Dopamine and Glutamate Dysfunction in a Rodent Model of Attention-Deficit/Hyperactivity Disorder: Implications for Future Neuropharmacology

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DOPAMINE AND GLUTAMATE DYSFUNCTION IN A RODENT MODEL OF ATTENTION-DEFICIT/HYPERACTIVITY DISORDER: IMPLICATIONS FOR FUTURE NEUROPHARMACOLOGY

Dissertation

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

By

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Lexington, KY
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Attention-deficit/hyperactivity disorder (ADHD) is one of the most common disorders of childhood. It is theorized to be caused by catecholamine dysfunction in the striatum (Str) and frontal cortex (FC). The spontaneously hypertensive rat (SHR) has been used as a model for ADHD because of its attention deficits, impulsiveness, and hyperactivity. Prior studies of dopamine (DA) in the Str and FC have revealed conflicting results in the SHR compared to control, indicative of a need for a better understanding of DA dynamics in this model. In addition to the DA hypothesis, studies have begun implicating glutamate in the etiology of ADHD. Previous evaluations of the SHR model of ADHD found that the SHR have increased α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activity and elevated calcium levels in the FC, suggesting that altered glutamatergic neurotransmission exists in the SHR.

The first set of studies presented here suggest that increased surface expression of DA transporters may exist in the SHR model of ADHD, lowering basal DA levels. Second, we discovered that the glutamate system in the FC of the SHR model of ADHD is hyperfunctional, thus raising the possibility that targeting glutamate dysfunction in the FC could lead to the development of novel therapeutics for the treatment of ADHD. The third and fourth set of studies explored glutamate signaling in the awake rodent to fully understand glutamate neurotransmission as well as the effects of methylphenidate (MPH) on glutamate signaling in the prelimbic cortex, a region heavily implicated in ADHD. The SHR displayed similar phasic glutamate signaling compared to control; however, in the SHR but not the WKY control, chronic treatment with MPH lowered phasic glutamate amplitude. Additionally, intermediate treatment with MPH increased tonic glutamate in the SHR only, whereas chronic MPH treatment increased tonic levels in both the SHR and WKY compared to saline.

Taken together, this body of work characterizes DA and glutamate signaling in the anesthetized SHR model of ADHD. Additionally, glutamate dynamics and the effects of the stimulant medication MPH were explored in the awake animal, providing evidence that glutamate is a likely target for future neuropharmacology for the treatment of ADHD.

KEYWORDS: ADHD, dopamine, glutamate, frontal cortex, methylphenidate
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Dedicated to my parents and my husband
Acknowledgments

What is a teacher? I'll tell you: it isn't someone who teaches something, but someone who inspires the student to give of her best in order to discover what she already knows. -Paulo Coelho

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*I sustain myself with the love of family.* -Maya Angelou

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Preface to Chapter One

The differences between human and rodent prefrontal cortices (PFC) are discussed through this chapter, and throughout this entire body of work. However, it must be noted that the two regions are not identical between species. Some do not even agree that the rodent has a PFC (Preuss 1995), but there is plenty of research to support the hypothesis that rodents possess a structure analogous to the human PFC (Seamans, Lapish et al. 2008). While referring to the PFC in the rodent in the following chapters, it’s important to note that the region being referred to is actually the frontal cortex.

Chapter One: Introduction

Attention-Deficit/Hyperactivity Disorder

Attention-deficit/hyperactivity disorder (ADHD) is characterized by impulsivity, hyperactivity, inattention and cognitive impairment. The DSM-V classifies ADHD into 3 subtypes: predominantly inattentive type (ADHD-PI), predominantly hyperactive-impulsive type (ADHD-HI, the least commonly diagnosed type) and the most common subtype – combined (ADHD-C) (Paternite, Loney et al. 1995, Morgan, Hynd et al. 1996) – see Figure 1.1. ADHD is estimated to affect 6% of children worldwide and a portion of those diagnosed as children will continue to suffer into adulthood, affecting roughly 5% of adults (Kessler, Adler et al. 2006, Polanczyk, de Lima et al. 2007, Biederman, Petty et al. 2011). Children are three times more likely to repeat a year of classes and are more likely to drop
out of school compared to their normal peers (Barbaresi, Katusic et al. 2007, Barbaresi, Katusic et al. 2007, Froehlich, Lanphear et al. 2007), whereas adolescents and adults are more likely to suffer from mood and anxiety disorders and to develop drug addiction (Biederman, Petty et al. 2010). Hyperactivity has been found to lessen in adolescence, though cognitive impairment persists (Brassett-Harknett and Butler 2007, Spencer, Biederman et al. 2007). Those diagnosed with ADHD combined type (ADHD-C), characterized by hyperactivity and inattention, had larger impairments including lower grades in school and more arrests during adolescence compared to the other ADHD subtypes (inattentive and hyperactive/impulsive) (Molina, Hinshaw et al. 2009). Thus, evidence suggests the efficacy of treatments for adolescents could be improved and calls for a better understanding of the pathophysiology of ADHD, particularly for those diagnosed with ADHD-C.

The Multimodal Treatment Study of Children with ADHD (MTA) followed children with ADHD for 14 months during treatment (behavior therapy alone, medication alone, or both) and roughly 8 years after its completion, when the participants were reaching late adolescence and young adulthood, it was discovered that more than 60% of children treated with medication during the initial 14 month long MTA study had stopped taking any medication for their ADHD symptoms (Molina, Hinshaw et al. 2009). For these reasons, investigations into the neurobiological basis of ADHD in the adolescent population would greatly benefit these individuals if it were possible to better target their ADHD symptoms using novel pharmacotherapies.
ADHD and the Link to Neurochemistry

When the Diagnostic and Statistical Manual of Mental Disorders (DSM-1) was first published in 1952, childhood psychiatric disorders were thought to be caused by environment and referred to as ‘reactions’ (Association 1952). It wasn’t until the DSM-2 was published in 1968 that ADHD began to be separated from general reactions and become its own diagnosis, referred to as the ‘hyperkinetic reaction of childhood.’ This reaction was characterized by a short attention span, hyperactivity, and restlessness (Association 1968), and in 1980, with the publication of the DSM-3, the ADHD diagnosis became more specific and was described as ADD (attention-deficit disorder) (Association 1980); however, by this time, this disorder was already being treated with stimulant medications, a treatment still used to this day.

Stimulant medications were initially discovered to treat hyperactivity in the early 1900s when the psychiatrist Charles Bradley used amphetamines to treat children with headaches caused by pneumoencephalography and found it improved their school performance, social interactions and emotional responses. However, amphetamine as a treatment for ADHD was ignored until years later due to a variety of reasons (Strohl 2011). In the 1950s, researchers were beginning to look for the underlying mechanisms causing behavioral problems and it was at this time that Bradley’s discovery of amphetamine as a treatment for hyperactivity was uncovered and investigations into the mechanism of action of amphetamine began. The amphetamine formulation Bradley used in his patients was called Benzedrine,
a racemic mixture of 50/50 d- and l-amphetamine, produced by the company Smith, Kline and French (Strohl 2011). Treatment with this medication, in a variety of experimental paradigms, reduced hyperactivity (Robbins and Sahakian 1979); however, of particular note is a study published in 1976 showing decreased hyperactivity when treated with amphetamine in rodents with dopamine depletion (Shaywitz, Klopper et al. 1976). This was the first time that hyperactivity was linked to dopamine, but far from the last. Figure 1.2 illustrates the inverted U bell curve, first suggested by Yerkes and Dodson in 1908, though it was modified for performance and dopamine levels (Yerkes and Dodson 1908). As dopamine levels increase in the normal brain, so does performance (represented by the red box); however, in the ADHD brain, it is theorized that decreased dopamine levels are the cause behind decreased performance.

**Dopamine**

Dopamine was first suggested to function as a signaling molecule by Arvid Carlsson (Carlsson 1959). Previously, it has been thought that the role of dopamine was purely as a metabolic intermediate in the synthesis of norepinephrine and epinephrine. Dopamine, now classified as a catecholamine neurotransmitter, is produced in the cells of the substantia nigra (SN, A9) and ventral tegmental area (VTA, A10) of the midbrain and project to numerous brain regions, including the prefrontal cortex (PFC), striatum and nucleus accumbens (NA, see Figure 1.3). Projections from the VTA to the NA are identified as the mesolimbic pathway, or the “reward pathway,” because these dopamine projections are involved in
rewarding behaviors, (Schultz 2001) firing when a reward is greater than expected (Schultz 1986, Schultz 1998, Arias-Carrion and Poppel 2007). Projections from SN to the striatum are collectively referred to as the nigrostriatal pathway and play a role in many aspects of motor control (Iversen 2009). The mesocortical system consists of dopaminergic projections from the VTA to the PFC, and is implicated in many cognitive functions including, but most certainly not limited to, attention and memory (Iversen 2009).

Dopamine is produced from tyrosine into 3,4-dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase. DOPA is then made into dopamine via DOPA-decarboxylase. Conversely, dopamine is enzymatically converted by a number of mechanisms: 1) dopamine-β-hydroxylase converts dopamine into norepinephrine, 2) monoamine oxidase (MAO) converts dopamine into 3,4-dihydroxyphenylacetic acid (DOPAC), and 3) catechol-o-methyltransferase (COMT) catalyzes the formation of homovanillic acid (HVA). Dopamine-β-hydroxylase only exists in norepinephrine neurons and thus will not be a focus in this work; however, MAO exists on the outer mitochondrial membrane in regions rich with catecholamines and is also thought to be in abundance extracellularly (Iversen 2009). Furthermore, COMT is mostly extracellular and plays a major role in regulating dopamine neurotransmission, especially in the prefrontal cortex (Iversen 2009). The final and most prominent method in which dopamine is cleared from the synapse is via the dopamine transporter (DAT). The DAT exists on the presynaptic neuron and can transport dopamine either into or out of the neuron, dependent upon the concentration gradient. It has been discovered that the
removal of dopamine from the synapse is predominantly performed by the DAT and not metabolism or diffusion (Cass, Zahniser et al. 1993).

Intracellularly, dopamine is packaged into vesicles via the vesicular monoamine transporter (VMAT-2). The release of dopamine from the vesicle is Ca\(^{2+}\)-dependent and occurs when an action potential raises the Ca\(^{2+}\) levels in the presynaptic neuron, causing vesicles stored with dopamine to bind to the cellular membrane and release its contents. The resulting synaptic dopamine is then able to bind to dopamine receptors on both the pre- and postsynaptic neurons. These receptors are classified into two major categories: 1) D\(_1\) receptors, consisting of D\(_1\) and D\(_5\) and expressed postsynaptically, and 2) D\(_2\) receptors expressed both pre- and postsynaptically, consisting of D\(_2\) (short), D\(_2\) (long), D\(_3\) and D\(_4\). Stimulation of D\(_1\)-type receptors causes increased cAMP production (activating), whereas stimulation of D\(_2\)-type receptors causes inhibition of cAMP production (inhibiting). The effects of these receptors give dopamine the classification of a modulatory neurotransmitter. For a simplified PFC dopamine synapse diagram, see Figure 1.4a.

**Glutamate**

Recent clinical evidence has implicated glutamate in the etiology of ADHD. This evidence stems from proton magnetic resonance spectroscopy studies of children and adults with ADHD. These studies have shown increased levels of a marker for combined glutamine/glutamate in the striatum and anterior cingulate cortex of the PFC compared to control peers (Carrey, MacMaster et al. 2002,
Based on this evidence, new investigations into glutamatergic functioning in ADHD are ongoing.

Glutamate is the major excitatory neurotransmitter in the central nervous system and must be tightly regulated for proper neuronal signaling to occur (Danbolt 2001). Unlike dopamine, glutamate is in abundance in most all areas of the brain. Glutamate projections originating in the PFC extend to the striatum, NA, and the VTA and SN of the midbrain (see Figure 1.3). Glutamate is produced in the nerve terminals of these projections from two sources: 1) the Krebs cycle and 2) glutamine transported via glial cells. Once produced, glutamate is transported into vesicles via the vesicular glutamate transporter (VGLUT) and when Ca^{2+} levels increase to cause an action potential, vesicles stored with glutamate bind to the cellular membrane and release their contents (Takamori 2006). Additionally, glutamate is released into the extracellular space via carrier mediated exchange with other amino acids (Jabaudon, Shimamoto et al. 1999, Jensen, Pickering et al. 2000). Unlike with dopamine, there are no extracellular enzymes to degrade glutamate. Concentrations of extracellular glutamate are regulated in part by the uptake of glutamate via five high affinity sodium-dependent excitatory amino acid transporters (EAAT1, GLAST) (EAAT2, GLT-1), (EAAT3, EAAC), EAAT4, and EAAT5 (Danbolt 2001). The majority of glutamate uptake in the rodent brain is performed by two glial transporters, GLAST and GLT-1 (Rothstein, Dykes-Hoberg et al. 1996, Danbolt 2001). The glutamate taken up by the EAATs located on these glial cells is converted by glutamine synthetase into glutamine and transported out of the glial cell by system N transporter and taken up by the system A transporter.
on the presynaptic neuron to help replenish glutamate levels through the mitochondrial bound glutaminase (Iversen 2009). Glutamate acts on synaptic glutamate receptors in the target brain region, which are classified into two major types: 1) ionotropic, which include the NMDA, AMPA and kainate receptors and 2) metabotropic, including the excitatory mGluRs 1 and 5 and the inhibitory mGluRs 2, 3, 4, 6, 7, and 8. AMPA and kainate receptors are involved in fast neurotransmission. Glutamate binds producing a conformational change in both receptors allowing Na\textsuperscript{2+} influx into the postsynaptic neuron. Conversely, the NMDA receptor requires the binding of two ligands, glutamate and glycine, and also requires depolarization to release a Mg\textsuperscript{2+} block and allow Ca\textsuperscript{2+} into the postsynaptic neuron. Glutamate binding to AMPA and kainate receptors allows Na\textsuperscript{2+} into the cell, depolarizing the postsynaptic neuron and removing the Mg\textsuperscript{2+} block. mGluRs are G-protein coupled receptors that use second messenger systems for signal transduction, and as such signaling is slower compared to the activation of ionotropic receptors. Group 1 (mGluR1 and mGluR5) is excitatory and located on postsynaptic glutamatergic neurons. Group 2 (mGluR2 and mGluR3) is inhibitory. mGluR2s are localized primarily on presynaptic glutamate neurons, whereas mGluR3s are located on glia and pre- and postsynaptic glutamatergic neurons. Finally, group 3 (mGluR4, mGluR6, mGlu7 and mGluR8) is inhibitory with mGluR4 and mGluR7 located on both pre- and postsynaptic neurons (Schoepp 2001). For a simplified PFC glutamate synapse diagram, see Figure 1.4b.
Dopamine and Glutamate Interactions

A dysfunctional interaction between the dopamine and glutamate systems has been implicated in numerous neuropsychiatric disorders including schizophrenia, Parkinson’s disease, Alzheimer’s disease, mood disorders, attention-deficit/hyperactivity disorder, and drug addiction (Brambilla, Perez et al. 2003, Moghaddam and Javitt 2012, Lesch, Merker et al. 2013, Paula-Lima, Brito-Moreira et al. 2013, Poletti and Bonuccelli 2013, Quintero 2013). The regions most often linked to these disorders and the dopamine-glutamate dysfunction include the PFC and striatum, as these regions both receive heavy innervation from the dopaminergic midbrain region (SN/VTA) as well as the thalamus and other glutamate rich regions, as described in the previous section. The PFC has been found to be heavily involved with behavior and attention in both humans and rodents, inhibiting inappropriate responses and sustaining attention over long delays (Goldman-Rakic 1996, Goldman-Rakic 1996, McCarthy, Puce et al. 1996, Robbins 1996, Robbins 2000, Khan, Koulen et al. 2001, Perry, Joseph et al. 2011). The striatum is involved with decision-making, sensorimotor and cognitive processes, goal-directed and habit learning, as well as spatial domains and motor activity (Brasted, Humby et al. 1997, Balleine, Delgado et al. 2007, Dunnett and Lelos 2010). Finally, the NA core (sometimes referred to as the ventral striatum) plays a role in appetitive and aversive motivational processes in both humans and rodents (Salamone 1994, Salamone, Cousins et al. 1994, Salamone and Correa 2012, Segovia, Correa et al. 2012, Salamone and Correa 2013).

