AP +1.0 mm, ML -2.5 mm, DV -5.0 mm; vs. bregma) based on coordinates provided by the rat brain atlas of Paxinos and Watson (1986). The assembly was secured with the addition of approximately four layers of Hygenic cold cure dental resin (Island Dental Co., Inc., Catalog #844-0794) mixed with Jet Acrylic Liquid (Lang Dental Manufacturing Co., Inc., Reference #1406) which adhered tightly to the skull screws. This dental resin mixture covered the microelectrode/connector
and all exposed wires, with great care taken to prevent resin from adhering to the threads of the microelectrode connector.

Rats were permitted to recover on a heating pad (37ºC) to maintain body temperature as the animals awakened from the anesthetic. Three subcutaneous injections of 1 ml Ringer’s Solution (Fisher Scientific, Catalog #S77939) were administered subcutaneously to enhance recovery. All animals were allowed three days to recover prior to the initial recording session.

**Basal Glutamate Recording Sessions**

Recording sessions were conducted on days four, five and six following microelectrode implantation. All animals were allowed 20 minutes to acclimate to the recording chamber prior to connecting them to the FAST-16 system. Following the initiation of the recording software, approximately one hour was necessary for all channels to attain a stable baseline reading, at which time data relating to basal glutamate concentration were collected. At the conclusion of each recording session (~2 hours), stress-evoked glutamate levels were measured as well. A clothespin was placed at the base of the tail for a five minute period, followed by a thirty minute stress-free time period in order to allow glutamate concentrations to return to basal levels.

**Data Analysis**

Basal and stress-evoked glutamate levels were obtained and analyzed from young (n = 7), late-middle aged (n = 6) and aged (n = 5) F344 rats. Basal glutamate concentrations were obtained by averaging glutamate levels over a ten second time period following the attainment of a stable baseline on channels rendered sensitive to glutamate, and then subtracting concentrations obtained on the accompanying self-referencing channel from this value. Due to the inherent redundancy of each microelectrode, each self-referenced recording pair yielded two striatal basal glutamate levels per day, per animal, resulting in a moderate number of values for each age group. Data were analyzed via a one-way
analysis of variance (ANOVA) followed by a Tukey's post-hoc test. Statistical significance was defined as \( p<0.05 \).

**Results**

The primary aim of these experiments was to validate the efficacy of the ceramic-based microelectrode array as a useful tool to study aging in the striatum of the freely moving F344 rat. As illustrated in Figure 5.5, self-referenced recording of basal glutamate in the striatum resulted in the production of dual representative tracings at each electrode recording site pair. A simple process of subtraction of these two values yielded average basal glutamate concentration (see Materials and Methods). While the average basal glutamate concentration of the late-middle aged striatum (6.27 ± 2.15 µM) was reduced in comparison to the young (7.20 ± 1.19 µM) and aged (7.09 ± 1.58 µM) animals, no significant difference existed amongst the rats of all three age groups (Fig. 5.5).

An inherent advantage that this technology affords us is the capability to further resolve these data to study alterations of basal glutamate levels within age groups on several days. While no significant difference existed amongst basal glutamate concentrations within age groups on the days selected for recording sessions, this data analysis highlighted an interesting age-related phenomenon. Basal levels of the young animals decreased slightly on day five (4.74 ± 1.51 µM) in comparison to the initial day of recording (5.30 ± 1.54 µM), yet basal glutamate concentration approximately doubled in magnitude on day six post-implantation (9.58 ± 2.38 µM). This phenomenon is also present in the striatum of the aged animals, as basal glutamate levels on day four (6.50 ± 0.82 µM) and five (6.15 ± 1.31 µM) were similar, while concentration on day six (10.00 ± 3.48 µM) was much greater in magnitude. In addition to highlighting a potential biological effect of a lack of acclimation to the recording process, this effect confirms that the microelectrodes did not exhibit a reduction in sensitivity throughout the three days selected for the recording sessions. This
phenomenon, however, is reversed in the late-middle aged rats. Basal glutamate levels were elevated on the initial day of recording (9.19 ± 3.86 µM) in relation to both day five (2.75 ± 2.12 µM) and six (3.94 ± 0.26 µM) (Fig. 5.6).

