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DYNAMIC L-GLUTAMATE SIGNALING IN THE PREFRONTAL CORTEX AND THE EFFECTS OF METHYLPHENIDATE TREATMENT

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DISSERTATION

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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
Catherine Elizabeth Mattinson

Lexington, Kentucky

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

Lexington, KY

2012

ABSTRACT OF DISSERTATION

DYNAMIC L-GLUTAMATE SIGNALING IN THE PREFRONTAL CORTEX AND
THE EFFECTS OF METHYLPHENIDATE TREATMENT

The prefrontal cortex (PFC) is an area of the brain that is critically important for learning, memory, organization, and integration, and PFC dysfunction has been associated with pathologies including Alzheimer's disease, schizophrenia, and drug addiction. However, there exists a paucity of information regarding neurochemical signaling in the distinct sub-regions of the PFC, particularly the medial prefrontal cortex (mPFC). The mPFC receives glutamatergic input from a number of brain areas, and functional glutamate signaling is essential for normal cognitive processes. To further understand glutamate neurotransmission, *in vivo* measurements of glutamate were performed in the cingulate cortex, prelimbic cortex, and infralimbic cortex of anesthetized rats using enzyme-based microelectrode array technology. Measurements of acetylcholine were also performed to examine the relationship between glutamate and other neurotransmitters in the mPFC. The described studies revealed a homogeneity of glutamate and acetylcholine signaling in the mPFC sub-regions, indicating somewhat uniform tonic and phasic levels of these two transmitters. In the infralimbic mPFC of awake freely-moving rats, rapid, phasic glutamate signaling events, termed "transients" were observed and *in vivo* glutamate signaling was successfully monitored over 24 hour time periods.

The effects of methylphenidate (MPH), a stimulant medication with abuse potential that is used in the treatment of attention-deficit hyperactivity disorder, were measured in mPFC sub-regions of anesthetized rats. Data revealed similar tonic and phasic glutamate levels between chronic MPH-treated rats and controls in all sub-regions. Locomotor data from the chronic treatment period supported the behavioral sensitization effects of multiple MPH treatments. Significant effects were observed in locomotor activity, resting levels of glutamate, and glutamate uptake rates in the infralimbic mPFC of awake, freely-moving animals that received chronic MPH treatment.

Taken together, this body of work characterizes glutamate signaling in the rat mPFC to a degree never before reported, and serves to report for the first time the effects of MPH on glutamate signaling in the mPFC.

Catherine Elizabeth Mattinson

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For Raymond & Helen, Maurice & Madalen, Paul & Marilyn, and Jason

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Chapter One: Introduction

The Functions and Anatomy of the Prefrontal Cortex

The term 'prefrontal cortex' was first used by Hines (Hines 1929) to describe the area of the human brain anterior to the premotor cortex, and the prefrontal area has been defined as the cortex "anterior to the arcuate sulcus on the lateral, medial, and ventral surfaces of the frontal lobe,"(Walker 1940). In the human brain, the PFC encompasses Brodmann's areas 9/46 (dorsolateral PFC) (Brodmann 1909; Rajkowska and Goldman-Rakic 1995) and 10 (rostral PFC) (Brodmann 1909; Semendeferi, Armstrong et al. 2001), as well as parts of other areas. The human medial prefrontal cortex (mPFC) is specifically composed of areas 25, 32, and 10 (Ongur and Price 2000). The monkey brain has similar boundaries, with the only difference being in the definition of Brodmann's area 32. In rats, cytoarchitectural differences make it difficult to assign comparable mPFC Brodmann's areas, and thus areas of the rat brain are demarcated by their connections with other brain structures (Rose and Woolsey 1948). It has been argued that the mPFC does not exist in the brains of mammals other than primates, (Preuss 1995), but within the neuroscience field, the overwhelming amount of literature and research support the hypothesis that rats possess a structure analogous to the primate mPFC (Kolb 1984; Heidbreder and Groenewegen 2003; Uylings, Groenewegen et al. 2003; Seamans, Lapish et al. 2008).

As a brain structure that is involved in many global functions, the prefrontal cortex (PFC) has long been an area of interest for neuroscientists. The PFC has been shown to be critical for higher cognitive processes including attention, memory, learning, decision making, and integration. However, many of these processes are interrupted or dysfunctional in pathologies such as Alzheimer's disease, schizophrenia, and drug addiction.

Dating back to the mid-twentieth century, studies by Jansen and colleagues found that electrical stimulation of the prefrontal cortex in cats resulted in "searching" and "attention" responses (Jansen, Andersen et al. 1955). Neurons in the prefrontal cortex of monkeys have been shown to have increased activation during an attentional task (Suzuki and Azuma 1977)

The PFC has been shown to be necessary for certain memory processes, specifically working memory. Lesions of the PFC have been demonstrated to drastically impair working memory performance in matching-to-sample tasks (Winocur 1992; Granon, Vidal et al. 1994), avoidance tasks (Fritts, Asbury et al. 1998), and visual object discrimination (Ragozzino, Detrick et al. 2002). Additionally, antagonism of the PFC glutamatergic system has been shown to impair spatial working memory performance (Jentsch, Tran et al. 1997) and human fMRI studies suggest that PFC neural activity is responsible for maintaining working memory and attention (Rypma and D'Esposito 1999; Hartley and Speer 2000; Osaka, Komori et al. 2007; Ikkai and Curtis 2011). Learning also involves the PFC, as illustrated in studies with non-human primates (Wise, Murray et al. 1996; Asaad, Rainer et al. 1998; Toni and Passingham 1999; Pasupathy and Miller 2005). Specifically, PFC neurons have been shown to be most strongly activated when cues are changed during a task (Asaad, Rainer et al. 1998), and the PFC has been implicated in learning a visuomotor conditional task during PET scans in non-human primates (Toni and Passingham 1999). PFC neuronal activity during a trial has also been shown to be dependent on the outcome of a previous trial (Histed, Pasupathy et al. 2009).

Decision making is another example of a PFC integration process, as decision making involves evaluating material, predicting outcomes, and then making a choice. In patients with frontal damage, novel choices are difficult (Godefroy and Rousseaux 1997), and decisions may be made with only immediate outcomes in mind (Bechara, Tranel et al. 2000; Manes, Sahakian et al. 2002). Studies using neuroimaging techniques during decision making tasks also implicate the PFC in the decision making process (Paulus, Hozack et al. 2002; Heekeren, Wartenburger et al. 2003; Rogers, Ramnani et al. 2004).

The mPFC has been implicated in the development and maintenance of drug addiction. Lesions of the mPFC have been shown to decrease lever pressing for cocaine in rats (Goeders and Smith 1986) as well as eliminate cocaine-induced sensitization (Li, Hu et al. 1999), and neuronal firing between the mPFC and nucleus accumbens, a structure in the brain involved in drug addiction, has been shown to be correlated just prior to the self-administration of cocaine (Chang, Janak et al. 2000). Withdrawl from amphetamines has been shown to alter glutamate receptor expression in the mPFC (Lu, Monteggia et al. 1999), and mPFC glutamate levels have been shown to increase following a cocaine challenge dose after a withdrawl period in rats previously treated with cocaine (Williams and Steketee 2004). Together, these data indicate that the mPFC at least plays some role in stimulant drug abuse.

The connections of the PFC are many and of differing modalities. The defining characteristic of the PFC is the projection it receives from the mediodorsal nucleus of the thalamus (Leonard 1969; Krettek and Price 1977; Divac, Kosmal et al. 1978; Groenewegen 1988). This projection is glutamatergic in nature, and the mPFC sends a reciprocal glutamatergic efferent to the mediodorsal nucleus as well (Pirot, Jay et al. 1994). Glutamatergic afferents arise from limbic structures such as the amygdala (Bacon, Headlam et al. 1996; McDonald 1996) and the hippocampus (Jay, Thierry et al. 1992), and these structures also receive reciprocal glutamatergic efferents from the mPFC. Within the mPFC, glutamatergic recurrent collaterals from pyramidal neurons synapse on each other (Pirot, Glowinski et al. 1995). The mPFC sends glutamatergic projections to a variety of brain regions including the nucleus accumbens (NAc) (Christie, James et al. 1985), periaqueductal grey matter (Christie, James et al. 1986), ventral tegmental area (VTA) (Taber, Das et al. 1995; Rossetti, Marcangione et al. 1998), and striatum (Karreman and Moghaddam 1996). A second neurotransmitter system, the dopaminergic system, is intimately related to the glutamatergic system in the mPFC. The mPFC receives dopaminergic projections from the VTA (Thierry, Blanc et al. 1973; Lindvall, Bjorklund et al. 1978) that serve to modulate mPFC pyramidal neuronal excitability (Gulledge

and Jaffe 2001). The mPFC can also indirectly influence the NAc (Ungerstedt 1971; Beckstead, Domesick et al. 1979), hippocampus (Gasbarri, Verney et al. 1994; Gasbarri, Sulli et al. 1997), and amygdala (Oades and Halliday 1987) via dopaminergic projections from the VTA (for a diagram of the mPFC/limbic system connections, see Figure 1.1). The neurotransmitter γ-aminobutyric acid (GABA) serves to modulate the mPFC as well. GABA in the mPFC arises from local circuit neurons within the mPFC (Retaux, Julien et al. 1992), as well as from VTA projections to the mPFC (Pirot, Godbout et al. 1992; Carr and Sesack 2000). For the purposes of this thesis, the glutamatergic system is the main focus.

Glutamate Regulation

Glutamate, an amino acid, is the major excitatory neurotransmitter of the central nervous system. Glutamate was first suggested as a neurotransmitter by Hayashi in 1954 (Hayashi 1954), and later confirmed through identification of glutamate receptors and specific antagonists (Davies, Evans et al. 1979; Watkins and Evans 1981).

Glutamate signaling involves multiple receptors, transporters, and neurons as well as glia (representative synapse is diagrammed in Figure 1.2). Glutamate is synthesized in two ways in the brain. Glutamate can be created from glucose via the Krebs cycle, and glutamate can be synthesized via the glutamate-glutamine cycle. Glutamine, a precursor for glutamate synthesis, is readily available in extracellular fluid (Hamberger and Nystrom 1984; Fonnum 1993). Glutamine is transported into the presynaptic neuron via low affinity transport (Erecinska and Silver 1990) and is converted to glutamate via phosphate activated glutaminase, an enzyme that is localized to the mitochondria (Laake, Takumi et al. 1999). Intracellular glutamate is then packaged into synaptic vesicles by a v-type proton-pump ATPase and a vesicular glutamate transporter (VGLUT) (Lewis and Ueda 1998; Bellocchio, Reimer et al. 2000). Calcium-triggered exocytosis allows for the delivery of glutamate from the vesicle into the synaptic cleft. It is important to note that glutamate may also enter the synapse

through calcium-dependent vesicular release from astrocytes (Montana, Ni et al. 2004) or through the actions of the cystine-glutamate exchanger, found on glia (Baker, Xi et al. 2002).

Once in the synaptic cleft, glutamate can act on two different types of receptors: ionotropic glutamate receptors and metabotropic glutamate receptors. Ionotropic glutamate receptors include N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, and kainate receptors (Watkins and Evans 1981) (see Table 1.1 for review). These receptors are all glutamate gated ion channels that conduct Na^+ and K^+, with NMDA receptors also being permeable to Ca^{2+} (Mayer and Westbrook 1987; Ascher and Nowak 1988). The AMPA and kainate receptors are responsible for voltage-independent fast signaling, and are often grouped together as non-NMDA receptors due to their similar responses to glutamate receptor agonists and antagonists (Bettler and Mulle 1995). These non-NMDA receptors also desensitize quickly (Trussell and Fischbach 1989). NMDA receptors, however, are slower to desensitize, and their signaling results in slower changes in postsynaptic current (Collingridge, Herron et al. 1988; Hestrin, Nicoll et al. 1990; Lester, Clements et al. 1990). Additionally, NMDA receptors require membrane depolarization to remove the Mg2+ block that inhibits ion channel permeability (MacDonald and Wojtowicz 1982; Mayer and Westbrook 1985; Flatman, Schwindt et al. 1986).

Metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors with seven transmembrane domains. These receptors signal slower due to their protein coupling, and are classified into three groups. Group I mGluRs consist of mGluR1 and mGluR5, group II includes mGluR2 and mGluR3, and group III is associated with mGluR4, mGluR6, mGluR7, and mGluR8. The groups differ by their methods of signal transduction and their responses to agonists (see Table 1.2 for review). Together, the ionotropic and metabotropic glutamate receptors are essential for glutamate signal transduction.

Glutamate signaling is tightly regulated within the brain, and glutamate uptake is a critically important process for preventing excitotoxicity associated

with excess glutamate. Glutamate is cleared from the synapse by high affinity sodium-dependent excitatory amino acid transporters (EAATs). Five EAATs have been identified to date: GLAST (EAAT1), GLT-1 (EAAT2), EAAC (EAAT3), EAAT4, and EAAT5. GLAST and GLT-1, are localized to astrocytes (Storck, Schulte et al. 1992) (Pines, Danbolt et al. 1992; Rothstein, Martin et al. 1994; Chaudhry, Lehre et al. 1995), while EAAC is localized to neurons (Kanai and Hediger 1992; Rothstein, Martin et al. 1994). EAAT4 is localized to Purkinje cells in the cerebellum (Furuta, Rothstein et al. 1997), and EAAT5 is localized to the retina (Pow and Barnett 2000). GLAST and GLT-1 are responsible for glutamate uptake into glia. Once in the glia, glutamate is converted to glutamine by glutamine synthase, and the resulting glutamine is released into the extracellular space to continue the cycle.

Methylphenidate and Alterations in Glutamate Neurotransmission

Drug abuse is a problem that has plagued humanity for as long as recorded history. In the United States in 2010, the Substance Abuse and Mental Health Services Administration estimated 23 million Americans had used an illicit substance within the month prior to data collection (approximately 8.9 percent of the population) (2011). Alarmingly, in 2011, 50% of high school students reported using an illicit drug by the time they left high school (Johnston and Schulenberg 2012). Illicit drug use has been shown to be associated with risky behaviors, including unprotected sex (DeHovitz, Kelly et al. 1994; Diaz, Chu et al. 1994; Hwang, Ross et al. 2000) and criminal activity (Mumola 1999; Friedman, Glassman et al. 2001). A 2009 study by the National Highway Traffic Safety Administration found 18% of fatally injured drivers tested positive for at least one substance (2010).   Illicit drug use was also associated with almost one million emergency department visits in the U.S. in 2009, and the percentage of pharmaceutical misuse or abuse emergency department visits increased 98% from 2004 to 2009 (2010). The costs associated with drug abuse totaled $140 billion in 1998, with the majority of the costs associated with lost productivity

(2001). Without a better understanding of brain changes associated with drug abuse and potential treatments, these costs will continue to climb as our population increases.

One drug with abuse potential that has gained popularity relatively recently is methylphenidate (MPH; Ritalin), a stimulant that was first synthesized in 1944. In the brain, the mechanism of action for MPH is similar to other stimulants. MPH binds with high affinity to the dopamine transporter (Ritz, Lamb et al. 1987; Heron, Costentin et al. 1994; Pan, Gatley et al. 1994; Wayment, Deutsch et al. 1999), and thus inhibits presynaptic reuptake of dopamine, allowing for increased concentrations of this neurotransmitter in the extracellular space (Hurd and Ungerstedt 1989; Butcher, Liptrot et al. 1991). This increase in extracellular dopamine concentration is responsible for the stimulant effects of the drug (Taylor and Snyder 1971; Roberts, Zis et al. 1975). MPH exerts indirect influence over dopamingergic signaling through the vesicular monoamine transporter -2 (VMAT-2). MPH has been shown to increase intracellular dopamine uptake into vesicles (Sandoval, Riddle et al. 2002), and redistribute VMAT-2 from synaptosomal membranes into the cytoplasm (Volz, Farnsworth et al. 2008). Increases in norepinephrine (NE) release have also been observed following MPH administration, as MPH acts on the norepinephrine transporter (Wall, Gu et al. 1995; Gatley, Pan et al. 1996). MPH differs from other stimulants, such as amphetamine, in that it has no effect on the serotonergic system (Kuczenski and Segal 1997).

The primary use of MPH is as a medication that is prescribed for the treatment of attention-deficit hyperactivity disorder (ADHD) (Sykes, Douglas et al. 1972; Porges, Walter et al. 1975; Charles, Schain et al. 1981). Although it seems counterintuitive to treat ADHD with a stimulant medication, low-level stimulant treatment has been shown to improve cognitive performance in both non-ADHD and ADHD-diagnosed children and adolescents (Gabrys 1977; Mohs, Tinklenberg et al. 1978; Rapoport, Buchsbaum et al. 1978; Balthazor, Wagner et al. 1991; Klein 1993; Gimpel, Collett et al. 2005). MPH is a schedule II controlled substance in the U.S. In 2010, approximately 1.9 million children received MPH

prescriptions (Chai, Governale et al. 2012), and it has been estimated that 5-15% of children and adolescents in the U.S. are taking MPH (Barbaresi, Katusic et al. 2002). MPH is typically taken orally (although transdermal patches are an option), and is available in immediate and extended release formulations. The standard treatment dose averages up to 1.0 mg/kg, although this varies among individuals (Greenhill, Abikoff et al. 1996), and MPH peak plasma concentrations appear roughly two hours after an oral dose is taken (Wargin, Patrick et al. 1983). MPH is generally accepted as one of the preferred treatments for ADHD (Wigal 2009; De Sousa and Kalra 2012).

However, there is some evidence that ADHD is over diagnosed (Meijer, Faber et al. 2009; Bruchmuller, Margraf et al. 2012; Morrow, Garland et al. 2012), thus contributing to an increase MPH and other ADHD stimulant medication prescriptions (Safer, Zito et al. 1996; Olfson, Gameroff et al. 2003). This is a problem, as misdiagnoses could lead to unnecessary exposure to stimulant – based pharmacotherapies, especially in children and adolescents. It has been shown that MPH serves as a reinforcer for children diagnosed with ADHD (MacDonald Fredericks and Kollins 2005), and animal studies have also established the reinforcing effects of MPH (Collins, Weeks et al. 1984; Nielsen, Duda et al. 1984). Rats will self-administer MPH (Botly, Burton et al. 2008; Marusich and Bardo 2009; Marusich, Beckmann et al. 2010), and rats exposed to MPH during adolescence adapt readily to cocaine self-administration as adults (Brandon, Marinelli et al. 2001; Crawford, Baella et al. 2011; Harvey, Sen et al. 2011). These experiments help to illustrate the serious abuse potential of MPH and highlight the importance of carefully considered ADHD diagnoses. Additionally, the physiological side effects of stimulant treatment can be serious. MPH is associated with minor increases in blood pressure and heart rate (Stiefel and Besag 2010), long term treatment has been shown to stunt physical height in children (Zhang, Du et al. 2010) and sleep disturbances are often reported (Greenhill, Puig-Antich et al. 1983; Corkum, Panton et al. 2008).

MPH abuse has been on the increase in college and adolescent populations in recent years (DeSantis, Webb et al. 2008; Dupont, Coleman et al.

2008; Bogle and Smith 2009; Setlik, Bond et al. 2009). MPH is abused in the college population, often as a "study drug" (Sussman, Pentz et al. 2006; Teter, McCabe et al. 2006). In animal studies, MPH has been shown to increase extracellular dopamine in the PFC in rat studies (Bymaster, Katner et al. 2002). Additionally, given the increased use of MPH in recent years, both legal and illicit, a greater understanding of the effects of MPH on neurochemical signaling is warranted.

Although many neurotransmitter systems have been implicated in the development of drug abuse and addiction, the actions of the glutamate system warrant further investigation due to the amount of glutamate signaling that occurs in the brain. Previous studies have linked alterations in glutamate neurotransmission with abuse of substances including cocaine (Pulvirenti, Swerdlow et al. 1989; Pulvirenti, Swerdlow et al. 1991; Witkin 1993), amphetamine (Pulvirenti, Swerdlow et al. 1989; Kelley and Throne 1992), methamphetamine (Hara, Akaike et al. 1987; Earle and Davies 1991; Witkin 1993), heroin (Pulvirenti, Swerdlow et al. 1991; Xi and Stein 2002), phencyclidine (Idriss and Albuquerque 1985; Loo, Braunwalder et al. 1987), and alcohol (Freed and Michaelis 1978; Michaelis, Michaelis et al. 1980; Ledig, M'Paria et al. 1982). In light of the fact that glutamate is responsible for some of the signaling within the reward circuitry in the brain, glutamate likely plays a role in the development and maintenance of addiction. As previously described, the mPFC maintains connections with the mesolimbic dopamine system, which is implicated in the reward circuitry of addiction.

Despite the amount of literature in the area of drug addiction research, MPH's effects on the glutamate system are not yet well understood. However, other stimulants have been reported to increase glutamate levels in the PFC (Reid, Hsu et al. 1997). In the studies detailed in this dissertation, the effects of MPH on the glutamate system in the mPFC were explored using an advanced neurochemical monitoring technique, the microelectrode array.

Microelectrode Array Measurements of Glutamate Neurotransmission

The ability to accurately measure *in vivo* levels of neurotransmitters has long been a topic of interest in the field of neuroscience. Microdialysis has been the preferred method for measuring neurochemistry since the early 1980s (Zetterstrom, Sharp et al. 1983; Benveniste and Huttemeier 1990). Until recently, microdialysis was unchallenged as the gold standard technique for measuring *in vivo* levels of neurotransmitters in the mammalian central nervous system, and the majority of what is known about mPFC glutamate and levels comes from microdialysis studies. However, microdialysis is limited in two critical areas 1) by its temporal resolution (minutes) (Nandi and Lunte, 2009) and 2) by its spatial resolution (millimeters) (Clapp-Lilly et al., 1999). Furthermore it has been shown to cause extensive brain damage (millimeters) around the probe (Clapp-Lilly et al., 1999; Georgieva et al., 1993; Grabb et al., 1998).

Our group has worked extensively on a microelectrode array (MEA) technology that allows for measurements of neurotransmitter synaptic spillover at rates of 1-4 Hz. With platinum recording sites having a small footprint (15 x 333 μm) and measuring neurotransmitters in the nanomolar range, the MEAs allow for the characterization of specific brain sub-regions, such as those in the prefrontal cortex (Bruno et al., 2006; Burmeister et al., 2008; Day et al., 2006; Nickell et al., 2005; Stephens et al., 2009). Additionally, the multiple Pt recording sites of the MEA allow for a self-referencing technique that subtracts background current and provides *in vivo* measurements of glutamate concentration. MEAs can readily be used in anesthetized or awake experiments, allowing for measurements of neurochemistry in multiple brain regions, or while simultaneously monitoring animal behavior.

The advanced spatial and temporal resolution of MEA technology has allowed us to characterize mPFC sub-regional glutamate neurotransmission to a degree never before reported. Additionally, we have been able to use this technique to examine the effects of MPH on the glutamate system, observe dynamic changes in phasic glutamate signaling, and measure tonic glutamate

levels during awake freely-moving animal experiments. The information gained from these experiments will help the neuroscience field to better understand the role of glutamate in the mPFC in drug addiction.

Portions of this chapter have been previously published in the manuscript:

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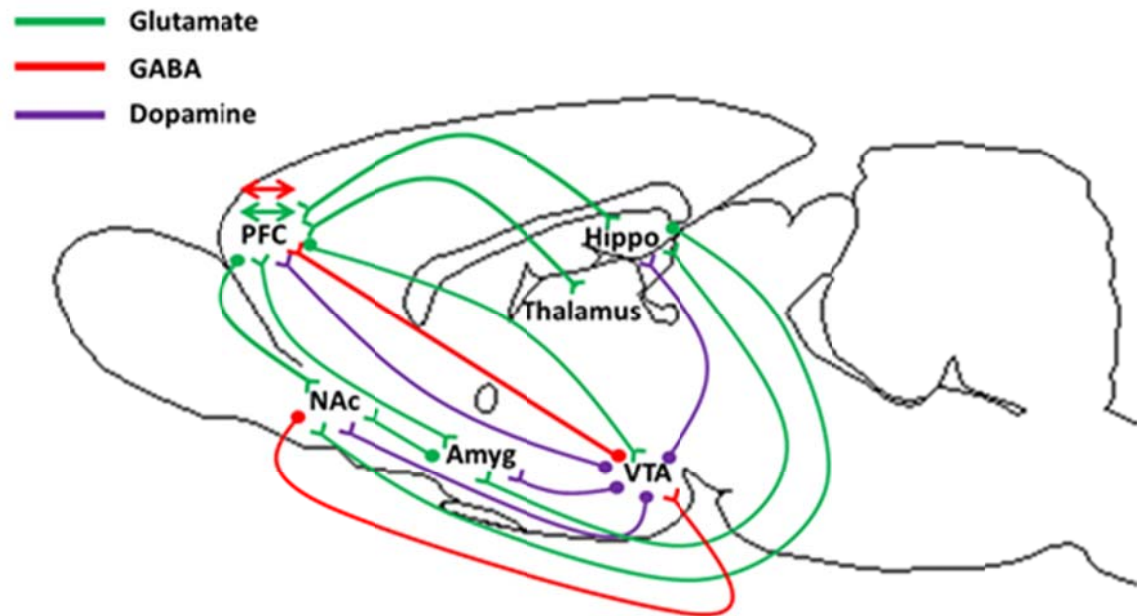
Figure 1.1 Diagram of Cortical and Mesolimbic Circuitry
The innervation of the prefrontal cortex (PFC) is composed of both direct and indirect influences on PFC excitability. The PFC receives glutamatergic input from the mediodorsal nucleus of the thalamus, the hippocampus (hippo), and the amygdala (amyg). All of these connections are reciprocated by the PFC. The PFC also sends glutamatergic projections to the ventral tegmental area (VTA) and the nucleus accumbens (NAc). The hippo and amyg communicate with each other through glutamate projections, and glutamatergic efferents leave the hippo and the amyg and travel to the NAc Within the PFC, pyramidal neurons signal to each other via glutamate, and γ-aminobutyric acid (GABA). The VTA inhibits the PFC via a GABAergic projection as well, and the NAc influences the VTA via a GABAergic projection. Dopaminergic projections originating in the VTA travel to the NAc, amyg, PFC, and hippo. The connections of the ventral pallidum are not pictured in this diagram. Figure adapted from (Paxinos 2009), and (Kelley and Berridge 2002)
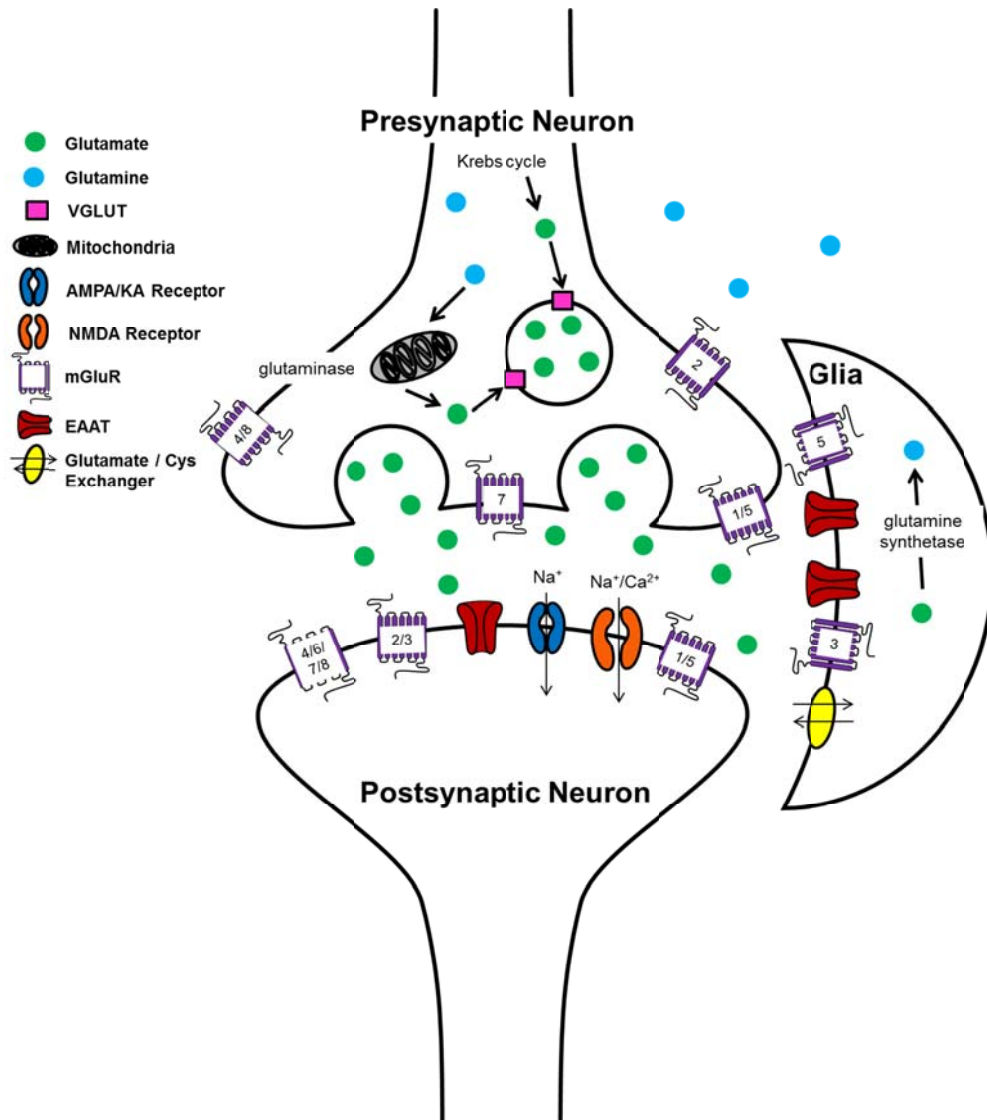
Figure 1.2 Schematic of a Glutamate Synapse

A general description of glutamatergic signaling is depicted in this figure. In the presynaptic neuron, glutamate from the Krebs cycle is loaded in synaptic vesicles by the vesicular glutamate transporter (VGLUT). Glutamate can also be synthesized from glutamine by glutaminase in the mitochondria, and this glutamate can be loaded into synaptic vesicles as well. Once docked at the synapse, vesicles release their stores of glutamate into the synapse, and glutamate is free to stimulate receptors on the presynaptic neuron, postsynaptic neuron, and glia. On the presynaptic neuron, glutamate can stimulate a variety of metabotropic glutamate receptors (mGluRs). On the postsynaptic neuron, glutamate can stimulate Group I, II, or III mGluRs, or ionotropic receptors such as

the NMDA receptor or AMPA/KA receptors. Glutamate can also be taken up into the postsynaptic neuron by the excitatory amino acid transporter EAAC. On the glia, glutamate can be cleared from the synapse by the excitatory amino acid transmitters GLT-1 and GLAST. Glia also possess mGluRs, and glutamate can be returned to the synapse via the actions of the glutamate/cysteine exchanger. Within the glia, glutamate is converted to glutamine via glutamine synthetase. Glutamine diffuses in the extracellular space, and is taken up by presynaptic neurons for further use. Figure adapted from (Schoepp 2001).