Studies of signaling interactions between the dopaminergic and
glutamatergic systems demonstrate that the NMDA receptor is crucial in activating the dopamine neurons in the VTA/SN (Martinez-Fong, Rosales et al. 1992, Warton, Howells et al. 2009). Also, it has been found that stimulation of the D₂-class dopamine receptor is involved in the downstream inhibition of the NMDA receptor, weakening the excitatory response to those neurons (Kotecha, Oak et al. 2002). Likewise, it was found that activation of D₄ receptors depressed AMPA receptor-mediated excitatory synaptic transmission in PFC pyramidal neurons, which was accompanied by a D₄-induced decrease of AMPA receptors at the synapse (Yuen, Liu et al. 2010).

ADHD is associated with a D₄ polymorphism that has been found to weaken D₄ function (Van Tol, Wu et al. 1992, LaHoste, Swanson et al. 1996, Rowe, Stever et al. 1998, Thapar 1998, Thapar, O'Donovan et al. 2005). The function of D₄ receptors has been found to play a role in hyperactivity and impulsivity in adolescent aged mice (Avale, Falzone et al. 2004). In the rodent, D₄ is located primarily in the prefrontal cortex (Wedzony, Chocyk et al. 2000) where the stimulation of D₄ by dopamine exerts regulation on CaMKII (Gu and Yan 2004, Gu, Jiang et al. 2006). CaMKII then in turn acts on intracellular stores of the AMPA receptor (Barria, Muller et al. 1997, Hayashi, Shi et al. 2000). CaMKII phosphorylates the AMPA receptor at the P2 serine 831 site, which increases channel conductance of the GluA1 subunit (Benke, Luthi et al. 1998). AMPA receptor activation then allows Ca²⁺ entry via removal of NMDAR Mg²⁺ block (Malenka and Nicoll 1999). These results provide substantial evidence that the dopamine and glutamate systems work in tandem to create a balance of neurotransmission in these regions, and in the
ADHD brain, these systems likely malfunction.

The hypodopaminergic theory of ADHD asserts that the hyperactive and inattentive behaviors are caused by depleted levels of dopamine. Decreased dopamine released in the striatum and PFC would then be expected to lead to more active NMDA and AMPA receptors based on the studies mentioned above resulting in increased glutamatergic output to the striatum and SN/VTA, as well as an increased glutamate signal to the prefrontal cortex. Glutamate coming into the SN/VTA would normally go on to release more dopamine (Warton, Howells et al. 2009); however, in the ADHD brain, this feedback does not seem to occur.

**Translational Neuropharmacology of ADHD Treatments**

Investigations into the effects of stimulant action on the dopaminergic system have revealed that these medications increase extracellular dopamine levels via numerous mechanisms. First, amphetamine has been found to increase dopamine through calcium-independent mechanisms, such as increased release of dopamine as well as blocking the uptake of dopamine through the DAT (Carboni, Imperato et al. 1989, Kahlig and Galli 2003). Methylphenidate (MPH), another stimulant medication commonly used to treat ADHD, increases dopamine levels by inhibiting dopamine reuptake via the DAT similarly to amphetamine (Kuczenski and Segal 1997, Gerasimov, Franceschi et al. 2000, Gerasimov, Franceschi et al. 2000, Kuczenski and Segal 2001, Volkow, Wang et al. 2001, Huff and Davies 2002, Marsteller, Gerasimov et al. 2002). Interestingly, norepinephrine levels have been found to be increased following MPH administration, as MPH has been
discovered to block the norepinephrine transporter (NET) in addition to the DAT (Gatley, Pan et al. 1996). MPH has been found to cause disinhibition of D<sub>2</sub> receptors, which are negatively coupled to cAMP, on the presynaptic dopaminergic neuron, and activation of D<sub>1</sub> receptors, which are positively coupled to cAMP, on the postsynaptic neuron (Seeman and Madras 2002). These interactions result in an amplification of DA activity and improvement of attentional deficits, cognitive functioning, and motor hyperactivity (Volkow, Wang et al. 2001).

Although stimulants are very effective in the treatment of ADHD, ~30% of children and ~50% of adults do not respond well to them (Schweitzer, Cummins et al. 2001). Moreover, the use of stimulants for treatment of ADHD is considered to be predominantly safe; however, concerns surround them because of the risk for cardiovascular problems, as well as amphetamine and MPH’s drug abuse liability (Kollins, MacDonald et al. 2001, Nissen 2006). The non-stimulant medication atomoxetine (ATX) has a unique niche as a treatment for ADHD compared to the stimulant medications because it has lower abuse liability than stimulants (Heal, Cheetham et al. 2009). ATX has been found to increase levels of dopamine and norepinephrine by selectively blocking the NET, which is also able to clear dopamine (Bymaster, Katner et al. 2002, Swanson, Perry et al. 2006, Newman, Darling et al. 2008) and is almost as effective as stimulants at assuaging ADHD symptoms (Michelson, Faries et al. 2001, Michelson, Adler et al. 2003, Adler, Spencer et al. 2005, Adler, Spencer et al. 2008). In vitro work has shown that ATX acts as an NMDA receptor antagonist (Ludolph, Udvardi et al. 2010), providing
preliminary evidence that current treatments for ADHD may have an effect on the glutamatergic system.

Using proton magnetic resonance spectroscopy, it was found that children treated with ATX, but not MPH, had decreased levels of a marker for glutamate/glutamine in the PFC compared to non-treated ADHD individuals (Carrey, MacMaster et al. 2002), though in a separate imaging study MPH was able to decrease glutamate in the anterior cingulate region (Hammerness, Biederman et al. 2012). In the striatum, both ATX and MPH decreased the glutamate/glutamine marker levels by 56% compared to controls (Carrey, MacMaster et al. 2002). These results suggest that ATX may be regulating and activating prefrontal cortex neurons. However, another clinical study using a similar technique found that chronic long-acting MPH decreased glutamate levels in the PFC of children with ADHD (Wiguna, Guerrero et al. 2012). Wiguna et al. (2012) also discovered that MPH treatment resulted in an increase in the amount and functional state of neurons in the PFC, supporting that the current ADHD stimulant treatment MPH can activate PFC neurons. Further evidence of PFC activation comes from a study of brain-derived neurotropic factor (BDNF), a marker for neuronal plasticity. ATX was found to increase BDNF expression in the PFC; however, MPH had the opposite effect and reduced BDNF expression in the PFC (Fumagalli, Cattaneo et al. 2010), though it must be noted that this was study was completed in naïve rodents and may explain why these results don’t match the results seen in ADHD patients.
Several unconventional treatments for ADHD targeting the dopamine or glutamate systems have been proven to improve ADHD symptoms. Memantine is an uncompetitive NMDA receptor antagonist (Rogawski and Wenk 2003) and has also been found to act as a D$_2$ receptor agonist (Seeman, Caruso et al. 2008). It has been approved and used as a treatment for Alzheimer’s disease; however, in an 8 week open-label pilot study in children with ADHD, memantine was found to improve ADHD symptoms (Findling, McNamara et al. 2007) (Findling et al, 2007). Surman et al. (2013) extended these findings to adults with ADHD in a separate open-label study lasting 12 weeks and found similar results, with memantine improving ADHD symptoms and neuropsychological performance (Surman, Hammerness et al. 2013). Deprenyl, an MAO-B inhibitor which stops the degradation of dopamine, is used as a treatment in Parkinson’s disease, and was found to alleviate ADHD symptoms (Jankovic 1993, Feigin, Kurlan et al. 1996). These clinical data using glutamate and dopamine altering drugs provide strong links for dysfunctional dopamine-glutamate interactions in ADHD, though the importance of this dysfunction is still unknown. Based on these data, we believe it’s important to not overlook the role of typical dopamine-glutamate interactions, but to instead focus on this relationship. Animal models of ADHD provide a unique opportunity to investigate neurotransmitter system dysfunction as well as to develop novel ways to treat ADHD targeting these systems.
The Spontaneously Hypertensive Rat Model of ADHD

The seminal work of Terje Sagvolden (1945-2011) has pioneered the way for the spontaneously hypertensive rat (SHR) to be used as a model of ADHD-C. The SHR has been used as an animal model for ADHD-C since the 1970’s because of its sustained attention deficits (Sagvolden 2000), motor impulsiveness (Sagvolden, Hendley et al. 1992, Sagvolden, Metzger et al. 1992, Wultz and Sagvolden 1992), and hyperactivity (Sagvolden 2000) with the hyperactivity absent in novel situations (Knardahl and Sagvolden 1979). The SHR is the most widely utilized animal model of ADHD (Russell 2011, Sagvolden and Johansen 2011); however, criticism of this strain lies in using the progenitor strain, the Wistar Kyoto (WKY), as a control (Alsop 2007). The NIH Animal Genetic Resource stock of the WKY was obtained in 1971 as outbred animals from the Kyoto School of Medicine in Japan. These animals were distributed to laboratories (i.e. Harlan and Charles River) before the F20 generation, resulting in multiple strains of the WKY (Sagvolden, Johansen et al. 2009). Two commonly used WKY control strains, WKY/NCrl (Charles River) and WKY/NHsd (Harlan), display wide genetic divergence (Sagvolden, Dasbanerjee et al. 2008). When contacted, both laboratories specified that inbred animals were subjected to single nucleotide polymorphism panels every quarter, thus the separate WKY strains should remain genetically different. However, a recent manuscript disputed this when they discovered that the strains at each individual laboratory produced different genetic profiles (Zhang-James, Middleton et al. 2013). Nonetheless, current behavioral evidence points to the SHR/NCrl being the most accurate animal model of ADHD-
C (Sagvolden, Russell et al. 2005, Russell 2011) and the WKY/NHsd serves as the most appropriate control strain. The WKY/NCrl strain is best suited as a model of ADHD-PI because of its behavioral and neurochemical abnormalities (Sagvolden, Dasbanerjee et al. 2008, Sagvolden, Johansen et al. 2009, Roessner, Sagvolden et al. 2010). It has been suggested that the outbred Sprague Dawley (SD) rat strain, used by some groups in the past, could be used as an additional control (Drolet, Proulx et al. 2002, Sagvolden, Johansen et al. 2009). Taken together, the SHR/NCrl and WKY/NCrl models of ADHD and their control strains, the inbred WKY/NHsd and outbred SD, seem to be valuable translational animal models to assess the neurobiological dysfunction associated with ADHD.

**Measuring Neurotransmitters *In Vivo***

Recent studies point to the importance of a dysfunctional relationship between dopamine and glutamate neurotransmission in ADHD, therefore new investigations into this relationship are necessary to improve our understanding and may lead to improved therapeutics for ADHD. Based on the development of novel and revolutionary methods of measuring dopamine and glutamate *in vivo*, we realize we are in a unique position to test our hypotheses that dopamine and glutamate regulation play a major role in the pathophysiology of ADHD. The development of carbon fiber microelectrodes and glutamate oxidase-coated microelectrode arrays (MEAs) provide improved spatial resolution, sub-second temporal resolution, and low limits of detection – <10 nM for dopamine (Miller, Pomerleau et al. 2012), <0.2 μM for glutamate (Miller, Pomerleau et al. 2014) –
over conventional techniques used in the past, such as microdialysis, which yields results only as quickly as every few minutes, missing much of the dynamic changes. Additionally, microdialysis varies across studies, with differing sampling times, flow rates, and probe sizes. The smaller size of the microelectrode probes used in the following studies causes decreased damage to tissue compared to microdialysis probes and allows for the in vivo characterization of dopamine and glutamate signaling closer to the synapse. Using these technologies, the exploration of dopamine and glutamate neurotransmission in the brain of the SHR model of ADHD is possible. The studies described here could potentially lead to the development of novel therapies for ADHD, which will be discussed in detail later.

*Portions of this chapter have been previously published in the open-access book chapter:*


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ADHD combined type (ADHD-C, red) is the most prevalent subtype of ADHD diagnosed, followed by ADHD predominantly inattentive type (ADHD-PI, blue). ADHD hyperactive-impulsive type (ADHD-HI, green) is the least common type of ADHD diagnosed.
Figure 1.2 The Dopamine Bell Curve and ADHD

Low levels of dopamine are thought to cause decreased performance (blue arrow) in ADHD. The red box signifies normal levels of dopamine, causing peak performance. Elevation of dopamine above normal, such as in drug abuse, decreases performance. Modified from (Yerkes and Dodson 1908).
Modulatory dopaminergic neurons (blue) project to the dorsal striatum via the substantia nigra (SN, A9) and the ventral striatum and prefrontal cortex (PFC) via the ventral tegmental area (VTA, A10) in the rodent brain. From the striatum, inhibitory GABA neurons (red) extend to multiple regions including the thalamus, which has reciprocal excitatory glutamate connections (green) to the striatum, as well as connections to the PFC. Prefrontal cortical efferent excitatory glutamate neurons extend to the striatum, nucleus accumbens (NA), SN, as well as the VTA (Miller, Thomas et al. 2013).
Figure 1.4 Dopaminergic and Glutamatergic Synapses in the PFC

A) Presynaptically, dopamine is transported into vesicles, which release their contents upon increase of the Ca2+ concentration. Synaptic dopamine is then able to stimulate dopamine receptors on both the pre- and postsynaptic neurons before it is cleared by the DAT or metabolism. B) Presynaptically, glutamate is stored in vesicles and then released into the extracellular space. Synaptic glutamate is then able to stimulate glutamate receptors (here represented as the NMDA and mGluR) on both the pre- and postsynaptic neurons before it is cleared by the EAAT located on nearby glial cells (Miller, Thomas et al. 2013).
Thesis Outline

In these studies, dysfunctional neurotransmission was examined in a rodent model of attention-deficit/hyperactivity disorder (ADHD). Many children, adolescents, and adults do not respond well to medications currently on the market or the side effects are too much to handle and research suggests that novel therapies would benefit these individuals. Most current medications for ADHD on the market target the catecholamine system. Thus, in Chapter Two, the vesicular release and uptake of dopamine, a modulatory catecholamine neurotransmitter present in brain regions implicated in ADHD, were investigated in an adolescent-aged rodent model of ADHD, the spontaneously hypertensive rat (SHR). Dysfunctional dopamine signaling was observed in the SHR compared to control, specifically the model of ADHD was found to have faster dopamine uptake in the ventral striatum, nucleus accumbens core, and regions within the frontal cortex compared to the control strain.

By the time Chapter Two had been completed, clinical research in ADHD had begun implicating glutamate in the etiology of the disorder. Because of this, Chapter Three focused on characterizing glutamate signaling in the same method dopamine was characterized from Chapter Two. Vesicular release and the uptake of glutamate were found to be aberrant in the striatum and the frontal cortex of the SHR model of ADHD compared to control. Additionally, tonic levels were found to be overall higher in the SHR.