Our data also demonstrate that the administration of a tail pinch stressor to the animals results in a strong response in the glutamate circuitry of the striatum. A typical bi-modal tail pinch stress response is depicted in Figure 5.7. Upon the application of the stress, extracellular glutamate levels increased rapidly to maximum amplitude (1-2 sec.) followed by a time of clearance and sharp fluctuations in baseline that customarily spanned the duration of the tail pinch stressor (Fig. 5.7).

**Discussion**

The utilization of ceramic-based microelectrode arrays for the study of glutamate dynamics in anesthetized animals has proven to be a powerful tool in prior aging research conducted in our laboratory. Several key issues regarding the current state of the technique, however, must be addressed in order to continue the evolution of this technology. For instance, while the urethane anesthetic selected for use in the non-survival surgeries described in Chapters Three and Four has been demonstrated to exert minimal influence over certain aspects of dopaminergic neurotransmission, emerging data within our laboratory have shown that the administration of urethane to awake, freely moving F344 rats reduces basal glutamate levels in the striatum upwards of 40% (unpublished observations). In addition, previous electrophysiological data suggest that alterations in the release and/or uptake properties of glutamatergic corticostriatal projections may play a prominent role in the motor dysfunction observed in normal aging (Stanford *et al*., 2001b), and the anesthetized animal preparation is limited in terms of its capability to properly study this hypothesis.

The current study validates that the modification of our ceramic microelectrodes for chronic implantation in the F344 rat is a feasible method whereby to research alterations in glutamate neurotransmission in the aging
striatum. Here we have demonstrated that, with the aid of self-referencing to enhance the selectivity of the microelectrode, basal glutamate levels are not significantly altered in either the late-middle aged or aged animals in comparison to young rats. Also, while not significant, basal levels tend to rise as a function of time in the young and aged animals, an effect that is reversed in the late-middle aged rats. Finally, our data prove that the effects of a manual stressor may be observed in the glutamatergic motor circuitry of striatum.

As mentioned previously, data obtained following the administration of urethane to the freely moving F344 rat suggest that glutamate release and/or uptake processes may indeed be impaired by this anesthetic. A recent investigation conducted by Day et al. (2006) that utilized the ceramic-based multisite microelectrode array technology documented throughout this dissertation validated this finding. Briefly, a basal glutamate level of ~2 µM in the striatum of the young, anesthetized F344 rat was determined via a variety of approaches, including the application of the universal glutamate transporter D,L-threo-β-benzyl oxyaspartate (TBOA) and implementation of the self-referencing technique outlined within this chapter. In addition, local application of tetrodotoxin resulted in no significant alteration in basal glutamate levels, indicating that the basal extracellular glutamate concentrations obtained in this study were derived almost exclusively from neuronal origin. These basal glutamate levels are approximately one-third of the magnitude of those calculated in the young, freely moving animal, thus further highlighting the importance of eliminating the confounding variable of anesthetic when studying basal glutamate release and clearance properties.

Many groups have recently attempted to investigate changes in glutamate regulation in the freely moving rat via microdialysis. Once again, however, it is crucial to consider the significant limitations imparted by this technique when interpreting these data. Microdialysis provides the user with a high degree of sensitivity (picomolar) and the ability to identify a number of analytes of interest, yet it is particularly lacking in terms of temporal resolution. The typical sample obtained by the performance of microdialysis is representative of analyte
concentration over a period of several minutes, yet there have been significant strides in recent studies to overcome this limitation; Kennedy et al. (2002) coupled microdialysis on-line with capillary electrophoresis to attain a temporal resolution of six seconds. Nonetheless, this study still lacks the subsecond time resolution necessary to study the rapid dynamics of glutamate and has yet to be conducted in an awake, freely moving animal.

Another important consideration when employing a technique that involves chronic implantation and neurotransmitter sampling in the brain of an awake animal is that of mechanical disruption and damage of surrounding neurons and glia. An intricate study conducted by Clapp-Lilly et al. (1999) investigated this phenomenon in the striatum of freely moving rats following microdialysis probe placement. Briefly, light and electron microscopy were utilized to examine the morphological and subcellular characteristics of neurons immediately surrounding the area of implantation. Evidence of non-excitotoxic damage to axons was found at the maximum distance observed from probe placement (1.4 mm), and an estimate of neuronal density revealed a significant loss in volume up to a distance of 400 µm. Cellular organelles such as mitochondria and endoplasmic reticula also displayed swelling and bloating, characteristics of necrosis-induced damage. These processes may be significant confounding factors in studies using microdialysis to study neurotransmitter release in freely moving animals.