Table 1.1: Characteristics of Ionotropic Glutamate Receptors

	Ionotropic Glutamate Receptors		
	NMDA	**AMPA**	**Kainate**
Receptor Selective Agonists	NMDA	AMPA	Kainic acid
Other Agonists	Ibotenate Quinolinate L-aspartate Homocysteate	Quisqualic acid	Domoic acid
Receptor Selective Antagonists	D-AP5 D-AP7 CGS-19755 CGP-37849 CGP-40116 CPP	CNQX DNQX	CNQX DNQX
Other Antagonists	CGS 19755	NBQX GYK1 52466	
Channel Blockers	MK-801 Phencyclidine		

Ionotropic glutamate receptors are divided into three types, based on their interactions with specific small signaling molecules. The discussion of agonist and antagonists is made more complicated by the glutamate and glycine binding sites on NMDA receptors, thus only the glutamate binding sites are described in this table. Abbreviations: NMDA, N-methyl-D-aspartate; D-AP5, (R)-2-amino-5-phosphonopentanoate; D-AP7, amino-7 phosphonoheptanoic acid; CGS-19755, (±)-cis-4-phosphonomethyl-2-piperidine carboxylic acid; CPP, 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; MK-801, dizocilpine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DNQX, 6,7-dinitro-quinoxaline-2,3-dione; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline; GYK1 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine. Adapted from Cooper et al., 2003.

Table 1.2: Characteristics of Metabotropic Glutamate Receptors

	Receptor	G Protein and Function	Location	Agonists	Antagonists
Group I	**mGlu1**	G_q; increased PLC	Postsynaptic neurons	3,5-DHPG quisqualate	CPCCOEt LY367385 EMQMCM JNJ16259685 YM298198
	mGlu5	G_q; increased PLC	Postsynaptic neurons, glia		MPEP MTEP Fenobam
Group II	**mGlu2**	G_i, G_o; decreased AC	Pre- and postsynaptic neurons	DCG-IV APDC LY379268 LY354740 LY404039 LY389795	EGLU LY341495 LY307452
	mGlu3	G_i, G_o; decreased AC	Postsynaptic neurons, glia		
Group III	**mGlu4**	G_i, G_o; decreased AC	Pre- and postsynaptic neurons	L-AP4 L-SOP PPG	CPPG MAP4
	mGlu6	G_i, G_o; decreased AC	Retinal tissue dendrites	3,4-DCPG (mGlu8)	
	mGlu7	G_i, G_o; decreased AC	Pre- and postsynaptic neurons		
	mGlu8	G_i, G_o; decreased AC	Pre- and postsynaptic neurons		

Metabotropic glutamate receptors consist of eight different receptors that are divided into three groups based on their characteristics. Abbreviations: PLC, phospholipase C; AC, adenyl cyclase; 3,5-DHPG, 3,5-dihydroxyphenylglycine; DCG-IV, (2S,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine; APDC, 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylic acid; L-AP4, (*S*)-2-amino-4-phosphonobutanoic acid; L-SOP, *S*-serine-*O*-phosphate PPG, (*RS*)-4-phosphonophenylglycine; 4,3-DCPG, CPCCOEt, 7-hydroxyiminocyclopropan[*b*]chromen-1a-carboxylic acid ethyl ester; EMQMCM, (3-ethyl-2-methyl-quinolin-6-yl)-(4-methoxy-cyclohexyl)-methanone methane sulfonate; MPEP, 2-methyl-6-(phenylethynyl)pyridine; MTEP, 3-[2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine; EGLU, (*S*)-α-ethylglutamic acid; CPPG, (*RS*)-α-cyclopropyl-4-phosphonophenylglycine; MAP4, (S)-α-methyl-2-amino-4-phosphonobutanoic acid. Adapted from Schoepp, 2001.

Thesis Outline

There exists a paucity of information in our current understanding of tonic and phasic glutamate signaling in the mPFC, and thus not much is known about how stimulant drug use affects glutamatergic signaling in this brain region. The studies detailed in this dissertation sought to further explore and characterize glutamate neurotransmission, and explore the effects of MPH on glutamate signaling in the mPFC. In Chapter Three, we investigated glutamate and acetylcholine (ACh) dynamics in the cingulate, prelimbic, and infralimbic sub-regions of the mPFC. The glutamatergic and acetylcholinergic neurotransmission of the individual sub-regions of the mPFC has not been well characterized in the literature, as microdialysis probe size is prohibitive for measuring these distinct brain regions. Our results revealed uniform resting levels for each neurotransmitter among the three sub-regions. KCl-evoked release of glutamate and ACh resulted in similar peak amplitude concentrations within each neurotransmitter among the sub-regions. However, comparison of peak amplitudes and T_{80} values between glutamate and ACh revealed significant differences. These data suggest an unexpected uniformity of both glutamate and ACh signaling in the mPFC.

Chapter Four chronicles the characterization of glutamate signaling in the infralimbic mPFC in the awake freely moving animal. We sought to identify and measure rapid and dynamic changes in glutamate signaling termed glutamate 'transients'. The infralimbic sub-region was identified as an area of interest due to its multiple roles in higher cognitive processes, as well as the potential it has for circadian influences. 24 hours recordings showed significant effects in glutamate resting levels, transient glutamate events, and peak amplitude concentrations. Correlations were found between glutamate transients and glutamate concentrations, as well as glutamate concentrations and locomotor activity. These data confirm the presence of transients in the infralimbic mPFC, and highlight relationships between glutamate signaling parameters during light and dark cycle phases.

The effects of MPH on glutamate signaling were explored in Chapter Five. A depth profile analysis was performed through three sub-regions of the mPFC following chronic treatment with MPH to identify any alterations in glutamate signaling. Rats chronically treated with MPH showed increased locomotor activity during the treatment period as compared to saline controls. Data did not reveal any significant differences in resting levels of glutamate among sub-regions or between treatment groups. Chronically MPH-treated animals trended towards higher glutamate concentration than their saline counterparts. KCl-evoked glutamate release did not differ in peak amplitude, T_{80} values, or uptake rates among sub-regions or between groups, but T_{80} values in the prelimbic and infralimbic sub-regions of chronically MPH-treated rats tended to be increased as compared to chronically saline-treated rats. These data indicate that MPH does produce significant locomotor effects, and that MPH treatment did not significantly alter glutamate neurotransmission. However, the effects of anesthetic are a possible confound, and further studies need to be performed in awake-freely moving animals.

Chapter Six examines the effects of MPH treatment on infralimbic glutamate neurotransmission in the awake freely-moving rat. In addition to avoiding the possible confounds of urethane anesthesia, unanesthetized experiments allow for the simultaneous measurement of behavior and glutamate signaling. Significant effects were found in locomotor activity from the chronic treatment period, as well as during saline, MPH and cocaine challenge doses. Significant effects were also seen in glutamate resting levels during these challenge doses. These data support behavioral sensitization associated with MPH administration, and indicate that infralimbic mPFC glutamate resting levels may play a role in the reinforcing effects of chronic MPH treatment.

Chapter Seven serves to provide a summation of the results and interpretations of this body of work. The findings from these experiments are discussed and future experiments are suggested to continue to expand upon this area of research.

Chapter Two: Materials and Methods

Chemicals

Reagents were purchased from Sigma-Aldrich (St. Louis, MI), except where noted.

Animals

Male Fischer 344 rats or male Sprague-Dawley rats were used for all experiments (Harlan, Indianapolis, IN). The animals were given access to food and water *ad libitum*, and were maintained on a 12 h light: 12 h dark cycle (lights on at 06:00 h). Animals were individually housed, and were monitored by the Department of Laboratory Animal Resources at the University of Kentucky. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Amperometry

Recordings were generated by applying a constant potential of +0.7 V vs. a Ag/AgCl reference electrode to a microelectrode array. The potential allowed for electroactive substances, such as the reporter molecule H_2O_2, to be oxidized at the platinum recording sites on the microelectrode array. The resulting current was digitized by a Fast Analytical Sensing Technology-16 mkII potentiostat (FAST-16 mkII; Quanteon LLC, Nicholasville, KY) into a signal that could be analyzed.

Microelectrode Array Configuration and Preparation

Microelectrode Array Fabrication

Microelectrode arrays were manufactured in conjunction with Thin Film Technologies, Inc. (Buellton, CA), and a detailed description of the process is available in previously published articles (Burmeister, Moxon et al. 2000; Burmeister and Gerhardt 2001). Briefly, a photomask was used to pattern

electrodes onto ceramic wafers (Coors Ceramics Co., Golden, CO). Titanium and platinum were sputter-coated onto the ceramic, and lift off revealed the recording sites and connecting lines. Ceramic electrodes were cut from the wafers and bonded to printed circuit boards (Hybrid Circuits, Inc., Sunnyvale, CA). Microelectrode surfaces, except for the recording sites, were insulated with a polyimide coating to minimize cross-talk between the recording sites.

Microelectrode Array Site Configuration

The S-2 microelectrode pad configuration was used for all experiments. The S-2 microelectrode array consists of four platinum recording sites, each measuring 15 x 333 μm. The sites are arranged two pairs, with 30 μm separating the sites within a pair, and 100 μm separating the two pairs vertically. The bottom pair of sites begins 100 μm from the tapered end of the ceramic tip. This configuration is pictured in Figure 2.1.

Modifications for Awake Freely-Moving Recordings

For unanesthetized experiments, the microelectrode array circuit board was modified to reduce the size of the implant. The process of fabricating this type of microelectrode array has been previously described (Rutherford, Pomerleau et al. 2007; Hascup, Hascup et al. 2008). Briefly, 30 gauge varnished copper wire (Radioshack, Ft. Worth, TX) was cut into one inch long pieces. The coating was removed approximately 1/8" in from either end. One end of the wire was soldered into a small gold-plated socket (Ginder Scientific, Nepean, ON). The other end of the wire was soldered to the "stub" type circuit board of the microelectrode array. After all four channels were soldered, the gold-plated sockets were inserted into a black nine pin ABS plug (Ginder Scientific, Nepean, ON). A miniaturized Ag/AgCl reference electrode was also inserted into the plug. The wires were arranged so that they did not touch each other or solder. Loctite Quickset Epoxy (Henkel Corp., Rocky Hill, CT) was used to cover and secure the wires and electrode to the plug. The epoxy was allowed to cure for a minimum of 24 hours before microelectrode array implantation.

Microelectrode Array Coatings

Microelectrode arrays were prescreened through a cleaning and preliminary testing process to minimize inherent platinum recording site differences. Enzyme coatings (Figure 2.1) were used to make the microelectrode arrays selective for and sensitive to glutamate. This process has been described previously (Burmeister and Gerhardt 2001; Burmeister, Pomerleau et al. 2002; Day, Pomerleau et al. 2006). Glutamate detecting microelectrode arrays were coated with a solution of 0.1 units/µL glutamate oxidase, 1% bovine serum albumin (BSA), and 0.125% glutaraldehyde on one pair of platinum recording sites, while the second pair of recording sites was coated with only the BSA/glutaraldehyde coating matrix solution Three coats of each solution were applied to their respective sites using 10 µl Hamilton syringes (Hamilton Co., Reno, NV), with one minute of curing time allowed between each coat. Microelectrode arrays cured at room temperature for a minimum of 48 hours before use.

Exclusion Layers

An exclusion layer was applied to the microelectrode surface to minimize potential electroactive interferents, such as ascorbic acid, from coming into contact with the platinum recording sites. Different exclusion layers were used for anesthetized versus awake freely-moving recordings. In the anesthetized preparation, a size exclusion layer of m-Phenylenediamine (mPD; 5 mM in 0.5 M phosphate-buffered saline; Acros Organics, Geel, Belgium) was electrodeposited on to the platinum recording sites prior to calibration. mPD excludes interferents based on the size of the molecule, thus excluding ascorbic acid and allowing H_2O_2 to reach the platinum recording sites. In preparation for awake freely moving experiments, Nafion®, a poly-sulfonated Teflon® derivative, was coated onto microelectrode arrays by swirling the ceramic tip of the microelectrode in Nafion® and then baking the microelectrodes for four minutes at 200˚C. Nafion®

excludes interferents based on the charge of the molecule, and is generally thought to be more stable for chronic *in vivo* measurements.

Microelectrode Array Calibrations

All microelectrode arrays were calibrated *in vitro* before implantation. Microelectrode arrays with a Nafion® exclusion layer were soaked in phosphate buffered saline (PBS) solution (0.05 M, pH = 7.4) for a minimum of 30 minutes prior to calibration to activate the exclusion layer. PBS (40 mL) was added to a 50 mL beaker, and placed in a water bath maintained at 37˚C by a recirculating pump (Gaymar Industries, Inc., Orchard Park, NY). A miniature stir bar was added to the beaker, and the solution was stirred by a battery-operated, portable magnetic stir plate (Barnant Co., Barrington, IL). A glass Ag/AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN) and approximately half of the ceramic tip of the MEA were lowered into the PBS. The calibration was performed using FAST system software. During the calibration, ascorbic acid (20 mM; 500 µL) was added as an interferent, and three serial additions of glutamate (20 mM; 40 µL) were added to establish the slope of the signal in response to glutamate (Figure 2.2). Dopamine (2 mM; 40 µL) and H_2O_2 (8.8 mM; 40 µL) were also used as a test substance and a positive control, respectively. Parameters of interest included slope, limit of detection (LOD), linearity of the response to the glutamate additions, and selectivity for glutamate over other substances.

Reference Electrode Preparation

For *in vivo* experiments, a miniature Ag/AgCl reference electrode was created using Teflon®-coated silver wire (A-M Systems, Inc., Sequim, Washington, 0.008" bare, 0.011" coated). The wire was stripped approximately 1/8" from each of the ends. For anesthetized preparations, one end of the wire was soldered into a gold pin amphenol (Mill-Max Mfg. Corp., Oyster Bay, NY). For awake freely moving preparations, one end of the wire was soldered into a gold-plated socket. The other end was placed in a 1 M HCl solution that was

saturated with NaCl. A platinum wire that served as the cathode was also submerged in the plating bath. A nine-volt power supply (Elenco Inc., Wheeling, IL) was used to anodize the silver wire for 15 minutes. After plating, the Ag/AgCl reference electrode was stored in an amber bottle containing 3 M NaCl until use.

Application of Intracranial Solutions

For anesthetized experiments, a glass micropipette was affixed to the microelectrode array to allow for pharmacological manipulation *in vivo.* A glass micropipette (A-M Systems, Inc.; 1 mm o.d., 0.58 i.d.,) was pulled using a vertical pipette puller (David Kopf Instruments, Tujunga, CA). The micropipette was bumped to an inner diameter of approximately 10 μm. Sticky wax (Kerr Corp., Orange, CA) was used to adhere the micropipette to the microelectrode array, with the micropipette tip centered between the four platinum recording sites and approximately 75 μm from the surface of the microelectrode array.

Microelectrode Array Implantation Surgery and Recordings

Anesthetized Surgery Procedures

On the day of surgery, rats were anesthetized with urethane (25%; 1.25 g/kg). Once the toe pinch response was absent, rats were secured in a stereotaxic frame (David Kopf Instruments). The rats were shaved at the surgery site, and then disinfected using ethanol (70%). A craniotomy was performed, and stereotaxic coordinates (Paxinos 2009) were used to determine the location of the mPFC (from bregma, AP: +3.2 mm; ML: ±0.8 mm; DV: -1.5 to -5 mm). A placement hole for the microelectrode array was drilled into the skull, and in the contralateral hemisphere, a hole was drilled for reference electrode placement. The micropipette was loaded with the solution of interest, and connected to a Picospritzer III (Parker Hannifin, Cleveland, OH). The microelectrode array assembly was then stereotaxically placed and lowered to each depth using a microdrive. The rat brain and surrounding tissues were kept moist during the experiment using cotton pellets (Richmond Dental, Charlotte, NC).

Anesthetized Recordings

Once the microelectrode array was lowered to its first depth, the FAST system software started recording. The recordings were allowed a minimum of one hour to reach baseline levels before pharmacological manipulations began. Each time the microelectrode array was lowered to a new depth, a minimum of ten minutes was given for baseline levels to reestablish. After baseline was achieved, multiple ejections of the substance of interest were performed with a minimum of one minute between each ejection. The rate, pressure, and volume of the ejections were controlled by the Picospritzer III. The volume of the ejections was confirmed using a stereomicroscope that was fitted with a calibrated reticule.

Awake Freely Moving Surgery Procedures

Prior to surgery, rats were given an injection of carprofen (Rimadyl®, Pfizer, Inc., New York City, NY; 10 mg/kg, s.c.). Isoflurane (Isothesia, Butler Schein, Dublin, OH) was used as the anesthetic for all survival surgeries. Initial anesthesia was induced with 5% isoflurane, and once the rat was secured in the stereotaxic frame, the isoflurane level was reduced to 2.5%. Artificial tears (Rugby Laboratories, Inc., Duluth, GA) were applied to the rat's eyes. The rat's head was shaved, and three applications each of Providone scrub (The Butler Co., Columbus, OH) and ethanol (70%) were used to disinfect the surgery site. A craniotomy was performed, and five holes were drilled: one for the microelectrode array placement, one for the reference electrode placement, and three for anchoring skull screws. After the skull screws were placed in the skull, dura was reflected and the microelectrode array was stereotaxically placed in the IL mPFC (from bregma, AP: +3.2; ML: ±0.8; DV: -4.5 mm), with the reference electrode positioned in its placement hole. Multiple applications of dental cement (Ortho Jet Powder and Jet Acrylic Liquid, Lang Dental Manufacturing Co., Inc., Wheeling, IL) were used to secure the microelectrode array assembly to the rat's skull. The animals were monitored in their home cages following surgery until

normal locomotion was observed. Animals received daily injections of carprofen for the three days following surgery.

Awake Freely-Moving Recordings

Recordings began a minimum of 72 hours after surgery. The animal was hooked up to the connector which consisted of a miniaturized potentiostat attached to a tether (Figure 2.3), which in turn was hooked up to a swivel in the recording chamber to allow the animal to move freely within the chamber. Within the recording chamber, a Digiscan Animal Monitoring device (Omnitech, Inc., Sioux Falls, SD) measured animal movement during the experiments. After hook up, rats were allowed at least one hour to baseline before data was collected. Rats were monitored during experiments to ensure that they did not interfere with their tethers. After the experiment, rats were returned to their home cages.

<div align="center">

Data Collection

</div>

During each experiment, parameters including resting levels of glutamate, peak amplitude of the glutamate signal (either evoked or spontaneous), T_{80} (time for 80% of the signal to decay back to baseline), and uptake rate were collected and analyzed (Figure 2.4) using a custom Matlab®-based (The Mathworks, Inc., Natick, MAJ) analysis package.   Statistics were performed using GraphPad Prism® 5 (GraphPad Software, La Jolla, CA).

<div align="center">

Histology

</div>

At the end of an experiment, the animal was euthanized under heavy anesthesia. The brain was removed and flash frozen with dry ice. Brains were stored at -80°C until sliced on a cryostat into 40 μm coronal sections. After being mounted on slides, brains were stained with cresyl violet and microelectrode placement in the mPFC was visually confirmed. If the placement could not be confirmed, the animal was excluded from the study.

Portions of these methods have been previously published in the manuscript:

Mattinson, C.E., Burmeister, J.J., Quintero, J., Pomerleau, F., Huettl, P., Gerhardt, G.A. 2011. Tonic and phasic release of glutamate and acetylcholine neurotransmission in sub-regions of the rat prefrontal cortex using enzyme based microelectrode arrays. J Neurosci Methods. 202 (2): 199-208.
Permission was obtained from Elsevier for use.
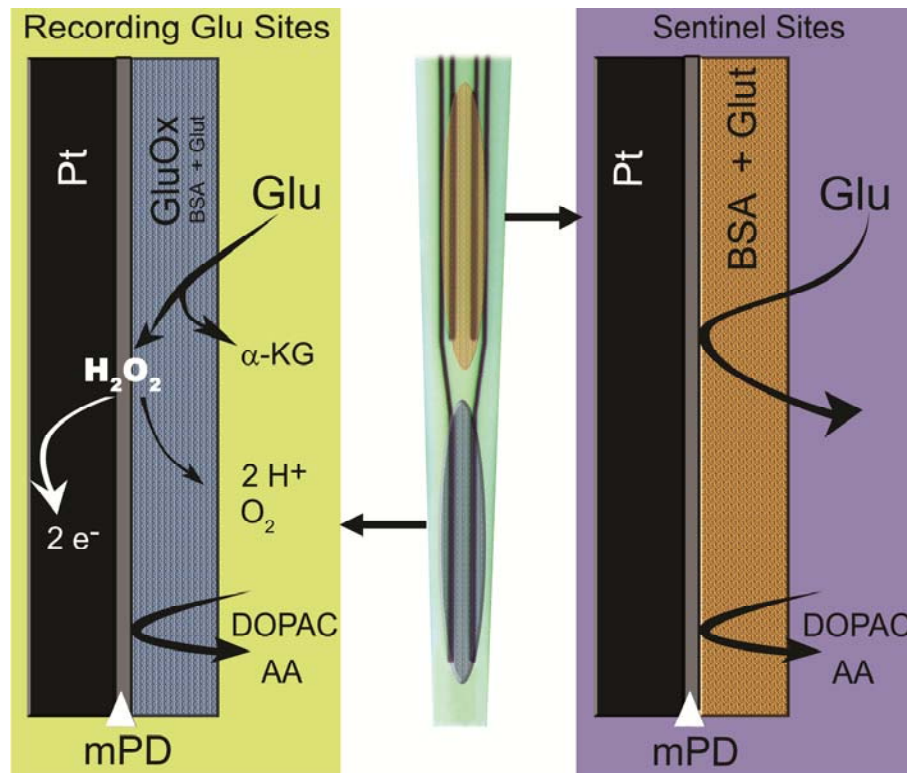
Figure 2.1 Microelectrode Array Glutamate Coating Schematic
The ceramic tip of the S-2 configuration of the microelectrode array features four platinum recording sites arranged in two pairs (center). On the bottom pair of sites, a glutamate oxidase (GluOx) coating is applied (left). When glutamate (Glu) comes into contact with the glutamate oxidase, it is oxidized into H_2O_2 and α-ketoglutarate. The H_2O_2 is free to diffuse, and when it comes into contact with a platinum recording site (Pt) while a potential of 0.7 V vs. Ag/AgCl reference is applied, the H_2O_2 is oxidized and donates 2 electrons to the site. An exclusion layer of *meta*-phenylenediamine (mPD) is electroplated onto the microelectrode array surface, and serves to insulate the sites from potentially electroactive substances such as DOPAC and ascorbic acid (AA). The top pair of sites (right) is coated with the coating matrix lacking enzyme. Thus, it cannot generate a glutamate signal. The signal from the top pair of sites is subtracted from the bottom pair of sites to isolate a self-referenced glutamate signal.

In Vitro Calibration
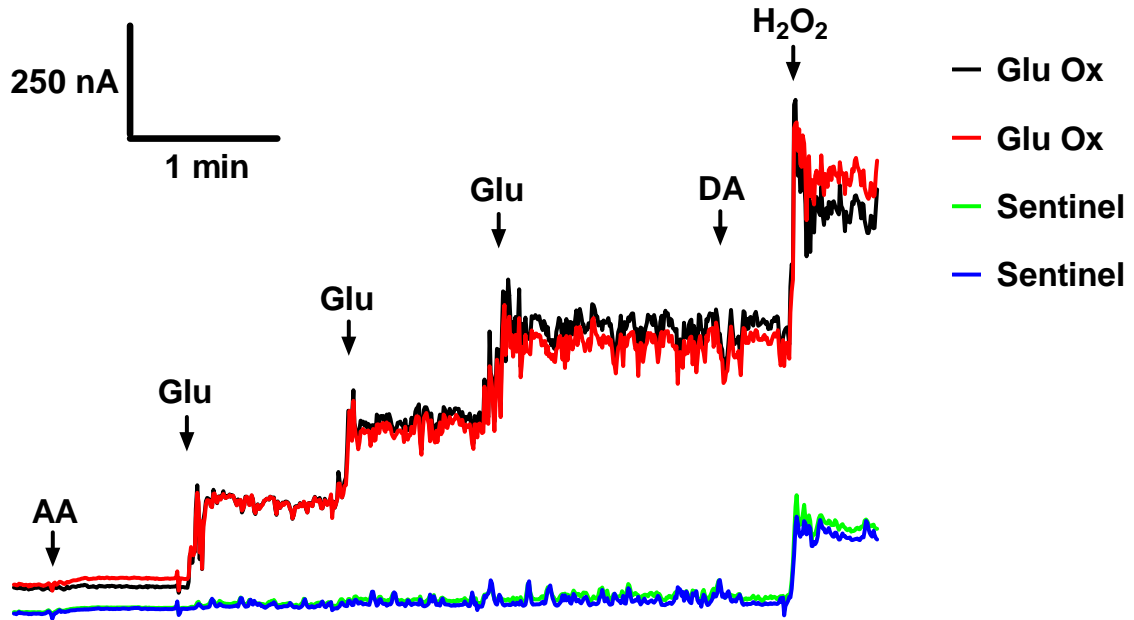
Figure 2.2 *In Vitro* Calibration of a Glutamate Selective Microelectrode Array
An *in vitro* calibration is performed before all experiments. Channels 1 and 2 are coated with glutamate oxidase, and channels 3 and 4 are only coated with the coating matrix. Phosphate buffered saline (PBS, 0.05M, 40 mL) is heated to 37°C in a water bath. A microelectrode array and a reference electrode are both submerged in the PBS, and the recording software is started. After baseline has been established, 250 µM ascorbic acid (AA; final beaker concentration) is added to ensure that the exclusion layer is intact. Next, three serial additions of glutamate (Glu) are pipetted into the beaker, totaling a concentration of 60 µM. These stepwise increases in glutamate are used to calculate the slope of the electrode. An addition of dopamine (DA; final beaker concentration of 2 µM) also serves to ensure that the exclusion layer is working. Finally, an addition of H_2O_2 (final beaker concentration of 8.8 µM) is made as a positive control, to ensure that all recording sites can measure the reporter molecule.
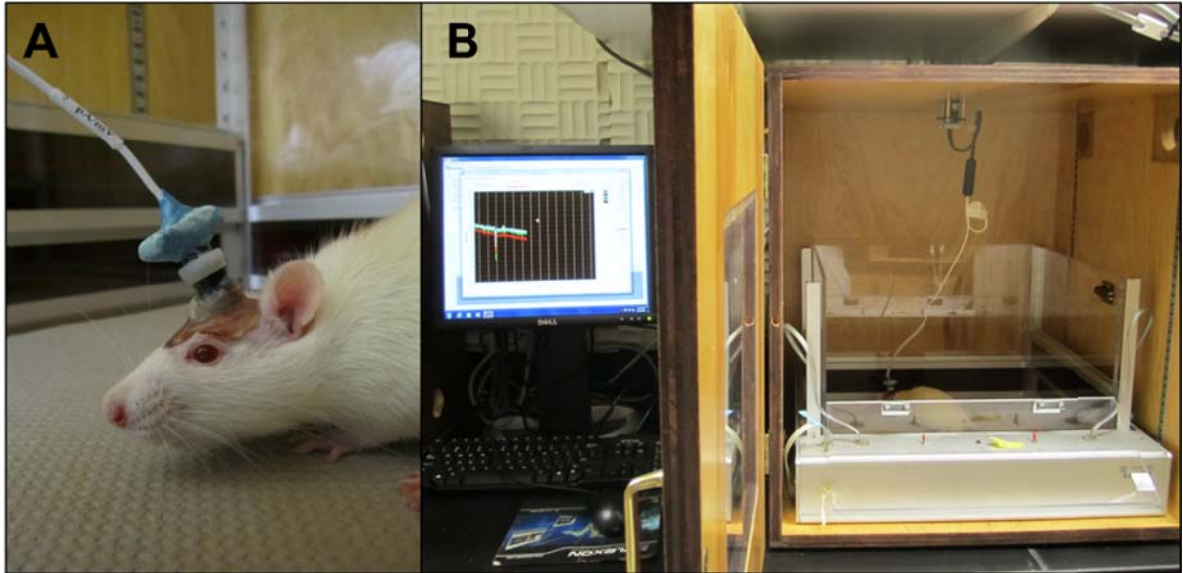
Figure 2.3 Awake Freely Moving Recordings
Prior to the start of the recordings, animals were connected to a miniaturized potentiostat (A). During the recording, rats were free to move in the activity monitoring device inside the wooden recording chamber (B). The recording could be monitored in real-time on the computer screen.
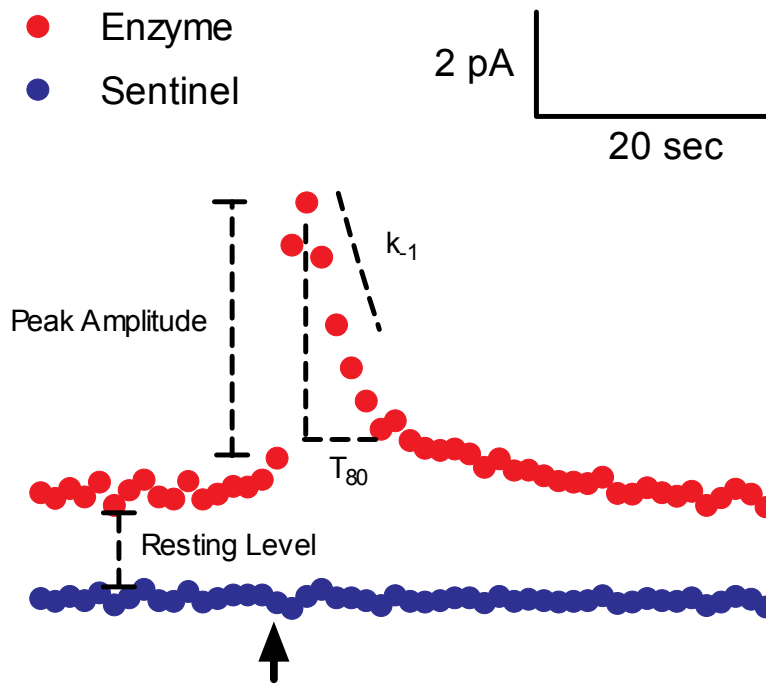
Figure 2.4 Data Parameters of Interest

During the experiment, resting levels of glutamate are calculated by subtracting the signal from the sentinel sites from the signal from the glutamate recording sites. Peak amplitude is the peak magnitude of the signal, either from an evoked release (75-100 nL of KCl (70 mM, pH 7.4); indicated by black arrow) or from a spontaneous release. The T_{80} value is the time it takes for 80% of the signal to decay back to baseline. The uptake rate is the product of the peak amplitude and the k_{-1} value. The k_{-1} value represents the best fit line of the first order decay of the signal.