As Chapter Three came to its conclusion, novel technology became available that allowed for the recording of multiple sub-regions along the dorsal-
ventral axis in the same animal in an awake, freely-moving paradigm, something which had never been accomplished before. This allowed the glutamate recordings to proceed as the animal was awake and freely-moving, instead of being anesthetized during the recording, as had been done in Chapters Two and Three. Because the SHR is a model of ADHD, the following was tested in Chapter Four: the hyperactive behaviors of this model of ADHD would link to the tonic and phasic glutamate signaling in the frontal cortex of the SHR. Moreover, methylphenidate, a commonly prescribed ADHD treatment, would have an effect on these tonic and phasic glutamate levels. Recordings were completed in the cingulate, prelimbic, infralimbic and dorsal peduncle cortices within the frontal cortex. However, because of the novelty of this technology, revisions in the electrodes used to measure neurotransmission in multiple brain regions became necessary. Chapter Five then evolved to use microelectrode arrays that had previously been used to characterize glutamate signaling in Chapter Three, though modified to allow for chronic freely-moving recordings. The purpose of this final chapter was to examine the acute, intermediate and chronic effects of methylphenidate on tonic and phasic glutamate signaling within the prelimbic region of the frontal cortex in the model of ADHD and the control WKY strain compared to saline treatment.

Finally, Chapter Six works to link all of the above data into a final conclusion and summation. The findings from these studies are discussed and the implications and future directions of this research are suggested.

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Chapter Two: The Spontaneously Hypertensive and Wistar Kyoto Rat Models of ADHD Exhibit Sub-Regional Differences in Dopamine Release and Uptake in the Striatum, Nucleus Accumbens, and Prefrontal Cortex

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is characterized by impulsivity, hyperactivity, inattention and cognitive impairment. The DSM-IV-TR classifies ADHD into 3 subtypes: predominantly inattentive type (ADHD-PI), predominantly hyperactive-impulsive type (ADHD-HI) and the most common subtype – combined (ADHD-C). The seminal work of Terje Sagvolden (1945-2011) has pioneered the way for the spontaneously hypertensive rat (SHR) to be used as a model of ADHD-C. Sagvolden and colleagues found sustained attention deficits (Sagvolden 2000), motor impulsivity (Sagvolden, Metzger et al. 1992, Wultz and Sagvolden 1992), and hyperactivity (Knardahl and Sagvolden 1979, Sagvolden 2000) in the SHR. The SHR is the most widely utilized animal model of ADHD (Russell 2011, Sagvolden and Johansen 2011); however, criticism of this strain lies in using the progenitor strain, the Wistar Kyoto (WKY), as a control (Alsop 2007). The NIH Animal Genetic Resource stock of the WKY was obtained in 1971 as outbred animals from the Kyoto School of Medicine in Japan. These animals were distributed to laboratories (i.e. Harlan and Charles River) before the F20 generation, resulting in multiple strains of the WKY (Sagvolden, Johansen et al. 2009). Two commonly used WKY control strains, WKY/NCrI (Charles River) and WKY/NHsd (Harlan), display wide genetic divergence (Sagvolden,
Dasbanerjee et al. 2008). When contacted, both laboratories specified that inbred animals were subjected to single nucleotide polymorphism panels every quarter, thus the separate WKY strains should remain genetically different. Current behavioral evidence points to the SHR/NCrI being the most accurate animal model of ADHD-C (Sagvolden, Russell et al. 2005, Russell 2011) and the WKY/NHsd serves as the most appropriate control strain. The WKY/NCrI strain is best suited as a model of ADHD-PI because of its behavioral and neurochemical abnormalities (Sagvolden, Dasbanerjee et al. 2008, Sagvolden, Johansen et al. 2009, Roessner, Sagvolden et al. 2010). It has been suggested that the outbred Sprague Dawley (SD) rat strain, used by some groups in the past, could be used as an additional control (Drolet, Proulx et al. 2002, Sagvolden, Johansen et al. 2009). Taken together, the SHR/NCrI and WKY/NCrI models of ADHD and their control strains, the inbred WKY/NHsd and outbred SD, seem to be valuable translational animal models to assess the neurobiological dysfunction associated with ADHD.

ADHD is thought to be caused by catecholamine dysfunction (Levy 1991, Solanto, Arnsten et al. 2001) in brain regions involved with attention and reward including the nucleus accumbens (NA) (Russell 2000, Podet, Lee et al. 2010) and striatum (Str) (Krause, Dresel et al. 2003). Prior studies examining dopamine (DA) function in the SHR and WKY rat brains are equivocal and have been shown to range from no differences (Versteeg, Van Der Gugten et al. 1976, Ferguson, Gough et al. 2003) to lower DA levels in the Str in vitro and in vivo in the SHR (Linthorst, Van den Buuse et al. 1990, Linthorst, De Lang et al. 1991). Furthermore, a recent microdialysis study comparing the SHR to the SD determined that the
SHR has 78% higher basal efflux of DA in the Str (Heal, Smith et al. 2008). Striatal uptake of DA in the SHR has been reported to be slower (Myers, Whittemore et al. 1981, Leo, Sorrentino et al. 2003) or not different (Linthorst, Van den Buuse et al. 1990, Li, Lu et al. 2007) versus the WKY, yet a higher concentration of DA transporters (DAT) in the Str of the SHR was found (Watanabe, Fujita et al. 1997, Roessner, Sagvolden et al. 2010). Additionally, it has been demonstrated that extracellular DA levels in the NA are higher in the SHR compared to the WKY (Carboni, Silvagni et al. 2003). The PFC of the SHR has been reported to have decreased dopamine uptake (Myers, Whittemore et al. 1981), yet a study found no differences in the levels of DAT, tyrosine hydroxylase, D₁, D₂, D₃, D₅ receptors, and dopamine-β-hydroxylase between the SHR and its progenitor strain, the WKY, in the PFC. Regional differences in the D₄ receptors in the PFC were found, providing evidence that the SHR’s D₄ levels are lower than those of the WKY (Li, Lu et al. 2007). Further, it was found that PFC AMPA receptor activity was increased in the SHR (Russell 2001) and inhibitory dopaminergic activity was found to be decreased while noradrenergic activity increased in the SHR (Russell 2002). These findings all convey a message that dopamine regulation is dysfunctional in the PFC of the SHR model of ADHD; however, direct observation of in vivo dopamine dynamics in the separate PFC sub-regions (cingulate, prelimbic, and infralimbic) of the SHR have not yet been accurately defined. Thus, there still remains controversy surrounding the regulation of DA release and uptake in the SHR.
One of the issues with the previously described studies is that the strains of the SHRs and WKYs were not properly defined, as the importance of the lineage of these strains was not yet understood. There might also be an issue with comparing these studies, as the techniques used to study DA regulation ranged from *in vitro* superfusion of brain slices to *in vivo* microdialysis. Microdialysis has been the dominant technique for *in vivo* measures in the SHR; however, this methodology varies across studies, with differing sampling times, flow rates, and probe sizes. Therefore, comparisons from prior studies can be compromised due to a variety of experimental variables. Furthermore, it has been shown that the microdialysis probes can cause extensive damage to the surrounding tissues (Clapp-Lilly, Roberts et al. 1999, Rutherford, Pomerleau et al. 2007), which can greatly affect neurotransmitter function. Recently, it was discovered that microdialysis probes significantly alter presynaptic dopaminergic dynamics in the rodent striatum (Wang and Michael 2012). Because of this, specialized techniques have been developed to evaluate DA dynamics in addition to microdialysis. These include electrochemical techniques such as fast-scan cyclic voltammetry, constant potential amperometry, and high-speed chronoamperometry (Lee, Blaha et al. 2006, Park, Takmakov et al. 2011, Zhang, Heien et al. 2011, Littrell, Pomerleau et al. 2012). Fast-scan cyclic voltammetry allows for high chemical and spatial resolution (Robinson, Venton et al. 2003, Owesson-White, Roitman et al. 2012, Owesson-White, Roitman et al. 2012), but it has rarely been used to map dopaminergic nerve terminal density profiles in discrete brain regions *in vivo* (Chadchankar and Yavich 2011, Zhang, Heien et al. 2011). Also, this technique
has rarely been used in conjunction with local application of chemicals from micropipettes placed adjacent to the microelectrodes in order to map the density of DA uptake and release from nerve terminals in a given brain area (Bergstrom, Sanberg et al. 2011, Howard, Keefe et al. 2011, Park, Takmakov et al. 2011, Owesson-White, Roitman et al. 2012, Sugam, Day et al. 2012, Wang and Michael 2012). Constant potential amperometry has exceptional temporal and spatial resolution but is incapable of identifying the predominant contributors to the electrochemical response as with both fast-scan cyclic voltammetry and high-speed chronoamperometry (Schonfuss, Reum et al. 2001, Lee, Blaha et al. 2006). Thus, researchers have begun to employ the power of high-speed chronoamperometry combined with local application of drugs from micropipettes to map the in vivo dynamics of release and uptake of dopamine in multiple sub-regions within specific brain regions, such as the striatum and nucleus accumbens (Womersley, Hsieh et al. 2011, Littrell, Pomerleau et al. 2012).

In this study, the use of carbon fiber microelectrodes coupled to pressure-ejection of drugs allowed for the sub-regional mapping of DA nerve terminal properties with rapid temporal and spatial resolution. This technique allowed for better in vivo characterization of DA signaling closer to the synapse than with other techniques (Joyce, Glaser et al. 2007, Littrell, Pomerleau et al. 2012). In the present study, the information concerning the best control animals for the SHR and WKY models of ADHD was used to study DA release and uptake in sub-regions within the striatum, nucleus accumbens, and PFC to better understand dopamine signaling and its regulation in animal models of ADHD-C and ADHD-PI.
Methods

Animal Preparation for Acute Electrochemical Recordings

Male, 8-10 weeks old, spontaneously hypertensive rats (SHR/NCrl, average 225 g, average PND 60), Wistar Kyoto rats (WKY/NCrl, average 210 g, average PND 61), and Sprague Dawley rats (SD/NCrl, average 289 g, average PND 69) were obtained from Charles River Laboratories (NCrl), Wilmington, Massachusetts. A second group of Wistar Kyoto rats (WKY/NHsd, average 202 g, average PND 62) was obtained from Harlan Laboratories (NHsd), Indianapolis, Indiana. Animals were given access to food and water ad libitum and housed in a 12 hour light/dark cycle. Rats were anesthetized intraperitonealy (i.p.) using a 25% urethane solution (1.25 g/kg) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, California). A circulating heating pad (Gaymar Industries, Inc., Orchard Park, New York) was used to maintain body temperature. The skull overlying the striatum was removed bilaterally for recordings in the striatum (Str, AP +1.0, ML ±2.5, DV -4.0 to -6.5 in 0.5 mm increments) and the nucleus accumbens core (NAc, AP +1.0, ML ±2.5, DV -7.0 to -7.5 in 0.5 mm increments), and the prefrontal cortex (AP +3.2, ML ±1.0, DV -2 to -6 in 0.5 mm increments) – see Figure 2.2A (Paxinos and Watson 2009). A small hole remote from the site of surgery was drilled for placement of the miniature Ag/AgCl reference electrode. Protocols for animal use were approved by the Institutional Animal Care and Use Committee, which is Association for Assessment and Accreditation of Laboratory Animal Care International approved. All procedures were carried out in accordance
with the National Institutes of Health Guide for Care and Use of Laboratory Animals and all efforts were made to minimize animal suffering and to reduce the number of animals used.

High-Speed Chronoamperometric Recordings of DA Release and Uptake

High-speed chronoamperometric measurements (1 Hz sampling rate, 200 ms total) were performed using the FAST16mkII recording system (Fast Analytical Sensing Technology, Quanteon, LLC, Nicholasville, Kentucky) as described previously (Gerhardt and Hoffman 2001, Littrell, Pomerleau et al. 2012). Single carbon fiber electrodes (SF1A; 30 µm outer diameter × 150 µm length; Quanteon, LLC, Nicholasville, Kentucky) were coated with Nafion® (5% solution, 1–3 coats at 180°C, Aldrich Chemical Co., Milwaukee, Wisconsin) prior to an in vitro calibration used to determine selectivity, limit of detection, and slope before use in vivo (Gerhardt and Hoffman 2001). The average selectivity for all microelectrodes used in these experiments was 1877 ± 664 µM for DA vs. ascorbic acid. The average limit of detection for the measurement of DA was 0.028 ± 0.008 µM (S/N of 3). The average slope for the electrodes was -0.492 ± 0.111 nA/µM DA. The average red/ox ratio measured during the peak response of the potassium-evoked DA signals was 0.51 ± 0.11, which is indicative of the detection of predominantly DA (Gerhardt and Hoffman 2001, Joyce, Glaser et al. 2007, Littrell, Pomerleau et al. 2012). Finally, miniature Ag/AgCl reference electrodes were prepared as previously described (Littrell, Pomerleau et al. 2012).
In Vivo Experimental Protocol

The carbon fiber microelectrode was affixed to a micropipette (10 µm inner diameter) which was positioned approximately 200 µm from the carbon fiber electrode tip using sticky wax (Kerr USA, Romulus, Michigan). The micropipette was filled with filtered isotonic KCl (120 mM KCl, 29 mM NaCl, 2.5 mM CaCl₂·2H₂O) solution (pH 7.2-7.4) using a 4 inch filling needle (Cadence Inc., Staunton, Virginia) and a 5 ml syringe (Figure 2.1). Experiments were initiated with the insertion of the micropipette/microelectrode assembly into a stereotactically selected region of the left or right hemisphere's Str. The system was allowed to reach a stable baseline dorsal to the first recording site. The micropipette/microelectrode assembly was then lowered to the target recording depth where the signal was again allowed to stabilize for an average of 5 minutes before the effect of a single local application of KCl on DA release was determined (Joyce, Glaser et al. 2007, Lundblad, af Bjerken et al. 2009, Thomas, Grandy et al. 2009, Morris, Bomhoff et al. 2011, Nevalainen, Af Bjerken et al. 2011). The potassium solution was locally applied by pressure ejection (5–25 psi for 0.5 seconds). A single application of a set volume of KCl (75–125 nl) was delivered to each sub-region and was measured by determining the amount of fluid ejected from the micropipette using a dissection microscope fitted with an eyepiece reticule that was calibrated so that 1 mm of movement was equivalent to 25 nl of fluid ejected (Cass, Gerhardt et al. 1992, Friedemann and Gerhardt 1992). If the volume was determined to be greater than or less than 75-125 nl, then that data point was excluded. After the Str and NAc were characterized, the whole assembly was
removed from the brain and reinserted into the same hemisphere’s PFC using the same experimental procedure.

After the KCl studies, the micropipette/microelectrode assembly was filled with filtered isotonic 200 μM DA solution containing 100 μM ascorbic acid (an antioxidant) in 0.9% saline (pH 7.2-7.4). The micropipette/microelectrode assembly was then inserted stereotactically into the contralateral Str. Again, once a stable baseline was achieved in a location dorsal to the first recording site, the micropipette/microelectrode assembly was lowered to the target recording depth where the signal was again allowed to stabilize for an average of 5 minutes before the DA solution was locally applied by pressure ejection (10-30 psi for 0.5-10 s) to achieve a maximum amplitude ranging from 0.5 to 1.0 μM DA (Littrell, Pomerleau et al. 2012). The maximum concentration of the DA in the extracellular space was measured by subtracting the apex of the recorded peak from the baseline recorded prior to the ejection. If the peak amplitude was greater than or less than 0.5 to 1.0 μM DA, then that data point was excluded. After the Str and NAc were characterized, the assembly was removed from the brain and reinserted into the same hemisphere’s PFC using the same experimental procedure as previously described.