In comparison, chronically-implanted ceramic-based microelectrodes induce minimal tissue damage in the striatum. Density of astrocytes surrounding the microelectrode tract remains unaltered over a period of eight weeks, and the number of activated microglia in the area are not significantly elevated (unpublished observations). Taken together, these observations indicate that data collected using these microelectrode arrays may be more indicative of normal physiological neurotransmitter release and clearance than those obtained using more traditional methods of analysis such as microdialysis.

Nonetheless, a preponderance of all previous freely moving aging studies have been conducted using microdialysis. Thus, we must interpret our results in
light of these studies while keeping these technical limitations in mind. Our primary finding of no alteration in striatal basal glutamate levels in aging is in accordance with the data obtained from a number of prior studies (Segovia et al., 1999, 2001a; Del Arco et al., 2001). As discussed previously, glutamate release in the striatum is not an isolated function, as corticostriatal projections receive a high degree of modulation from adjacent dopaminergic projections. A recent study by Segovia et al. (1999) examined this neurotransmitter interaction in the context of aging in awake rats. Briefly, a universal glutamate uptake inhibitor, L-trans-pyrrolidine-3,4-dicarboxylic acid (PDC), was perfused throughout the rat striatum in varying concentrations to increase extracellular glutamate levels, and the effects of these increased glutamate concentrations on dopamine release were observed. It was discovered that while glutamate has a slightly elevated ability to facilitate dopamine release in the middle-aged animals, there were no significant alterations in glutamate-dependent dopamine release characteristics in all age groups in comparison to young rats. This finding, coupled with data that demonstrate no changes in basal dopamine levels in the aging striatum (Segovia et al., 1999; Santiago et al., 1993; Kametani et al., 1995), suggests that if dopamine’s inhibitory influence on glutamate release remains unaltered in late-middle aged and aged rats it is not beyond the realm of possibility that basal glutamate levels remain consistent as well.

It is important to note that while both microdialysis and current voltammetric freely moving studies remove the confounding factor of anesthetic, they lack the capability to study basal glutamate concentration as a function of depth. Due to the heterogeneous nature of the striatum as described in Chapters Three and Four, it may be incorrect at this point in time to deem the basal glutamate levels obtained in our study as representative of the entire striatum. Rather, they are indicative of basal levels at a depth of five millimeters in the dorsal striatum. There also remains the possibility that regional compensatory mechanisms could temporarily mask reduced glutamate clearance or release at this depth, resulting in no net alteration in basal glutamate levels (please refer to Chapter Three for compensation discussion). Thus, it will be necessary to
investigate phasic glutamate release and clearance in the freely moving rat in future studies.

Several key additions to the freely moving recording apparatus will make such studies a possibility in the near future. To address the issue of the current apparatus' inability to accurately eject minute volumes of solution (i.e. < 1 µl potassium and glutamate) into the vicinity of the microelectrode, a fluid commutator will be implemented. The stationary nature of the current microelectrode must also be remedied in order to better study the regional aspects of glutamate regulation in the layered striatum. This will be accomplished following completion of the development of a microdrive that will be compatible with the microelectrode/connector assembly and allow for dorsal/ventral movement of the microelectrode within an animal.