**Chapter Three: Tonic and Phasic Release of Glutamate and
Acetylcholine in the Rat Prefrontal Cortex Sub-Regions using
Enzyme-Based Microelectrode Arrays**

Introduction

The mPFC is an area of the brain that is critically involved in higher cognitive processes such as learning, memory, and attention, and within the mPFC exist multiple sub-regions, including the cingulate, prelimbic, and infralimbic mPFC. Glutamate and ACh in combination with monoamine neurotransmitters (Berger et al., 1976; Brown et al., 1979; de Brabander et al., 1993; Gratton et al., 1989; Perry et al., 2011; Thierry et al., 1983) are essential for prefrontal cortical functioning. The mPFC receives glutamatergic inputs from the thalamus (Gigg et al., 1992; Leonard, 1969; Pirot et al., 1994), basal forebrain (Henny and Jones, 2008), hippocampus (Gigg et al., 1994; Jay et al., 1992), and amygdala (McDonald, 1996). This brain area also receives ACh input from the basal forebrain as part of the basal forebrain cortical cholinergic system (Mesulam et al., 1983). The loss or antagonism of these specific glutamatergic and acetylcholinergic afferents has been shown to result in deficits in spatial working memory (Bailey and Mair, 2005; Romanides et al., 1999), passive avoidance tasks (Torres et al., 1994), and attention (McGaughy et al., 1996), highlighting the importance of these neurotransmitter systems in daily cognitive functioning. Dysregulation of the glutamatergic and/or acetylcholinergic circuitry in the mPFC has been implicated in disorders such as schizophrenia (Bauer et al., 2008; Benes et al., 1992; Crook et al., 2001; Olbrich et al., 2008; Oni-Orisan et al., 2008; Sarter et al., 2005; Tsai et al., 1995; Woo et al., 2008; Zmarowski et al., 2009), Alzheimer's disease (Burbaeva et al., 2005; Francis et al., 1999; Kashani et al., 2008; Perry et al., 2011; Trabace et al., 2007), and drug addiction (Arnold et al., 2003; Lu et al., 1999; McFarland et al., 2003; Sofuoglu and Mooney, 2009; Williams and Steketee, 2004). The overlap and possible interactions of these two neurotransmitter systems underscores the importance of understanding both the tonic and phasic aspects of glutamate and ACh

neurochemical signaling in the mPFC and could lead to a better understanding of alterations associated with neuropathologies.

In this chapter, we report experiments that used our MEA technology to gain insight into potential sub-regional differences in tonic and phasic release of glutamate and ACh in the rat mPFC. We first measured resting neurotransmitter levels within the cingulate, prelimbic, and infralimbic mPFC. Secondly, we examined the dynamics of KCl-evoked release of glutamate and ACh in the cingulate, prelimbic and infralimbic to investigate additional transient or phasic release of these neurotransmitters. These experiments provide new information regarding the regional signaling properties of glutamate and ACh in different sub-regions of the mPFC, an area of the brain that is implicated in many neuropathologies.

Materials and Methods

Animals

Male Fischer 344 rats (Harlan, Indianapolis, IN), aged 3 – 6 months, were used for all experiments (n = 17). For care, see Animals.

Microelectrode Array Preparation

Ceramic based conformal MEAs were made sensitive to and selective for glutamate or ACh detection using enzyme layers coated onto the surface of platinum (Pt) recording sites (Day, Pomerleau et al. 2006; Burmeister, Pomerleau et al. 2008). Glutamate coatings are detailed Microelectrode Configuration and Preparation – Microelectrode Coatings. MEAs configured for ACh detection (Figure 3.1) required a dual enzyme coating scheme to produce the H_2O_2 reporter molecule, as ACh is also non-electroactive. Acetylcholinesterase was used to hydrolyze ACh into acetate and choline, and then choline was catalytically oxidized by choline oxidase to produce H_2O_2 (Bruno et al., 2006). MEAs were coated with a solution of 0.2 units/µl choline oxidase, 0.9% BSA and

0.11% glutaraldehyde on all four sites and allowed to cure at 4°C for a minimum of 48 hours. MEAs were then coated with 0.83 units/µl human recombinant acetylcholinesterase, 0.83% BSA, and 0.10% glutaraldehyde on one pair of sites, and the BSA/glutaraldehyde solution on the other pair of sites to ensure comparable coating thickness between the pairs of sites and to allow for self-referencing (Burmeister et al., 2008). In order to enhance selectivity a size exclusion layer of mPD was electrodeposited on to the Pt recording sites prior to calibration. Each of the MEA sites were tested to determine selectivity vs. dopamine during an in vitro calibration to ensure that the mPD electrodeposited onto sites was uniform. The signal from the choline oxidase-coated sites was subtracted from the combined acetylcholinesterase/choline oxidase sites to determine the self-referenced ACh signal.

All recordings were performed at a final output frequency of 1 Hz. Before each experiment, MEAs were calibrated in vitro using stepwise additions to increase concentrations of glutamate or ACh, as well as, electroactive interferents as previously described (Burmeister and Gerhardt, 2001; Burmeister et al., 2002). Sensitivity, linearity, limit of detection, and selectivity over electroactive interferents were calculated for all MEAs using the in vitro calibration data prior to experimentation. MEAs used in glutamate experiments had an average slope of -4.2 ± 2.0 pA/µM, an average limit of detection of 0.99 ± 0.9 µM, a linearity $R^2 > 0.99$, and an average selectivity of 57:1 ± 37. In ACh experiments, MEAs averaged a slope of -5.8 ± 2.6 pA/µM, a limit of detection of 0.30 ± 0.44 µM, a linearity R2> 0.99, and a selectivity of 420:1 ± 720.

In Vivo Recordings

Animals were anesthetized for *in vivo* recordings, and surgery was performed (Microelectrode Array Implantation Surgery and Recordings – Anesthetized Surgery Procedures; Anesthetized Recordings). The micropipette attached to the MEA was filled with an isotonic KCl solution (70 mM KCl, 79 mM NaCl, 2.5 mM $CaCl_2$ • $2H_2O$; pH = 7.4) and connected to a Picospritzer III (Parker Hannifin Corp, NJ) to allow for local applications of KCl into the extracellular

space . The MEA/micropipette assembly was lowered into the mPFC (AP: +3.2 mm; ML: ±0.8 mm; DV: -1.5 to -5 mm; from bregma (Paxinos and Watson, 2009) using a Narishige MO-10 microdrive. The MEA assembly was lowered in 0.5 mm increments and recordings were taken at each depth to record glutamate or ACh levels along the dorsal-ventral axis of the mPFC. Once lowered into place, basal levels were established over ten minute recording durations, and then KCl solution was ejected (75-100 nl) to evoke glutamate or ACh signals

Histology

At the end of the experiment, animals were euthanized and histology was performed to confirm MEA placement (Histology; Figure 3.3). Only data collected from confirmed electrode placements in the different sub-regions of the mPFC were included in the final data analysis; data from two animals were excluded in these studies.

Data Analysis and Statistics

Collected data were processed using a custom Matlab®-based analysis package (Data Collection). Data parameters analyzed were resting (tonic release) neurotransmitter levels (average of the ten data points prior to the first ejection at each depth) and data from KCl-evoked release (phasic release) of glutamate or ACh that included: peak amplitude, T_{80} (time for 80% of the signal to decay to baseline), and k-1, best fit line of first order rate constant of the signal return to baseline. Glutamate and ACh KCl-evoked peaks were volume matched (75-100 nL) for data analysis. Outliers were eliminated from data using a Grubb's test before analyses were conducted. As data was not always attainable at every depth throughout the depth profile due to clogging of the tips of the micropipettes, occasional data drop out occurred. Thus, a one-way ANOVA with a Bonferroni *post hoc* test was used to analyze the KCl-evoked data and resting levels between glutamate and ACh were analyzed using a two-way ANOVA. Correlations were also performed to compare the log of KCl-evoked data and resting data for each neurotransmitter. Significance was defined as $p < 0.05$.

Results

Glutamate and Acetylcholine Resting Levels Are Stable Across Sub-Regions

Glutamate and ACh recordings in the mPFC focused on three sub-regions: the cingulate, prelimbic, and infralimbic. For both neurotransmitters, resting levels were calculated using the self-referenced signals. When average resting levels of glutamate were compared among brain regions (Figure 3.3), they were found to be similar ($F(2,18) = 0.069$; $p = 0.93$), with only a 0.70 µM difference between the highest and lowest levels. Interestingly, analyses of average ACh resting levels revealed comparable resting levels (Figure 3.3; $F(2,17) = 0.47$; $p = 0.63$) of 0.60 – 1.0 µM for the cingulate, prelimbic and infralimbic mPFC. There was a significant main effect between the two neurotransmitters ($F(1,35) = 16$; $p = 0.0003$), however, the resting levels of glutamate and ACh were not significantly different within sub-regions of the mPFC.

KCl-Evoked Glutamate Release is Similar Among Sub-Regions in the Medial Prefrontal Cortex

KCl-evoked release of glutamate was successfully carried out in the cingulate, prelimbic and infralimbic sub-regions of the mPFC. Local application of KCl was seen to produce detectable and transient increases in extracellular glutamate that lasted for, on average, 3-5 seconds. Representative tracings of KCl-induced glutamate release from each brain region were compared (Figure 3.4) to illustrate the similar peak amplitudes among the sub-regions of the mPFC. The tracings indicate the uniformity among signals, with similar peak rise times, peak amplitudes, and similar decay profiles.

The glutamate peak amplitudes, T_{80} values, and k-1 values were used to characterize KCl-evoked release of glutamate. Measures of glutamate release revealed no significant differences in the peak amplitudes of glutamate (Figure 3.4; $F(2,15) = 1.8$; $p = 0.21$) or T_{80} values (Table I; $F(2,16) = 1.9$; $p = 0.18$) in the

different sub-regions. Glutamate k-1 values were also comparable among brain regions (Table 3.1; k-1: $F_{(2,16)} = 0.31$; $p = 0.74$). Correlations of the log of KCl-evoked release of glutamate with resting levels of glutamate were not significant for any sub-region of the mPFC (Figure 3.8; cingulate, $p = 0.97$; prelimbic, $p = 0.12$; infralimbic, $p = 0.20$).

Acetylcholine Signaling Parameters are Similar Among Brain Regions

Studies were carried out to investigate KCl-evoked release of ACh in the sub-regions of the mPFC. Similar to the glutamate studies, KCl was seen to produce detectable and transient release of ACh lasting 5-10 seconds in the mPFC. The similarity among ACh release and uptake from each brain sub-region are further illustrated in Figure 3.5. KCl-evoked ACh peaks rose to peak amplitudes in a similar manner, and decayed at similar rates, resulting in comparable signaling parameters among the cingulate, prelimbic, and infralimbic mPFC. When ACh signaling parameters were compared among the three brain sub-regions, no significant differences were found suggesting homogeneity of ACh neurotransmission in the mPFC.   Peak amplitudes showed uniformity, (Figure 3.5; $F_{(2,26)} = 1.1$; $p = 0.35$) thus further characterizing KCl-induced ACh release among the brain sub-regions. When the log of KCl-evoked releases of ACh were correlated with ACh resting levels within each sub-region, a significant difference in the cingulate was found (Figure 3.7; Cg1, $p = 0.049$).   No significance was found in either the prelimbic (Figure 3.7; $p = 0.93$) or the infralimbic (Figure 3.7; $p = 0.29$). ACh uptake parameters including T_{80} values were also homogeneous (Table 3.2; $F_{(2,23)} = 0.32$; $p = 0.73$), as were k-1 values (Table 3.2; k-1: $F_{(2,26)} = 0.096$; $p = 0.91$).

Comparisons of KCl-Evoked Glutamate and Acetylcholine Reveal Significant Differences Between the Neurotransmitters

We compared the peak amplitudes of both neurotransmitters to contrast mPFC glutamate and ACh KCl-evoked release (Figure 3.6).   While significant

differences were not found within each neurotransmitter, significant differences did exist between neurotransmitters. ACh peak amplitude measurements ranged from 5.3 – 7.1 µM, and glutamate peak amplitude concentrations measured 1.8 – 2.9 µM. Analyses revealed that the peak levels of ACh in the cingulate were significantly higher than glutamate signals in all sub-regions ($F(5,41) = 5.4$; $p = 0.0007$). As compared to glutamate levels in the cingulate sub-region, cingulate ACh levels were greater by 144%. KCl-evoked ACh release in the cingulate was also greater by 255% and 294% compared to glutamate levels in the prelimbic and infralimbic, respectively.

We also compared T_{80} values between the two neurotransmitters, and found significant differences in decay times between glutamate and ACh ($F(5,39) = 11$; $p < 0.0001$). In the cingulate mPFC, ACh decay times were increased 200% as compared to glutamate decay times in the same brain sub-region (Figure 3.6). ACh T_{80} values in the prelimbic and infralimbic were increased 190% and 330% as compared to glutamate values, respectively.

Discussion

These are the first studies comparing both tonic and phasic release of glutamate and ACh levels on a second-by-second time scale in individual sub-regions of the anesthetized rat mPFC. The results of our studies demonstrate that we can reliably measure both tonic and phasic glutamate and ACh release *in vivo* using the MEA technology. The spatial resolution of our MEAs allowed for the measurement of these neurotransmitters in discrete mPFC sub-regions such as the cingulate, prelimbic, and infralimbic, and the sampling rate offered the ability to measure individual KCl-induced glutamate and ACh peaks. Interestingly, our data support that the glutamate and ACh neurotransmitters systems have tonic and phasic release that are uniform within the sub-regions, but there were significant differences in both resting and KCl-evoked release of glutamate vs. ACh in the mPFC.

Resting Levels of Glutamate and Acetylcholine

These studies did not find significant differences in resting glutamate levels within the cingulate, prelimbic, and infralimbic mPFC, although there was marked variability within each mPFC sub-region. We had initially hypothesized that we would see differences among the sub-regions for glutamate resting levels, as glutamate is tightly regulated in the mPFC (Hascup et al., 2010). Thus, the relative homogeneity of our glutamate resting levels within the mPFC sub-regions was unexpected, but not unprecedented. Previous MEA work within our lab has shown that glutamate resting levels in anesthetized animals do not differ greatly between brain regions. We have reported resting glutamate levels in anesthetized rats of 1.6 ± 0.30 μM in the frontal cortex and 1.4 ± 0.20 μM in the striatum, and in the hippocampus, we have measured glutamate concentrations ranging from 2.5 μM in the CA1 region to 3.4 μM in the dentate gyrus (Stephens et al., 2009). Our glutamate resting levels of 3.7 - 4.3 μM in mPFC sub-regions are higher than previously reported levels from MEA studies in other brain regions, but not unreasonable. Resting levels of glutamate that have been measured using microdialysis in the mPFC have ranged from 0.23 ± 0.061 μM to 3.8 ± 0.40 μM (Del Arco et al., 1998; Ferraro et al., 2001; Hugues et al., 2007; Karreman and Moghaddam, 1996; Lupinsky et al., 2010) and 3.0 ± 0.60 μM in the striatum (Miele et al., 1996). Compared with microdialysis measures, our resting levels of glutamate are over 600% higher than previously reported microdialysis glutamate levels in the mPFC of the same strain (Selim and Bradberry, 1996). However, this difference in concentration between MEA technology and microdialysis measures can be explained because the MEAs sample the extracellular space of the brain directly rather than being separated by a membrane containing a net analyte concentration of zero driving diffusion into the microdialysis probe. Also, far less edema or gliosis occurs with MEAs than microdialysis probes, allowing for better estimates of neuronally-based release vs. release from other sources.

The Pt recording sites on the MEA only measure neurotransmitters that come into contact with the surface of the electrode (Burmeister et al., 2000). We

have hypothesized that our increased glutamate resting levels and higher ACh resting levels may be the result of the enhanced spatial resolution of our MEAs that allows us to be closer to synaptic spillover, as evidenced by studies using tetrodotoxin (Day et al., 2006). Microdialysis probes may be able to pick up some spillover, but the size of the probe membranes and the inherent time constant of the microdialysis membrane (Schultz and Kennedy, 2008) are prohibitive for measuring neurochemical activity at the level of synapses (Timmerman and Westerink, 1997). Thus, the MEAs measure the concentration of the neurotransmitter that resides in the extracellular space and do not record a fractional level. Our recently reported studies support that this translated to a 5-10 fold difference of resting levels of glutamate measured by MEAs versus microdialysis in the awake animal and is largely due to the direct measurement of the neurotransmitter concentration in the extracellular space by the MEA technology (see Hascup et al., 2010).

In our first paper using MEA technology to measure ACh (Bruno et al., 2006), we did not report resting levels. However, in this set of studies we are now able to report for the first time in vivo resting levels of ACh using MEA technology. Similar to our glutamate hypothesis, we predicted that we would see significant differences in ACh resting levels among the sub-regions of the mPFC. We did not find any significant differences in resting levels of ACh among the mPFC sub-regions, and little variability existed within each sub-region. Reported resting levels of ACh in the mPFC have widely varied from 0.58 ± 0.14 nM to 3.0 ± 0.3 µM in microdialysis studies (Arnold et al., 2001; Hernandez et al., 2008; Huang et al., 2008; Mork et al., 2009; Neigh-McCandless et al., 2002). Our ACh measurements of 0.59 – 0.96 µM fall into the higher end of the range of these previously reported microdialysis ACh concentrations. Microdialysis, with its collection times on the scale of several seconds to minutes, may allow for more ACh hydrolysis by acetylcholinesterase, resulting in lower reported concentrations. Thus, many scientists who use microdialysis for measuring ACh levels also perfuse an acetylcholinesterase inhibitor, such as neostigmine, to prevent ACh breakdown (Nirogi et al., 2010; Ragozzino et al., 1996). However,

this technique may artificially affect the measured ACh levels in the extracellular space. The detection of higher levels of both glutamate and ACh can be attributed to the improved spatial and temporal resolution of the MEA.

The present series of studies was carried out in anesthetized animals to take advantage of the brain mapping capability of the MEAs to record glutamate and ACh levels in different sub-regions of the mPFC in the same rat. Work from our group has shown that the administration of urethane anesthesia to awake, freely moving rats resulted in a 58% decrease in resting glutamate levels (Rutherford et al., 2007). Thus, it is likely that the anesthetic attenuated glutamate resting levels in these studies and possibly affected ACh levels as well. Even with this possible attenuation, our glutamate resting levels are still relatively high compared to microdialysis studies, but this likely relates to the aforementioned properties of the MEA measurements as compared to microdialysis. By contrast, to date we do not have any indication that urethane affects KCl-evoked release of glutamate based on our work (data not shown). Urethane has also been shown to enhance the actions of the nicotinic ACh receptor, but only to a small degree (Hara and Harris, 2002). Future studies will be carried out in the awake animal to circumvent the potential effects of anesthesia. It should also be noted that glutamate and ACh levels in the mPFC vary with rat strain. For example, glutamate levels in awake rats have been reported from 0.22 µM in Sprague-Dawley rats (Ferraro et al., 2001) to 4.2 µM in Wistar-King rats (Abekawa et al., 2000). ACh levels in rats exhibit an even greater range of resting levels, from 0.58 nM in awake Sprague-Dawley rats (Huang et al., 2008) to 3.0 µM, also in awake Sprague-Dawley rats (Mork et al., 2009). Thus, the concentrations reported in the present studies were likely affected by anesthesia and were likely dependent on the strain used.

A comparison of our resting levels of glutamate and ACh in mPFC sub-regions did not reveal any differences within neurotransmitters, but we did see a significant main effect between neurotransmitters, suggesting that the neurotransmitter resting levels do vary between glutamate and ACh within the mPFC. This may be due to the differential innervation of the mPFC by theses

neuronal systems and/or the regulation of these neurons by other neurons and signaling molecules. Interestingly, our resting levels of ACh were extremely similar between the prelimbic and infralimbic sub-regions, suggesting that ACh regulation is uniform in these areas. This is supported by microdialysis experiments that have sought to characterize sub-regions of the mPFC, and have reported similar resting levels between the prelimbic and infralimbic mPFC (Hedou et al., 2000). However, it should be noted that there was overlap between sub-regions due to the spatial resolution of the microdialysis technique.

Measurements of KCl-Evoked Release of Glutamate and Acetylcholine in the Rat Medial Prefrontal Cortex

Although no significant differences were found in KCl-evoked glutamate release, both the peak amplitudes and the T_{80} values followed trends along the dorsal-ventral axis in the mPFC (Table 3.1). In previous microdialysis studies that used KCl to evoke neurotransmitter release, the infusion of KCl into the mPFC resulted in an increase in glutamate levels of 90% (Frantz et al., 2002). KCl has also been reported to have no effect on glutamate levels (Welty and Shoblock, 2009). However, these studies were not specific to sub-regions of the mPFC, and they did not measure rapid changes in glutamate or ACh. We saw that glutamate peak amplitudes decreased approximately 38% from the cingulate to the infralimbic mPFC, while T_{80} values in the infralimbic mPFC were also decreased by 44% as compared to T_{80} values in the cingulate sub-region. These findings are suggestive of subtle variance in the amounts of glutamate released in response to KCl, and in the time needed for 80% signal decay among mPFC brain sub-regions that also could relate to the extent of glutamate regulation by glutamate transporters located primarily on glia in sub-regions of the mPFC (Danbolt, 2001). Additional studies are needed to further investigate this finding.

Our studies showed that potassium stimulation was seen to produce increases in extracellular ACh that ranged from roughly 500-1000% of the baseline or tonic ACh levels. A previous microdialysis study showed that potassium stimulation produced an increase in ACh levels of 650% (Herzog et

al., 2003). Thus, the relative change from baseline or tonic ACh levels seen from potassium stimulation was in the range of that reported by microdialysis. Interestingly, our KCl-evoked ACh peak amplitude values followed a pattern similar to that of glutamate along the dorsal-ventral axis of the mPFC. Both ACh peak amplitudes and ACh uptake rates were decreased in more ventral areas as compared to dorsal areas. Our T_{80} values in the infralimbic sub-region were found to be consistent with a previous study using our ceramic based MEAs (Bruno et al., 2006).

When we compared our glutamate and ACh peak amplitudes, we found that the ACh peak amplitude concentrations were significantly higher in the cingulate region of the mPFC (7.1 µM) than the glutamate concentrations in all sub-regions (1.8-2.9 µM). Acetylcholinesterase distribution in the mPFC is known to be heterogeneous (Mrzljak and Goldman-Rakic, 1992; Rajkowska et al., 1993). Thus, the varying amounts of this enzyme may be responsible for the significant ACh concentration differences.   It has also been shown that the basal forebrain projects directly to the cingulate, among other areas of the mPFC (Bigl et al., 1982; Houser et al., 1985), and this may account for increased release of ACh. Additionally, the tight regulation of the glutamate signaling (Hascup et al., 2010) may result in decreased amounts of glutamate release. Our higher observed glutamate resting levels may be sufficient to maintain glutamatergic tone within the mPFC. Our studies also found significant differences in T_{80} values between glutamate and ACh within all sub-regions. ACh T_{80} values in the cingulate, prelimbic, and infralimbic mPFC were all significantly increased as compared to glutamate T_{80} values in the same brain sub-regions (Figure 3.6). These increased decay times seen in KCl-evoked release of ACh suggest that glutamate is being cleared more rapidly than ACh. This may be due to the separate processes that govern the breakdown of these neurotransmitters. Glutamate clearance from the extracellular space is dictated by a number of factors, including diffusion and the presence of high affinity glutamate transporters (Danbolt, 2001). Extracellular ACh is rapidly broken down by endogenous acetylcholinesterase. In addition to diffusion, choline is removed

from the extracellular space via sodium dependent high affinity choline transporters (Okuda et al., 2000) and through low affinity transport (Meyer et al., 1982). Our data suggests that, based on our T_{80} values, glutamate transport may be occurring on a faster time scale than the hydrolysis of ACh and transport of choline.

For these ACh experiments, we used a human recombinant source of acetylcholinesterase in our coating solution. Many studies (Bruno et al., 2006; Giuliano et al., 2008) used acetylcholinesterase from an eel source, which became unavailable from vendors for a period of time in 2006. When the eel source of acetylcholinesterase was reintroduced, we observed that it had markedly lower unit activity than before (≥1000 units/mg of protein), thus limiting the ability of the MEAs to measure ACh. An SDS-PAGE gel analysis of the eel source of AChE revealed the presence of possible impurities and/or degradation products, in addition to the AChE protein (Figure 3.9; Dr. Luke Bradley, unpublished work). This less reliable form of the enzyme was used for a number of experiments, with fairly poor results. Thus, we were forced to look for an alternative source of acetylcholinesterase. The human recombinant version of this enzyme proved to be a viable option for coating, with its higher unit activity (≥2000 units/mg of protein). We feel that the human recombinant acetylcholinesterase corrected the previous problems of measuring ACh associated with the eel source of acetylcholinesterase. The human recombinant acetylcholinesterase, as well as our curing protocol, has allowed us to optimize MEAs for in vivo ACh detection (Burmeister et al., 2008) and to carry out the studies described in this study.

The cholinergic system is a complex network that uses and ACh for neurochemical signaling. It has been postulated that the *in vivo* hydrolysis of ACh by acetylcholinesterase occurs so rapidly (Lawler, 1961) that little ACh can escape the synaptic cleft intact, and thus measuring choline is a suitable alternative (Giuliano et al., 2008). Our data presented in this paper contradicts this hypothesis, as evidenced by our prelimbic resting ACh concentrations of 1.0 ± 0.7 µM, which are at least one order of magnitude higher than the levels of

0.058 – 120 nM (Brooks et al., 2007; Del Arco et al., 2007; Huang et al., 2008; Prus et al., 2007) reported in recent microdialysis studies performed in the mPFC of awake rats. The amplitudes of ACh measured from the KCl-evoked measures in this study yielded peak amplitude concentrations of 7.1 ± 3.9 μM, which were similar to those previously reported using MEA technology (Bruno et al., 2006). Even more impressive, these calculations are based on a self-referenced signal that subtracts the background current produced by the breakdown of choline. Thus, we can conclude that the subtracted signal likely reflects ACh concentrations in the mPFC, and that these ACh levels are robust enough to warrant investigations separate from those of choline when they are measured on a second-by-second time scale.

Finally, we sought to examine the relationships between resting levels of neurotransmitters and the KCl-evoked release of neurotransmitters. Over the course of analyzing our data, we noticed increased resting levels of glutamate with decreased KCl-evoked release of glutamate compared to ACh. The two neurotransmitters almost seemed to have an inverse relationship to each other in these two parameters. We were interested in whether the KCl-evoked release of a neurotransmitter was correlated to its resting levels, so we performed correlations for each neurotransmitter within the three sub-regions (Figure 3.7). The correlation between the log of the evoked release of ACh and resting levels of ACh in the cingulate was found to be significant. We did not see any significant correlations in our glutamate data, nor did we find significance in the prelimbic and infralimbic sub-regions for ACh. These findings suggest that the tonic and phasic aspects of signaling in the mPFC are independent of each other. However, resting ACh levels may be predictive of KCl-evoked ACh release in the cingulate mPFC.

Conclusions

In conclusion, we believe that this series of studies is critical to furthering our understanding of prefrontal cortex neurochemistry. We observed no significant differences in tonic and phasic release of glutamate or ACh in sub-

regions of the mPFC. However, we did see greater amounts of resting glutamate compared to resting ACh levels in the three sub-regions of the mPFC. In addition, the amount of KCl-evoked ACh release was greater as compared to KCl-evoked glutamate release in all sub-regions of the mPFC. Finally, when the KCl-evoked signals of glutamate or ACh were expressed relative to baseline, it was found that KCl-evoked ACh release was 500-1000 % of baseline whereas glutamate was no greater than 100% of baseline, supporting that there was evidence for potential autoregulation of both neurotransmitters. The next step is to continue these recordings in awake, freely moving animals with the goal of exploring glutamate and ACh signaling in the prefrontal cortex during behavior without the potential confound of anesthesia. Also planned are experiments that use drugs such as TTX and TBOA to determine the neuronal contribution to glutamate and ACh signals in the mPFC. Additionally, while these studies used separate MEAs to measure glutamate and ACh, the development of a novel MEA design that allows for measurement of multiple chemicals of interest at the same time (Hascup et al., 2007) should prove to be very useful for determining relationships between different neurotransmitter systems *in vivo*.