Materials

Urethane, dopamine, ascorbic acid, sodium chloride, potassium chloride, calcium chloride and Nafion® were obtained from Sigma (St. Louis, MO). Carbon
fiber microelectrodes (SF1A’s) were fabricated by the Center for Microelectrode Technology.

**Histology**

Brains were removed and processed (frozen) for histological evaluation of microelectrode recording tracks. Only data from histologically confirmed placement of microelectrodes into the Str, NAc, and PFC were used for final data analysis. Based on histological analyses, no animals were excluded due to microelectrode placement errors (see Figure 2.2A).

**Data Analyses**

Collected data were processed using a custom Matlab®-based analysis package. For the potassium-evoked DA release portion of the study, maximum amplitude of the evoked DA peak was used as the major analysis parameter, with the volume of a single ejection of the KCl and the red/ox ratio – used to confirm the detection of DA by the Nafion®-coated microelectrodes – represented in Table 2.1. The volume of KCl applied was kept constant across depths and strains (75–125 nl). For DA uptake studies, the primary parameters used were the time to 80% decay of the DA signal ($T_{80}$) and the first order rate constant of the signal ($k_{-1}$), as well as the amount of time it took to reach the peak amplitude ($T_{rise}$) – see Table 2.2. DA signals were amplitude matched (ranging from 0.5 to 1.0 µM DA) to ensure accurate measurement of DA uptake kinetics (Littrell, Pomerleau et al. 2012). All data were averaged to one point per sub-region of the Str (dorsal, intermediate,
and ventral) and the NAc – see Figure 2.2A. For a graphic representation of all parameters, see Figure 2.2B. Outliers were excluded via the Grubb’s test before averaging if the conditions for homogeneity of variance were met. To compare KCl-evoked DA release in the separate ADHD models to the control strains, as well as DA uptake, two-way repeated measures ANOVAs followed by Bonferroni post-hoc comparisons were used. Significance was set at p<0.05 (GraphPad Prism 5.0).

Results

Evoked Dopamine Release in the Striatum and NA Core

High-speed chronoamperometry coupled with carbon fiber microelectrodes was used to evaluate KCl-evoked DA release because of its capability to record DA release within sub-regions of the Str and the NAc (Littrell, Pomerleau et al. 2012) using a local application of 75-125 nl KCl applied in 500 µm increments along the dorso-ventral axis. A two-way repeated measures ANOVA revealed a significant effect of depth (F\textsubscript{3,27}=33.03, p<0.0001) and interaction between strain and depth (F\textsubscript{9,81}=2.04, p<0.05) in the peak amplitude of KCl-evoked DA release. Bonferroni post-hoc comparisons revealed that the SHR model of ADHD-C (n=8) displayed significantly decreased KCl-evoked DA release versus the WKY model of ADHD-PI (n=8) in the dorsal Str (Figure 2.3A, 2.3B: p<0.01). Differences in KCl-evoked DA release were also observed between the control SD (n=7) and WKY model of ADHD-PI in the dorsal Str (Figure 2.3A, 2.3B: p<0.01), and the control SD and control WKY (n=8) strains in the intermediate region of the Str (Figure 2.3A, 2.3C: p<0.05). There were no significant differences in volumes applied
between strains. In addition, there were no significant differences in the red/ox ratios (usually 0.4-0.6) throughout the Str and NAc in all strains, supporting the detection of predominantly DA in all sub-regions (Gerhardt and Hoffman 2001, Joyce, Glaser et al. 2007, Littrell, Pomerleau et al. 2012) – see Table 2.1. It should be noted that the amplitudes of the KCl-evoked signals decreased as the electrode moved ventrally in all strains, consistent with previous reports that the ventral Str has reduced vesicular-released concentrations of DA when compared to the dorsal Str (Gerhardt, Rose et al. 1986).

Dopamine Uptake in the Striatum and NA Core

Local applications of exogenous DA were applied in the separate strains to study the functional properties of the dopamine transporter (Littrell, Pomerleau et al. 2012). A two-way repeated measures ANOVA revealed a significant effect of depth (F₃,₃₀=7.49, \( p<0.001 \)), strain (F₃,₃₀=3.93, \( p<0.05 \)), and interaction between strain and depth (F₉,₉₀=3.16, \( p<0.01 \)) in the length of time required to clear 80% of the locally applied exogenous DA (or T₈₀). Bonferroni post-hoc comparisons revealed that the SHR model of ADHD-C (n=9) displayed significantly faster DA uptake in the ventral Str versus both the control WKY (n=8) (\( p<0.05 \)) and control SD strains (Figures 2.4A, 2.4B: \( p<0.01 \)). The SHR model of ADHD-C also displayed significantly faster uptake in the NAc versus both the control WKY (\( p<0.01 \)) and control SD (Figure 2.4A, 2.4C: \( p<0.001 \)). The WKY model of ADHD-PI was found to exhibit significantly faster DA uptake in the NAc versus the control SD (Figure 2.4A, 2.4C: \( p<0.001 \)). In the control strains, the time to clear the locally
applied exogenous DA was slower in the NAc when compared to the dorsal Str, supporting previous data that in the rodent, the NAc has lower concentrations of the dopamine transporter and therefore slower DA uptake rates (Cass, Gerhardt et al. 1992, Cass, Gerhardt et al. 1993, Cass, Zahniser et al. 1993, Calipari, Huggins et al. 2012, Mitch Taylor, Jaquins-Gerstl et al. 2012). Interestingly, the rodent models of ADHD did not display a dorsal-ventral change in dopamine uptake. There were no differences between the maximum amplitude achieved nor were there any differences in the k-1 values. Finally, there were no differences in the time required to reach peak amplitude (T_{rise}) between the models of ADHD and control strains – see Table 2.2.

Prefrontal Cortex

High-speed chronoamperometry coupled with carbon fiber microelectrodes was used to evaluate KCl-evoked DA release because of its capability to record DA release within sub-regions of the striatum and the NAc (Littrell, Pomerleau et al. 2012, Miller, Pomerleau et al. 2012) using a local application of 75-125 nl KCl applied in 500 μm increments. To examine potential differences in evoked dopamine release in the separate sub-regions of the PFC between the outbred SD, the WKY progenitor, and the SHR model of ADHD, one-way ANOVAs were used. No significant differences were found between strains (cingulate cortex, p=0.1295; prelimbic cortex, p=0.1998; infralimbic cortex, p=0.1050). These data suggest that the cingulate, prelimbic and infralimbic regions in all three strains have a similar capacity to release dopamine during an action potential event. It’s
important to note that in both the SD and SHR strains, dopamine peak amplitudes increased as the microelectrode was moved ventrally; however, the WKY strain displayed the opposite effect. See Figure 2.5. Note that all dopamine signals were indicative of the detection of dopamine and/or norepinephrine based on the reduction/oxidation ratios of the signals that averaged ~0.8-1.0 for all recordings.

To examine differences in dopamine uptake in the separate prefrontal cortical sub-regions, we used local applications of dopamine to directly observe the functional properties of the dopamine and norepinephrine transporters – see Figure 2.6. One-way ANOVAs followed by Bonferroni post-hoc comparisons were used in each sub-region. It was discovered that the SHR model of ADHD ($p<0.05$) and the outbred SD control strain ($p<0.001$) displayed significantly faster dopamine uptake compared to the WKY strain in the cingulate cortex ($F_{(2,23)}=11.11$). The average dopamine uptake times in the cingulate cortex were: SD, $30.8 \pm 2$ seconds; WKY, $79.1 \pm 10$ seconds; and SHR, $44.8 \pm 3$ seconds. No dopamine uptake differences were observed in the prelimbic cortex ($p=0.9605$); however, the SHR exhibited significantly faster dopamine uptake compared to both the SD control ($p<0.01$) and the WKY strain ($p<0.05$) in the infralimbic cortex ($F_{(2,28)}=6.53$). The average dopamine uptake times in the infralimbic cortex were: SD, $61.6 \pm 8$ seconds; WKY, $49.8 \pm 11$ seconds; and SHR, $18 \pm 4$ seconds. These data reveal that the dopamine and norepinephrine transporters clear dopamine faster in the SHR in the cingulate and infralimbic cortices compared to control, but not the prelimbic cortex. It's important to note that as the microelectrode was moved ventrally in the control SD strain, the dopamine uptake became slower; however,
in the WKY and SHR strains, dopamine uptake became faster as the electrode moved ventrally.

**Discussion**

*Striatum and NA Core*

The SHR has been used for decades as a model of ADHD because of its various behavioral phenotypes that mimic the symptoms of ADHD. Its progenitor strain, the WKY, has been used as a control for the SHR in the past with no regard for where the strain originated. Recent evaluations of this control strain have revealed that not all WKY strains are identical, genetically or behaviorally. The NIH stock of the WKY strain was obtained in 1971 as outbred animals from the Kyoto School of Medicine. These animals were distributed to laboratories such as Harlan and Charles River before the F20 generation, which is considered to be the gold standard in obtaining a pure inbred animal (Sagvolden, Johansen et al. 2009). It has recently been shown that two commonly used WKY strains, the WKY/NCrl from Charles River Laboratories and the WKY/NHsd from Harlan Laboratories, display wide genetic divergence (Sagvolden, Dasbanerjee et al. 2008). When Harlan and Charles River Laboratories were contacted, both specified that all of their inbred animals, both at the USA and international laboratories, were subjected to single nucleotide polymorphism panels every quarter, thus these strains from the different laboratories will remain genetically separate. Although no behavioral data were gathered in this study, multiple studies have concluded that the WKY/NCrl from Charles River is not a valid control for the SHR due to its
behavioral abnormalities (i.e. inattention) and is more appropriate as a model of ADHD inattentive type (Drolet, Proulx et al. 2002, Sagvolden, Dasbanerjee et al. 2008, Sagvolden, Johansen et al. 2009, Roessner, Sagvolden et al. 2010, Sagvolden and Johansen 2011). Terje Sagvolden (1945-2011), a leading researcher in the SHR/ADHD field, proposed this change and concluded that the most appropriate models of ADHD are the SHR/NCrl (ADHD-C) and the WKY/NCrl (ADHD-PI) (Sagvolden, Johansen et al. 2009, Roessner, Sagvolden et al. 2010). As for the most suitable control strain, Sagvolden and colleagues determined that the inbred WKY/NHsd strain from Harlan Laboratories, not the outbred Sprague Dawley (SD) (Sagvolden, Johansen et al. 2009, Sagvolden and Johansen 2011), was the best fit control for the models of ADHD. However, the outbred SD strain from Charles River (SD/NCrl) was tested in this study as a potential control because some researchers have supported it as a way around the WKY control strain confusion (Drolet, Proulx et al. 2002). Although the present study does not discount the many behavioral studies that have used the outbred SD as a control for the SHR, it does suggest that studies looking at neurochemical differences in ADHD should focus on the inbred WKY due to a difference observed in the evoked DA release parameter in the intermediate Str between these control strains. Based on this difference in DA regulation, this study proposes that the inbred SHR progenitor strain, the WKY/NHsd, is the most appropriate control for neurochemical investigations in the SHR.

A major purpose of this research was to help resolve the confusion over DA regulation in the SHR model of ADHD-C compared to the novel WKY model of
ADHD-PI and the control strains by exploring DA release and uptake dynamics. The current experiments were performed using high-speed chronoamperometry coupled with Nafion®-coated carbon fiber microelectrodes. This technique has higher spatial and temporal resolution, decreased damage to surrounding tissue and samples a much smaller field of DA nerve terminals along the dorso-ventral axis compared to most techniques used in the past (e.g. in vivo microdialysis) (Joyce, Glaser et al. 2007, Littrell, Pomerleau et al. 2012). The reported neurochemical studies in the SHR prior to 2008 were unable to distinguish between sub-regions along the dorsal-ventral axis in the Str due to the size of the microdialysis probes; however, the technology used in this study was able to provide a higher resolution representation of the DA dynamics within the heterogeneous Str (Lindvall and Bjorklund 1974, Veening, Swanson et al. 1982, Gerfen, Baimbridge et al. 1987, Gerfen, Herkenham et al. 1987).

To determine differences in DA release, a single local ejection of a set volume of potassium chloride was used to cause calcium-dependent vesicular release of DA. The results of this study reveal that the SHR model of ADHD-C exhibits decreased depolarization-evoked DA release in the dorsal Str versus the WKY model of ADHD-PI. This decrease in DA release could be due to a decrease in stored vesicular DA (Russell, de Villiers et al. 1998) because of a potential dysfunction of monoamine oxidase-B (MAO-B), an enzyme that catalyzes the oxidation of DA in DA-producing neurons. It was recently found that an MAO-B inhibitor, deprenyl, significantly improved ADHD symptoms (Jankovic 1993, Feigin, Kurlan et al. 1996, Mohammadi, Ghanizadeh et al. 2004, Rubinstein, Malone et al. 2004.
2006). Moreover, it is possible that the vesicular monoamine transporter (VMAT) may be dysfunctional and as a result, DA may not be efficiently transported into the vesicles. It has been found that a common ADHD treatment, d-amphetamine, causes increased VMAT trafficking to the vesicular membrane within monoaminergic nerve terminals ex vivo (Riddle, Hanson et al. 2007), thus supporting a possible role for VMAT dysfunction in ADHD.

Previous investigations have implicated the dopamine transporter (DAT) in the DA dysfunction of the SHR model of ADHD—see Table 2.3 (Watanabe, Fujita et al. 1997, Leo, Sorrentino et al. 2003, Viggiano, Vallone et al. 2004, Roessner, Sagvolden et al. 2010, Simchon, Weizman et al. 2010). In the SHR brain, there have been reports of increased DAT expression (Watanabe, Fujita et al. 1997, Roessner, Sagvolden et al. 2010) and one likely explanation for the decreased DA release observed in the dorsal Str is over-abundance or over-activity of DAT. It has been shown that clearance of DA is primarily performed by DAT rather than metabolism or diffusion of DA (Cass, Zahniser et al. 1993). In this study, potassium stimulation in the dorsal Str of the SHR produced a significantly smaller signal amplitude than in the WKY model of ADHD, suggesting that the DA was likely cleared through uptake by DAT before it reached our microelectrode. To the best of our knowledge, no study has been able to quantify levels of DAT in specific striatal sub-regions on the dorso-ventral axis; however, the ratio of DA to DAT to DA receptors is similar in the naïve rodent Str and NAc (Madras, Miller et al. 2005), supporting that DAT is the major regulator of DA neuron signaling strength and duration. However, when DA uptake was examined in the dorsal Str
sub-region by locally applying exogenous DA, no differences in DA uptake were observed between these strains. It is worth noting that DA uptake was not studied directly after the local application of KCl because KCl stimulation allows for the direct examination of the release capacity output of the surrounding terminals. If the uptake dynamics of these signals were studied, changes in uptake may not be seen due to the overwhelming concentrations, or ‘ceiling effect,’ of the neurotransmitter and this effect would mask DAT productivity. In order to fully study DA regulation in these strains, exogenously applied DA was used to examine uptake dynamics. While no differences in uptake were found in the dorsal Str, it is possible that exogenously applied DA may not be as efficiently removed by DAT as the DA released inside the synapse. In addition, KCl depolarizes the DA nerve terminal membrane causing synaptic release of DA, creating the possibility that the action potential could potentiate some of the differences observed in DA uptake. The DAT is known to be electrogenic and depolarization causes the DAT to exist in a different state than the basal state (Reith, Jacobson et al. 1991, Zahniser, Gerhardt et al. 1998, Hoffman, Zahniser et al. 1999, Kandasamy 2000, El Ayadi, Afailal et al. 2001). By testing with local applications of DA rather than by using depolarization alone, the current study was able to prevent the activation of DAT to fully focus on the uptake capability of the protein, which showed no differences in the SHR versus the other strains in the dorsal Str. While this KCl-evoked difference was not observed in the other striatal sub-regions in our study, DAT location is not homogenous throughout the Str (Hebert et al. 1999) and the possibility exists that when the KCl-evoked DA is released from the nerve terminals
in the dorsal Str of the SHR model of ADHD-C, the DAT is the primary mechanism to clear the released DA.