These preliminary results have reliably shown that the modification and implementation of ceramic-based microelectrodes for the study of basal glutamate levels in the awake, freely moving animal is both practical and necessary. The ultimate goal for this technology is to study glutamate temporal release and uptake dynamics during the performance of a behavioral task designed to examine motor efficiency. There is strong electrophysiological evidence implicating dysfunctional glutamate neurotransmission in age-related motor deficiencies. Nonlocomotor-related striatal neurons were found to exhibit increased firing rates and bursting activity during both bouts of locomotion and periods of non-movement, suggesting that tonic hyperactivation of the striatopallidal pathway of the basal ganglia may play a prominent role in the bradykinesia observed in aged animals (Stanford and Gerhardt, 2004). This increased activity is believed to be due in large part to the unchecked release of glutamate at the level of the medium spiny neuron, as both dopamine release (Friedemann and Gerhardt, 1992; Kametani et al., 1995; Rose et al., 1986) and the D₂ receptors responsible for mediating their inhibitory action on glutamate release are diminished in aging (Crawford and Levine, 1997; Han et al., 1989; Hyttel, 1987). Clearly, additional studies are warranted to determine glutamate release and uptake dynamics in the striatum of the freely moving, aged F344 rat.
Figure 5.1 Ceramic-Based Pt Microelectrode (S2 Configuration)

This arrangement consists of four paired microelectrode recording sites in a rectangular formation. Each recording site has dimensions of 15 x 333 µm, with 30 µm spacing between sites in a pair and a distance of 100 µm between pairs.
Figure 5.2 Freely Moving Microelectrode Array Preparation
Series of photographs illustrating the various alterations necessary to convert an S2 microelectrode to the lighter model utilized for freely moving recordings. A) The printed circuit board and attached ceramic microelectrode tip. Numbers denote individual electrode recording site channels. B) Scored and cut circuit board holder with galvanized copper wire soldered into each channel. C) Amphenols containing wires corresponding to each channel positioned in connector. D) Fully assembled connector/microelectrode array and freely moving rat connector (top). The headstage circuit board is illustrated at center.
Figure 5.3 Self-Referencing S2 Microelectrode Layer Schematic
Photomicrograph (center) of S2 microelectrode array and schematics (left, right) describing preparation of electrode for self-referencing recordings. Recording site pair depicted by (A.) are prepared as described in Figure 2.5, while the remaining recording site pair (B.) is prepared in a similar fashion, the notable exception being the lack of glutamate oxidase. This technique provides the investigator with another means to validate the purity of all signals obtained via a method of subtraction. Please refer to Materials and Methods for further discussion.
Figure 5.4 Sample Self-Referencing Microelectrode Calibration
Representative tracings illustrating the enhanced selectivity of self-referencing microelectrodes. The black tracing represents a channel coated with Nafion and the glutamate oxidase/glutaraldehyde/BSA mixture, while the blue tracing is representative of a channel coated with simply the exclusion layer beneath the glutaraldehyde/BSA mixture (sentinel channel). Note that while both channels lack a response to the ascorbic acid interferent, only the channel rendered sensitive to L-glutamate via the application of the enzyme layer responds to subsequent additions of glutamate. The application of peroxide confirms that the sentinel site's lack of response to glutamate is due to the absence of the enzyme layer and not simply a malfunction of the microelectrode recording site.
Figure 5.5 Basal Glutamate Recordings in the F344 Striatum in Aging

Sample striatal basal glutamate concentration recording from a young F344 rat as a function of time. As described in the text, basal glutamate levels were calculated by subtracting the average concentrations recorded on the sentinel sites from those recorded on the glutamate oxidase-coated sites over a period of ten seconds upon the attainment of a stable baseline. As a result, the disparity between these values (as shown above) is a visual representation of the basal glutamate concentration. The inset displays the average striatal basal glutamate concentrations amongst the three age groups. All data represent mean (µM) ± SEM from seven young, six late-middle aged and five aged F344 rats.
Figure 5.6 Time-Dependent Alterations in Basal Glutamate in Aging

Data representative of basal glutamate alterations over the course of the three day recording sessions. Note the sharp increase in basal glutamate concentration on the final day of recording in both the young and aged animals, while basal levels in the late-middle aged rats are reduced over time. All data represent mean (µM) ± SEM from seven young, six late-middle aged and five aged F344 rats.
Figure 5.7 Stress-Evoked L-Glutamate Release

Sample representative tracing of stress-evoked glutamate release upon the application of a manual stressor. A clothespin was placed at the base of the tail following the attainment of a stable baseline, resulting in a rapid and robust increase (~80 µM) in extracellular glutamate concentration, followed by a period of clearance from the synapse. Sharp fluctuations in basal glutamate are present throughout the duration of the tail pinch stressor following the initial rise in glutamate levels.


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