Portions of this chapter have been previously published in the manuscript:

Mattinson, C.E., Burmeister, J.J., Quintero, J., Pomerleau, F., Huettl, P., Gerhardt, G.A. 2011. Tonic and phasic release of glutamate and acetylcholine neurotransmission in sub-regions of the rat prefrontal cortex using enzyme based microelectrode arrays. J Neurosci Methods. 202 (2): 199-208.
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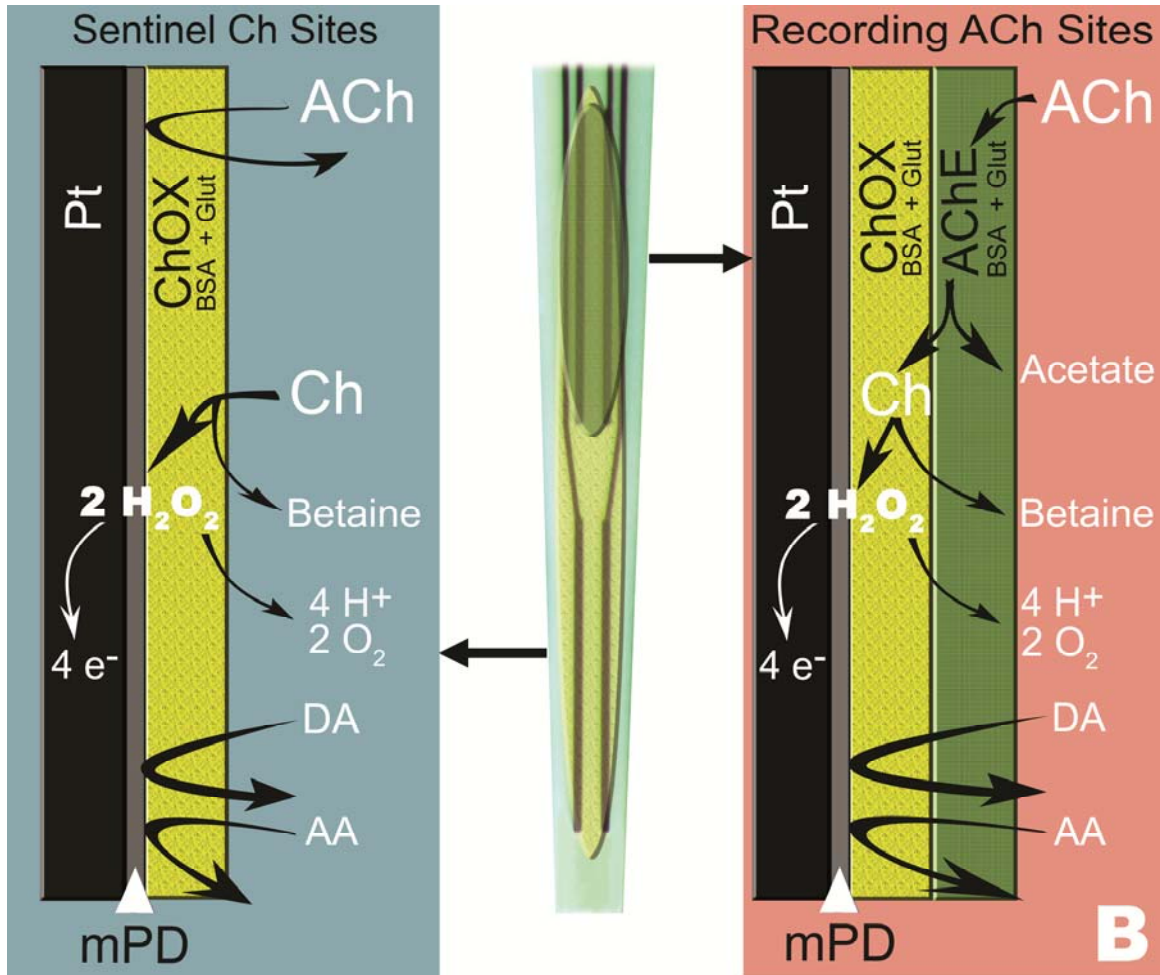
Figure 3.1 Microelectrode Array Acetylcholine Coating Schematic

For the detection of ACh, one pair of sites is coated with human recombinant acetylcholinesterase (AChE) and choline oxidase (ChOx), while the other is coated with only ChOx. When ACh comes into contact with the enzyme layers, AChE hydrolyzes ACh into choline and acetate. The choline is in turn oxidized by the ChOx into H2O2 and betaine. Similar to the glutamate MEA, the H2O2 is oxidized at the Pt site producing a signal current that is directly proportional to the concentration of choline. mPD is used to exclude electroactive interferents such as AA. The sentinel ChOx coated sites generate a choline signal, which is subtracted from the combined ACh/choline signal, resulting in a self-referenced ACh signal for measures of ACh resting levels.
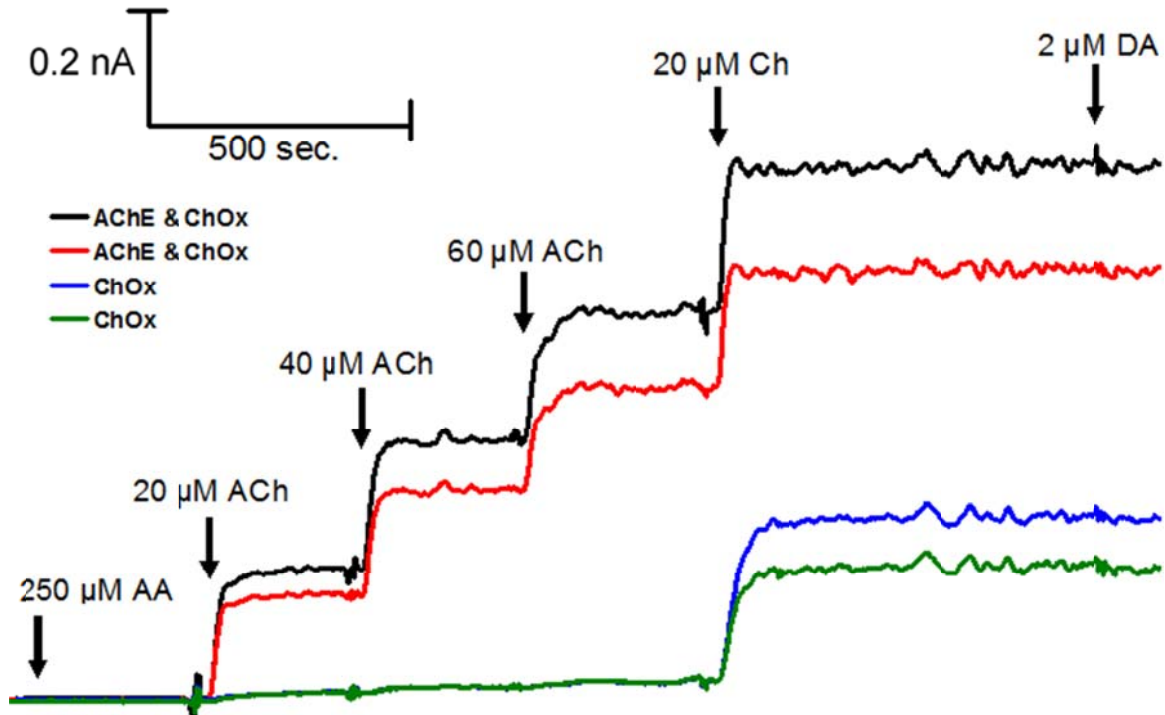
Figure 3.2 *In Vitro* Calibration of an Acetylcholine Selective Microelectrode Array
An *in vitro* calibration was performed prior to all experiments. Channels 1 (black) and 2 (red) were coated with acetylcholinesterase and choline oxidase, while channels 3 (blue) and 4 (green) were only coated with the choline oxidase. After baseline was been established, 250 µM ascorbic acid (AA; final beaker concentration) was added to ensure that the exclusion layer was intact. Next, three serial additions of acetylcholine (ACh) were pipetted into the beaker, totaling a concentration of 60 µM. These stepwise increases in ACh were used to calculate the slope of the electrode. An addition of choline (Ch) was added to the beaker (final beaker concentration of 20 µM) to confirm that the choline oxidase was working on all channels. A final addition of dopamine (DA; final beaker concentration of 2 µM) also served to ensure that the exclusion layer is working.
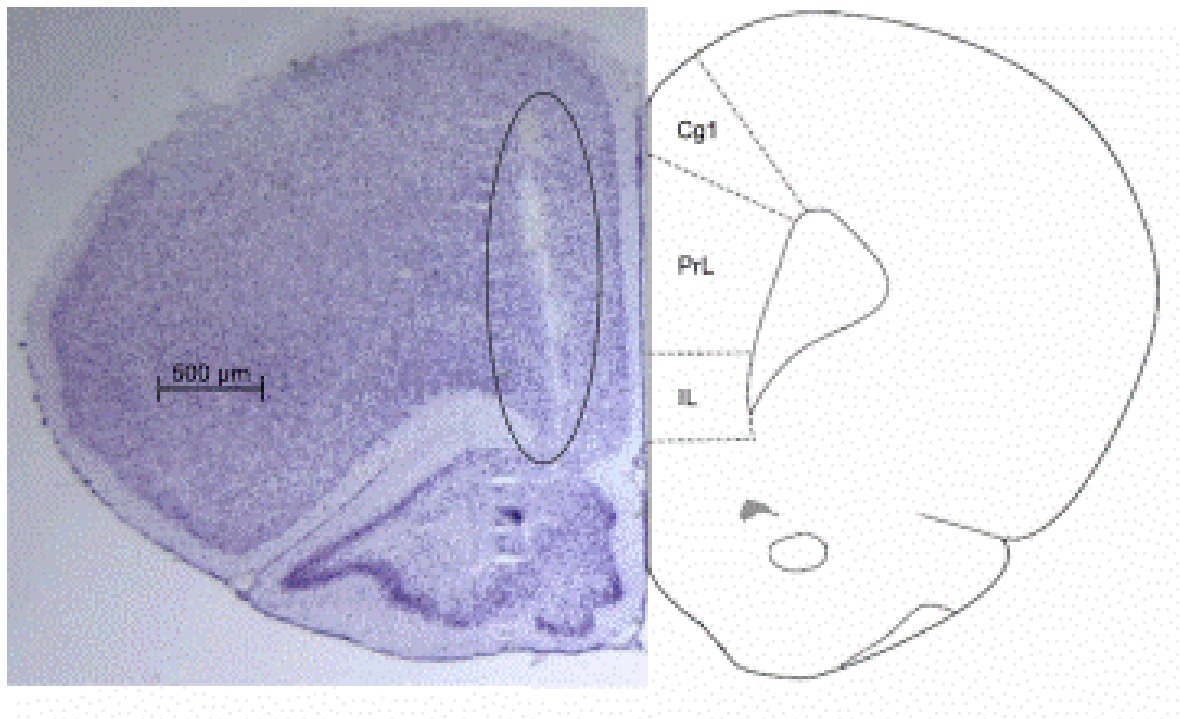
Figure 3.3 Microelectrode Array Placement in the Medial Prefrontal Cortex
Histological section with cresyl violet staining showing minimal damage produced
by the MEA. Histological sections were used to confirm placement in the mPFC
cingulate (Cg1), prelimbic (PrL), and infralimbic (IL). Adapted from Paxinos and
Watson, 2009.
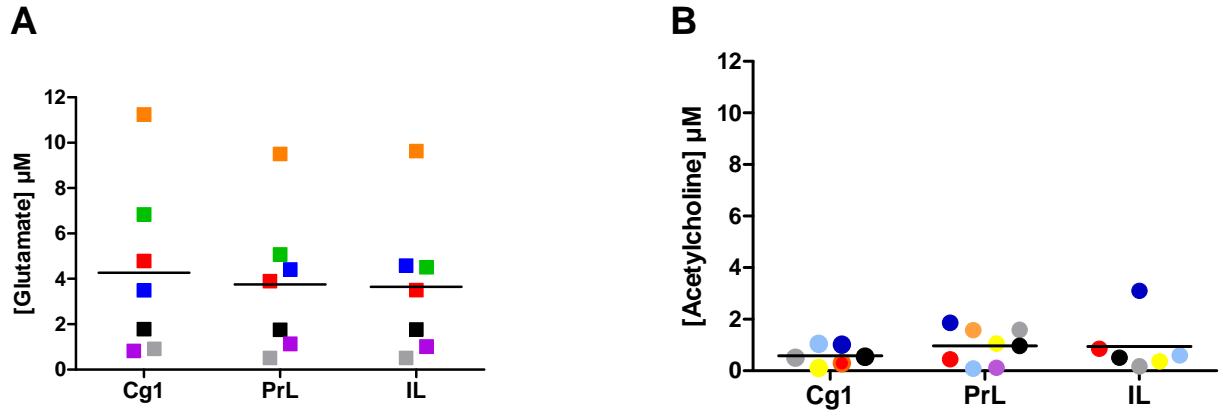
Figure 3.4 Resting or Tonic Levels of Glutamate and Acetylcholine in Sub-
Regions of the Medial Prefrontal Cortex
A) Resting levels of glutamate were homogeneous and not significantly different
within sub-regions of the mPFC. (n = 7 for all regions). B) Resting levels of ACh
were not significantly different within sub-regions of the mPFC (Cg1 and IL, n = 6;
PrL, n = 8). Each color represents a different animal within both
neurotransmitters.

A

B

Peak Amplitude
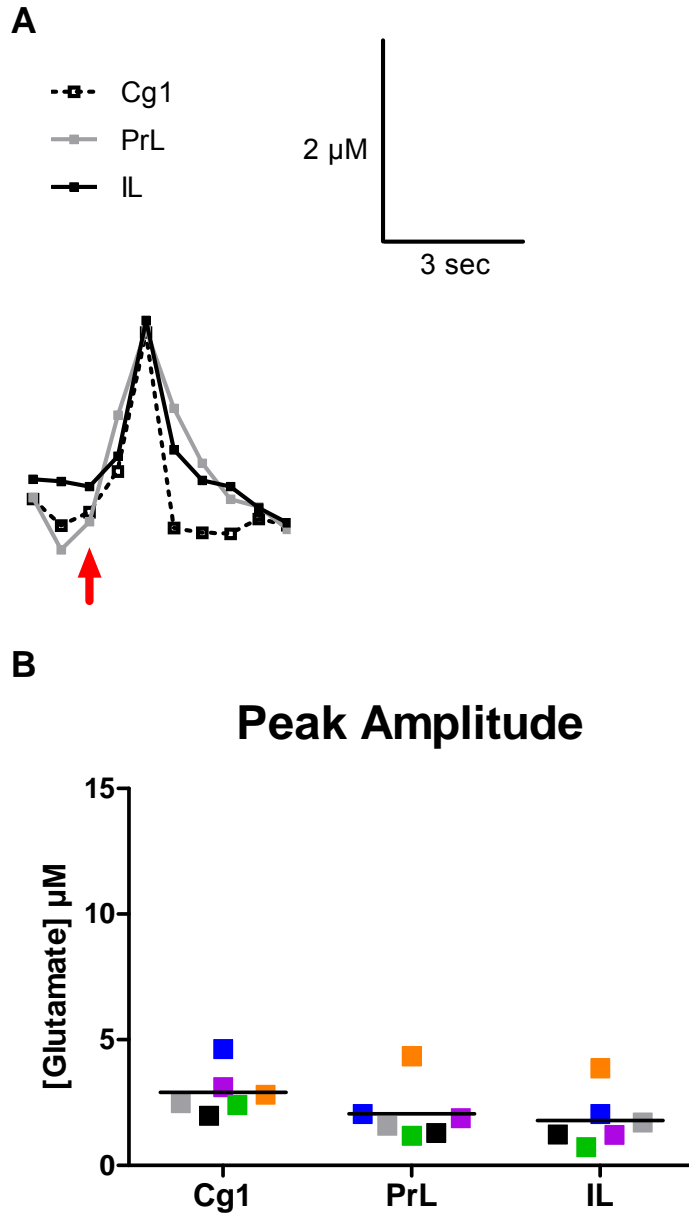
Figure 3.5 KCl-Evoked Glutamate Release in the Medial Prefrontal Cortex
A) Shown are representative signals from the Cg1, PrL and IL of the mPFC. The arrow marks the ejection of KCl to evoke phasic release of glutamate. B) Graphs showing the maximum amplitudes of KCl-evoked release in the different sub-regions of the mPFC. The average amplitudes of KCl-evoked glutamate release were not significantly different in the three brain regions. Each color represents a different animal.
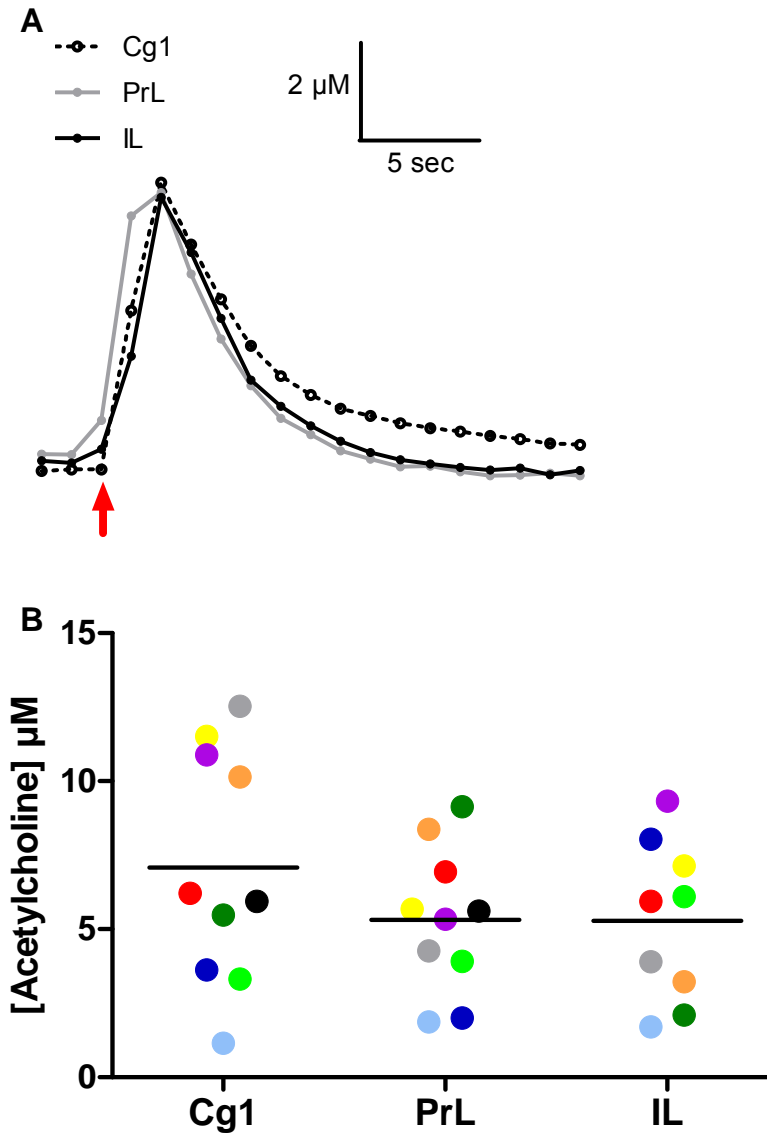
Figure 3.6 KCl-Evoked Acetylcholine Release in Sub-Regions of the Medial Prefrontal Cortex

A) Shown are single traces of KCl-evoked ACh release from the Cg1, PrL and IL of the mPFC. The arrow marks the ejection of KCl to evoke phasic release of Ach. B) Graphs showing average amplitudes of KCl-evoked ACh release in the 3 sub-regions of the mPFC. There was no significant difference in the ACh amplitudes within the different sub-regions along the dorsal-ventral axis of the mPFC. (Cg1 and PrL, n = 10; IL, n = 9). Each color represents a different animal.
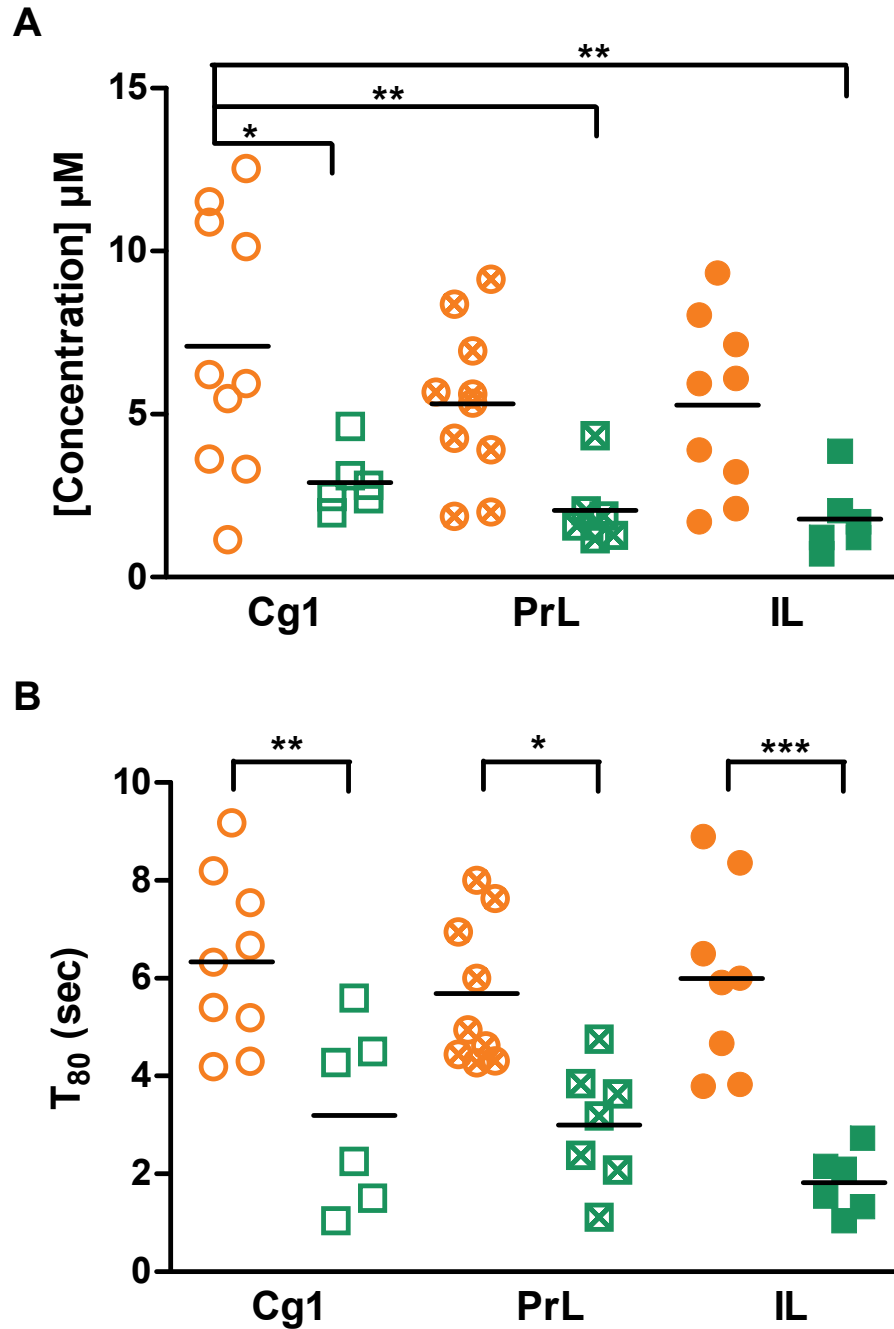
Figure 3.7 Comparisons of Glutamate and Acetylcholine Peak Amplitudes and T80 Values in Different Sub-Regions of the Medial Prefrontal Cortex

A) Significant differences were found between the KCl-evoked ACh signal amplitudes (orange circles) in the Cg1 (**$p < 0.01$; *$p < 0.05$; Glu: n = 6; ACh: Cg1 and PrL, n = 10, IL, n = 9) as compared to glutamate signaling (green squares) in the three sub-regions of the mPFC. B) T_{80} values differ significantly

between glutamate and ACh in each sub-region (***p < 0.001; **p < 0.01; *p < 0.05; Glu: Cg1 and IL, n = 6, PrL, n = 7; ACh: Cg1 and PrL, n = 9, IL, n = 8).
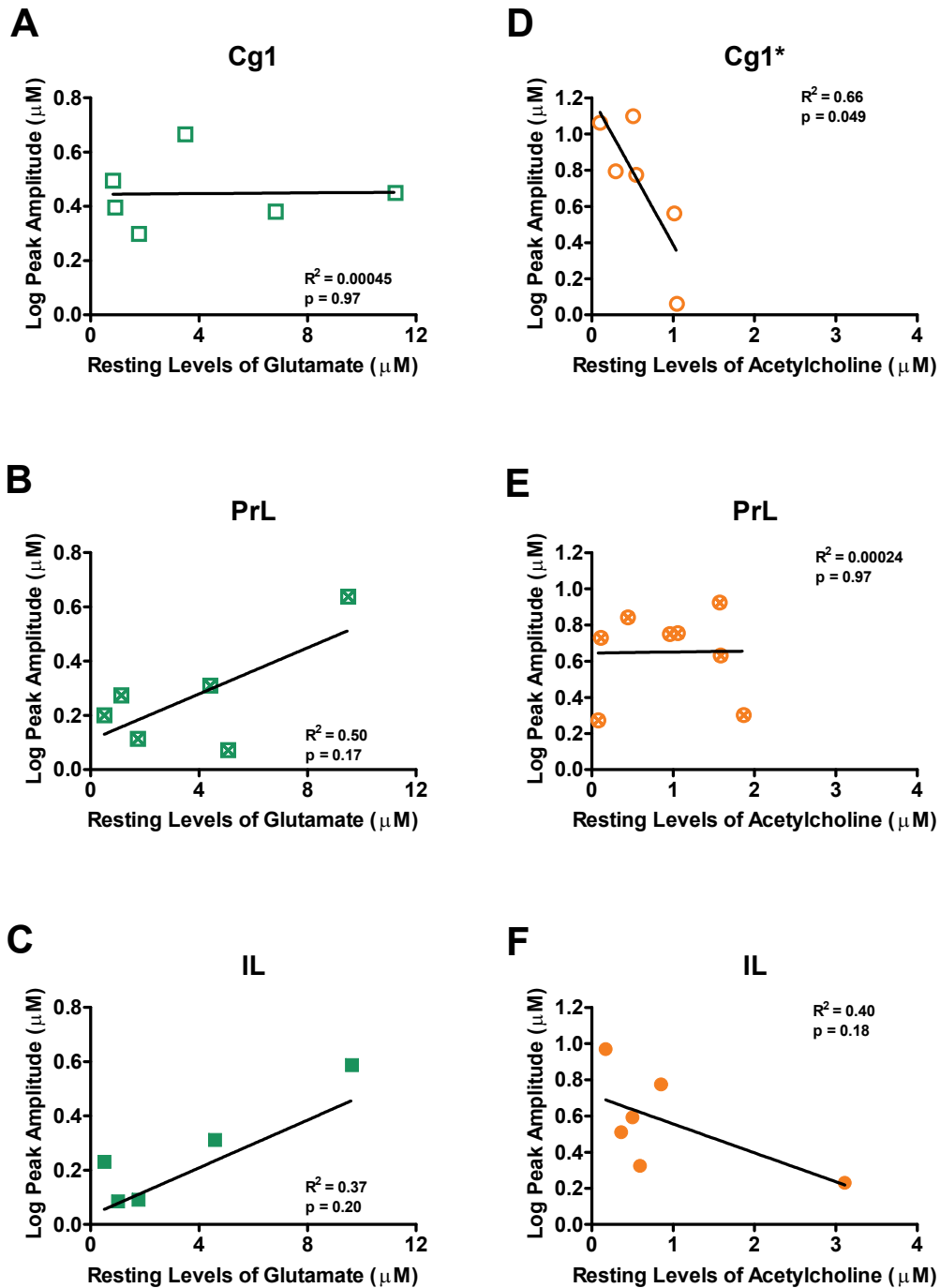
Figure 3.8 Correlations of Glutamate and Acetylcholine Peak Amplitudes and Resting Levels

A, B, C) The correlations of the log of KCl-evoked glutamate peak amplitudes and glutamate resting levels were not significant (Cg1 and PrL, n = 5; IL, n = 5).

D, E, F) The log of KCl-evoked ACh peak amplitudes and ACh resting levels showed a significant correlation in the Cg1 sub-region of the mPFC ($p < 0.05$). Other sub-regions did not show any significant correlations (Cg1 and IL, n = 6; PrL, n = 8).
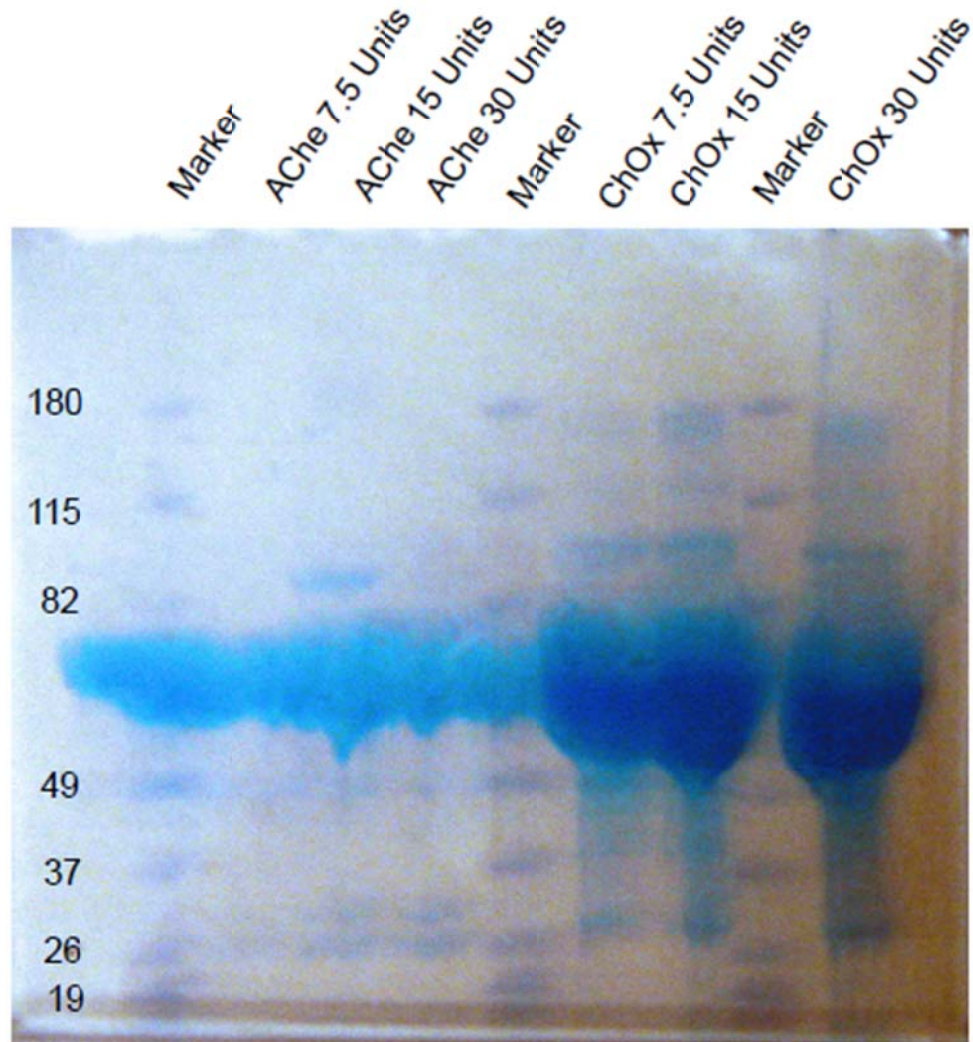
Figure 3.9 Eel Source Acetylcholinesterase Shows Impurities in SDS-PAGE Gel
Samples are given with their total protein added per lane (estimated). The
molecular weight values for the marker are listed on the left in kDa. The AChE
lanes show a smear near the expected weight of the protein (66 kDA), but also
show contaminants at approximately 45, 32, and 28 kDA (unpublished data from
the lab of Dr. Luke Bradley).

Table 3.1 KCl-Evoked Glutamate Release Data

Sub-region	Peak Amplitude	T_{80} Value	k_{-1}
Cg1	2.9 ± 0.9 µM	3.2 ± 1.8 s	0.7 ± 0.7 s^{-1}
PrL	2.0 ± 1.1 µM	3.0 ± 1.2 s	0.6 ± 0.9 s^{-1}
IL	1.8 ± 1.1 µM	1.8 ± 0.6 s	0.8 ± 0.9 s^{-1}

Peak amplitudes, T_{80} values, and k-1 values for each of the three sub-regions highlight the homogeneity of the mPFC (mean ± SD; Cg1 and IL: n = 6; PrL: peak amplitude, n = 6, T_{80} value and k-1, n = 7).

Table 3.2 KCl-Evoked Acetylcholine Release Data

Sub-region	Peak Amplitude	T_{80} Value	k_{-1}
Cg1	7.1 ± 3.9 µM	6.3 ± 1.7 s	0.3 ± 0.4 s^{-1}
PrL	5.3 ± 2.4 µM	5.7 ± 1.5 s	0.3 ± 0.3 s^{-1}
IL	5.3 ± 2.7 µM	6.0 ± 1.9 s	0.3 ± 0.3 s^{-1}

Peak amplitudes, T_{80} values, and k_{-1} values for each of the three sub-regions highlight the homogeneity of the mPFC, and ACh release data (mean ± SD; Cg1 and PrL: peak amplitude and k_{-1}, n = 10, T_{80} value, n = 9; IL: peak amplitude, and k_{-1}, n = 9, T_{80}, n = 8).

**Chapter Four: Glutamatergic Recordings in the Infralimbic Prefrontal Cortex of Awake Freely Moving Rats:
Circadian Data and Phasic Glutamate Signaling**

Introduction

Microdialysis has traditionally been used to measure *in vivo* neurotransmission. However, with collection times in the range of minutes (Nandi and Lunte 2009), microdialysis is unable to measure phasic changes in glutamate signaling. MEA technology can sample glutamate concentrations *in vivo* on a sub-second time scale (Burmeister, Pomerleau et al. 2002), thus greatly improving upon the temporal resolution of microdialysis. This advanced temporal resolution is necessary for the detection of glutamate 'transients'. Transients are rapid, dynamic increases in glutamate that have been observed in multiple brain regions and multiple rat strains using MEAs (Hascup, Hascup et al. 2011).

In addition to the temporal advantages of MEAs, this technology allows for the measurement of distinct brain sub-regions including the infralimbic mPFC, as the MEA only measures substances that come into contact with the surface of the Pt recording sites (Burmeister, Moxon et al. 2000). Rutherford et al. demonstrated the ability to modify MEA technology for use in the awake freely-moving animal, thus allowing for simultaneous measurements of glutamate and behavior (Rutherford, Pomerleau et al. 2007). The advanced spatial resolution of the MEA and these modifications will allow us to record glutamate signaling from the infralimbic mPFC on a sub-second time scale without the confound of anesthetic.