Interestingly, in the ventral striatum and nucleus accumbens core (NAc), it was discovered that the SHR model of ADHD-C demonstrated faster DA uptake compared to both of the control strains, both of which are areas highly implicated in impulsive ADHD behaviors (Basar, Sesia et al. 2010). These results may provide neurochemical evidence for this ADHD-like behavior observed in the SHR (Sagvolden, Metzger et al. 1992, Wultz and Sagvolden 1992, Sagvolden 2000, Johansen and Sagvolden 2004, Russell 2011). As mentioned previously, clearance of DA is primarily performed by DAT rather than metabolism or diffusion (Cass, Zahniser et al. 1993). Therefore, it is reasonable to assume that the faster uptake observed in the rodent model of ADHD-C is due to differences in DAT expression, activity, and/or affinity for dopamine. Michaelis-Menten kinetics dictates that two variables affect an enzyme’s productivity, affinity and velocity. The $k^{-1}$ parameter (the first-order rate constant) is a measure of velocity and remained unchanged in all strains. This suggests that the affinity of DAT for DA is increased in the ventral Str and NAc of the rodent model of ADHD-C. Increased affinity of DAT for DA could be due to increased concentrations of the DAT at the synaptic membrane resulting from increased DAT trafficking, which, to our knowledge, has not been investigated in specific striatal sub-regions. Because the SHR has been found to possess increased DAT expression (Watanabe, Fujita et al. 1997, Roessner, Sagvolden et al. 2010, Wallis 2010), the results from this study suggest that increased DAT expression is coupled to increased DAT function at a
synaptic level. No strain differences were observed in depolarization-evoked DA release in the NAc; however, the faster DA uptake when exogenous DA was applied supports that the DA is cleared by an amplified number of DATs or enhanced DAT activity. Future studies should focus on quantifying the levels of DAT in the separate striatal sub-regions to obtain more precise evidence of the role of DAT in the rodent models of ADHD.

Finally, in the dorsal Str, the WKY model of ADHD-PI displayed increased evoked DA release as well as faster DA uptake in the NAc when compared to the control SD strain. These findings are in agreement with a previous study which showed that behavioral activity in running wheels was negatively correlated with in vitro K⁺-stimulated DA release in rat NAc and dorsal Str but not in the shell division of the NA (Tarr, Kellaway et al. 2004).

The present study uses a neurochemical technique to complement the established behavioral evidence indicating that the SHR/NCrl is a useful rodent model of ADHD-C. The decreased KCl-evoked DA release observed in the dorsal sub-region of the Str may aid in the explanation of why the SHRs express some behavioral aspects of ADHD, as the dorsal Str of the rat is akin to the human putamen (Grahn, Parkinson et al. 2008) and is believed to play a role in motor activity (Grahn, Parkinson et al. 2008). The results from this study reveal that the hyperactive SHR has DA dysfunction in this region compared to the less active WKY/NCrl. The findings of DA dysfunction in the ventral Str and NAc of the SHR is consistent with the understanding that the more ventral Str is associated with impulsive behaviors (Basar, Sesia et al. 2010). Finally, the NAc may play a role in
the inattention observed in individuals with ADHD (Volkow, Wang et al. 2009, Volkow, Wang et al. 2011) and the differences in DA uptake between the SHR/NCrl and WKY/NCrl models of ADHD and the control strains provide preliminary neurochemical evidence for the inattentive behavior observed in these strains; however, more evidence is needed to confirm the speculation that DAT is altered in discrete sub-regions of the Str and NAc in the SHR/NCrl and WKY/NCrl.

**Prefrontal Cortex**

Previous investigations have implicated the dopamine transporter (DAT) in the DA dysfunction of the SHR model of ADHD (Watanabe, Fujita et al. 1997, Leo, Sorrentino et al. 2003, Viggiano, Vallone et al. 2004, Roessner, Sagvolden et al. 2010, Simchon, Weizman et al. 2010, Womersley, Hsieh et al. 2011) and our data revealing differences in dopamine regulation in the striatum can be attributed to increased activity of the DAT in the striatum of the SHR. It is reasonable to assume that if the striatum has increased DAT activity, it’s likely that similar dopamine dysfunction exists in the PFC of the SHR. It’s important to clarify that the norepinephrine transporter (NET) is present in the PFC in much greater concentrations than the DAT and dopamine uptake in the PFC is preferentially due to the NET instead of the DAT (Moron, Brockington et al. 2002), so investigations into the mechanism of dopamine clearance in the PFC of the SHR should be examined in the future.

Using similar volumes of a potassium solution, evoked overflow of catecholamine nerve terminals surrounding the tip of the carbon fiber
microelectrode was used to attempt to locate differences in vesicular dopamine storage in the different PFC sub-regions. Upon stimulation, no differences were observed between the inbred SHR model of ADHD, the inbred progenitor WKY and the outbred SD control strains. The lack of differences signifies to us that the separate PFC sub-regions all have the same capacity to store and release dopamine and/or norepinephrine in these strains. MAO and VMAT, both implicated in ADHD, can then be considered to be functional in the PFC of the SHR model of ADHD and drugs targeting these proteins, such as deprenyl, may not be useful in this model.

Though no differences were observed in the KCl-evoked dopamine signals, there were significant differences in the length of time required to clear exogenous dopamine applications between the SHR and control strains. Similar maximum dopamine amplitudes were achieved by applying various volumes of an exogenous dopamine solution in order to evaluate the uptake kinetics of the signals. The DAT is electrogenic and depolarization causes the DAT to change from the basal state (Reith, Jacobson et al. 1991, Zahniser, Gerhardt et al. 1998, Hoffman, Zahniser et al. 1999, Kandasamy 2000, El Ayadi, Afailal et al. 2001) and in order to test the full uptake capabilities of the transporters, including both the DAT and NET, it was necessary to prevent the action potentials induced with potassium. Utilizing this approach, it was discovered that the SHR displayed faster uptake in the cingulate and infralimbic cortices compared to the WKY strain, but not the prelimbic cortex. The SHR model of ADHD was also discovered to have faster dopamine uptake compared to the SD strain in the infralimbic cortex. These
results are significant because the cingulate cortex is involved with learning and memory, playing a vital role in Papez circuit and the cortical control of emotions in humans (Granziera, Hadjikhani et al. 2011). This data further demonstrates that there exists a neurochemical dysfunction in a region important for linking behavioral outcomes to motivation (Adey 1951, Adey and Meyer 1952) in the SHR. Also, the infralimbic cortex in rodents is known to be involved with attention to stimulus features, task contingencies, and attentional set-shifting (Dalley, Cardinal et al. 2004) – all behaviors known to be affected in individuals with ADHD (Krusch, Klorman et al. 1996, Mehta, Goodyer et al. 2004, Klimkeit, Mattingley et al. 2005).

The SHR has previously been found to have dysfunctional dopamine dynamics in the striatum and NA core (Miller, Pomerleau et al. 2012), but here we describe evidence for faster dopamine uptake in the cingulate and infralimbic cortices of the medial PFC. These regions are heavily implicated in ADHD (Arnsten and Dudley 2005, Arnsten and Li 2005, Arnsten 2009) and this data gives further evidence for use of the SHR as a model of ADHD. Therapeutics targeting this dysfunction may prove to be useful in the SHR. However, MPH, a DAT blocker, has been investigated and found to not be useful in this model because instead of calming these animals as it does in humans, it increased locomotion in clinically relevant doses (Sagvolden, Metzger et al. 1992, Sagvolden 2000, Sagvolden and Johansen 2011). This signifies to us that targeting the NET instead of the DAT, such as with the use of ATX, may provide a more useful option of targeting PFC dopamine dysfunction in the SHR model of ADHD.
Conclusions

In conclusion, the results from this study demonstrate that the SHR/NCrl model of ADHD-C and the WKY/NCrl model of ADHD-PI have distinct differences in the regulation of DA release and uptake in the Str and NAc compared to each other as well as compared to the WKY and SD control strains. Additionally, the data presented above in the SHR model of ADHD provides evidence for dopaminergic system dysfunction in the PFC. This study further reveals that the SD strain may not be a useful control when investigating neurochemical changes in the rodent models of ADHD. However, because of increasing evidence that ADHD is not limited to purely DA dysfunction, it will be valuable to study other neurotransmitter systems in the future, such as norepinephrine and glutamate (Russell 2001, Kotecha, Oak et al. 2002, Russell 2002, Madras, Miller et al. 2005, Heal, Smith et al. 2008), and how they relate to the SHR and WKY rodent models of ADHD. Glutamate regulation in the SHR may be impaired, creating the possibility that targeting the dopamine-glutamate interaction in this model of ADHD may prove useful with novel therapeutics.

Animal models grant us the ability to investigate neurotransmitter system regulation in vivo, creating a more accurate depiction of the dysfunction in multiple sub-regions throughout the brain. Using these rodent models of ADHD, common ADHD treatments, such as MPH and ATX, as well as unconventional treatments, such as memantine and deprenyl, should be used to examine the effects of these drugs on the dopamine and glutamate systems with the ultimate goal of discovering novel ways to treat ADHD with minimal side-effects and clear long-
term safety and efficacy. It is our belief that targeting the interaction between the dopamine and glutamate systems will provide an avenue not yet understood to achieve this goal.

As more and more research is beginning to implicate a dysfunctional glutamate system in ADHD, it’s hard to ignore that glutamate may be playing some role in the pathophysiology of ADHD. It is thought that the relationship between the dopamine and glutamate systems is a likely target for future pharmacotherapies. It is our hope that through modification of these interactions, we will be able to better treat individuals with ADHD and greatly improve their quality of life.
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Figure 2.1 Carbon Fiber Electrochemistry

The micropipette was placed 200 µm away from the carbon fiber tip, filled with either KCl (for release studies) or dopamine (DA, for uptake studies). When dopamine (either endogenous or exogenous) came into contact with the carbon fiber, a voltage of +0.55 V was applied (100 ms) vs. a Ag/AgCl reference electrode, dopamine was oxidized into dopamine-ortho-quinone and 2 electrons were measured and amplified by the FAST16mkII recording system. The voltage was changed to 0.0 V (100 ms) vs. a Ag/AgCl reference electrode and some of the dopamine-ortho-quinone product was reduced back into dopamine, while some diffused away, resulting in a red/ox ratio. A charge exclusion layer of Nafion® excluded electroactive interferent molecules, such as ascorbic acid (AA).
Figure 2.2 Experimental Methodology

A) Carbon fiber microelectrode placement within the rodent Str and NAc. The diagram on the right shows the approximate location of the tip of the carbon fiber microelectrode, and the left side shows the actual carbon fiber track (highlighted with the black box) from a 20 μm slice stained with cresyl violet. The carbon fiber microelectrode was lowered in 500 μm increments in both hemispheres (one hemisphere for KCl recordings and one for DA) and the average of these recordings was used to give one data point per sub-region. ac, anterior commissure. B) In Vivo Parameters. The arrow on the x axis indicates the time point of local application of KCl or DA. The KCl parameters used were the maximum amplitude of evoked DA release, represented by the purple dashed lines, and the ratio of the reduction/oxidation current (solid black line divided by the solid grey line). DA parameters included the time it took to clear 80% of the exogenous DA in reference to the maximum amplitude (T80), the curve fit based on first order fitting of the signal decay (k-1), and the amount of time it took to reach the peak amplitude (Trise), which are all represented by the orange dashed lines (Miller, Pomerleau et al. 2012).
Figure 2.3 Decreased KCl-Evoked Dopamine Release in the SHR

The model of ADHD-C demonstrated significantly decreased KCl-evoked DA release versus the model of ADHD-PI in the dorsal Str (**p<0.01). The control SD also displayed decreased KCl-evoked DA versus the model of ADHD-PI in the dorsal Str (‡‡p<0.01). In the intermediate Str, it was observed that the control WKY displayed significantly increased KCl-evoked DA release versus the control SD strain (§p<0.05). Mean ± SEM. B) Representative tracing of the difference in KCl-evoked DA release in the dorsal Str between the model of ADHD-C, the model of ADHD-PI, and the control SD. C) Representative tracing of the difference in KCl-evoked DA release in the intermediate Str between the control SD and control WKY strains. Arrows indicate local application of KCl (Miller, Pomerleau et al. 2012).
Figure 2.4 Dopamine Uptake in the SHR Model of ADHD-C, WKY Model of ADHD-PI and Control Strains

A) A DA uptake depth profile through the Str and NA core revealed that B) in the ventral Str, the model of ADHD-C displayed faster DA uptake versus both the control SD ($^{XX}p$<0.01) and the control WKY ($^{\dagger}p$<0.05). C) In the NAc, the model of ADHD-C exhibited faster DA uptake versus the control SD ($^{XXX}p$<0.001) and the control WKY ($^{\dagger\dagger}p$<0.01). The model of ADHD-PI displayed faster DA uptake versus the control SD ($^{+++}p$<0.001) in the NAc. Arrows indicate local applications of DA. Mean ± SEM (Miller, Pomerleau et al. 2012).
Figure 2.5 KCl-Evoked Differences in the PFC

No differences were observed between the outbred SD control strain, the WKY progenitor strain, and the SHR model of ADHD in the KCl-evoked dopamine peak amplitudes following a local application of KCl in any of the prefrontal cortical sub-regions. Values represent the mean ± SEM (Miller, Thomas et al. 2013).
Figure 2.6 Dopamine Uptake in the PFC

The SHR model of ADHD and the outbred SD control strain exhibited significantly faster dopamine uptake than the WKY strain in the cingulate cortex (*p<0.05, ***p<0.001). No dopamine uptake differences were observed in the prelimbic cortex; however, the SHR exhibited significantly faster dopamine uptake in the infralimbic cortex compared to both the SD control and WKY strain (*p<0.05, **p<0.01). Values represent the mean ± SEM (Miller, Thomas et al. 2013).
### Table 2.1 KCl-Evoked Dopamine Release

<table>
<thead>
<tr>
<th>Sub-Region</th>
<th>SHR/NCrl ADHD-C</th>
<th>WKY/NCrl ADHD-Pi</th>
<th>WKY/NHsd Inbred Control</th>
<th>SD/NCrl Outbred Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal Str</td>
<td>0.58 ± 0.07</td>
<td>0.53 ± 0.11</td>
<td>0.47 ± 0.12</td>
<td>0.50 ± 0.08</td>
</tr>
<tr>
<td>Intermed Str</td>
<td>0.47 ± 0.06</td>
<td>0.58 ± 0.11</td>
<td>0.51 ± 0.07</td>
<td>0.65 ± 0.05</td>
</tr>
<tr>
<td>Ventral Str</td>
<td>0.45 ± 0.06</td>
<td>0.64 ± 0.20</td>
<td>0.55 ± 0.09</td>
<td>0.62 ± 0.10</td>
</tr>
<tr>
<td>NA Core</td>
<td>0.52 ± 0.10</td>
<td>0.57 ± 0.07</td>
<td>0.51 ± 0.06</td>
<td>0.66 ± 0.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Volume (nl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal Str</td>
</tr>
<tr>
<td>Intermed Str</td>
</tr>
<tr>
<td>Ventral Str</td>
</tr>
<tr>
<td>NA Core</td>
</tr>
</tbody>
</table>