The infralimbic sub-region of the mPFC was investigated in these studies. The infralimbic mPFC is a ventral portion of the mPFC that has been shown to be important for working memory (Ragozzino and Kesner 1998; Delatour and Gisquet-Verrier 1999), attention (Birrell and Brown 2000), goal-directed behavior (Killcross and Coutureau 2003), impulsivity (Murphy, Dalley et al. 2005), and extinction (Morgan and LeDoux 1995; Morgan and LeDoux 1999; Peters, LaLumiere et al. 2008). Glutamatergic neurotransmission in the infralimbic

mPFC has not been well characterized in the awake, freely moving animal. The poor spatial resolution of microdialysis probes has been prohibitive in measuring specific sub-regions of brain structures. Thus the infralimbic and prelimbic areas of the mPFC have typically been grouped together as the ventral mPFC (Dalley, Cardinal et al. 2004), despite the possibility that differential glutamate signaling may exist between these two regions.

The infralimbic mPFC receives input from the suprachiasmatic nucleus (SCN) via the paraventricular thalamic nucleus (Sylvester, Krout et al. 2002). As the SCN serves as the circadian pacemaker of the brain (Kalsbeek, Palm et al. 2006), we extended our awake freely-moving glutamate recordings to 24 hour time periods to investigate the possibility of circadian influences on glutamate neurotransmission in the infralimbic mPFC.

We hypothesized that we would be able to detect rapid, dynamic glutamate transients in the infralimbic mPFC using MEA technology, as well as measure tonic glutamate levels. We also hypothesized that we would see changes in glutamate signaling related to the light and dark cycles during a 24 hour time period.

Materials and Methods

Animals

Male F344 rats, aged 3-6 months were used in these studies (for care, see Animals). Animals were maintained on a 12 h light: 12 h dark cycle (lights on at 06:00 h). During the 24 hour recordings, animals had *ad libitum* access to water and food in the recording chambers. Following MEA implant surgery, animals were monitored and weighed daily. If an animal weighed less than 80% of its pre-surgery weight, recordings were not performed that day.

Microelectrode Array Preparation

To optimize the MEA for chronic recordings, the MEA circuit board was minimized, and the MEA was epoxied to a plug/connector assembly (Microelectrode Array Configuration and Preparation – Modifications for Awake Freely-Moving Recordings). MEAs were made selective for glutamate through the use of glutamate oxidase coating layers (Microelectrode Array Configuration and Preparation – Microelectrode Array Coatings). For *in vivo* chronic recordings, Nafion® was used as the exclusion layer (Microelectrode Array Configuration and Preparation – Exclusion Layers) to prevent electroactive interferents from coming into contact with the Pt recording sites. Prior to implantation, MEAs were calibrated *in vitro* (Microelectrode Array Calibrations). For awake freely-moving MEAs, the calibrations were performed within the recording chamber to best simulate the environment in which the recordings would take place. After calibration, MEA ceramic tips and reference electrodes were soaked in phosphate buffered saline (PBS; 0.05 M) until the implantation surgery.

Microelectrode Array Implant Surgery

MEA implant surgery procedures are described in Microelectrode Array Implant Surgery and Recordings – Awake Freely-Moving Surgery Procedures. Surgical tools were autoclaved for the first surgery of the day, and sterilized in a glass bead sterilizer for any subsequent surgeries. Total time for each surgery was approximately one hour.

Awake Freely-Moving Recordings

All recordings were performed at 4 Hz. Typically, each animal underwent two 24 hour recording sessions. Recordings were carried out as described in Microelectrode Array Implant Surgery and Recordings – Awake Freely-Moving Recordings. Animals were connected and allowed to baseline for approximately one hour before the 24 hour recording session began. During the recording session, animals remained in the recording chambers within a sound-isolated

room. The experimenter occasionally entered the room during the light cycle to monitor the animal and check to ensure that the tether was not tangled. After the 24 hour recording session, the animal was returned to its home cage for a minimum of 20 hours before the second 24 hour recording session was carried out.

Locomotor Activity Monitoring

During the experiments, animals were placed in a Digiscan animal activity monitoring device inside of the recording chamber. Multiple parameters of locomotion, including distance traveled and number of movements, were measured in five minute time bins over the course of the 24 hour sessions using Oasis software. Locomotor activity was measured for the entire duration of electrochemical recording sessions. After a recording session, the recording chamber was cleaned with Roccal disinfectant (Pfizer, Inc., New York City, NY).

Histology

After the experiments, animals euthanized under heavy anesthesia and their brains were removed to check for MEA placements. For details, see Histology. Animals with MEA placements outside of the IL cortex were excluded from data analysis.

Data Analysis and Statistics

Collected data were processed using a custom Matlab®-based analysis package (Data Collection). Data parameters of interest included resting levels of glutamate, glutamate peak amplitudes, T_{80} values, and uptake rates. To examine phasic glutamate activity, recordings were scanned using a peak finder tool with a threshold set at a minimum of a signal-to-noise ratio of 2.5. A one-way repeated measures ANOVA with a Bonferroni post hoc test was used for analyses, as were correlation statistics. Significant was defined as $p < 0.05$.

Results

24-Hour Signaling Parameters Show Significant Effects

Variable resting levels of glutamate (15 min bins of average glutamate resting levels were averaged together for the light and dark cycles; average ± SD: light 10 ± 20 µM, dark 8.7 ± 12 µM) both increase and decrease over a 24-hour period, and a significant effect of time was found on glutamate resting levels ($F_{(4,95)}$ = 220; $p < 0.0001$; n = 5). Bonferroni *post hoc* comparisons revealed significant differences between all animals. 24-hour recordings in the infralimbic area of the mPFC show the number of transients (Figure 4.1) in a 15 minute period varying, on average, between 10 and 40 events. Analyses revealed a significant effect of time on number of glutamate transients ($F_{(4,95)}$ = 37; $p < 0.0001$; n = 5), and Bonferroni *post hoc* comparisons showed significant differences between all animals, except for green and blue, green and black, and purple and orange. Additionally, the peak amplitude concentration of the transients showed a significant effect of time on glutamate transient peak amplitudes ($F_{(3,95)}$ = 180; $p < 0.0001$; n = 4). Bonferroni *post hoc* comparisons revealed significant differences between all animals, except purple and orange.

Significant Correlations Exist Between Glutamate Signaling Parameters

Negative correlations exist between glutamate concentrations and the number of glutamate events (Figure 4.2) during both the light (R^2 = 0.15; $p = 0.0001$; n = 4) and dark cycles (R_2 = 0.047; $p = 0.044$; n = 4). Distance traveled and glutamate concentrations also negatively correlated during the light cycle (Figure 4.3; R^2 = 0.099; $p = 0.0010$; n = 5), but did not correlate during the dark cycle. Distance traveled did not correlate with the number of glutamate events during either portion of the light cycle.

Discussion

This set of experiments sought to further our understanding of tonic and phasic aspects of glutamate signaling in the infralimbic sub-region of the mPFC

over the course of a circadian cycle. Our studies confirmed the existence of rapid, dynamic transient glutamate signaling events in the infralimbic mPFC. We also observed significant effects over the 24 hour recording period. Finally, correlations were revealed between transient events and transient glutamate peak amplitude concentrations, and between locomotor activity and transient glutamate peak amplitude concentrations. These findings suggest relationships among aspects phasic glutamate signaling and locomotion in the infralimbic mPFC.

Glutamate Resting Levels Vary Among Individual Animals

When glutamate resting levels were analyzed over a 24 hour time period, we did not find patterns of signaling that indicated a circadian influence on glutamate neurotransmission. Previous work using microdialysis has resulted in mixed outcomes. Using polygraphic recordings to determine activity states, studies have reported no change in glutamate levels in the mPFC (Lena, Parrot et al. 2005), as well as an increase in glutamate levels during REM sleep in the orbitofrontal cortex (Lopez-Rodriguez, Medina-Ceja et al. 2007). Another study using MEA technology to measure glutamate during the sleep cycle showed increased glutamate levels during waking and REM sleep in the infralimbic mPFC, and reported resting glutamate concentrations of 15 ± 4.1 μM (Dash, Douglas et al. 2009). Our reported resting levels of glutamate in the infralimbic sub-region ranged from approximately 1 μM to 20 μM among five animals, with averages of 10 μM and 9 μM during the light and dark cycles, respectively. Our averages differ from those of Dash et al. due to differences in circadian quantification and strain. We analyzed our data in terms   of the light and dark cycles of a 24 hour time period, whereas Dash and colleagues used EEG and EMG to monitor animal activation states. Additionally, our experiments were performed in F344 rats while their research used Wistar Kyoto rats. Wistar Kyoto rats originating at Charles River Laboratories have been postulated to be a model of the inattentive type of ADHD, and unpublished work from our lab has reported increased resting glutamate levels and evoked glutamate release in the PFC as compared to control strains. When we examined individual animals

(Figure 4.1, D; each color represents a different animal, and the color was consistent over all figures), we found that two animals showed decreasing glutamate levels over the 24 time period (purple decreased by 54%, blue decreased by 33%), one animal showed increasing levels (black increased by 38%), one animal remained relatively constant (orange decrease by 11%), and one animal had relatively large fluctuations in resting levels of glutamate (green). These differences underscore the potential for variability among individual animals, and the MEA's ability to measure these differences.

We have previously reported glutamate resting levels of 3.7 µM in the infralimbic mPFC of urethane-anesthetized rats (Mattinson, Burmeister et al. 2011). The 270% increase in our freely-moving resting glutamate levels of 10 µM as compared to anesthetized resting glutamate levels can be accounted for by the effects of anesthesia. Our laboratory has shown that urethane anesthesia administration resulted in significantly decreased resting levels of glutamate (Rutherford, Pomerleau et al. 2007). Additionally, it should be noted that anesthetized studies sampled the infralimbic mPFC from two depths, -4.5 and -5 mm, while awake freely-moving studies only sampled from one depth, -4.5 mm. This difference in the area of the infralimbic mPFC that was sampled for these experiments may account for some of the differences in glutamate resting levels between the anesthetized and awake studies as well.

Glutamate Transients and Peak Amplitudes Are Not Influenced By the Circadian Cycle

Following recording sessions, data were examined for the presence of transient glutamate signaling events. These phasic changes in glutamate are characterized by their rapid time course, their intermittent appearance during a recording, and their signal that is only recorded on MEA sites that are coated for glutamate detection. It is hypothesized that these glutamate transients are the result of synaptic spillover. Due to the advanced spatial and temporal resolution of the MEA, synaptic spillover can be detected to a degree never before reported. Hascup and colleagues were the first to report these dynamic changes

in glutamate neurotransmission as measured by MEA technology (Hascup, Hascup et al. 2011), but another group using biosensors has also recently reported the presence of these transients in the amygdala (Wassum, Tolosa et al. 2012). This group also reported an increase in the number of transients during a lever pressing task, and concluded that glutamate transients may be important for decision making processes. The experiments in this chapter sought to measure glutamate transients over the course of a 24 hour time period. Our data did not reveal any significant patterns in transient glutamate signaling corresponding to the circadian cycle. However, our studies provide further support for the existence of this dynamic phenomenon (see inset of Figure 4.4).

Peak amplitude concentrations of glutamate transients were also quantified over the circadian cycle. Once again, patterns associated with the light and cycle did not emerge during analysis. Our peak amplitude concentrations averaged 1.9 µM for the light cycle, as well as 1.9 µM for the dark cycle, thus indicating a uniformity of glutamate transient peak amplitudes over the course of 24 hours. Interestingly, these concentrations are similar to the KCl-evoked 1.8 µM average peak amplitude concentration we reported in a previous study in the infralimbic mPFC (Mattinson, Burmeister et al. 2011). This indicates that glutamate release in the infralimbic sub-region is not affected by urethane anesthesia, as the concentrations are consistent between anesthetized and awake freely-moving experiments. Additionally, the similar peak amplitudes of KCl-evoked release and glutamate transients suggest a similar regulation.

Correlations Exist Between Glutamate Signaling Parameters and Locomotion

To further examine relationships between glutamate signaling parameters, we performed a series of correlations on our data for both the light and dark portions of the circadian cycle. When the number of glutamate transients was compared to the peak amplitudes of those transient events, we found significant negative correlations in both the light and dark cycles. Glutamate transients have been shown to be TTX dependent, thus suggesting

vesicular glutamate release (Wassum, Tolosa et al. 2012). As glutamate transients increased in frequency in our experiments, transient peak amplitude concentrations decreased, most likely due to the decreased time available to repackage glutamate before the next transient event. This finding is consistent with the tight regulation associated with *in vivo* glutamate signaling (Danbolt 2001).

Locomotor activity, defined as distance traveled, was also compared to glutamate transient peak amplitudes, and a significant negative correlation was only found during the light cycle. Thus, we observed a decrease in transient peak amplitude associated with increased movement. This relationship was not observed during the dark cycle. From examining the data, we can see that overall movement was increased during the dark cycle, as expected with a nocturnal species such as rats, but transient glutamate concentrations remained relatively consistent. Locomotor activity was also compared to the number of glutamate transients during the dark cycle, and no significant relationships were found (data not shown). These findings indicate that locomotor activity associated with dark portion of the circadian cycle is independent from transient glutamate signaling. As transient increases in glutamate are only recently reported, and only one other study has used MEAs to measure glutamate over a 24 hour time period (Dash, Douglas et al. 2009), it is difficult to compare our findings to existing literature.

Conclusion

These experiments have not only confirmed the presence of transient glutamate signaling in the infralimbic mPFC, but have also characterized glutamate signaling over a 24 time course. While a consistent pattern of glutamate signaling corresponding to changes in the circadian cycle did not emerge, we did observe significant relationships between signaling parameters during both the light and dark cycles of a 24 time period. These data indicate that the circadian influence on infralimbic glutamate levels should not be

considered a confound in future experiments, and the SCN may not exert a strong impact on glutamatergic signaling in the infralimbic mPFC.

Chapter Four: Figures

A

B

C

D
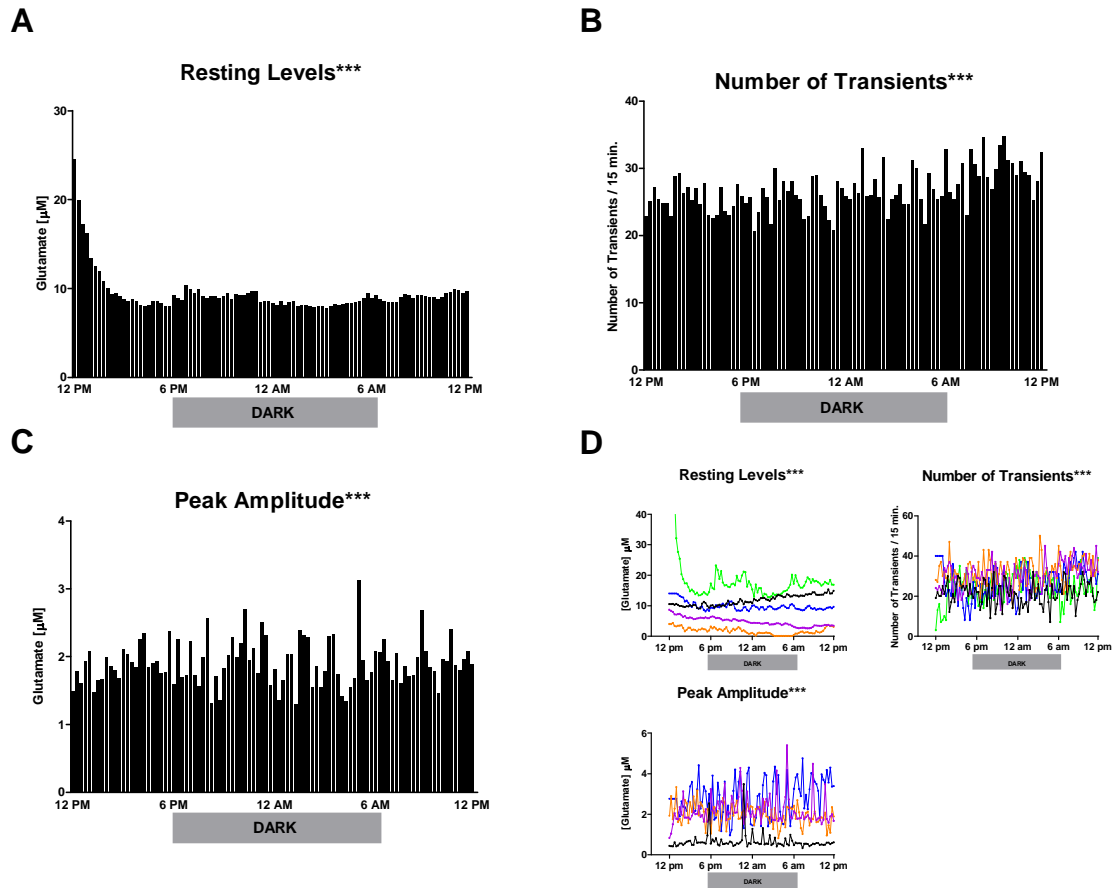
Figure 4.1 24-Hour Glutamate Neurotransmission Signaling Parameters

24-hour traces of glutamate signaling parameters revealed significant effects. A significant effect of time on glutamate resting levels (A; all animals averaged; $p < 0.0001$; n = 5) was found, and a significant effect of time on the number of glutamate transients was also revealed (B; all animals averaged; $p < 0.0001$; n = 5). There was also a significant effect of time on the transient peak amplitude concentrations (C; all animals averaged; ***$p < 0.001$; n = 4). Individual traces of each animal's signaling parameters reveal heterogeneity among animals (D; each color represents a different animal).

A

Light Cycle***

B

Dark Cycle*
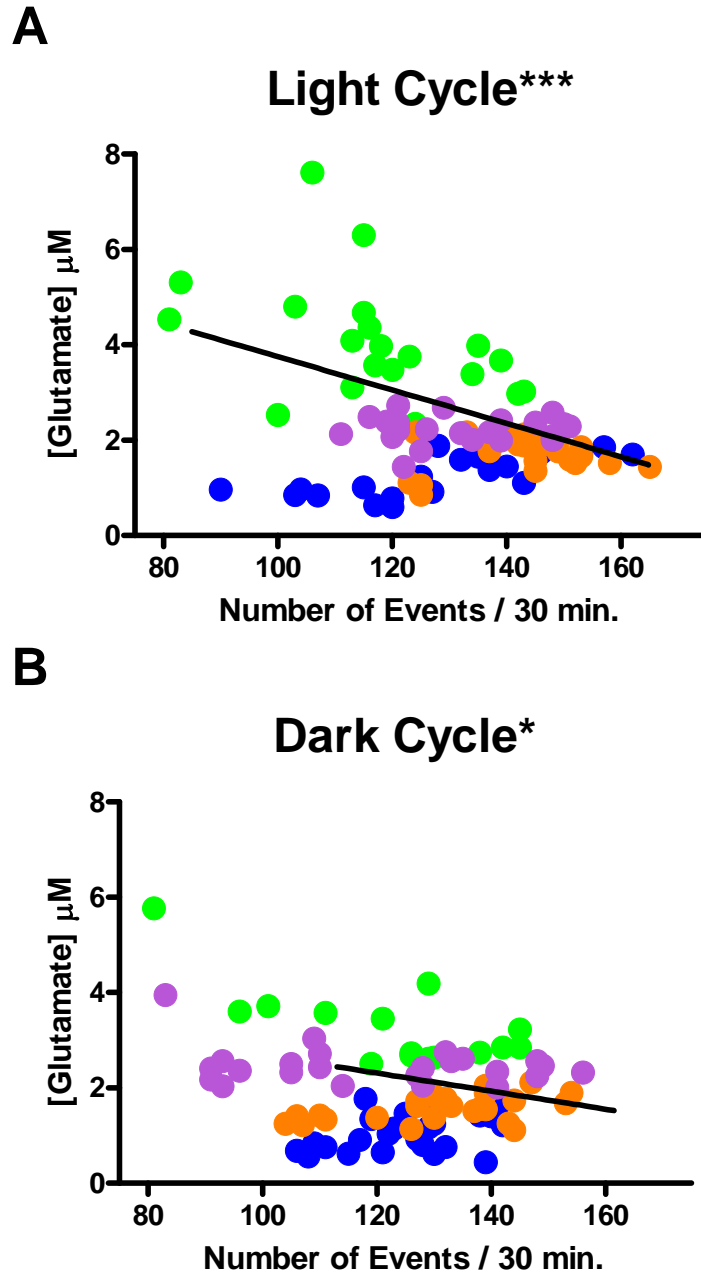
Figure 4.2 Number of Glutamate Events and Concentration Correlate
The number of transient glutamate events was correlated to the concentration of those glutamate events in both the light (A; $R^2 = 0.15$; ***$p < 0.001$; n = 4) and dark (B; $R^2 = 0.047$; *$p < 0.05$; n = 4) portions of the 24-hour cycle. Each color represents a different animal.

A

Light Cycle**

B

Dark Cycle
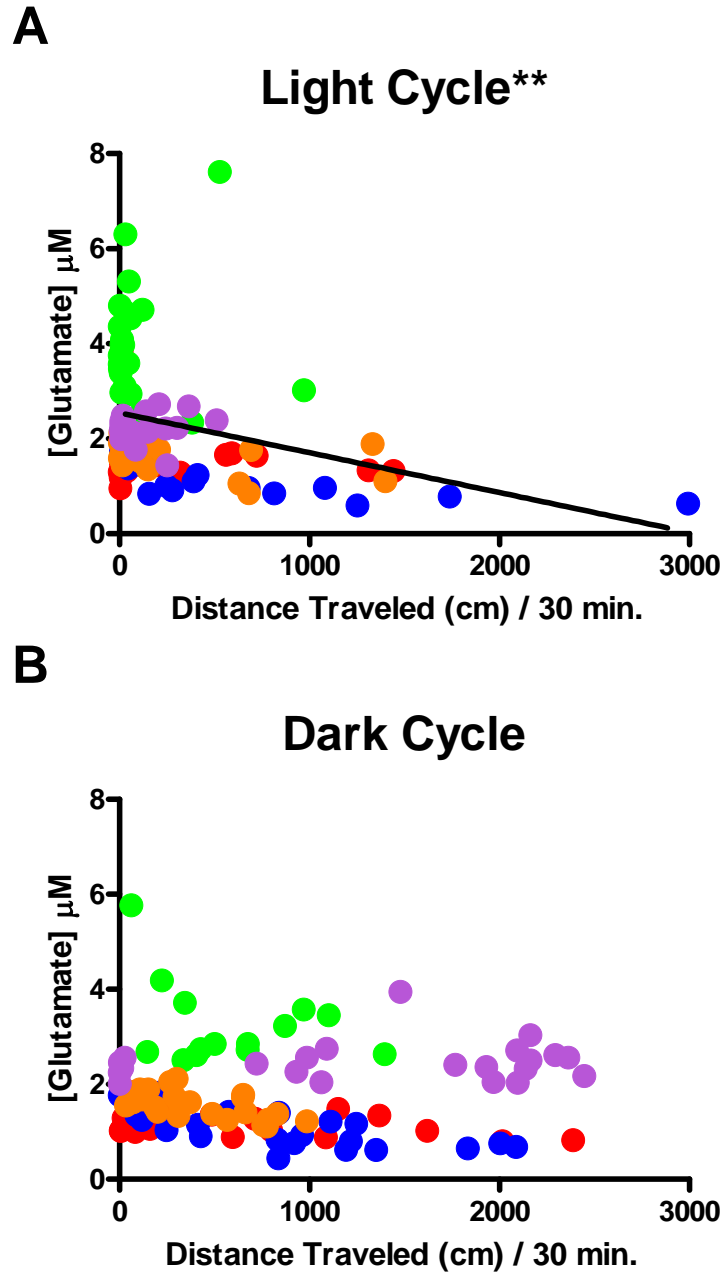
Figure 4.3 Locomotor Activity Correlates with Glutamate Concentration During the Light Cycle

When the distance traveled by an animal was correlated to the concentrations of the glutamate peaks, a negative correlation was found during the light cycle (A; R^2 = 0.099; **p < 0.01; n = 5). The dark cycle did not show a significant correlation. Each color represents a different animal.
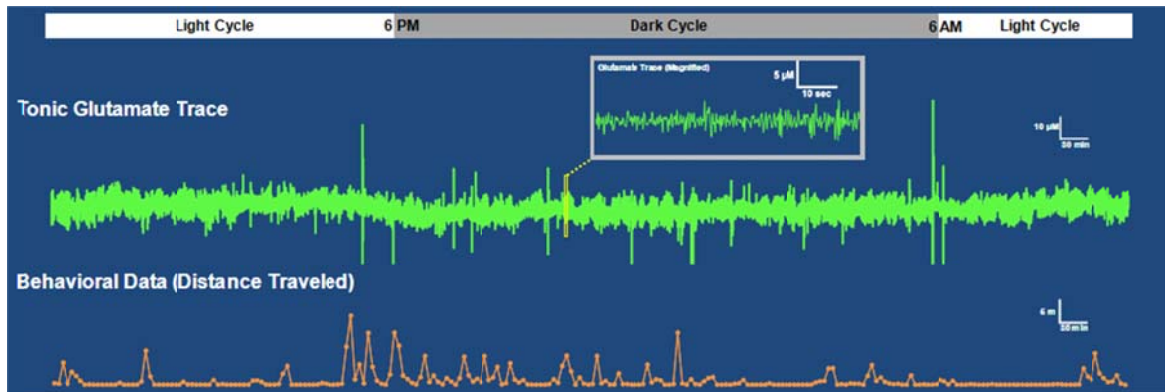
Figure 4.4 24-Hour Data Traces Illustrate the Relationships Between Glutamate
    Signaling and Locomotor Activity

A representative tonic glutamate trace shows the relationship between glutamate

resting levels and behavioral data over a 24-hour recording period.

Chapter Five: The Effects of Methylphenidate Treatment on Tonic and Phasic Glutamate Levels in Sub-Regions of the Rat Medial Prefrontal Cortex

Introduction

The mPFC is an area of the brain that is critically important for complex cognitive processes including attention, memory, learning, organization, and integration. These processes are often interrupted or dysfunctional in the development and maintenance of drug addiction. The abuse of stimulants in particular may result in behavioral sensitization, a hypersensitivity caused by repeated psychostimulant administration (Kalivas and Stewart 1991; Gaytan, al-Rahim et al. 1997; Dafny and Yang 2006). Thus, behavioral sensitization has been proposed as a model of drug seeking (Robinson and Berridge 1993; Hotsenpiller and Wolf 2002). Cocaine and amphetamine studies have implicated the PFC in the development of behavioral sensitization (Pierce, Reeder et al. 1998; Tzschentke and Schmidt 1998; Cador, Bjijou et al. 1999; Li, Hu et al. 1999; Liao and Lin 2008). Recently, Lee and colleagues also demonstrated that the PFC was required for the development of behavioral sensitization associated with MPH administration (Lee, Swann et al. 2008).

MPH is a stimulant drug that is commonly prescribed for the treatment of attention-deficit hyperactivity disorder (Challman and Lipsky 2000; Swanson and Volkow 2002; Askenasy, Taber et al. 2007). MPH has also been shown to have abuse potential. Rats will self-administer MPH (Botly, Burton et al. 2008; Marusich and Bardo 2009; Marusich, Beckmann et al. 2010), and rats exposed to MPH as adolescents will more readily adapt to cocaine administration as adults (Brandon, Marinelli et al. 2001; Crawford, Baella et al. 2011; Harvey, Sen et al. 2011).

To further investigate the effects of an abused stimulant medication on the mPFC, a high dose of MPH was chronically administered to rats. After an administration period and a rest period, glutamate was measured in three sub-regions of the mPFC: the cingulate cortex, the prelimbic cortex, and the infralimbic cortex. Glutamate is the major excitatory neurotransmitter in the

central nervous system, and mPFC glutamate is intimately associated with and influenced by brain circuitry that is implicated in the development of drug addiction. Stimulants such as cocaine and amphetamine have both been shown to increase PFC glutamate (Reid, Hsu et al. 1997). Three sub-regions of the mPFC were examined in an effort to detect any heterogeneous responses to MPH treatment within the mPFC.

These experiments employed the use of MEA technology to measure both tonic and phasic aspects of glutamate signaling in the mPFC. We hypothesized that MPH would differentially affect glutamate neurotransmission in mPFC sub-regions. The improved spatial and temporal resolution of the MEA allowed for an advanced characterization of resting glutamate levels and KCl-evoked glutamate release in the three distinct sub-regions of the mPFC. Thus, we were able to report, for the first time, the effects of MPH treatment on tonic and phasic glutamate signaling in the mPFC.

Materials and Methods
Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) aged 3-6 months were used for all experiments (n=11). For care, see <u>Animals</u>.

Methylphenidate Chronic Treatment

Chronic treatment of the rats with MPH prior to *in vivo* MEA recordings was carried out by Dr. Julie Marusich of the University of Kentucky. Rats received daily MPH (10 mg/kg in saline, s.c.) or saline injections for ten consecutive days. Following the ten day treatment period, rats rested for seven days. Recordings were performed the day after the rest period (day 18 from the start of the MPH injections).

Locomotor Monitoring

Activity monitoring was performed by Dr. Julie Marusich of the University of Kentucky. After receiving their daily injection, rats were placed in activity

monitoring chambers for one hour. The distance traveled in centimeters was averaged in five minute bins over the course of the hour, and then the bins were averaged to obtain the average distance traveled per five minutes for each rat on each day.

Microelectrode Preparation

MEAs for acute anesthetized recordings were made selective for glutamate through enzyme coatings (see <u>Microelectrode Array Configuration and Preparation – Microelectrode Array Coatings</u>), and an electroplated layer of mPD (described in Microelectrode Array Configuration and Preparation – Exclusion Layers). Prior to the experiment, *in vitro* calibrations (detailed in <u>Microelectrode Array Calibrations</u>) were performed to determine the slope, selectivity, limit of detection, and linearity of the MEAs. After calibration, a glass micropipette was affixed to the MEA to allow for intracranial delivery of KCl (see Application of Intracranial Solutions).

In Vivo Recordings

Acute anesthetized recordings were performed as previously described (see <u>Microelectrode Array Implantation Surgery and Recordings – Anesthetized Surgery Procedures and – Anesthetized Recordings</u>). On the day of surgery, rats were anesthetized with urethane and the surgery was performed. The micropipette attached to the MEA was filled with an isotonic KCl solution (70 mM KCl, 79 mM NaCl, 2.5 mM $CaCl_2$ • $2H_2O$; pH = 7.4) and connected to a Picospritzer III (Parker Hannifin Corp, NJ) to allow for local applications of KCl into the extracellular space . The MEA/micropipette assembly was lowered into the mPFC (AP: +3.2 mm; ML: ±0.8 mm; DV: -1.5 to -5 mm; from bregma (Paxinos and Watson, 2009) using a Narishige MO-10 microdrive. The MEA assembly was lowered in 0.5 mm increments and recordings were taken at each depth to record glutamate levels along the dorsal-ventral axis of the mPFC. Once lowered into place, basal levels were established over ten minute recording

durations, and then KCl solution was ejected (75-100 nl) to evoke glutamate release.

Histology

After the experiment, rats were euthanized under heavy anesthesia, and microelectrode array placements were confirmed. For detailed procedures, see Histology.