Similar reduction/oxidation ratios were obtained when the KCl was applied, all indicative for the presence of dopamine. The volumes of KCl applied between strains were not significantly different (mean ± SEM).
### Table 2.2 Dopamine Uptake

<table>
<thead>
<tr>
<th>Sub-Region</th>
<th>SHR/NCrl</th>
<th>WKY/NCrl</th>
<th>WKY/NHsd</th>
<th>SD/NCrl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADHD-C</td>
<td>ADHD-PI</td>
<td>Inbred</td>
<td>Outbred</td>
</tr>
<tr>
<td>K-1 (sec⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal Str</td>
<td>0.007 ± 0.001</td>
<td>0.009 ± 0.003</td>
<td>0.014 ± 0.003</td>
<td>0.014 ± 0.003</td>
</tr>
<tr>
<td>Intermed Str</td>
<td>0.011 ± 0.003</td>
<td>0.009 ± 0.004</td>
<td>0.013 ± 0.003</td>
<td>0.009 ± 0.001</td>
</tr>
<tr>
<td>Ventral Str</td>
<td>0.010 ± 0.003</td>
<td>0.009 ± 0.004</td>
<td>0.011 ± 0.003</td>
<td>0.009 ± 0.001</td>
</tr>
<tr>
<td>NA Core</td>
<td>0.008 ± 0.002</td>
<td>0.008 ± 0.003</td>
<td>0.006 ± 0.001</td>
<td>0.008 ± 0.001</td>
</tr>
<tr>
<td>T-rise (sec)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal Str</td>
<td>7.95 ± 1.15</td>
<td>7.07 ± 0.94</td>
<td>7.85 ± 1.51</td>
<td>7.85 ± 1.51</td>
</tr>
<tr>
<td>Intermed Str</td>
<td>5.71 ± 0.41</td>
<td>7.28 ± 0.75</td>
<td>8.24 ± 0.78</td>
<td>8.91 ± 3.38</td>
</tr>
<tr>
<td>Ventral Str</td>
<td>6.71 ± 1.38</td>
<td>7.64 ± 0.79</td>
<td>9.43 ± 0.83</td>
<td>10.3 ± 2.73</td>
</tr>
<tr>
<td>NA Core</td>
<td>9.25 ± 0.50</td>
<td>9.36 ± 0.85</td>
<td>12.9 ± 1.71</td>
<td>12.6 ± 3.5</td>
</tr>
<tr>
<td>Peak Amplitude (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal Str</td>
<td>0.89 ± 0.04</td>
<td>0.83 ± 0.04</td>
<td>0.75 ± 0.04</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td>Intermed Str</td>
<td>0.74 ± 0.03</td>
<td>0.79 ± 0.04</td>
<td>0.77 ± 0.04</td>
<td>0.73 ± 0.02</td>
</tr>
<tr>
<td>Ventral Str</td>
<td>0.72 ± 0.04</td>
<td>0.74 ± 0.04</td>
<td>0.69 ± 0.03</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>NA Core</td>
<td>0.73 ± 0.06</td>
<td>0.79 ± 0.02</td>
<td>0.66 ± 0.04</td>
<td>0.74 ± 0.03</td>
</tr>
</tbody>
</table>

k-1 values and the amount of time it took to reach the peak amplitude (T-rise) did not vary between strains. Similar amplitudes of exogenous DA were applied to the Str and NAc and did not significantly differ (mean ± SEM).
Table 2.3 Dopamine Transporter Function in the SHR

<table>
<thead>
<tr>
<th>DAT Function</th>
<th>Study</th>
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</thead>
<tbody>
<tr>
<td>↑</td>
<td>Current</td>
</tr>
<tr>
<td>↑</td>
<td>Roessner, Sagvolden et al. 2010</td>
</tr>
<tr>
<td>↑</td>
<td>Watanabe, Fujita et al. 1997</td>
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<tr>
<td>=</td>
<td>Li, Lu et al. 2007</td>
</tr>
<tr>
<td>=</td>
<td>Linthorst, Van den Buuse et al. 1990</td>
</tr>
<tr>
<td>↓</td>
<td>Leo, Sorrentino et al. 2003</td>
</tr>
</tbody>
</table>

Present and past data on dopamine transporter function in the SHR model of ADHD reveal conflicting results versus control, likely due to unknown strains and variable techniques.
Chapter Three: Aberrant Glutamate Signaling in the Prefrontal Cortex and Striatum of the Spontaneously Hypertensive Rat Model of Attention-Deficit/Hyperactivity Disorder

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is estimated to affect 6% of children worldwide and a portion of those will continue to suffer into adulthood (Polanczyk, de Lima et al. 2007). Hyperactivity lessens in adolescence, though cognitive impairment persists (Brassett-Harknett and Butler 2007, Spencer, Biederman et al. 2007). Those diagnosed with ADHD combined type (ADHD-C), characterized by hyperactivity and inattention, had larger impairments including lower grades in school and more arrests during adolescence compared to the other ADHD subtypes (inattentive and hyperactive/impulsive) (Molina, Hinshaw et al. 2009). Thus, evidence suggests the efficacy of treatments for adolescents could be improved and calls for a better understanding of the pathophysiology of ADHD, particularly for those diagnosed with ADHD-C.

ADHD has been posited to be caused by hypofunctional catecholamine systems (Rastogi and Singhal 1976, Robbins and Sahakian 1979, Levy 1991, Solanto, Arnsten et al. 2001) in various brain regions including the nucleus accumbens (Russell 2000, Podet, Lee et al. 2010), striatum (Krause, Dresel et al. 2003) and prefrontal cortex (PFC) (Halperin and Schulz 2006, Arnsten 2009). However, recent clinical evidence has implicated glutamate, the main excitatory neurotransmitter in the CNS (Danbolt 2001), in ADHD. Imaging studies of children
and adults with ADHD revealed increased levels of glutamate/glutamine in the striatum and anterior cingulate cortex of the PFC (Moore, Biederman et al. 2006, Moore, Biederman et al. 2007, Dramsdahl, Ersland et al. 2011). Furthermore, treatment with the ADHD medications methylphenidate and atomoxetine were found to lower these levels (Hammerness, Biederman et al. 2012). Memantine, an NMDA receptor antagonist, was found to improve ADHD symptoms in both children and adults (Findling, McNamara et al. 2007, Surman, Hammerness et al. 2012). Together, these data provide evidence for altered glutamate regulation in the ADHD brain.

Studies of signaling interactions between dopamine and glutamate demonstrate that stimulation of the D2-class dopamine receptor is involved in the downstream inhibition of the NMDA receptor, weakening the excitatory response (Kotecha, Oak et al. 2002). Likewise, activation of D4 dopamine receptors depresses AMPA receptor-mediated excitatory synaptic transmission in PFC pyramidal neurons, which is accompanied by a D4-induced decrease of AMPA receptors at the synapse (Yuen, Zhong et al. 2010). A hypodopaminergic state in the ADHD brain may then be expected to lead to more active NMDA/AMPA receptors and a subsequent increase in glutamate output.

Behavioral studies have demonstrated that the spontaneously hypertensive rat (SHR) exhibits key symptomatic aspects of ADHD-C, including hyperactivity, inattention and impulsivity (Knardahl and Sagvolden 1979, Knardahl and Sagvolden 1981, Sagvolden and Johansen 2012). Previous work from our lab has revealed hypofunctional dopamine systems in the ventral striatum, nucleus
accumbens core, and PFC of the SHR compared to the Wistar Kyoto (WKY) control (Miller, Pomerleau et al. 2012, Miller, Thomas et al. 2013). Therefore, due to the current understanding of the dopamine/glutamate relationship, we hypothesized that a hyperglutamatergic state exists in the striatum, nucleus accumbens core and PFC of the SHR. Understanding glutamate signaling in the SHR could provide us with an opportunity to study novel therapeutics for the treatment of ADHD.

**Materials and Methods**

*Animal Preparation for Acute Electrochemical Recordings*

Male, 7 week old spontaneously hypertensive rats (SHR) were obtained from Charles River Laboratories (NCrl), Wilmington, Massachusetts, while Wistar Kyoto (WKY) rats were obtained from Harlan Laboratories (NHsd), Indianapolis, Indiana (Sagvolden, Johansen et al. 2009). Animals were given access to food and water *ad libitum* and housed in a 12 hour light/dark cycle. All animals remained in a quarantine period for one week to ensure no travel-related stress confounds were possible.

On the day of recording, 8 week old rats were anesthetized using a 25% urethane solution (1.25 g/kg i.p.) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, California). A circulating heating pad (Gaymar Industries, Inc., Orchard Park, New York) was used to maintain body temperature. A skin incision was made and muscle/connective tissue were reflected. The skull overlying the striatum (Str) and prefrontal cortex (PFC) was removed bilaterally for
recordings in the Str (from bregma: AP +1.0 and ML ±2.5; from surface of the brain: DV -4.0 to -6.5 in 0.5 mm increments), nucleus accumbens core (NAc, from bregma: AP +1.0 and ML ±2.5; from the surface of the brain: DV -7.0 to -7.5 in 0.5 mm increments), and PFC (from bregma: AP +3.2 and ML ±1.0; from surface of the brain: DV -2.5 to -5 in 0.5 mm increments) (Paxinos and Watson 2009) – see Figure 3.1d for a reference to the brain regions studied. A small hole remote from the site of surgery was made for placement of the miniature Ag/AgCl reference electrode. Protocols for animal use were approved by the Institutional Animal Care and Use Committee, which is Association for Assessment and Accreditation of Laboratory Animal Care International approved. All procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

High-Speed Amperometric Recordings of Glutamate

High-speed amperometric recordings, displayed at a frequency of 4 Hz, were performed using the FAST16mkIII recording system (Fast Analytical Sensing Technology, Quanteon, LLC, Nicholasville, Kentucky). Glutamate-oxidase (GluOx) coated microelectrode arrays (MEAs) consisting of 4 Pt sites measuring 15 x 333 µm arranged vertically in dual pairs (S2 conformation) were used as previously described (Hinzman et al. 2012; Thomas et al. 2012; Hinzman et al. 2010). Briefly, 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), 0.125% glutaraldehyde (Glut; Sigma-Aldrich), and 1% glutamate oxidase (GluOx; US biological, Salem, MA) were prepared and coated onto the bottom pair of recording
sites to allow for the conversion of glutamate to α-ketoglutarate and the reporter molecule, H$_2$O$_2$. The top pair of recording sites, or the sentinel sites, only received a coating of the BSA/Glut matrix to allow for background measures and subtraction to obtain a self-referenced glutamate signal (Hinzman et al. 2010; Burmeister and Gerhardt 2001) – see Figures 3.1a and 3.1c. To eliminate interferent molecules such as ascorbic acid and dopamine from reaching the Pt sites, 1,3-phenylenediamine (mPD), a molecular size exclusion layer, was electroplated onto both the GluOx and sentinel sites (Hinzman et al. 2010) – see Figure 3.1b. Immediately before in vivo use, an in vitro calibration, as described previously (Hinzman et al. 2010), was used to determine selectivity (glutamate vs. ascorbic acid), limit of detection (in µM, S/N=3), and sensitivity (slope, nA/µM). The average slope, limit of detection and selectivity for all electrodes used are presented in Table 3.1. No significant differences in the performance of the MEAs used between strains were observed.

In Vivo Experimental Protocol

After calibration, a micropipette (A-M Systems, Inc., Everett, WA) was pulled and bumped to an inner diameter of 10 µm and placed atop and centered between the 4 Pt sites and positioned approximately 100 µm from the MEA surface using sticky wax (Kerr USA, Romulus, Michigan). The micropipette was filled with filtered isotonic KCl (70 mM KCl, 79 mM NaCl, and 2.5 mM CaCl$_2$), using a 4 inch filling needle (Cadence Inc., Staunton, Virginia) and a 5 ml syringe.
Experiments were initiated with the insertion of the MEA/micropipette assembly into a stereotactically selected region of the left or right hemisphere’s Str. The system was allowed to reach a stable baseline dorsal to the first recording site for an average of 45 minutes. The MEA/micropipette assembly was then lowered to the target recording depth where the signal was again allowed to stabilize for five minutes before five local applications of KCl, separated by an average of 60 seconds each, were used to determine the effects on glutamate release (Hinzman, Thomas et al. 2010). Only the first three KCl-evoked peaks were used for data analyses because of their reproducibility (see Figure 3.2d for an example). The KCl solution was applied by pressure ejection (5–25 psi for 0.5 seconds) to achieve a set volume of KCl (75–125 nl) which was measured by determining the amount of fluid ejected from the micropipette using a dissection microscope fitted with an eyepiece reticle that was calibrated so that 1 mm of movement was equivalent to ~25 nl of fluid ejected (Cass, Gerhardt et al. 1992, Friedemann and Gerhardt 1992). If the volume was determined to be greater or less than 75-125 nl, then that data point was excluded. Tonic glutamate levels were obtained by averaging the 10 seconds immediately prior to the first KCl-evoked glutamate peak in each sub-region. After the Str and NAc were characterized, the MEA/micropipette assembly was removed from the brain and reinserted into the same hemisphere’s PFC using the same experimental procedure.

After the KCl and tonic glutamate studies, the micropipette was filled with filtered isotonic 200 μM glutamate solution in 0.9% saline (pH 7.4). The MEA/micropipette assembly was inserted stereotactically into the contralateral Str.
Again, once a stable baseline was achieved in a location dorsal to the first recording site, the MEA/micropipette assembly was lowered to the target recording depth where the signal was allowed to stabilize for an average of five minutes before the exogenous glutamate solution was locally applied by pressure ejection (8-30 psi for 0.5-10 s) to achieve a range of maximum amplitudes between 1 and 50 µM glutamate (Hinzman, Thomas et al. 2010, Hinzman, Thomas et al. 2012). If the peaks obtained were less than or greater than 1 to 50 µM glutamate, then those data points were excluded (Hinzman, Thomas et al. 2010). After the Str and NAc were characterized, the MEA/micropipette assembly was removed from the brain and reinserted into the same hemisphere’s PFC using the same experimental procedure as previously described.

**Materials**

Urethane, L-glutamic acid, ascorbic acid, bovine serum albumin, gluteraldehyde, sodium chloride, potassium chloride, and calcium chloride were obtained from Sigma-Aldrich (St. Louis, MO). Glutamate oxidase was obtained from US Biological (Salem, MA).

**Histology**

Brains were removed and processed (frozen) for histological evaluation of microelectrode recording tracks. Only data from histologically confirmed placements of microelectrodes into the striatum, nucleus accumbens core, and prefrontal cortex in order to have a repeated measures design were used for final
data analysis. No animals were excluded due to placement errors.

Data Analyses

Collected data were processed using a custom Matlab®-based analysis package. Two-way repeated measures ANOVAs were used to examine differences between strain and depth for tonic glutamate, KCl-evoked glutamate, and glutamate uptake parameters followed by Bonferroni post-hoc comparisons if significant effects existed. Significance was set at p<0.05 (GraphPad Prism 6.0).