Data Analysis

Collected data were processed using a custom Matlab®-based analysis package (Data Collection). Data parameters analyzed were resting (tonic release) glutamate levels (average of the ten data points prior to the first ejection at each depth) and data from KCl-evoked release (phasic release) of glutamate that included: peak amplitude, T_{80} (time for 80% of the signal to decay to baseline), and k-1, best fit line of first order rate constant of the signal return to baseline. Glutamate KCl-evoked peaks were volume matched (75-100 nL) for data analysis. As data was not always attainable at every depth throughout the depth profile due to clogging of the tips of the micropipettes, occasional data drop out occurred. For locomotor data, a two-tailed paired t-test was used for analysis. Resting glutamate levels and phasic glutamate signaling parameters were analyzed using both a one-way repeated measures ANOVA and individual t tests. Significance was defined as $p < 0.05$.

Results
Methylphenidate Treated Rats Show Increased Locomotor Activity

After receiving their MPH or saline injections, rats were placed in locomotor activity monitoring chambers to investigate the effects of treatment on behavior. Rats treated with MPH showed significant increases in distance traveled as compared to saline controls (Figure 5.1; $t(9) = 12$; $p < 0.0001$). On average, rats treated with MPH moved approximately 1200 cm more than their saline treated counterparts within each five minute time bin.

Resting Levels of Glutamate Are Similar Between Treatment Groups

When resting levels in the cingulate, prelimbic, and infralimbic cortex were compared between rats chronically treated with MPH and their saline counterparts, no significant differences were found (Figure 5.2). Both a one-way repeated measures ANOVA ($F_{(5,21)} = 2.0$; $p = 0.12$; saline, n = 4; MPH, n = 5) and individual t tests were employed to check for significant differences between the groups. On average, the difference between the two treatment groups' glutamate resting levels was 1.8 µM, although this difference was not significant.

Phasic Glutamate Signaling Shows Homogeneity in All Sub-Regions

Phasic glutamate signaling parameters including peak amplitudes, T_{80} values, and uptake rates were analyzed within each sub-region of the mPFC. Peak amplitudes (Figure 5.2) did not differ significantly between MPH chronically treated rats and saline controls in any of the three sub-regions explored ($F_{(5,21)}$ = 0.30; $p = 0.91$; saline, n = 4; MPH, n = 5). T_{80} values were also found to be similar among the three sub-regions and between the treatment groups (Figure 5.3; $F_{(5,21)} = 1.1$; $p = 0.40$; saline, n = 4; MPH, n = 5). Uptake rates did not vary significantly between the two treatment groups, nor among any of the sub-regions as well (Figure 5.3; $F_{(5,21)} = 0.44$; $p = 0.82$; saline, n = 4; MPH, n = 5). For a summary of these data, see Table 5.1.

Discussion

These studies, to our knowledge, are the first to characterize glutamate signaling in the mPFC after chronic MPH treatment. The use of MEA technology, with its advanced spatial resolution, allowed for measurements of glutamate in distinct sub-regions of the mPFC in the same animal. The temporal resolution of the MEA technology allowed us to detect second-by-second changes in KCl-evoked phasic glutamate signaling within the cingulate, prelimbic, and infralimbic mPFC, thus revealing a more complete picture of dynamic changes in glutamate neurotransmission. Our results reflect our previous work (Mattinson, Burmeister

et al. 2011), with similar glutamate levels and signaling parameters among the three sub-regions investigated. Significant differences in locomotor behavior existed between chronic MPH-treated and saline-treated groups, but no differences in glutamate signaling existed.

Locomotor Effects of MPH Treatment

The behavioral effects of stimulant treatment are generally predictable, and have been well established. Cocaine, methamphetamine, and amphetamine were all shown to increase locomotor activity in mice in early studies (Dews 1953; Smith 1965), and Hughes and others reported increased motor activity and rearing in rats receiving MPH (Hughes 1972; Bhattacharyya, Ghosh et al. 1980). MPH administration increases extracelluar dopamine by blocking the dopamine transporter (Ritz, Lamb et al. 1987; Heron, Costentin et al. 1994; Pan, Gatley et al. 1994; Wayment, Deutsch et al. 1999), and this increase in extracellular dopamine is thought to be responsible for increases in motor activity (Rebec 2006). As lesion studies have shown, stimulant-induced increases in locomotion are associated with these drugs' effects on the mesolimbic dopaminergic system, particularly in the nucleus accumbens (Kelly 1975; Joyce and Koob 1981; Koob, Stinus et al. 1981). We also observed that over the ten day treatment period, distance traveled increased an average of 1000 cm in the MPH treated animals. This increase in locomotor activity is consistent with behavioral sensitization in response to chronic MPH treatment (Crawford, McDougall et al. 1998; Yang, Amini et al. 2003; Wooters, Dwoskin et al. 2006; Wooters, Bardo et al. 2011). Thus, the increased locomotor behavior in chronically MPH-treated rats in this study was as we anticipated. This increase in activity served as a positive control, assured that the dose of MPH was appropriate, and that the animals were responding as expected to the drug.

Resting Levels of Glutamate Show Similarity Between Treatment Groups

In these experiments, we examined the glutamate resting levels of chronically MPH-treated rats and controls in three sub-regions of the mPFC. Chronically MPH-treated rats averaged resting glutamate levels of 4.8 μM, 4.1 μM and 3.2 μM in the cingulate, prelimbic, and infralimbic mPFC, respectively. In comparison, chronically saline-treated rats showed glutamate resting levels of 2.8 μM in the cingulate, 2.0 μM in the prelimbic, and 2.0 μM in the infralimbic. No significant differences were found among mPFC brain sub-regions within each treatment group. However, this information is consistent with our previous work (Mattinson, Burmeister et al. 2011). Measurements of glutamate in the mPFC of F344 rats revealed a homogeneity of glutamate resting levels among mPFC sub-regions. We have postulated that these similarities are due to uniform regulation of tonic and phasic glutamate dynamics within the mPFC.

Our chronically saline-treated animals showed glutamate resting levels that were increased by a minimum of 100% as compared to other reported mPFC resting glutamate levels in Sprague-Dawley rats (Welty and Shoblock 2009; Roenker, Gudelsky et al. 2011). The difference in these glutamate resting level concentrations can best be accounted for by the difference in technologies used for measurement. Our reported concentrations of neurotransmitters *in vivo* are often increased as compared to those concentrations obtained using microdialysis (Hascup, Hascup et al. 2010). Our MEAs offer the advantage of improved spatial resolution over microdialysis probes, allowing for measurements of synaptic spill-over (Day, Pomerleau et al. 2006). Additionally, our probes have been shown to cause minimal damage compared to microdialysis probes (Hascup, af Bjerken et al. 2009), and only measure substances that come into contact with the surface of the Pt recording site (Burmeister, Moxon et al. 2000).

Experiments in the mPFC of F344 rats (Mattinson, Burmeister et al. 2011) revealed glutamate resting levels of 3.7 - 4.3 μM. These levels are increased as compared to our glutamate resting levels in Sprague-Dawley rats from these

experiments. As both sets of these experiments were performed under urethane anesthesia and using MEA technology, the logical explanation for these differences would be the strain of the animals. F344 rats are an in-bred strain typically used for aging and cancer research (Cameron, Hickman et al. 1985), whereas Sprague-Dawley rats are an out-bred strain commonly used in behavior and drug studies. Differences in genetics may account for the marked differences in mPFC glutamate concentrations between these two strains.

Interestingly, the F344 levels are similar to the levels observed in our Sprague-Dawley chronically MPH-treated rats. Although chronically-MPH treated rats do not vary significantly from the controls, the trend towards augmented glutamate levels in the MPH treatment group is apparent. An increased n value could provide significant results. As MPH is known to increase extracellular concentrations of dopamine (Hurd and Ungerstedt 1989; Butcher, Liptrot et al. 1991), it is possible that increased concentrations of dopamine within cortical and mesolimbic circuitry are responsible for potential increases in glutamate release in the mPFC.

It should also be noted that urethane anesthesia was used in these experiments, and Rutherford et al. has shown that urethane administration significantly reduces resting levels of glutamate (Rutherford, Pomerleau et al. 2007). Thus, it is possible that our reported resting levels of glutamate in the mPFC are actually significantly higher in both the MPH and saline treated groups when urethane is not present, and further studies without the effects of anesthetics are needed.

Dynamic Glutamate Signaling Parameters Are Homogenous

Dynamic glutamate signaling parameters including peak amplitudes, T_{80} values, and uptake rates were analyzed, and all parameters were found to be similar between chronically MPH-treated and saline-treated groups, as well as similar among mPFC sub-regions. Peak amplitudes (see Table 5.1) only varied by 1.2 µM between the highest and lowest reported average values. This indicates that KCl-evoked glutamate release in all three mPFC sub-regions is

relatively uniform. Additionally, MPH did not have an effect on stimulated phasic glutamate release as compared to controls. This differs from previous work by Stephans and Yamamoto (1995), who measured an increase in KCl-evoked glutamate release in the mPFC after chronic administration of methamphetamine (Stephans and Yamamoto 1995). As discussed in Chapter Three, microdialysis studies have reported both an increase (Frantz, Harte et al. 2002) and no change (Welty and Shoblock 2009) in mPFC glutamate levels in response to intracranial KCl administration. The rapid, phasic glutamate signal produced by KCl ejections cannot be measured using microdialysis due to this technique's poor temporal resolution, and therefore, our results provide a better characterization of KCl-evoked glutamate release in the mPFC.

Chronically MPH-treated rats' T_{80} values in both the prelimbic and infralimbic mPFC showed a trend toward increased time for 80% of signal decay to occur. These results were not significant, but the times averaged approximately five and four seconds for the prelimbic and infralimbic, respectively. This increase in decay times may be due to drug-induced differences in glutamate transporter dynamics (Danbolt 2001). Extinction from cocaine administration has been shown to alter EAAT binding in the infralimbic mPFC (Miguens, Crespo et al. 2008), however, acute and chronic amphetamine administration has been shown to have no effect on EAAT levels in the PFC (Sidiropoulou, Chao et al. 2001). Additionally, although MPH has not been shown to act on the glutamate transporter (Iversen 2006), it has been linked to a down regulation of GLAST in the striatum (Cavaliere, Cirillo et al. 2012). Uptake rates do not follow the same trend as T_{80} values, and remain relatively uniform despite differing treatments and mPFC sub-regions.

Conclusions

From this set of experiments, we can conclude that chronically MPH-treated animals do not differ significantly from saline controls in both tonic and phasic measurements of glutamate in the cingulate, prelimbic, and infralimbic sub-regions of the mPFC. Although not significant, resting levels of glutamate

trended towards increased glutamate concentrations in chronically MPH-treated rats in all mPFC sub-regions, and a trend of increased T_{80} values in the prelimbic and infralimbic mPFC of chronically MPH-treated rats was observed. Further experiments are needed to discern whether these trends are potential sources of significant differences. We did find, as predicted, significantly increased amounts of locomotor activity in animals chronically treated with MPH as compared to saline controls. Future experiments in awake freely-moving animals would allow for the simultaneous comparison of MPH treatment and animal behavior, thus providing a better characterization of the effects of MPH treatment on glutamate signaling in the mPFC.
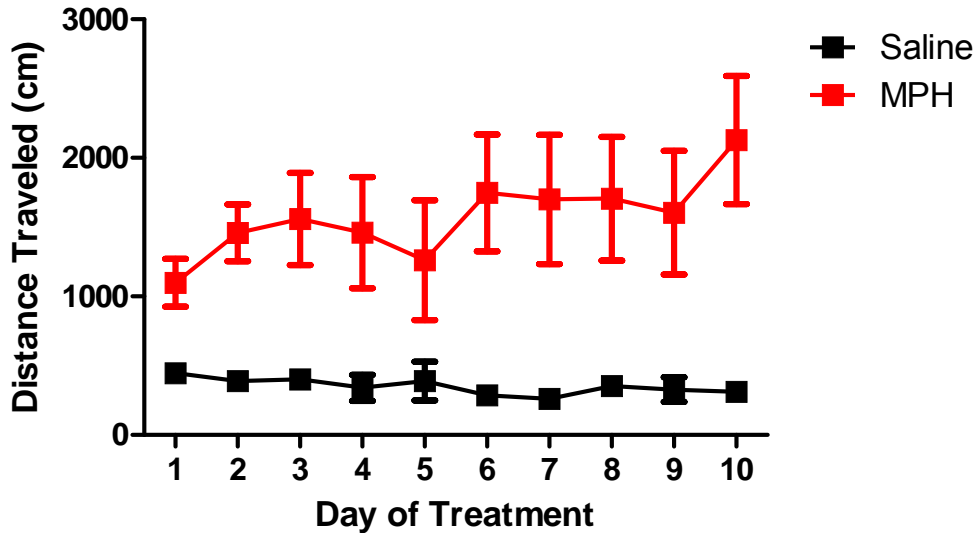
Distance Traveled****

Figure 5.1 Locomotor Activity Following Methylphenidate Treatment
After the daily treatment injection, rats were placed in locomotor chambers for one hour (saline, n = 4; MPH, n = 5). Distance traveled in five minute bins was averaged for each rat on each day. Rats treated with MPH had significantly increased locomotor activity as compared to the saline controls (t(9) = 12; ****p < 0.0001; two-tailed paired t test). Data points are shown as averages ± SEM.

A

Resting Levels

B
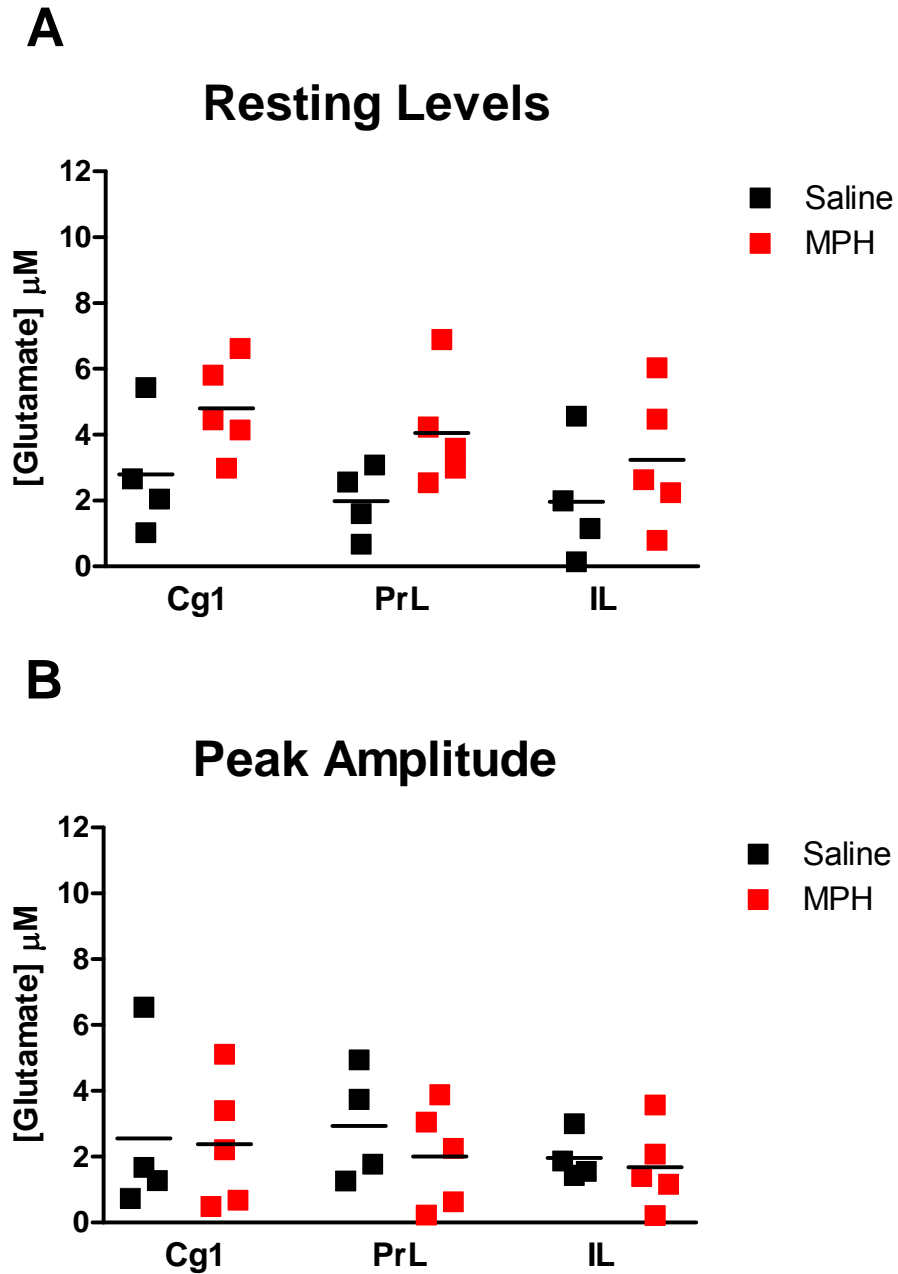
Peak Amplitude

Figure 5.2 Glutamate Resting Levels and Peak Amplitudes in Prefrontal Cortex Sub-Regions

Analyses of resting levels of glutamate levels (A) revealed similar concentrations between treatment groups and among sub-regions (saline, n = 4; MPH, n = 5). Peak amplitude measurements (B) were also fairly homogenous.

A

T_{80} Values

B
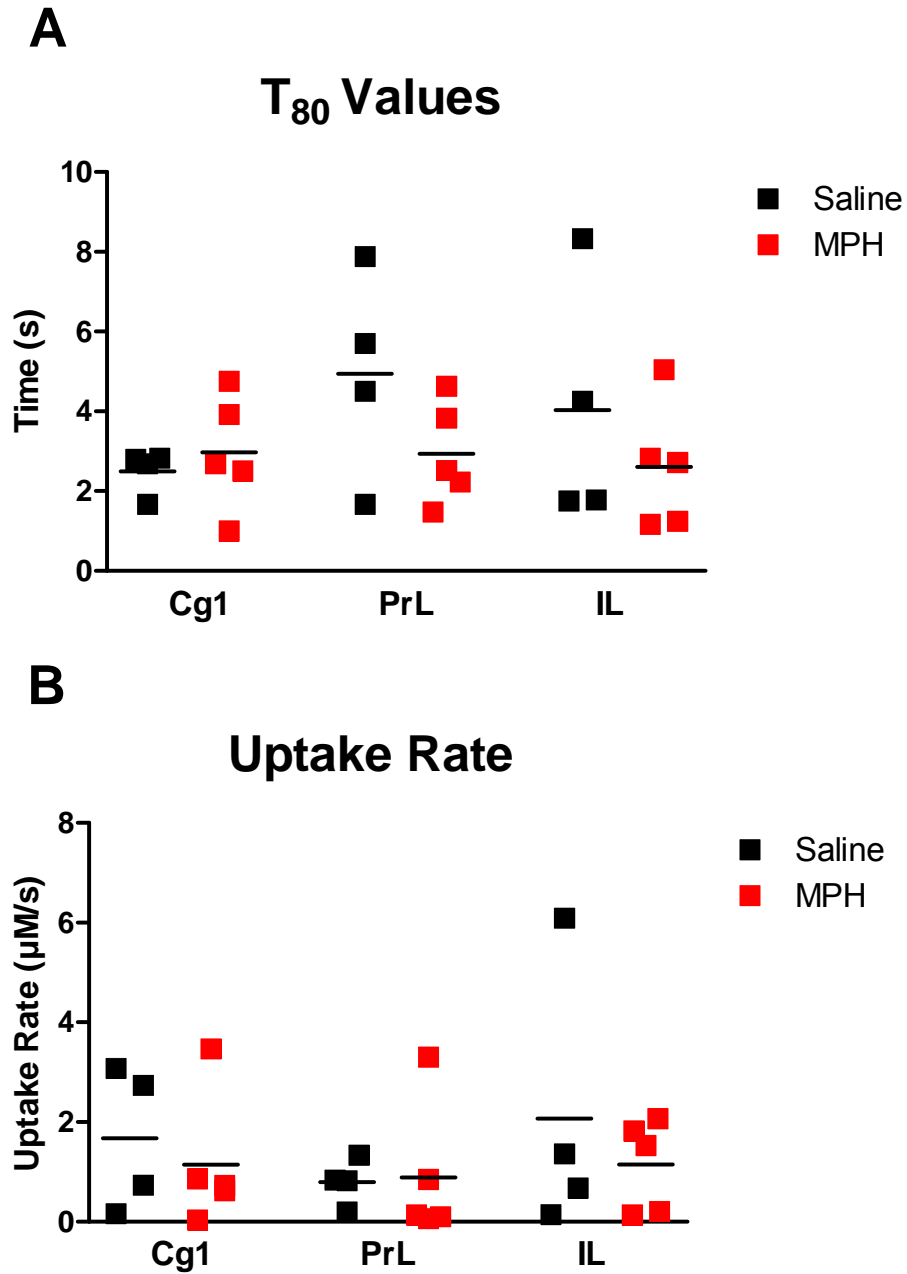
Uptake Rate

Figure 5.3 GlutamateT_{80} Values and Uptake Rates in Medial Prefrontal Cortex Sub-Regions
Both T_{80} values (A) and uptake rates (B) showed homogeneity between chronic treatment groups and among sub-regions (saline, n = 4; MPH, n = 5).

Table 5.1 Comparison of Glutamate Signaling Parameters Between MPH Treated Rats and Controls

Brain Sub-Regions	Chronic Treatment	Peak Amplitudes (µM)	T_{80} Values (s)	Uptake Rates (µM /s)
Cg1	Saline	2.6 ± 2.7	2.5 ± 0.56	1.7 ± 1.4
	MPH	2.4 ± 1.9	3.0 ± 1.4	1.1 ± 1.3
PrL	Saline	2.9 ± 1.7	4.9 ± 2.6	0.80 ± 0.47
	MPH	2.0 ± 1.6	2.9 ± 1.3	0.89 ± 1.9
IL	Saline	2.0 ± 0.71	4.0 ± 3.1	2.1 ± 2.7
	MPH	1.7 ± 1.3	2.6 ± 1.6	1.2 ± 0.92

Phasic glutamate signaling parameters of peak amplitudes, T_{80} values, and uptake rates show similarity between the treatment conditions in each mPFC sub-region (mean ± SD; saline, n = 4; MPH, n = 5).

Chapter Six: The Effects of Methylphenidate on Infralimbic Prefrontal Cortex Glutamate Signaling in the Awake Freely-Moving Rat

Introduction

MPH is a stimulant medication that used in the treatment of attention-deficit hyperactivity disorder. As a stimulant, MPH is also abused, particularly in the adolescent and college age populations (DeSantis, Webb et al. 2008; Dupont, Coleman et al. 2008; Bogle and Smith 2009; Setlik, Bond et al. 2009). It has been reported that approximately 20% of children and adolescents taking MPH have been approached to sell, give away or trade their medication (Musser, Ahmann et al. 1998; Kollins, MacDonald et al. 2001). Additionally, in human discriminative-stimulus studies, MPH has been shown to produce high levels of amphetamine- and cocaine-appropriate responding (Heishman and Henningfield 1991; Rush, Kollins et al. 1998; Rush and Baker 2001), highlighting the subject-perceived similarity between MPH and known stimulants of abuse.

The infralimbic mPFC was chosen as the site of interest in these studies because of its involvement in drug addiction. The infralimbic sub-region specifically has been shown to be critical for suppressing drug seeking behavior following extinction in rats (Peters, LaLumiere et al. 2008; Peters, Kalivas et al. 2009; LaLumiere, Smith et al. 2012). Additionally, data from our anesthetized chronic MPH treatment studies suggest that the infralimbic mPFC may be an area that differs in glutamate signaling parameters such as resting glutamate levels and T_{80} (time for 80% of a phasic signal to decay to baseline) between chronically MPH-treated and saline-treated rats. Rapid, transient glutamate events (Hascup, Hascup et al. 2011) were also examined, as our advanced MEA technology offers the ability to sample glutamate signaling on a sub-second time scale.

Our earlier work sought to characterize the effects of MPH on glutamate signaling in three sub-regions of the mPFC through a depth profile analysis. To gain further insight into the effect of MPH on infralimbic glutamatergic transmission without the confound of anesthesia, we chronically treated rats with

MPH, implanted MEAs in the infralimbic mPFC for glutamate detection, and recorded glutamatergic neurotransmission and behavior during an MPH challenge dose and a cocaine challenge dose. The challenge dose paradigm was used to investigate the possibility of sensitization to MPH following chronic treatment and a rest period, as it has been shown that behavioral responses to psychomotor stimulants exist long after drug treatment (Pierce and Kalivas 1997). Thus, we hypothesized that we would see behavioral sensitization, as well as a sensitization of tonic and phasic glutamate signaling to MPH administration.

Materials and Methods

Animals

Male Sprague-Dawley rats, aged 3-6 months were used for these experiments (for care, see <u>Animals</u>). Following MEA implant surgery, animals were monitored and weighed daily. If an animal weighed less than 80% of its pre-surgery body weight, recordings were not performed that day.

Methylphenidate Administration

Rats received injections for ten consecutive days. Animals were injected with either MPH (10 mg/kg in saline; s.c.) or saline (s.c.), and then placed in a locomotor activity monitoring chamber for one hour. Following activity monitoring, animals were given saline injections (s.c.) before being returned to their home cages.

Locomotor Activity Monitoring

After receiving the initial MPH or saline injection, animals were placed in a Digiscan animal activity monitoring device inside of the recording chamber. Multiple parameters of locomotion, including distance traveled and number of movements, were measured in five minute time bins over the course of one hour using Oasis software. Locomotor activity was measured for the entire duration of

electrochemical recording sessions. After a recording session, the recording chamber was cleaned with Roccal disinfectant (Pfizer, Inc., New York City, NY).

Experimental Timeline

The total length of each experiment was 20 days. Animals received either MPH or saline injections for ten consecutive days, and then were given a seven day rest period. During the rest period, on day 14 of the experiment, MEAs were implanted. Animals were given 3 days to recover from surgery before recordings were started. Recordings were performed on day 18, when rats received a MPH challenge dose, and on day 20, when they received a cocaine challenge dose. After the last recording, animals were euthanized and brains were collected.

Microelectrode Array Preparation

To optimize the MEA for chronic recordings, the MEA circuit board was minimized, and the MEA was epoxied to a plug/connector assembly (Microelectrode Array Configuration and Preparation – Modifications for Awake Freely-Moving Recordings). MEAs were made selective for glutamate through the use of glutamate oxidase coating layers (Microelectrode Array Configuration and Preparation – Microelectrode Array Coatings). For *in vivo* chronic recordings, Nafion® was used as the exclusion layer (Microelectrode Array Configuration and Preparation – Exclusion Layers) to prevent electroactive interferents from coming into contact with the Pt recording sites. Prior to implantation, MEAs were calibrated *in vitro* (Microelectrode Array Calibrations). For awake freely-moving MEAs, the calibrations were performed within the recording chamber to best simulate the environment in which the recordings would take place. After calibration, MEA ceramic tips and reference electrodes were soaked in phosphate buffered saline (PBS; 0.05 M) until the implantation surgery.

Microelectrode Array Implant Surgery

MEA implant surgery procedures are described in <u>Microelectrode Array Implant Surgery and Recordings – Awake Freely-Moving Surgery Procedures</u>. Surgical tools were autoclaved for the first surgery of the day, and sterilized in a glass bead sterilizer for any subsequent surgeries. Total time for each surgery was approximately one hour.

Awake Freely-Moving Recordings

All recordings were performed at 4 Hz. For details regarding recording protocols, see <u>Microelectrode Array Implant Surgery and Recordings – Awake Freely-Moving Recordings</u>. On day 18 of the experiment, rats were connected to their tethers outside of the recording chambers, in their home cages. This allowed for the signal to baseline before injections and placement into the recording chamber. After approximately one hour of baseline, the rats were given a saline injection and placed in the recording chamber. At this point, the activity monitoring software was also started. One hour following the saline injection, rats in both groups were given MPH challenge doses (10 mg/kg, s.c.). Recordings were performed for three hours following the challenge dose injections. Animals were monitored during the recordings, and if the tether became tangled due to locomotor activity, the experimenter unobtrusively unwound the line using the swivel. At the end of the experiment, animals were returned to their home cages.

On day 20, similar procedures were followed for a cocaine challenge. Both the saline injection and cocaine injection (10 mg/kg) were given i.p. Recordings were only continued for 90 minutes after the cocaine challenge injection, due to the shorter half-life of cocaine. After the experiments, animals were returned to their home cages, and then later sacrificed.

Histology

After the experiments, animals euthanized under heavy anesthesia and their brains were removed to check for MEA placements. For details, see

<u>Histology</u>. Animals with MEA placements outside of the infralimbic cortex were excluded from data analysis.

Data Analysis and Statistics

Collected data were processed using a custom Matlab®-based analysis package (<u>Data Collection</u>). Data parameters of interest included resting levels of glutamate, glutamate peak amplitudes, T_{80} values, and uptake rates. To examine phasic glutamate activity, recordings were scanned using a peak finder tool with a threshold set at a minimum of a signal-to-noise ratio of 2.5. A two-way repeated measures ANOVA was used for data analysis, with a Bonferroni *post hoc* test. Data points more than two standard deviations away from the mean were excluded from analyses. Significance was defined as $p < 0.05$.

Results
Chronic Treatment with Methylphenidate Increases Locomotor Activity

After receiving a daily injection of either MPH (n = 9) or saline (n = 8), animals' locomotor activity was monitored. Significant effects were found in distance traveled (Figure 6.1) including effects of time ($F_{(9,15)}$ = 1.96; p = 0.050) and treatment with MPH ($F_{(1,15)}$ = 110; $p < 0.0001$), and an interaction was also found to be significant ($F_{(9,135)}$ = 4.1; p = 0.0001). The number of movements (Figure 6.1) also showed significant effects of time ($F_{(9,15)}$ = 2.8; p = 0.0047) and treatment with MPH ($F_{(1,15)}$ = 26; p = 0.0001).

Saline Injections Before Challenge Doses Reveal Significant Effects

On drug challenge days, saline injections were administered prior to the MPH or cocaine injections. On MPH challenge days, saline injections showed a significant effect of time on distance traveled (Figure 6.2; $F_{(5,12)}$ = 15; p = 0.0001; n = 7), while no effects were seen in number of movements (Figure 6.2). On cocaine challenge days, saline injections also showed a significant effect of time (Figure 6.8; $F_{(5,12)}$ = 5.8; p = 0.0002; n = 7). Similar to MPH challenge days, no effects were seen in the number of movements (Figure 6.8).

Resting levels of glutamate following these saline injections were also analyzed. On MPH challenge days, resting levels of glutamate showed a significant effect of time (Figure 6.3; $F_{(7,11)}$ = 2.3; p = 0.037; saline, n = 6; MPH, n = 7), and on cocaine challenge days, resting levels of glutamate showed a significant interaction (Figure 6.9; $F_{(7,84)}$ = 2.6; p = 0.017; n = 7) as well as a significant effect of time ($F_{(7,12)}$ = 2.8; p = 0.011).

Glutamate signaling parameters including number of peaks, peak amplitude, T_{80} values, and uptake rates were calculated for saline injections on drug challenge days. Following saline injections on MPH challenge days, none of the parameters of interest showed significant effects, indicating a relative uniformity of data (Figure 6.4). On cocaine challenge days, saline injections resulted in a significant effect of time on number of glutamate peaks (Figure 6.10; $F_{(7,12)}$ = 2.5; p = 0.023; n = 7). There were no effects in peak amplitudes, T_{80} values, or uptake rates following saline injections on cocaine challenge days.