Results

Tonic and KCl-Evoked Glutamate Release in the Prefrontal Cortex

Exploring glutamate dynamics in the prefrontal cortex (PFC) is crucial to fully understanding the etiology of ADHD (Arnsten and Li 2005, Arnsten 2009), specifically with regards to using the SHR as a model of ADHD (Sagvolden, Johansen et al. 2005, Johansen, Killeen et al. 2009). First, tonic glutamate levels were examined between the SHR, a model of ADHD (n=8), and its progenitor strain, the WKY control (n=8). Multiple regions of the PFC, including the cingulate, prelimbic and infralimbic cortices, were sampled. No significant differences in tonic glutamate in any of these regions were observed between strains (average tonic glutamate – SHR: cingulate 3.7 ± 1.4 µM, prelimbic 3.2 ± 1.2 µM, infralimbic 3.1 ± 1.2 µM; WKY: cingulate 1.8 ± 0.24 µM, prelimbic 1.6 ± 0.36 µM, infralimbic 2.0 ± 0.53 µM) – Figure 3.2a.
Next, local applications of KCl were used to evoke glutamate release in order to examine the release properties of glutamatergic neurons terminating in the different PFC sub-regions. Using a two-way repeated measures ANOVA, a significant interaction between strain and depth was found ($F_{(2,30)}=3.94$, $p=0.03$). Bonferroni post-hoc comparisons revealed that the differences between the SHR (n=8) and WKY (n=9) existed in both the cingulate and infralimbic cortices, where the SHR strain had significantly increased glutamate release in response to the KCl than the WKY control (Figure 3.2b). Average evoked glutamate – SHR: cingulate $22.5 \pm 5.31$ µM, prelimbic $18.0 \pm 4.04$ µM, infralimbic $17.1 \pm 3.95$ µM; WKY: cingulate $8.17 \pm 2.24$ µM, prelimbic $12.3 \pm 2.86$ µM, infralimbic $3.50 \pm 1.55$ µM. Also discovered were a significant effect of depth ($F_{(2,30)}=3.82$, $p=0.03$) and strain ($F_{(1,15)}=5.99$, $p=0.02$) – see Figure 3.2c. Figure 3.2d represents an average KCl-evoked glutamate release event in each of the three separate PFC sub-regions (only the background subtracted traces are shown).

**Tonic and KCl-Evoked Glutamate Release in the Striatum and NA Core**

Tonic levels of glutamate were obtained to evaluate the basal dynamics of the glutamatergic system in both the SHR (n=8) and WKY (n=9) strains within the striatum. Using a two-way repeated measures ANOVA, no significant interaction between strain and depth of the electrode in the Str was found, nor were there any differences in the effect of strain nor the effect of depth (average tonic glutamate – SHR: dorsal $3.4 \pm 1.2$ µM, intermediate $4.9 \pm 2.1$ µM, ventral $4.3 \pm 1.7$ µM, NA core $4.0 \pm 1.3$ µM; WKY: dorsal $1.9 \pm 0.64$ µM, intermediate $1.7 \pm 0.49$ µM, ventral
1.7 ± 0.45 µM, NA core 1.5 ± 0.40 µM). See Figure 3.3a.

Using KCl to evoke glutamate release in the SHR (n=8) and WKY (n=9) enabled the examination of the vesicular storage and release capabilities of glutamatergic neurons. No significant interaction between strain and depth was found (F(3,45)=1.56, p=0.21). A significant effect of strain was discovered in the Str (F(1,15)=9.21, p=0.008), revealing that the SHR strain had significantly higher KCl-evoked glutamate in this region as a whole compared to the control WKY. See Figures 3.3b and 3.3c (average evoked glutamate – SHR: dorsal 10.5 ± 1.95 µM, intermediate 13.5 ± 2.42 µM, ventral 18.8 ± 4.89 µM, NA core 13.5 ± 2.51 µM; WKY: dorsal 5.61 ± 0.764 µM, intermediate 6.20 ± 0.921 µM, ventral 6.75 ± 0.927 µM, NA core 8.91 ± 1.75 µM). Figure 3.3d represents the three local applications of KCl which resulted in reproducible glutamate release events in the Str (only the background subtracted traces are shown). No significant effect was seen with respect to depth (F(3,45)=2.18, p=0.10).

Glutamate Uptake

Local applications of exogenous glutamate were used to examine differences in the uptake kinetics of the glutamate system in the different sub-regions of the PFC and striatum within the SHR (n=6) and WKY (n=6). There were no differences found in the amplitude of the glutamate signal achieved following these local applications in the PFC (F(2,20)=0.90, p=0.42) nor the striatum (F(3,33)=0.38, p=0.76). A significant effect of strain was found in both the PFC (F(1,10)=5.37, p=0.04) and striatum (F(1,11)=5.43, p=0.03) when examining the
volume of exogenous glutamate applied to attain similar amplitudes in these regions, though no significant interaction between strain and depth was found in either of these brain areas – see Table 3.2. In the PFC, it was discovered that a significant effect of strain existed in the amplitude per volume glutamate applied (Figure 3.4a, $F_{(1,10)}=5.88, \ p=0.03$), illustrating that the SHR strain had a significantly smaller amplitude/volume ratio in the PFC as a whole region; however, no differences were discovered in the amplitude per volume glutamate applied in the striatum (Figure 3.4b).

**Discussion**

Using microelectrode array (MEA) technology, aberrant glutamate signaling was discovered in the spontaneously hypertensive rat (SHR) model of ADHD-C compared to its progenitor strain, the Wistar Kyoto (WKY). Glutamate, an excitatory neurotransmitter that functions on a sub-second timescale, must be tightly regulated for proper neuronal control (Danbolt 2001). Rapid temporal resolution cannot be achieved using microdialysis, which yields results only as quickly as every few minutes, missing much of the dynamic changes. Direct glutamate measures using MEAs allow for the spatial and temporal resolution necessary to examine this system in a quasi-physiologically relevant state, enabling the discovery of quickly changing glutamate dynamics in anesthetized rodents. Thus, direct glutamate signaling was able to be fully investigated in the eight week old SHR. This age was used because it has previously been found that the age of eight weeks in rodents is analogous to late adolescence and/or young
adulthood in humans (Andreollo, Santos et al. 2012). The Multimodal Treatment Study of Children with ADHD (MTA) followed children with ADHD for 14 months during treatment (behavior therapy alone, medication alone, or both) and roughly 8 years after its completion, when the participants were reaching late adolescence and young adulthood, it was discovered that more than 60% of children treated with medication during the initial 14 month long MTA study had stopped taking any medication for their ADHD symptoms (Molina, Hinshaw et al. 2009) and there was no long term benefit found in the medication group. For that reason, investigations into the neurobiological basis of ADHD in the adolescent population would greatly benefit these individuals if it were possible to better target their ADHD symptoms using novel pharmacotherapies.

Previous investigations into the neurochemistry of the SHR, which has been discovered to exhibit hyperactive/impulsive and inattentive behaviors (Russell 2011), have revealed that the SHR has a hypodopaminergic state in the PFC and ventral striatum (Miller, Pomerleau et al. 2012, Miller, Thomas et al. 2013). To our knowledge, there are currently no studies reporting on direct glutamate neurotransmission in these strains, although multiple studies have implicated altered glutamate dynamics in the SHR. Activation of NMDA receptors in the SHR resulted in less calcium uptake into PFC slices compared to the WKY (Lehohla, Kellaway et al. 2004) and glutamate in PFC slices was found to enhance norepinephrine release by activation of AMPA receptors and this enhancement was augmented in the SHR (Russell 2001). Also found was increased glutamate-stimulated release of dopamine in the substantia nigra of the SHR compared to
the WKY, suggesting altered glutamate regulation of dopaminergic neurons in the SHR (Warton, Howells et al. 2009). However, in order to better understand the pathophysiology of ADHD and lead us to new categories of medications for the treatment of ADHD, direct glutamate signaling must be investigated.

The PFC is involved in a number of executive functions in both humans and rodents, including reward processing and attention (Perry, Joseph et al. 2011), as well as learning and memory (Kahn, Ward et al. 2012) and a possible dysfunctional role of the PFC in ADHD has been implicated in recent studies (Arnsten 2009). In this study, the SHR was found to have higher potassium-evoked glutamate release in the cingulate and infralimbic cortices, but not the prelimbic cortex, compared to the WKY control. The glutamate release differences in these brain regions furthers the theory that the PFC is heavily involved in ADHD, as the cingulate cortex plays a vital role in emotional regulation in humans (Granziera, Hadjikhani et al. 2011) and is thought to link behavioral outcomes to motivation (Adey 1951, Adey and Meyer 1952), while the infralimbic cortex is involved with attention to stimulus features, task contingencies, and attentional set-shifting (Dalley, Cardinal et al. 2004) – all behaviors known to be affected in individuals with ADHD (Krusch, Klorman et al. 1996, Mehta, Goodyer et al. 2004, Klimkeit, Mattingley et al. 2005). The increase in potassium-evoked glutamate is likely due to increased stores of vesicular glutamate or increased levels of docked and primed vesicles. Although there was observed a difference in glutamate release between strains, no differences in tonic glutamate were found. While applications of potassium evoked distinct differences in glutamate release between the SHR and WKY, the evoked
increase in glutamate was transient and the tonic levels of glutamate were quickly restored. As our lab has shown previously (Hascup, Hascup et al. 2010), only about 50% of tonic glutamate is neuronal in origin and thus may not be reflected in the levels measured here. Furthermore, tonic glutamate has been shown to be negatively affected by the anesthetics used, specifically by urethane (Rutherford, Pomerleau et al. 2007). Future studies are needed in awake animals (to evaluate differences in tonic glutamate between these strains without the use of anesthesia during the neurochemical measurements, though all of the animals in this study were anesthetized to the same degree with urethane and can be considered comparable to each other. Moreover, the results from this anesthetized study were needed to help locate the best brain region to place the MEA for un-anesthetized studies moving forward. Because we didn’t see any differences in glutamate uptake kinetics in this region, it’s unlikely that the similarities in tonic glutamate between strains are due to faster glutamate uptake in the SHR, but instead are caused by a similar resting state of the glutamate systems in these strains. For this reason, the observed differences in vesicular storage capabilities of the neurons terminating in the PFC likely contribute to the increased release of glutamate in the PFC during potassium stimulation in the SHR.

Interestingly, we discovered differences in the volume of glutamate solution needed to achieve similar amplitudes following local applications with exogenous glutamate in the PFC of the SHR compared to the WKY control, suggesting that the uptake mechanisms in the PFC of the SHR are more active. Another important finding discovered while examining glutamate uptake in the PFC was the
difference in the amplitude per volume of solution applied in this brain area, a parameter that was unchanged in the striatum. Previous work from our lab has shown that the relationship between amplitude and amount of locally applied dopamine solution has a positive linear correlation (Cass and Gerhardt 1995, Hebert, Larson et al. 1999), indicative of normal dopamine transporter function, and reflects an \textit{in vivo} \( B_{\text{max}} \), which allows for the examination of unoccupied transporter sites (Cass, Zahniser et al. 1993, Cass and Gerhardt 1995, Hoffman and Gerhardt 1998). Assuming that the glutamate transporters obey similar principles as the dopamine transporter, the amplitude of glutamate per volume applied is a useful tool to investigate glutamate uptake dynamics. In that regard, the SHR had a lower amplitude/volume ratio compared to the WKY control, indicative of increased glutamate uptake activity. Additionally, the time to clear 80\% of the exogenous glutamate was shorter in the PFC of the SHR, though no differences in distinct sub-regions within the PFC were observed. These findings could be due to feedback from the increased activity of the NMDA and AMPA receptors in the PFC of the SHR (Russell 2001, Lehohla, Kellaway et al. 2004) and it’s possible that this feedback occurred prior to our uptake recordings to compensate for the increased sensitivity to glutamate. Furthermore, future experiments should explore the role of glial uptake in the PFC of these strains, perhaps by using the GLT-1 activator ceftriaxone. Finally, both the excitatory and inhibitory sub-populations of the metabotropic glutamate receptors (mGluRs) may be dysfunctional due to the increased vesicular glutamate storage and the potential for release, though the role of these receptors have yet to be investigated in these
strains. The results from the PFC in this study reveal that it is imperative to study the neurochemical link to ADHD-like behaviors in the SHR. Specifically, the glutamate-selective microelectrode array should likely be placed in the PFC region, though within which subregion is still open to interpretation. When the animal has recovered from the implantation, it should be required to perform some PFC-dependent cognitive task, such as sustained attention and attentional set shifting (Berridge, Shumsky et al. 2012), while glutamate measurements are taken.

In addition to the PFC, the striatum was included because of its involvement with movement processing (dorsal) and the reward circuitry (ventral), both features disrupted in ADHD (Balleine, Delgado et al. 2007, Dunnett and Lelos 2010, Salamone and Correa 2012). This study discovered that the SHR displayed increased evoked glutamate release compared to the WKY control in the striatum as a whole; however, no differences in tonic glutamate in any of the striatal sub-regions were found. The increase in glutamate release, much like in the PFC, is likely due to increased intracellular stores of vesicular glutamate. This is the first study to uncover direct glutamate signaling differences in the striatum between the SHR model of ADHD-C. Previously, our lab discovered decreased potassium-evoked release of dopamine in the dorsal striatum in the SHR compared to the WKY model of ADHD inattentive type (Sagvolden, Dasbanerjee et al. 2008), as well as faster uptake in the ventral striatum and nucleus accumbens compared to the WKY control strain (Miller, Pomerleau et al. 2012). It has been shown that increased stimulation of the D4 and D2 receptors resulted in decreased AMPA and NMDA receptor signaling, respectively (Kotecha, Oak et al. 2002, Yuen, Zhong et
al. 2010). Decreased activation of these receptors is likely causing hyperactivity of the AMPA and NMDA receptors, resulting in increased sensitivity to the release of glutamate in the striatum of the SHR. Moreover, an increase in the glutamate-stimulated release of dopamine in the SHR substantia nigra was discovered in vitro (Warton, Howells et al. 2009), which would ordinarily project to the more dorsal regions of the striatum, though how this translates to in vivo glutamate signaling in the striatum is not fully understood. Future research should examine more closely the role of the ionotropic receptors (AMPA and NMDA) as well as the mGluRs in this rodent model of ADHD.

Conclusions

Although current medications for ADHD appear to be largely successful on the surface, many problems still exist (Charach, Yeung et al. 2011). Stimulant medications, such as methylphenidate and amphetamines, do not successfully treat all people with ADHD and occasionally need to be stopped due to negative side effects (Cascade, Kalali et al. 2010). Adolescents and adults with ADHD notoriously do not like how stimulants make them feel and “change” their personality. This same population is also at risk for abusing their medication for a high or diverting/selling their medicine to others (Cowles 2009). In addition, the Multimodal Treatment Study of Children with ADHD, a 14-month long study following children diagnosed with ADHD, found that eight years post-study, when the participants with ADHD were reaching late adolescence, over 60% of participants had stopped taking any medication for their ADHD symptoms (Molina,
Hinshaw et al. 2009). These data suggest that adolescents with ADHD would benefit from medications targeting neurotransmitter systems other than catecholamines. Understanding the role of glutamate in ADHD and drugs that can modulate it properly without direct involvement of the catecholamine pathways should open a new mechanism of action for the treatment of ADHD.

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Chapter Three: Figures

Figure 3.1 Glutamate-Selective Measurements

**a** Glutamate oxidase enzyme was mixed into a BSA/gluteraldehyde matrix and coated onto the bottom pair of Pt recording sites. When glutamate came into contact with the enzyme, it was converted into α-ketoglutarate, which was blocked, and the reporter molecule H$_2$O$_2$. An applied potential of +0.7 V was used to oxidize the H$_2$O$_2$ into two electrons and measured by the FAST16mkIII recording system. The top pair of Pt recording sites only received the BSA/gluteraldehyde matrix, which was not selective for glutamate. The pipette was placed in the center of the 4 Pt sites, allowing for the even diffusion of locally applied solution across all 4 sites.