Methylphenidate Challenge Dose Affects Locomotor Activity, Glutamate Resting Levels, and Phasic Glutamate Signaling

When MPH was administered as a challenge dose, significant effects were found in both measures of locomotor activity (Figure 6.5). Significant effects of time ($F_{(31, 12)}$ = 18; p < 0.0001; n = 7) and chronic treatment ($F_{(1,12)}$ = 5.5; p = 0.038) were shown, and a significant interaction was also present ($F_{(31, 372)}$ = 5.1; p < 0.0001) for distance traveled. Number of movements also displayed significant effects of time ($F_{(31,12)}$ = 4.4; p < 0.0001; n = 7) and chronic treatment ($F_{(1,12)}$ = 9.3; p = 0.010).

The significance found in resting levels of glutamate after MPH challenge dose injection includes an effect of time (Figure 6.6; $F_{(31,12)}$ = 7.5; p < 0.0001; n = 7) and an interaction ($F_{(31, 372)}$ = 4.2; p < 0.0001).

Uptake rates of phasic glutamate signaling associated with an MPH challenge dose showed a significant interaction (Figure 6.7; $F_{(31, 341)}$ = 1.6; p = 0.026; saline, n = 6; MPH, n = 7). No significant effects or interactions were

found for the number of glutamate peaks, peak amplitudes, and T_{80} values. A two-way repeated measures ANOVA was used for all analyses.

Significant Effects Seen in Locomotor Activity, Glutamate Resting Levels, and Peak Amplitudes Following Cocaine Challenge Dose

The administration of a cocaine challenge dose resulted in significant effects of time on both distance traveled (Figure 6.11; $F_{(19,12)} = 3.4$; $p < 0.0001$; n = 7) and number of movements ($F_{(19,12)} = 2.9$; $p < 0.0001$) over 90 minutes.

Glutamate resting levels showed a significant interaction (Figure 6.12; $F_{(19, 228)} = 4.0$; $p < 0.0001$; n = 7) associated with the cocaine challenge dose.

Phasic glutamate signaling parameters including number of peaks, peak amplitudes, T_{80} values, and uptake rates (Figure 6.13) did not show any significant effects or interactions with the cocaine challenge dose. A two-way repeated measures ANOVA was used for all analyses.

Discussion

This set of experiments illustrates the effects of chronic MPH treatment on locomotor behavior and glutamate signaling in the infralimbic sub-region of the prefrontal cortex. These studies reveal, for the first time, the *in vivo* glutamatergic response to MPH in the awake freely-moving animal. Challenge doses of MPH and cocaine were used to gain insight into possible behavioral or pharmacological sensitization that may have occurred following chronic treatment with MPH. Data indicated the presence of behavioral sensitization (see Chapter 5) during the chronic MPH treatment period, and revealed significant effects in locomotor behavior and glutamate resting levels following saline injections, MPH injections, and cocaine injections.

Chronic MPH Treatment Significantly Increases Locomotor Activity

Animals were treated with an abuse-level dose of MPH for ten days as a chronic treatment period. During this time, animals receiving MPH showed consistently increased levels of locomotor activity. As previously discussed in

Chapter 5, stimulant drugs predictably produce increased amounts of locomotor activity following administration (Dews 1953; Smith 1965; Hughes 1972; Bhattacharyya, Ghosh et al. 1980), and this is attributable to the behavioral sensitization associated with stimulant drug administration. It should be noted that on day 1 of treatment, both chronically MPH-treated and saline-treated animals showed similar numbers of movements. As the number of movements decreased and remained stable for chronically saline-treated rats, it can be assumed that the increase in locomotor activity was most likely due to exploring a novel environment.

Saline Control Injections Are Associated with Significant Effects on Locomotor Activity and Glutamate Resting Levels

After the chronic treatment period and the subsequent rest period, animals were given a control saline injection one hour prior to their MPH or cocaine challenge doses. Animals were connected to their tethers while in their home cages outside of the recording chamber to ensure that the glutamate signal had time to baseline before any pharmacological manipulations took place. After the baseline period, rats received saline injections and were placed in the recording chamber.

Both chronically MPH-treated and saline-treated animals showed similar significant effects of time on locomotor activity after the saline injection on day 18, the MPH challenge dose day. These patterns of locomotor activity between the two groups were surprisingly similar. Both groups of animals started with distance traveled total of around 1500 cm that decreased to approximately 500 cm, with similar slopes, over the course of 30 minutes. As the response of the chronically MPH-treated animals closely matched that of the chronically saline-treated animals, we can conclude that the injection and subsequent placement in the recording chamber did not lead to the behavior sensitization response previously seen in the MPH-treated animals during their chronic treatment period. On day 20, prior to cocaine administration, a saline injection was administered and resulted in locomotor levels slightly less than the saline injections given on

day 18.   It should be noted that this was the animals' first exposure to an i.p. injection, and this may account for the decreased levels of locomotion as compared to day 18.

Resting levels of glutamate were recorded from the infralimbic mPFC during the saline injection administration on both MPH and cocaine challenge dose days. On day 18, the s.c. saline injection resulted in a significant effect of time. Chronically MPH-treated animals averaged glutamate resting levels of 14 µM, and chronically saline-treated animals averaged glutamate resting levels of 22 µM following the saline injection. Other groups have reported glutamate resting level concentrations ranging from 0.15 to 1.0 µM in the mPFC of awake freely-moving Sprague-Dawley rats (Yamamoto and Cooperman 1994; Welty and Shoblock 2009; Roenker, Gudelsky et al. 2011). The more than 20-fold difference between our chronically saline-treated animals' resting levels of glutamate and the levels of these microdialysis studies can best be explained by the advanced spatial resolution of our MEAs. The possibility of the saline injection itself or the change in environment from home cage to recording chamber being the reasons for the increased resting levels as compared to the microdialysis studies does not seem probable, as glutamate resting levels did not change significantly from pre-injection glutamate resting levels. The 38% decrease in resting levels of glutamate of the chronically MPH-treated rats as compared to saline controls is not what we would have anticipated based on our anesthetized results (see Chapter 5). As the resting levels of glutamate of the chronically MPH-treated rats did not change significantly following the saline injection, it appears that the decrease in resting levels may be due to long term effects of chronic MPH treatment. In other stimulant literature, cocaine has been shown to significantly decrease mPFC glutamate levels during self-administration of the drug, while increasing dopamine levels (Ben-Shahar, Szumlinski et al. 2012), and amphetamine has been shown to decrease excitatory glutamatergic transmission in the PFC (Mair and Kauer 2007). Therefore it has been hypothesized that increased dopamine levels may be responsible for decreasing glutamate release via activation of D1 receptors in the mPFC (Abekawa, Ohmori

et al. 2000; Mair and Kauer 2007). Additionally, MPH administration has been shown to significantly decrease synaptic active zone length in the PFC and alter GLAST levels in the striatum (Cavaliere, Cirillo et al. 2012). Thus, it could also be possible that alterations in GLT-1 and GLAST in the mPFC are responsible for the observed decreased glutamate resting levels. Taken together, these findings provide support for our observed decrease in resting glutamate levels in chronically MPH-treated rats.

The saline injection given prior to the cocaine challenge dose on day 20 showed similar results to that of the saline injection given on day 18. The glutamate resting levels of the chronically MPH-treated rats were comparable to those observed on day 18. The significant effect of time is evident in the decrease in resting levels of glutamate of the chronically saline-treated rats, which were attenuated from 28 µM to 22 µM over the 30 minute time period. This decrease in resting levels associated with the saline injection may be due to the first exposure to an i.p. injection, which would cause stress to the animal. This injection was also the first injection following their previous exposure to MPH. Thus, the decrease in glutamate resting levels may be a sign of sensitization to the effects of the MPH injection. Additionally, a significant interaction was observed, indicating that there is a complex relationship between chronic treatment and time. Chronic treatment and time serve as independent variables that work together to produce effects on our measurements of glutamate resting levels.

Parameters of phasic glutamate signaling did not show any significant differences following the saline injection on day 18, although a significant effect of time on number of glutamate peaks or transients existed on day 20. This is not surprising, given our anesthetized data. Thus, it appears that the saline injections were not capable of influencing dynamic glutamate signaling in the mPFC, although they did affect tonic glutamate signaling.

MPH Challenge Injection

The administration of the MPH challenge dose resulted in significant effects of time and chronic treatment in both distance traveled and number of movements, with a significant interaction also present for distance traveled. The sensitized locomotor response to our MPH challenge injection from the chronically MPH-treated animals was apparent in the distance traveled within the first hour following the injection. Within sixty minutes, we observed increased levels of motor activity in the chronically MPH-treated group as compared to the chronically saline-treated control group, followed by a plateauing of distance traveled by the chronically MPH-treated group and a dramatic increase in distance traveled by the chronically saline-treated group. The locomotor sensitization seen in the chronically MPH-treated group of animals supports the previous findings that stimulants can induce behavioral sensitization. However, the chronically saline-treated animals achieved amounts of distance traveled higher than those of the chronically MPH-treated animals during their chronic treatment period, as well as during the MPH challenge dose. This result was unexpected, given that the chronically saline-treated animals were drug naïve until this MPH administration.

The resting levels of glutamate of the chronically MPH-treated rats following the MPH injection remained relatively constant and did not vary from pre-injection levels. Following the MPH injection, chronically saline-treated rats showed a marked decrease in glutamate resting levels that approached the levels of the chronically MPH-treated rats after approximately two hours. As MPH increases dopamine levels, this decrease in glutamate resting levels could be attributed to dopamine hyperactivity stimulating D1 receptors.

Dynamic glutamate signaling did not show significant effects following MPH administration, except in the case of uptake rates which showed a significant interaction. It appears from our results that MPH administration acts on resting glutamate levels more so than phasic aspects of glutamate signaling. This makes sense in light of second messenger systems involved in D1 receptor

activation, which would lack the temporal resolution needed to induce changes in rapid, phasic glutamate signaling.

Cocaine Challenge Injection

Following cocaine injection administration, significant effects of time were seen in both distance traveled and number of movements, and behavioral sensitization was evident in the chronically MPH-treated animals in distance traveled. Twenty minutes after cocaine injections, chronically MPH-treated animals peaked in distance traveled as compared to their saline counterparts. In addition to behavioral sensitization, this is an example of cross sensitization, the increase in locomotor activity that occurs when treatment with one substance (in this case, MPH) leads to sensitization effects when another substance is presented (in this case, cocaine) (Aizenstein, Segal et al. 1990). Cross sensitization has been well documented in numerous cases among MPH and other drugs of abuse (Schenk and Izenwasser 2002; Yang, Swann et al. 2003; Valvassori, Frey et al. 2007; Wooters, Neugebauer et al. 2008; Wanchoo, Swann et al. 2009). Cross sensitization experiments are essential for understanding the likelihood of the use and/or abuse of one drug leading to the abuse of another.

Resting levels of glutamate after cocaine administration showed a significant interaction, with chronically MPH-treated animals showing a slight increase in resting levels, and chronically saline-treated animals showing a decrease in resting levels. The decrease in resting levels could once again be account for by D1 activation, but the increase in glutamate resting levels in the chronically-MPH treated group show the opposite effect. There has been a report of amphetamine increasing glutamate levels in the mPFC (Del Arco, Martinez et al. 1998), but this effect was not seen previously with the MPH challenge dose on day 18. It could be that with the shorter half-life of cocaine as compared to MPH, we recorded long enough to see resting levels begin to return to levels closer to those of the chronically saline-treated animals.

Once again, no significant differences in dynamic glutamate signaling parameters were observed. If the animals had received injections, and then

performed a task, it is possible that we may have observed differences between chronically MPH- and saline-treated animals, as the infralimbic mPFC has been implicated in working memory and extinction. Thus, future experiments should include a task component to allow us to better characterize chronic treatment effects on phasic signaling during goal-directed behavior.

Conclusions

This set of experiments sought to characterize glutamate signaling in the awake freely-moving animal following chronic treatment and subsequent challenge with MPH. Our results suggest that MPH increases locomotor activity, in accordance with previous studies. Additionally, chronic MPH treatment resulted in decreased resting levels of glutamate, and following MPH administration, the resting levels of chronically saline-treated animals attenuated to levels similar to the chronically MPH-treated animals. This observation provides support for the hypothesis that increased dopamine levels lead to D1 activation and subsequent glutamate suppression in the mPFC. For the majority of the experiments, we did not observe differences in dynamic signaling parameters. This may be due to the fact that we did not employ a task that would require rapid glutamate neurotransmission, and thus we did not see differences between the chronic treatment groups. Overall, this data suggests that the glutamate system should not be overlooked in stimulant abuse studies, particularly in the infralimbic mPFC. Further work examining the relationships between glutamate and dopamine signaling in the infralimbic mPFC are needed to fully understand the neurochemical changes associated with drug addiction. The studies in this chapter provide a possible target for the therapeutic treatment of drug addiction.

Chapter Six: Figures
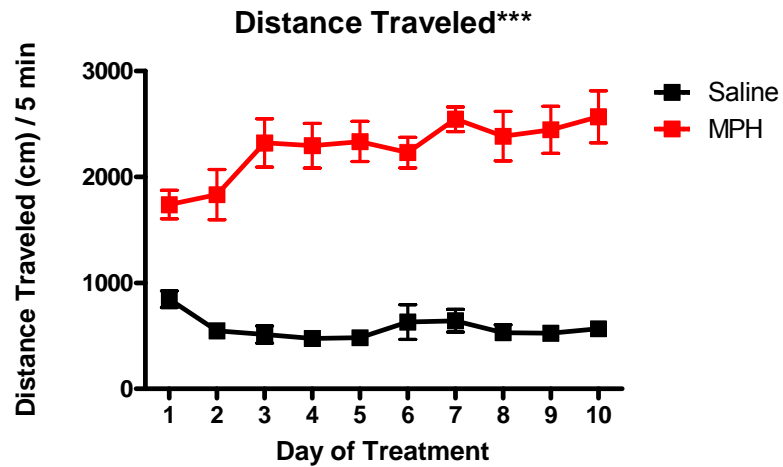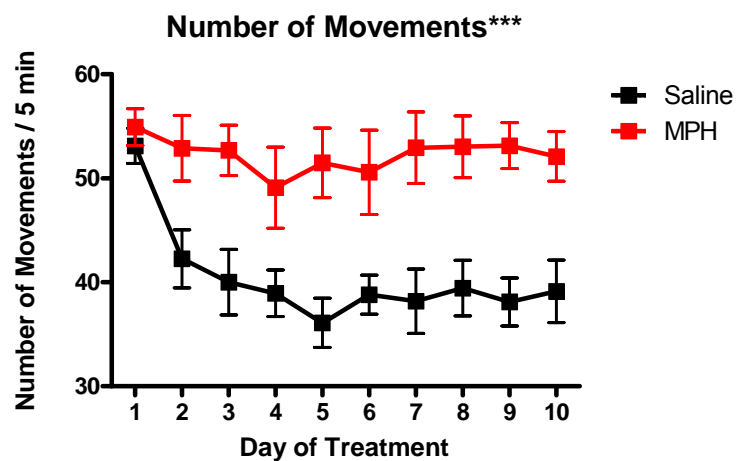
Figure 6.1 Locomotor Activity During Chronic Treatment Period
During the ten day treatment period, rats that received MPH injections (n = 9) showed significantly increased distance traveled (A; ***$p < 0.001$) and number of movements (B) as compared to saline controls (n = 8). Both sets of data were analyzed with a two-way repeated measures ANOVA. Data shown are mean ± SEM.

A

Saline Injection - MPH Challenge Day
Distance Traveled***

B
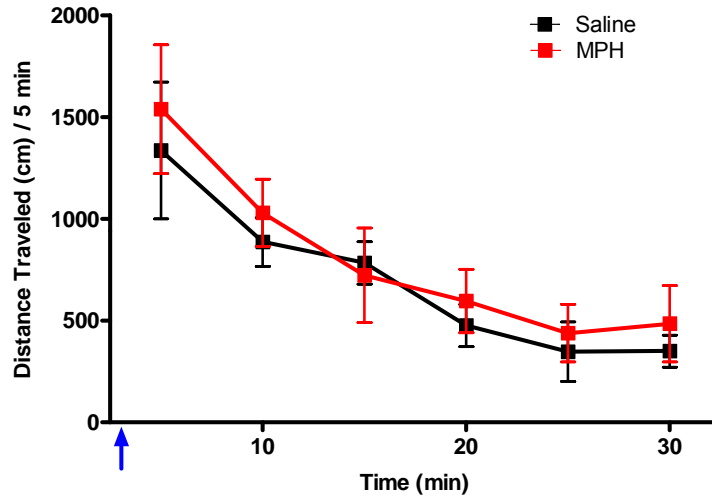
Saline Injection - MPH Challenge Day
Number of Movements
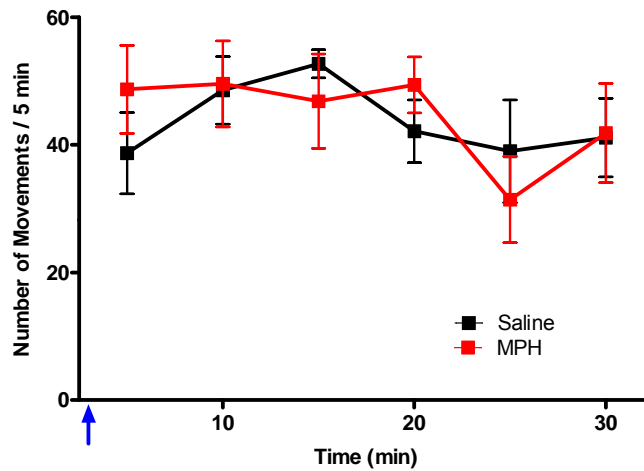
Figure 6.2 Motor Activity Decreases After Saline Injection on MPH Challenge
   Day

After receiving a saline injection (blue arrow) and being placed into the recording
chambers, both MPH and saline treated rats (n = 7) showed a significant effect of
time during distance traveled (A; ***$p < 0.001$). There were no differences in the
number of movements following the injection (B). Data were analyzed with a
two-way repeated measures ANOVA. Data are shown as mean ± SEM.

Figure 6.3 Glutamate Resting Levels Show A Significant Effect of Time After Saline Injection on MPH Challenge Day

Resting levels of glutamate (saline, n = 6; MPH, n = 7) were measured following a saline injection (blue arrow), and analysis revealed a significant effect of time (*$p < 0.05$). Data were analyzed using a two-way repeated measures ANOVA. Data are shown as mean ± SEM.

A

Saline Injection - MPH Challenge Day
Number of Peaks

B

Saline Injection - MPH Challenge Day
Peak Amplitudes

C

Saline Injection - MPH Challenge Day
T80 Values

D

Saline Injection - MPH Challenge Day
Uptake Rate
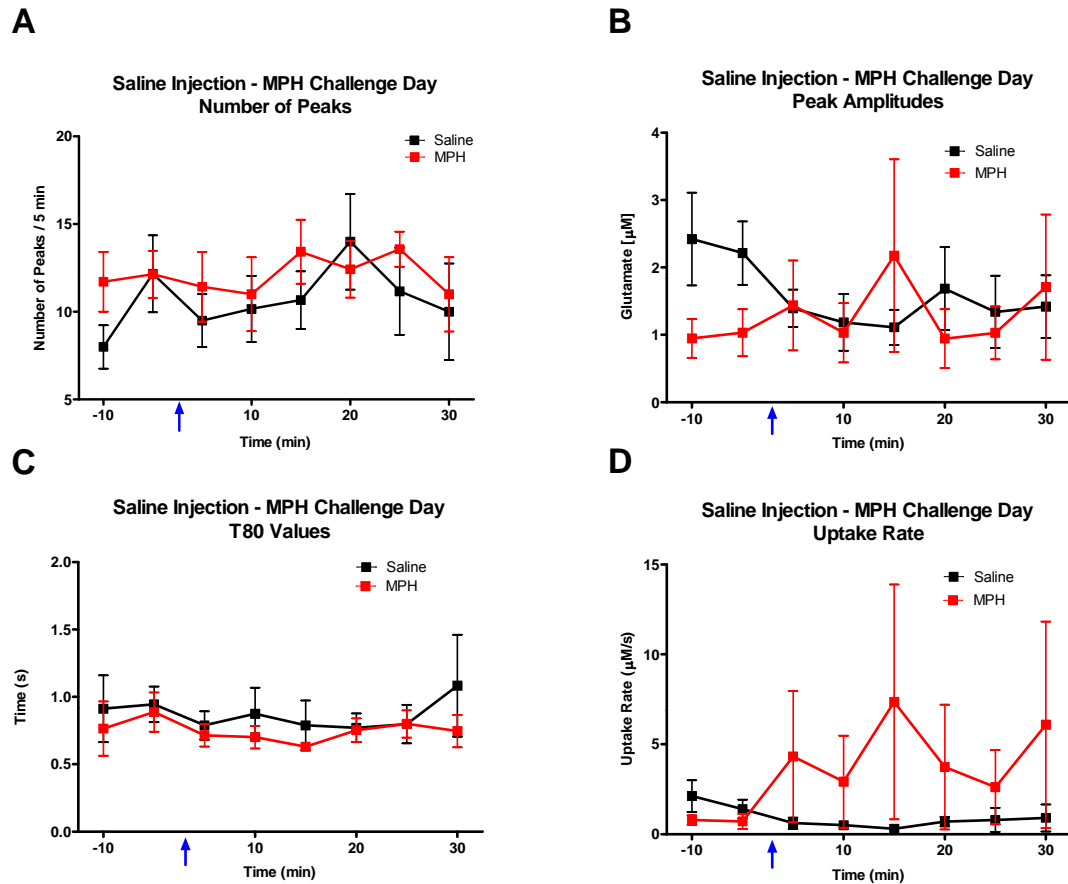
Figure 6.4 Phasic Glutamate Signaling Following Saline Injections on MPH Challenge Day

The number of peaks (A), peak amplitudes (B), T_{80} values (C), and uptake rates (D) were examined for both MPH and saline treated rats (saline, n = 6; MPH, n = 7) following a saline injection (blue arrow). No significant differences were found. Data were analyzed using a two-way repeated measures ANOVA. Data are shown as mean ± SEM.

A

MPH Injection
Distance Traveled***

B

MPH Injection
Number of Movements***
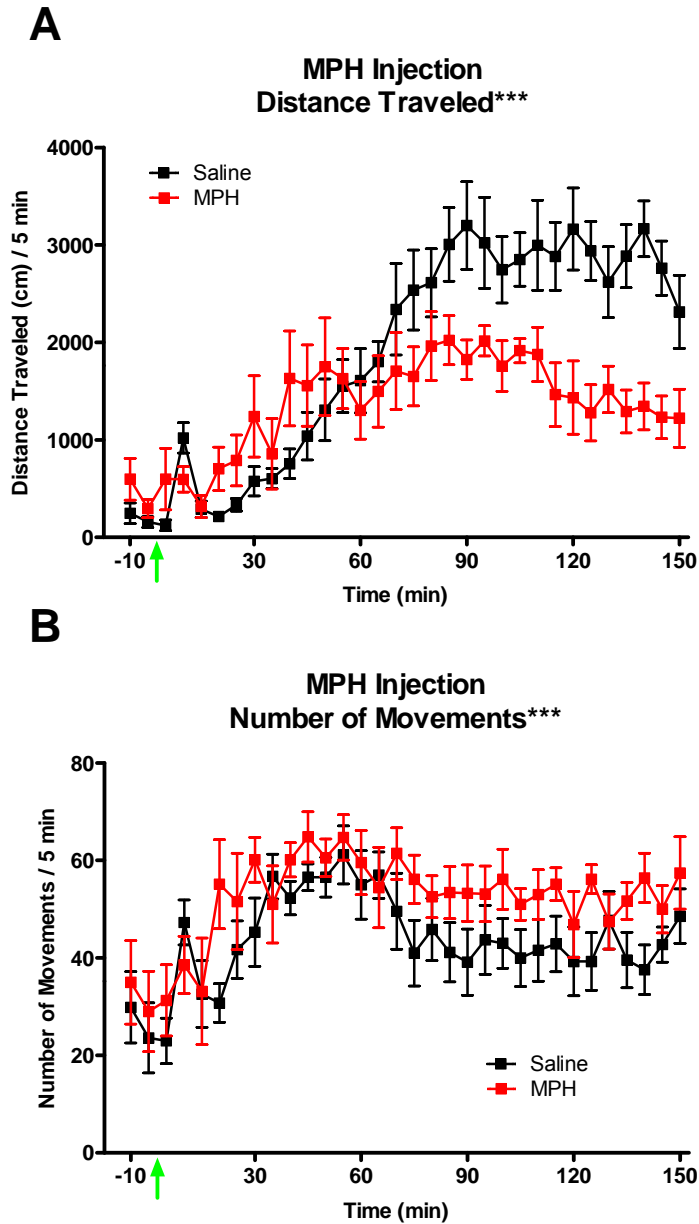
Figure 6.5 Methylphenidate Injections Result in Increased Locomotor Activity
After the administration of MPH injections (green arrow), significant effects of time (***$p < 0.001$), chronic MPH treatment ($p < 0.05$), and an interaction ($p < 0.001$) were seen in the amount of distance traveled (A; n = 7). The number of movements (B) shows significant effects of time (***$p < 0.001$) and chronic MPH treatment ($p < 0.05$). Data were analyzed using a two-way repeated measures ANOVA. Data are shown as mean ± SEM.

MPH Injection
Resting Levels***
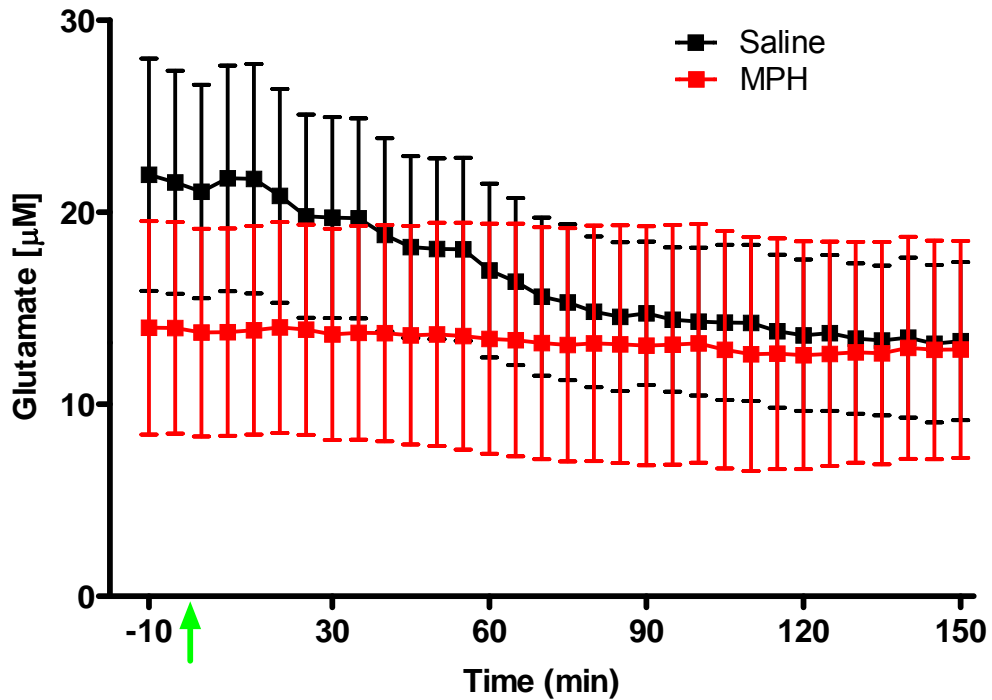
Figure 6.6 Glutamate Resting Levels Following MPH Injections
MPH injections (green arrow) resulted in a significant interaction (***$p < 0.001$)
and a significant effect of time (***$p < 0.001$) between glutamate resting levels of
saline and MPH treated rats (n = 7).   Data were analyzed using a two-way
repeated measures ANOVA. Data are shown as mean ± SEM.
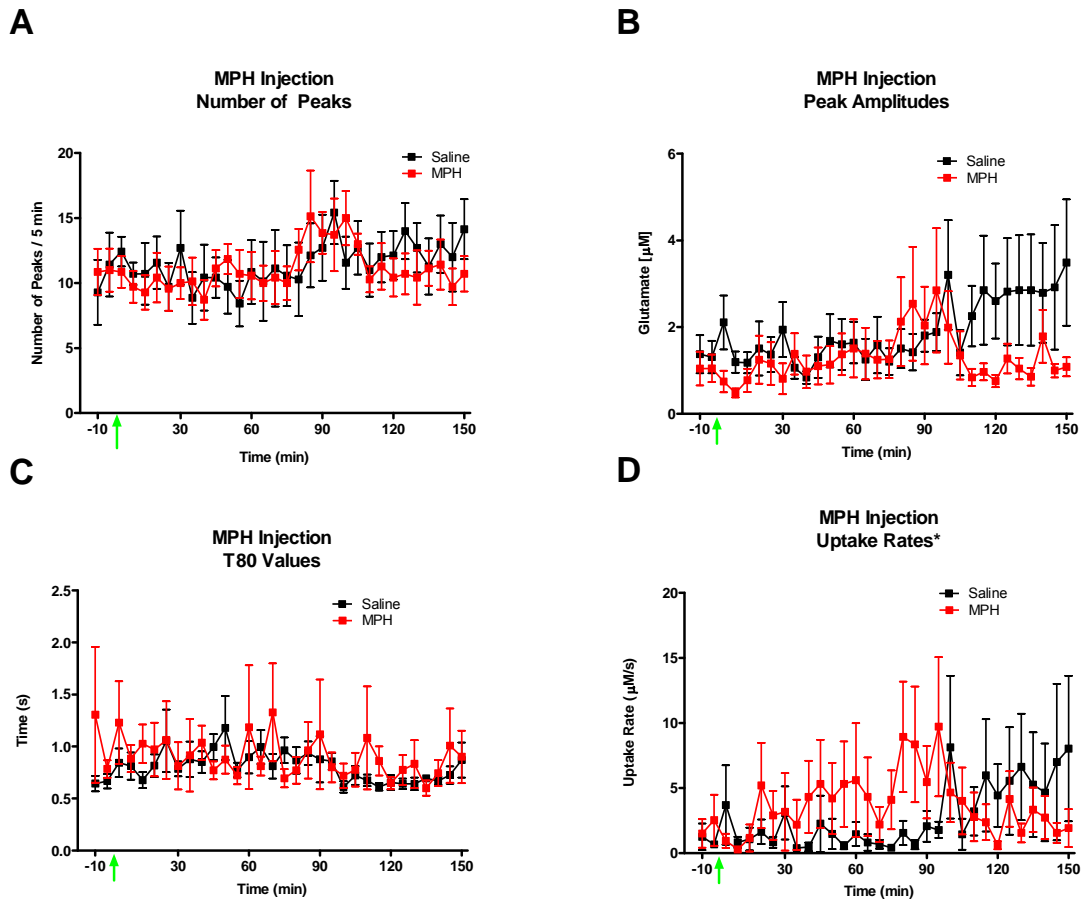
Figure 6.7 Phasic Glutamate Signaling Following MPH Injections

Following MPH injections (green arrow), rats displayed relatively homogenous numbers of peaks (A), peak amplitudes (B), and T_{80} values (C) in both saline and MPH treated groups (saline, n = 7 for number of peaks and peak amplitudes, n = 6 for T_{80} values and uptake rates; MPH, n = 7).   There was a significant interaction for uptake rates (D; *$p < 0.05$). Data were analyzed using a two-way repeated measures ANOVA. Data are shown as mean ± SEM.

A

Saline Injection - Cocaine Challenge Day
Distance Traveled***

B

Saline Injection - Cocaine Challenge Day
Number of Movements
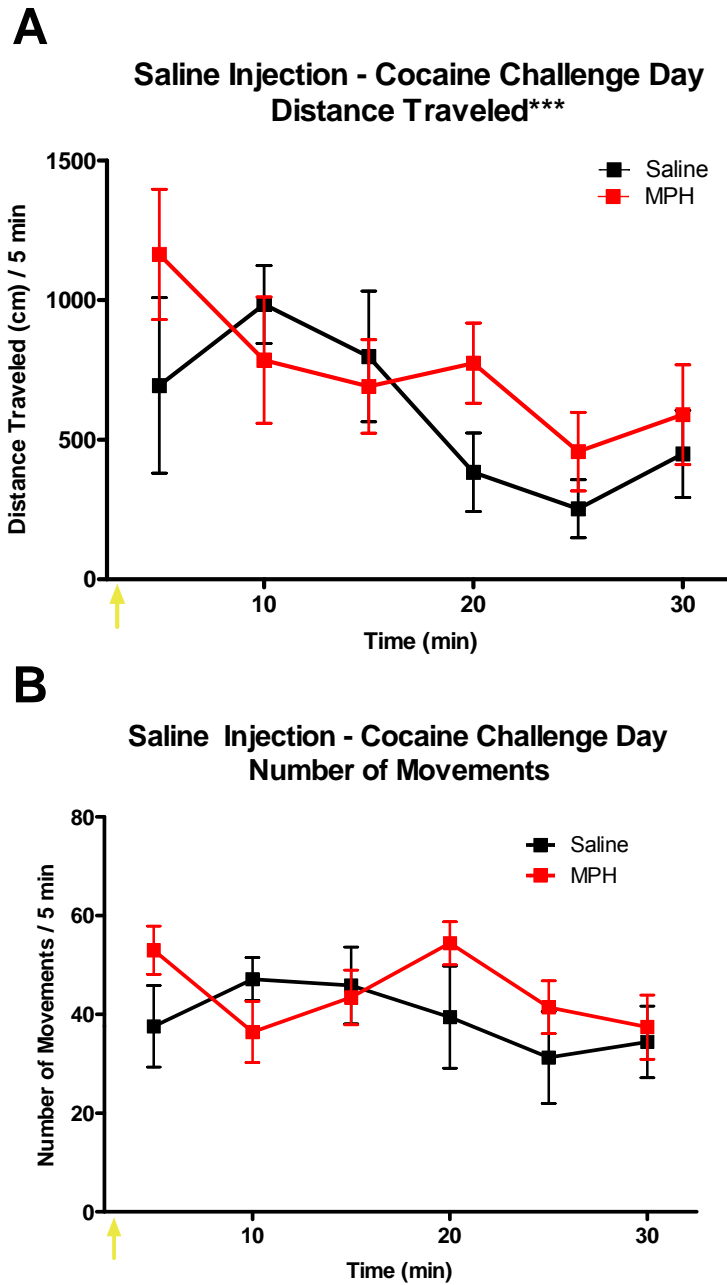
Figure 6.8 Locomotor Activity Following Saline Injections on Cocaine Challenge
Day

On the cocaine challenge day, saline and MPH treated rats (n = 7) were injected
with saline (yellow arrow). Data revealed a significant effect of time (***$p <$
0.001) on distance traveled (A) after the injection. This time effect was not seen
in number of the animals' movements (B). Data were analyzed using a two-way
repeated measures ANOVA. Data are shown as mean ± SEM.

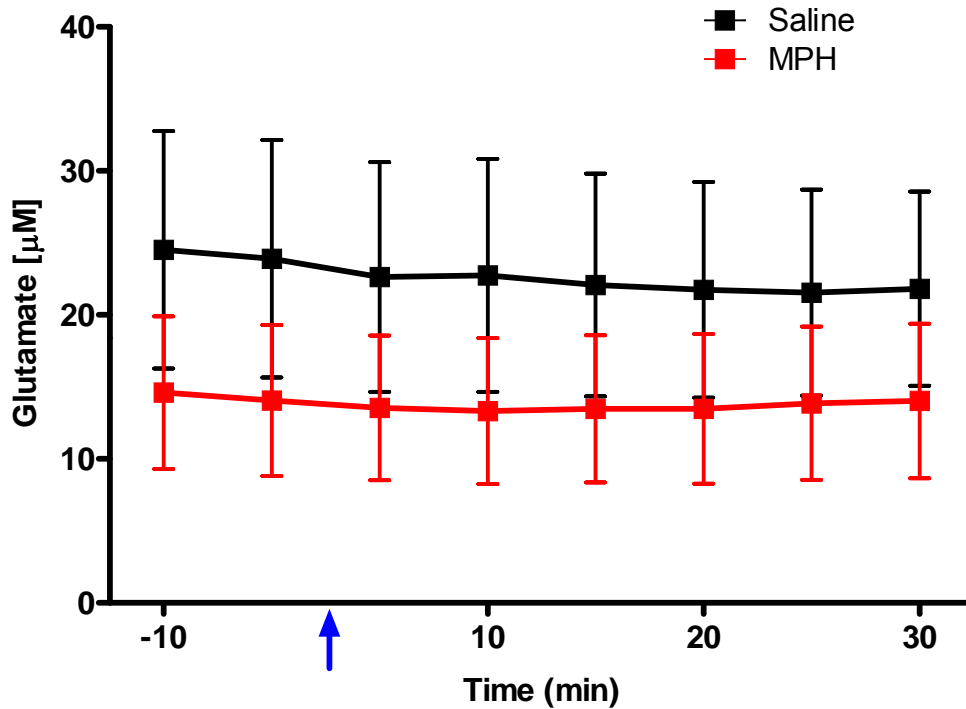**Saline Injection - Cocaine Challenge Day
Resting Levels***
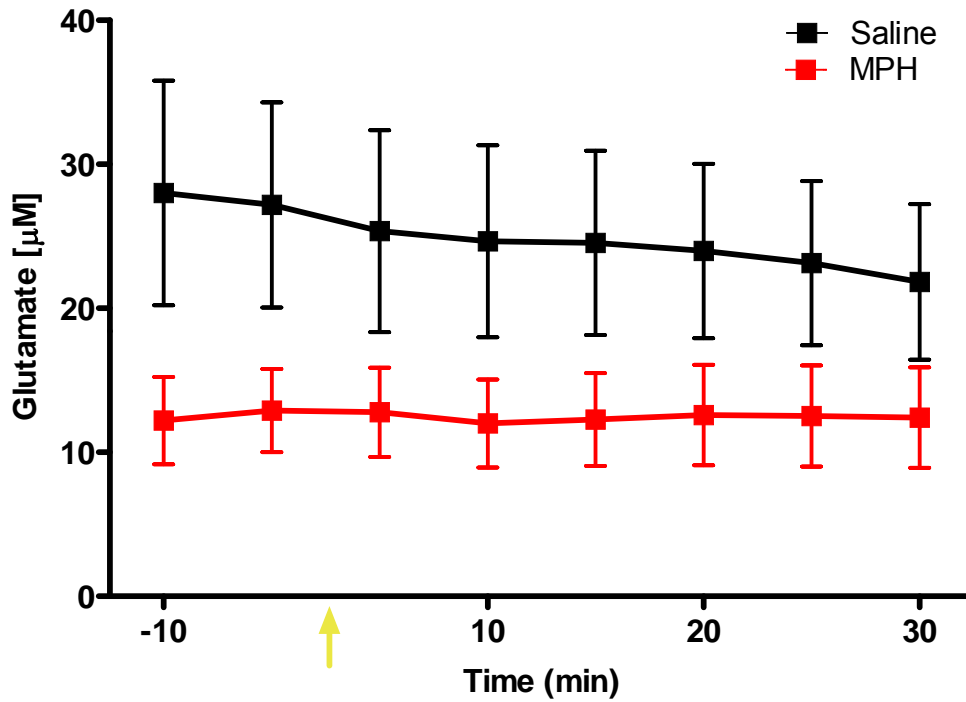
Figure 6.9 Glutamate Resting Levels Following Saline Injections on Cocaine
   Challenge Day

Saline injections (yellow arrow) administered before the cocaine challenge resulted in a significant interaction (*$p < 0.05$) and a significant effect of time (*$p < 0.05$) on glutamate resting levels in rats (n = 7). Data were analyzed using a two-way repeated measures ANOVA. Data are shown as mean ± SEM.
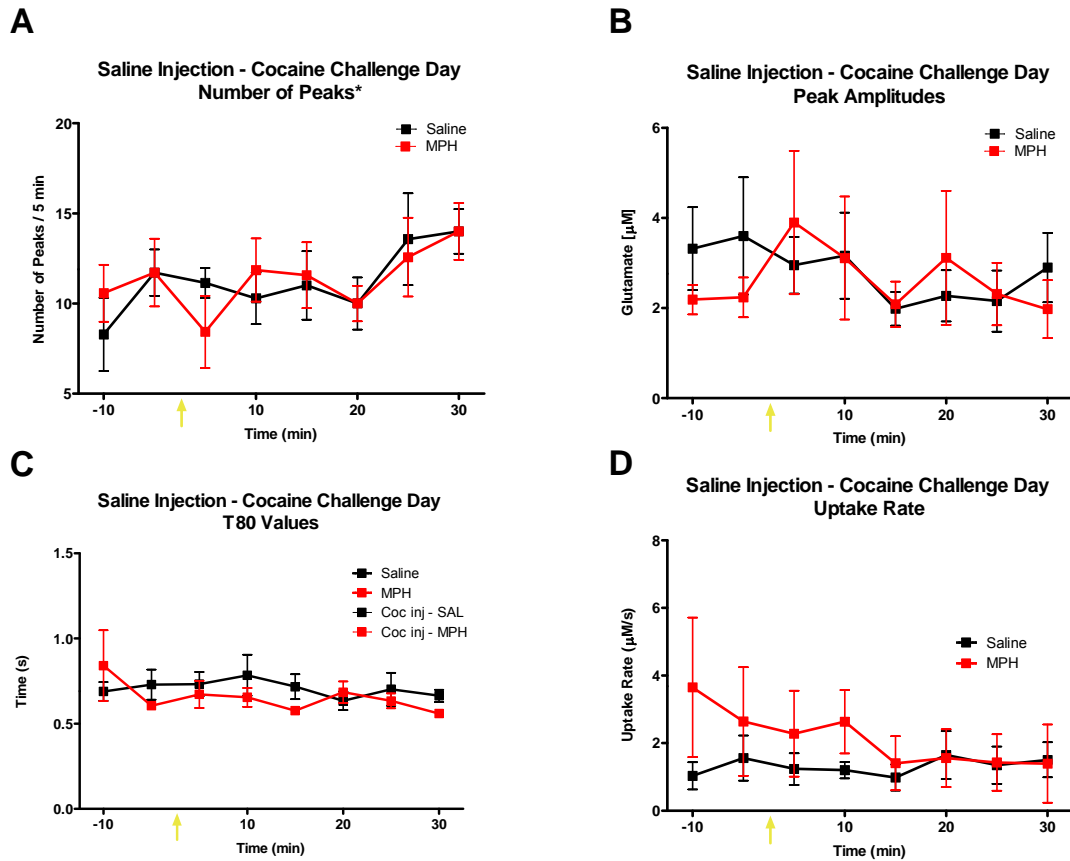
Figure 6.10 Phasic Glutamate Signaling After Saline Injections on Cocaine Challenge Day

A significant effect of time was seen in the number of peaks (A; *p < 0.05; n = 7) following saline injections (yellow arrow). Other phasic glutamate signaling parameters such as peak amplitudes (B; n = 7), T_{80} values (C; saline, n = 7; MPH, n = 6), and uptake rates (D; saline, n = 7; MPH, n = 5) did not reach significance. Data were analyzed using a two-way repeated measures ANOVA. Data are shown as mean ± SEM.

A

**Cocaine Injection
Distance Traveled*****

B

**Cocaine Injection
Number of Movements*****
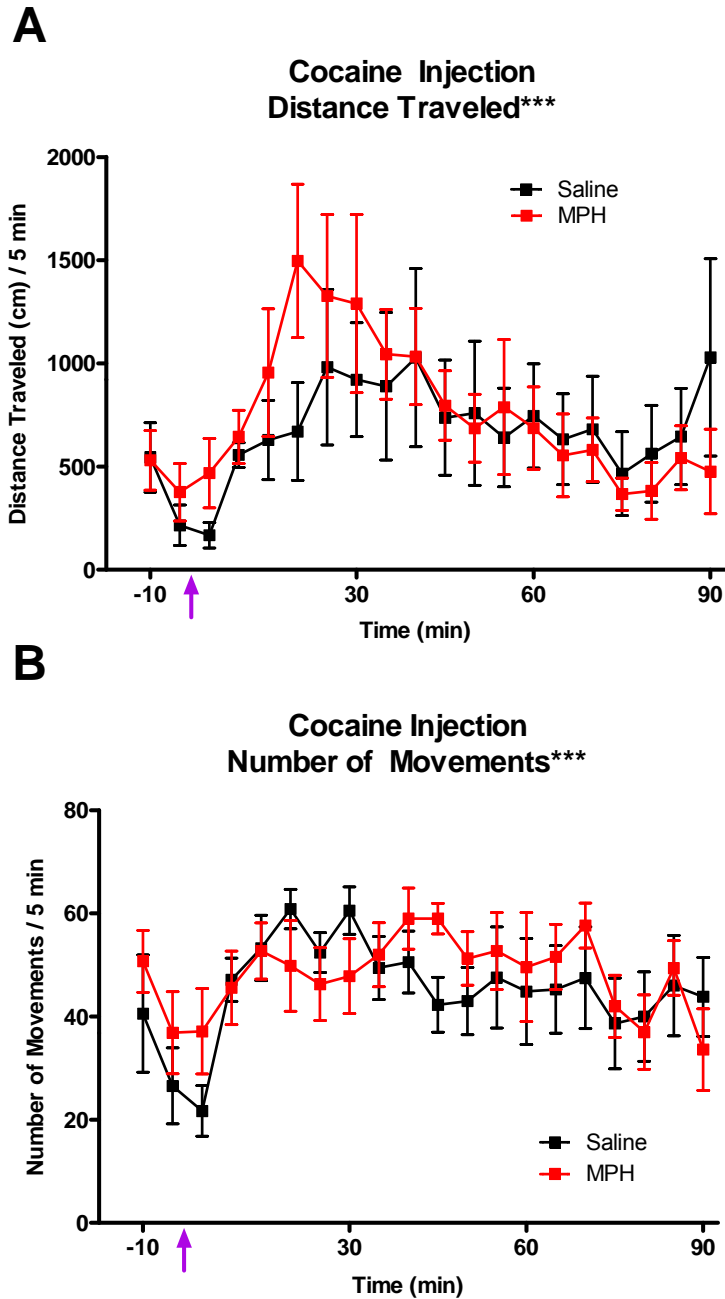
Figure 6.11 Locomotor Activity Following Cocaine Injections

Following cocaine injections (purple arrow), significant effects of time (***$p <$ 0.001) were observed for both distance traveled (A) and number of movements (B; n = 7). Data were analyzed using a two-way repeated measures ANOVA. Data are shown as mean ± SEM.
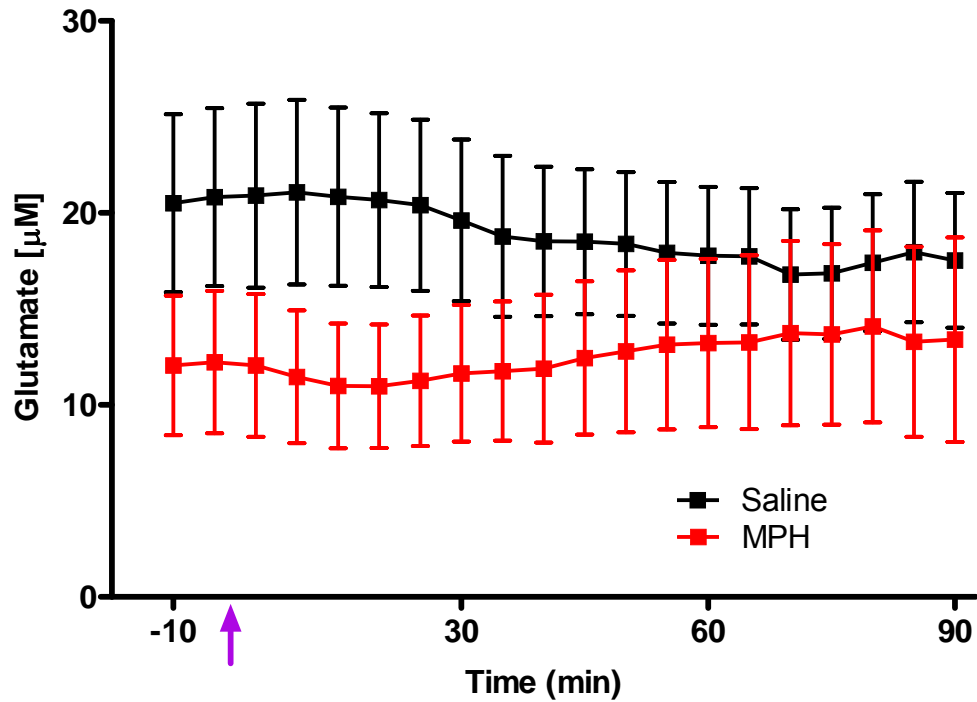
Figure 6.12 Tonic Glutamate Levels Following Cocaine Injections
A significant interaction (****$p < 0.001$; n = 7) was seen in resting glutamate levels following cocaine injections (purple arrow). Data were analyzed using a two-way repeated measures ANOVA. Data are shown as mean ± SEM.
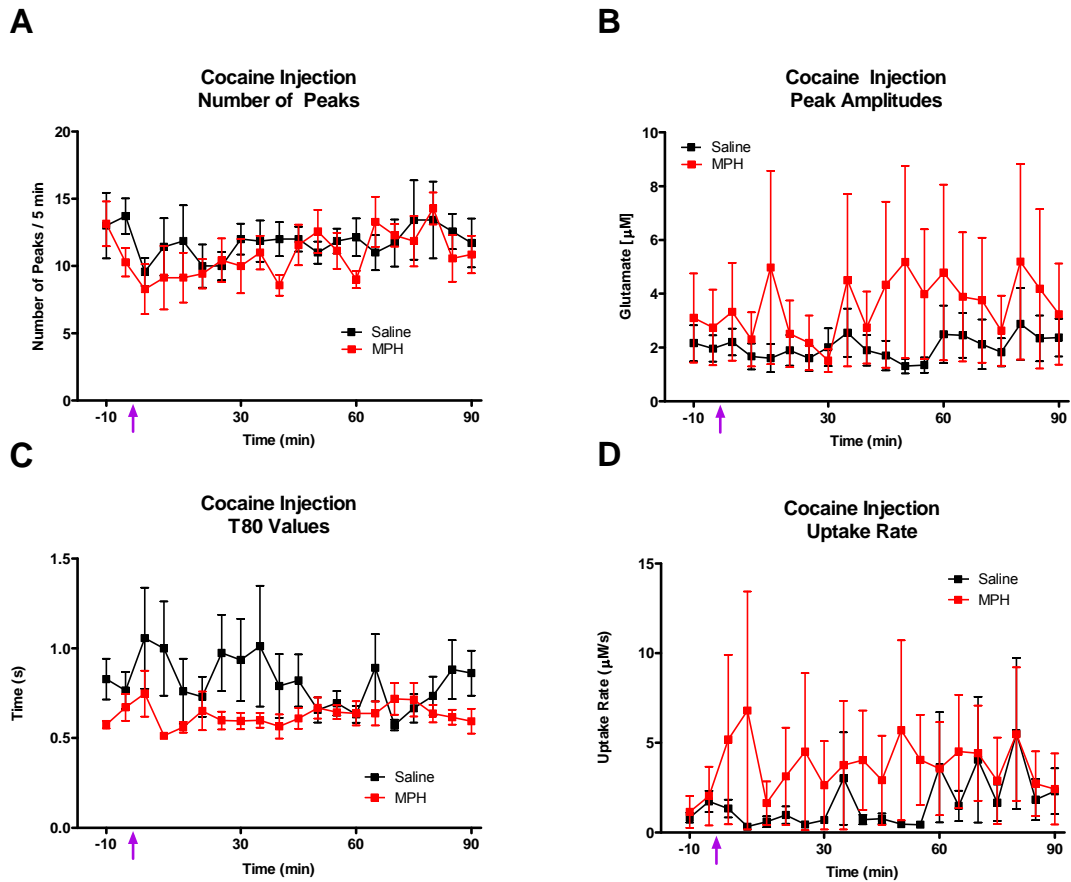
Figure 6.13 Phasic Glutamate Signaling After Cocaine Injections

Following cocaine injections (purple arrow), no significant effects were observed in the number of peaks (A; n = 7), peak amplitudes (B; n = 7), T_{80} values (C; saline, n = 7; MPH, n = 6) or uptake rates (D; saline, n = 7; MPH, n = 5). Data were analyzed using a two-way repeated measures ANOVA. Data are shown as mean ± SEM.

Chapter Seven: Final Conclusions

There is a lack of understanding of glutamate neurochemistry in the medial prefrontal cortex (mPFC), despite the critical role that this brain structure plays in higher cognitive processes and neuropathologies. Initially, this body of work characterized glutamatergic signaling in the mPFC, and from our mapping studies, we found that glutamate levels in the cingulate, prelimbic, and infralimbic mPFC were relatively uniform, as were ACh levels in these three sub-regions. The similarity in glutamate and ACh neurotransmission within the mPFC underscores a somewhat homogenized regulation of the glutamatergic and cholinergic systems in this brain area.

Our awake freely moving studies in the infralimbic mPFC did not find evidence of circadian effects on glutamate signaling. However, we did establish relationships between glutamate signaling parameters and locomotion during the light and dark cycles of the circadian cycle. Importantly, we observed rapid and dynamic glutamate transients. These findings support other recent studies that have reported this aspect of phasic glutamate signaling (Hascup, Hascup et al. 2011; Wassum, Tolosa et al. 2012)

Our second mapping study looked at the effects of chronic MPH treatment on glutamate signaling in the mPFC, and did not find that the drug had any significant effect on glutamate resting levels or glutamate signaling parameters. Locomotor monitoring did reveal behavioral sensitization, which is thought to model drug-induced plasticity. Due to the effects of anesthesia on glutamate resting levels, we then carried out this experiment, with a challenge dose paradigm, in the awake freely-moving animal.

The final study explored the effects of a challenge stimulant dose on glutamate neurotransmission following chronic MPH treatment in the awake freely-moving rat. Once again, locomotor behavioral sensitization was observed during the chronic treatment period in MPH-treated animals, and we confirmed the presence of glutamate transients. Significant effects were observed in

glutamate resting levels, although most other glutamate signaling parameters did not show significance. From our data, it appears that chronic MPH treatment resulted in decreased glutamate resting levels, and the MPH challenge dose given to chronically saline-treated animals decreased resting glutamate concentrations to similar levels.

Taken together, this set of experiments provides data on mPFC glutamate signaling to a degree never before reported, and also advances our understanding of the effects of chronic MPH treatment on glutamate signaling in the mPFC. This is the first report of mPFC sub-regional levels of glutamate and ACh using MEA technology, thus providing insight into neurotransmission in the discreet areas of a complex brain structure involved in many aspects of cognitive functioning. Additionally, the characterization of the effects of MPH on glutamate signaling in the mPFC eliminate the paucity of information regarding the actions of this drug of abuse on excitatory neurochemistry in a brain region implicated in higher cognitive processes.

With the information gained from our studies, scientists have an additional source of *in vivo* glutamate concentration levels in the mPFC, as measured using MEA technology. It is our hope that these data will provide insight into typical glutamate neurotransmission, as well as glutamate neurotransmission under the chronic influence of a drug of abuse. This information can be used to help develop pharmacotherapies that target the glutamatergic system and improve the lives of drug addicts.

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<p style="text-align: center;">**Vita**</p>

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Maiden Name: Catherine Elizabeth Werner
Date of Birth: 03/30/1983
Birthplace: Cleveland, OH USA

Education

1997-2001	High School Diploma, St. Augustine Academy (Phi Beta Kappa), Lakewood, OH USA
2001-2006	B.S. (with distinction), Psychology, The Ohio State University, Columbus, OH USA
2007-	Ph.D., University of Kentucky College of Medicine, Department of Anatomy and Neurobiology, Lexington, KY USA

Professional Experience

2007-2012	Training Course Instructor. Annual "Second-by-Second Electrochemical Measurements in Biological Systems" University of Kentucky, Lexington, KY. Center for Microelectrode Technology.
2009-2012	Graduate Certificate in Anatomical Sciences. University of Kentucky, Lexington, KY.
Fall 2009	Teaching Assistant: ANA 209 – *Principles of Human Anatomy.* University of Kentucky, Lexington, KY. Lectures given: Special Senses I
Spring 2010	Teaching Assistant: ANA 209 – *Principles of Human Anatomy.* University of Kentucky, Lexington, KY. Lectures given: Cardiovascular System II
Fall 2010	Teaching Assistant: ANA 209 – *Principles of Human Anatomy.* University of Kentucky, Lexington, KY. Lectures given: Endocrine I, Female Reproduction I
Spring 2011	Guest lecturer: ANA 209 – *Principles of Human Anatomy.* University of Kentucky, Lexington, KY. Lectures given: Muscle Biology, Digestive System I & II
Fall 2011	Teaching Assistant: ANA 209 – *Principles of Human Anatomy.* University of Kentucky, Lexington, KY.

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Lectures given: Cartilage, Urinary System II

Spring 2012   Guest lecturer: ANA 209 – *Principles of Human Anatomy.* University of Kentucky, Lexington, KY.
Lectures given: Nervous System V & VI

Spring 2012   Teaching Practicum: ANA 611 – *Regional Human Anatomy.* University of Kentucky, Lexington, KY
Assist in cadaver-based anatomy course for PA/PT graduate students
Lectures given: Cranial Contents

Scholastic and Professional Honors

2002-2006   Dean's list (five quarters). The Ohio State University, Columbus, OH.

2007   First place in the graduate student category of the Institute of Biological Engineering Student Poster Presentation and Competition. St. Louis, MO.

2008   Outstanding graduate student poster presentation at the annual Neuroscience Day hosted by the Bluegrass Chapter of the Society for Neuroscience. Lexington, KY.

2010-2012   Predoctoral Fellowship: NIH Training Grant 5T32 DA016176, "Training in Drug Abuse Related Research". University of Kentucky, Lexington, KY.

2011   Outstanding graduate student poster presentation at the annual Neuroscience Day hosted by the Bluegrass Chapter of the Society for Neuroscience. Lexington, KY.

Publications

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C.E. Werner, J.J. Burmeister, C.R. Gash, F. Pomerleau, P. Huettl, J.P. Bruno, and G.A. Gerhardt. Regional analysis of acetylcholine neurotransmission in rat prefrontal cortex and striatum. Monitoring Molecules in Neuroscience. 12th International Conference on *In Vivo* Methods. Vancouver, British Columbia, Canada, 2008.

C.E. Mattinson, J.E. Quintero, F. Pomerleau, P. Huettl, and G.A. Gerhardt. Regional depth analysis in rat PFC of glutamate neurotransmission *in vivo*: effects of anesthetics and diurnal rhythms. Monitoring Molecules in Neuroscience. 13th International Conference on *In Vivo* Methods. Brussels, Belgium, 2010.

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Poster Presentations

2007   **C.E.Werner**, J.J. Burmeister, C.R. Gash, F. Pomerleau, P. Huettl, J.P. Bruno, G.A. Gerhardt. Second-by-Second Microelectrode Array Measurements of Acetylcholine. 12th annual meeting - Institute of Biological Engineering. St. Louis, MO.

2007   **C.E.Werner**, J.J. Burmeister, C.R. Gash, F. Pomerleau, P. Huettl, J.P. Bruno, G.A. Gerhardt. Second-by-Second Microelectrode Array Measurements of Acetylcholine Release and Uptake in Cortex and Striatum. 37th annual meeting – Society for Neuroscience. San Diego, CA.

2008   **C.E. Werner**, J.J. Burmeister, C.R. Gash, F. Pomerleau, P. Huettl, J.P. Bruno, G.A. Gerhardt. Regional Analysis of *In Vivo* Acetylcholine Neurotransmission in Rat Prefrontal Cortex and

Striatum. Neuroscience Day - Bluegrass Chapter for the Society of Neuroscience. Lexington, KY.

2008   **C.E. Werner**, J.J. Burmeister, C.R. Gash, F. Pomerleau, P. Huettl, J.P. Bruno, G.A. Gerhardt. Regional Analysis of Acetylcholine Neurotransmission in Rat Prefrontal Cortex and Striatum. Monitoring Molecules in Neuroscience. 12th International Conference on *In Vivo* Methods. Vancouver, British Columbia, Canada,

2010   **C.E. Mattinson**, J.E. Quintero, F. Pomerleau, P. Huettl, G.A. Gerhardt. Regional Analysis of *In Vivo* Glutamate Neurotransmission in Rat Prefrontal Cortex. Neuroscience Day – Bluegrass Chapter for the Society of Neuroscience. Lexington, KY.

2010   **C.E. Mattinson**, J.E. Quintero, F. Pomerleau, P. Huettl, G.A. Gerhardt. Regional Analysis of *In Vivo* Glutamate Neurotransmission in Rat Prefrontal Cortex. Center for Clinical and Translational Science Conference. Lexington, KY.

2010   **C.E. Mattinson**, J.E. Quintero, F. Pomerleau, P. Huettl, G.A. Gerhardt. Regional Depth Analysis in Rat PFC of Glutamate Neurotransmission *In Vivo*: Effects of Anesthetics and Diurnal Rhythms. Monitoring Molecules in Neuroscience. 13th International Conference on *In Vivo* Methods. Brussels, Belgium.

2011   **C.E. Mattinson**, J.S. Beckmann, J.A. Marusich, F. Pomerleau, P. Huettl, M.T. Bardo, G.A. Gerhardt. Prefrontal Cortex Glutamate: Transient Signaling and the Effects of Methylphenidate. Neuroscience Day – Bluegrass Chapter of the Society for Neuroscience. Lexington, KY.

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2011   **C.E. Mattinson**, J.E. Quintero, F. Pomerleau, P. Huettl, G.A. Gerhardt. Diurnal Recordings of Second-by-Second Measurements of Tonic and Phasic Glutamate Release in the Prefrontal Cortex of Awake Rats Using Microelectrode Arrays. 41st annual meeting – Society for Neuroscience. Washington, D.C.

2012   **C.E. Mattinson**, J.S. Beckmann, F. Pomerleau, P. Huettl, M.T. Bardo, G.A. Gerhardt. The Effects of Methylphenidate on Prefrontal Cortex Glutamate Signaling in Awake Freely Moving

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