**b** An exclusion layer of mPD was electroplated onto all four Pt recording sites. This enabled the blockage of large molecule interferents, such as dopamine...
and ascorbic acid. c Signals were achieved on both the glutamate oxidase coated (glutamate selective) Pt recording sites (green) and the matrix-only (background selective) Pt recording sites (gray). When the background was subtracted from the glutamate signal, tonic levels of glutamate were observed (black). Only the traces from 1 glutamate oxidase coated and 1 matrix-only coated Pt sites are shown. d MEA placement into the striatum and e PFC. Cell bodies observed via Nissl stain (Miller, Pomerleau et al. 2014).
Figure 3.2 Glutamate Dynamics in the Prefrontal Cortex

a No differences in tonic glutamate levels between the SHR (red) and WKY (black) strains were observed in the PFC sub-regions. b The SHR strain was found to have significantly increased KCl-evoked glutamate release in the cingulate and infralimbic cortices of the PFC (*p<0.05), but not the prelimbic region, compared to the WKY control strain. c When examining the PFC as a whole, the SHR strain exhibited higher evoked glutamate release than the WKY control. d Comparisons of the representative KCl-evoked (black arrows) glutamate release events in each of the PFC sub-regions between strains. Mean ± SEM (Miller, Pomerleau et al. 2014).
Figure 3.3 Glutamate Dynamics in the Striatum

a No differences in tonic glutamate levels between the SHR (red) and WKY (black) strains were observed in the striatal sub-regions. b No differences in evoked glutamate release existed between strains in the striatal sub-regions, c though it was discovered that the SHR had increased evoked glutamate release in the striatum as a whole compared to the control WKY (**p<0.01). d Representative traces from the background subtracted signals showing three reproducible KCl-evoked (black arrows) glutamate peaks in the Str. Mean ± SEM (Miller, Pomerleau et al. 2014).
Figure 3.4 Glutamate Uptake

a An effect of strain in the PFC when the amplitude per nanoliter of exogenous glutamate applied was found, revealing the SHR model of ADHD had a smaller amplitude/volume. b No differences existed in the size of the glutamate signal per volume applied in the striatum. Mean ± SEM (Miller, Pomerleau et al. 2014).


## Table 3.1 S2 Microelectrode Array Specifications

<table>
<thead>
<tr>
<th></th>
<th>WKY Control</th>
<th>SHR Model of ADHD-C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Slope (nA/µM)</strong></td>
<td>0.0075 ± 0.00098</td>
<td>0.011 ± 0.00012</td>
</tr>
<tr>
<td><strong>Limit of Detection (µM)</strong></td>
<td>0.99 ± 0.23</td>
<td>0.44 ± 0.090</td>
</tr>
<tr>
<td><strong>Selectivity</strong></td>
<td>223 ± 108</td>
<td>373 ± 89</td>
</tr>
</tbody>
</table>

No significant differences were observed in the performance of the S2 microelectrode arrays used between strains. Mean ± SEM.
Table 3.2 Glutamate Uptake

<table>
<thead>
<tr>
<th></th>
<th>WKY Control</th>
<th>SHR Model of ADHD-C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Striatum</td>
<td>PFC</td>
</tr>
<tr>
<td><strong>Amplitude (µM)</strong></td>
<td>17 ± 7.0</td>
<td>24 ± 11</td>
</tr>
<tr>
<td><strong>Volume (nl)</strong></td>
<td>148 ± 60</td>
<td>161 ± 72</td>
</tr>
</tbody>
</table>

No differences were found in the size of the glutamate signals achieved following local application with exogenous glutamate. A significant effect of strain was found to exist in both the striatum and PFC when examining the volume required to achieve similar sized glutamate peaks, revealing that the SHR strain required significantly more glutamate (*p<0.05).
Chapter Four: Multi-Site Neurochemical Recordings: The Pathway to Understanding Systems Neuroscience in Freely-Moving Animals

Introduction

The aberrant regulation of neurotransmitters has been implicated in numerous disease states including schizophrenia, Parkinson’s disease, Alzheimer’s disease, mood disorders, attention-deficit/hyperactivity disorder (ADHD), and drug addiction (Brambilla, Perez et al. 2003, Moghaddam and Javitt 2012, Lesch, Merker et al. 2013, Paula-Lima, Brito-Moreira et al. 2013, Poletti and Bonuccelli 2013, Quintero 2013). Investigations into these molecules via neuronal signaling allows for the recording of a signal from an individual neuron (unit recordings) or the examination of an entire brain network (local field potentials) (McCracken and Grace 2013, Younts, Chevaleyre et al. 2013). While each method has its own advantages and disadvantages, they each provide a unique pathway to understanding neurotransmitter systems.

Glutamate, the major excitatory neurotransmitter in the CNS, is present throughout the brain (Danbolt 2001). One glutamate-rich region is the frontal cortex (FC). Deficits in this area have been found to cause poor impulse control, distractibility, hyperactivity, forgetfulness and poor organization and planning (Stuss and Levine 2002). The FC is organized into different cortical layers, including the cingulate cortex, prelimbic cortex, infralimbic cortex, and the dorsal peduncle. Each of these sub-regions within the FC has interneurons that can communicate with other FC regions, in addition to the afferent and efferent
connections to numerous brain structures (Dalley, Cardinal et al. 2004). To understand this complex cortical system and how glutamate dysfunction in these region facilitates behavior and disease, it is crucial to understand glutamate’s modulating system in this region with minimal disruption. The development of microelectrode arrays (MEAs) that can record neurotransmitters in multiple sub-regions along the dorsal-ventral axis simultaneously in discrete brain regions in freely-moving animals during an array of behavior tasks allows us the unique opportunity to examine neurotransmitter system dysfunction in an intact system.

Glutamate dysfunction has been implicated in ADHD in numerous studies, including recent clinical proton magnetic resonance spectroscopy studies which have discovered elevated levels of a marker for glutamate/glutamine in the frontal cortex (FC) of ADHD individuals compared to their normal peers (Moore, Biederman et al. 2006). The spontaneously hypertensive rat (SHR), a model of ADHD, has been discovered to have aberrant glutamate signaling in the FC in an anesthetized experimental paradigm (Miller, Pomerleau et al. 2014); however, no studies have focused on glutamate signaling in the freely-moving SHR while observing the ADHD-like behavior of the SHR. The purpose of this study was to use novel linear array, double sided MEA technology, which allows us to measure glutamate dynamics simultaneously within the cingulate, prelimbic, infralimbic and dorsal peduncle cortices of the FC. To our knowledge, this was the first time that glutamate was recorded simultaneously in multiple brain regions in the freely-moving SHR.
Materials and Methods

Animals

Male, 7 week old spontaneously hypertensive rats (SHR) were obtained from Charles River Laboratories (NCrl, Wilmington, MA). Male, 7 week old Wistar Kyoto (WKY) rats were obtained from Harlan Laboratories (NHsd, Indianapolis, IN). Animals were given access to food and water ad libitum and housed in a 12 hour light/dark cycle. All animals remained in a quarantine period for one week before experimental procedures to ensure no travel-related stress confounds were possible. During this time, each animal was handled daily to eliminate handling stress during the experiment.

Electrode Preparation

Double-sided paired-row 8 (DSPR8) microelectrode arrays (MEAs) were used for all described experiments and consisted of 4 Pt sites on the front and 4 separate Pt sites on the back, each measuring 15 x 333 µm arranged vertically (Figure 4.1A). A 100 µm space separated the tip of the MEA from the first Pt sites, 1 mm separated the 1st and 2nd pairs as well as the 2nd and 3rd pairs. Finally, 2 mm separated the 3rd and 4th pairs. This spacing scheme allowed for simultaneous electrochemical detection in multiple brain regions along the dorsal-ventral axis. To make the DSPR8s sensitive to glutamate, 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), 0.125% glutaraldehyde (Glut; Sigma-Aldrich), and 1% glutamate oxidase (GluOx; US biological, Salem, MA) were prepared and
coated onto the front 4 recording sites to allow for the conversion of glutamate to α-ketoglutarate and the reporter molecule, H₂O₂ (Burmeister and Gerhardt 2001). The 4 back recording sites, or the sentinel sites, only received a coating of the BSA/Glut matrix to allow for background measures and subtraction to obtain a self-referenced glutamate signal (Burmeister and Gerhardt 2001, Hinzman, Thomas et al. 2010).

After the DSPR8 MEAs were made sensitive for glutamate, they were built into an implantable head-cap (Figure 4.1B). Both ends of a piece of 1 inch long 30 American wire gauge varnished copper wire (RadioShack, Fort Worth, TX) were scraped (~0.25 cm) and fluxed (#186 Rosin flux type RMA, Kester). One end of the copper wire was soldered using eutectic solder and a low heat soldering iron (200°C) to a gold plated-socket (Ginder Scientific, Nepean, ON). The other end of the copper wire was soldered to holes within the stubbed paddle portion of the DSPR8. The 8 gold pins (one for each Pt recording site) were inserted into a 9 pin ABS plug (Ginder Scientific, Nepean, ON) and the copper wires were wrapped around the body of the ABS plug. A reference electrode, consisting of a 1 inch Teflon-coated silver wire (200 µm bare, 275 µm coated; A-M Systems, Carlsberg, WA), was scraped similar to the copper wires and soldered into a gold plated-socket and then placed into the ABS plug. The entire assembly was then finished with a heavy layer of marine-quality epoxy (Loctite Quick Set Epoxy, Home Depot, Atlanta, GA) and allowed to cure for at least 24 hours to ensure a water-proof product (Rutherford, Pomerleau et al. 2007, Hascup, Hascup et al. 2011). Finally, at least 2 days before the MEAs were surgically implanted, 1,3-phenylenediamine
(mPD, Acros, Fisher Scientific, Waltham, MA), a molecular size exclusion layer, was electroplated onto both the GluOx and sentinel sites to eliminate interferent molecules such as ascorbic acid and dopamine from reaching the Pt sites (Hinzman, Thomas et al. 2010).

**In Vitro Calibration**

High-speed amperometric recordings, displayed at a frequency of 40 Hz, were performed using the FAST16mkIII electrochemical recording system (Fast Analytical Sensing Technology, Quanteon, LLC, Nicholasville, KY). Immediately before in vivo implantation, an in vitro calibration, as described previously (Hinzman, Thomas et al. 2010), was used to determine selectivity (glutamate vs. ascorbic acid), limit of detection (in µM, S/N=3), and sensitivity (slope, pA/µM). The mean slope, limit of detection and selectivity for electrodes used are presented in Tables 4.1 and 4.2 for experiments 1 and 2, respectively. No significant differences in the performance of the MEAs used between strains were observed. An example in vitro calibration is represented in Figure 4.2. The 4 front recording sites, which are glutamate-sensitive, responded only to glutamate and the reporter molecule H2O2, whereas the 4 back sentinel recording sites only responded to the reporter molecule H2O2.

**Implantation Surgery**

After calibration and selection of MEAs, animals were prepared for surgery. Immediately before surgery, rats were given injections of carprofen (Rimadyl®,
Pfizer, Inc., New York City, NY; 10 mg/kg, s.c.) and 1 ml of 0.9% NaCl (s.c.).
Isoflurane (Isothesia, Butler Schein, Dublin, OH) was used as the anesthetic for all survival surgeries. Initial anesthesia was induced with 4% isoflurane, and once the rat was secured in the stereotaxic frame (David Kopf Instruments, Tujunga, California), the isoflurane level was reduced to 2.5%, but the levels of isoflurane ranged between 3% and 1% during surgery depending on the breathing style of each animal. Artificial tears (Rugby Laboratories, Inc., Duluth, GA) were applied to the rat's eyes. The rat's head was shaved, and 3 applications each of Povidone scrub (The Butler Co., Columbus, OH) and ethanol (70%) were used to disinfect the surgery site. A craniotomy was performed, and 5 holes were drilled: 1 for the MEA, 1 for the reference electrode, and 3 for anchoring skull screws (Amazon, part #B00FN0K02). After the skull screws were placed in the skull, dura was reflected and the MEA was stereotaxically placed in the FC (from bregma: AP +3.2 mm and ML -0.8 mm; from the surface of the brain: DV -5.5 mm) (Paxinos and Watson 2009). The reference electrode was positioned in its placement hole. Multiple applications of dental cement (Ortho Jet Powder and Jet Acrylic Liquid, Lang Dental Manufacturing Co., Wheeling, IL) were used to secure the microelectrode array assembly to the rat's skull. The animal was then monitored for the next two days and given daily injections of 10 mg/kg carprofen and 1 ml 0.9% NaCl (both administered s.c.). On the third day post-surgery, behavior and glutamate recordings began. Protocols for animal use were approved by the Institutional Animal Care and Use Committee, which is Association for Assessment and Accreditation of Laboratory Animal Care International approved. All procedures
were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Recording Apparatus

The recording apparatus consisted of a large wooden box with an open-field activity box (Omnitech Electronics Inc, Columbus, OH) inside and was used for all behavior measures. Additionally, a clear insert was used to allow the animals to have unrestricted movement. The recording head-stage consisted of a miniature connector with 9 connector pins (one connecting each of the 8 channels and one connecting the reference electrode). The connector pins lead to a novel grounded 8-channel low-noise potentiostat to reduce noise (Rat-hat; Quanteon). Connecting wires, encompassed by an insulator, lead to the low torque commutator at the top of the box. The recording assembly hung from the top center of the box from an 18 lead commutator (Airflyte, Bayonne, NJ) – see Figures 4.1C and 4.1D. This allowed the animal to freely move to all areas of the box (Rutherford, Pomerleau et al. 2007). Figure 4.2B represents an in vivo recording performed at 40 Hz.

Methylphenidate Administration

Methylphenidate (MPH) HCl [threo-methyl-α-phenyl-α-(2-piperidyl)acetate hydrochloride; M2892; Sigma-Aldrich, St. Louis, MO] was dissolved in 0.9% saline (2 mg/ml solution). Clinically, stimulants are administered orally and exert therapeutic actions at low doses that result in peak plasma concentrations within the range of 8 to 40 ng/ml (Swanson and Volkow 2002). Although MPH is
administered orally for the treatment of ADHD, the majority of studies that have examined the behavioral, neurochemical, and electrophysiological actions of moderate and high dose stimulants in rodents used intraperitoneal (i.p.) or subcutaneous (s.c.) administration (Kuczenski and Segal 2005). Moreover, s.c. administration has more rapid pharmacokinetics than oral administration (Wargin, Patrick et al. 1983), allowing changes in glutamate to occur while the animal is tethered, and is easiest to administer while the animal is tethered and in the open-field box.

Experiment 1: Daily Behavior and Glutamate Measurements

Behavior and glutamate recordings began on the third day post-MEA implant. Recordings were performed during the animal’s light cycle to reduce any effects of foraging (Jensen, Collier et al. 1983). Additionally, the majority of studies focused on SHR behavior have been completed during the light cycle (Kuczenski and Segal 2001, Mc Fie, Sterley et al. 2012) and studies have shown that MPH administration during the dark cycle failed to produce sensitization (Kuczenski and Segal 2002). The animal was removed from the home cage, tethered to the commutator, and placed into the behavior box. The total distance traveled was measured in 5 minute time bins over the course of the session using Oasis software. A single injection of MPH (2 mg/kg, s.c.) was administered 3 hours after the animal was tethered and a stable baseline was reached. 3 hours following MPH, the animal was removed from the recording apparatus and returned to its home cage. After each recording session, the recording chamber was cleaned with
Roccal disinfectant (Pfizer, Inc., New York City, NY). Additionally, the experimenter occasionally entered the room to monitor the animal and check to ensure that the tether was not tangled. This procedure was repeated for up to 2 weeks (Table 4.3).

**Experiment 2: Alternate Days of Behavior and Glutamate Measurements**

Behavior and glutamate recordings began on the third day post-MEA implant. The animal was removed from the home cage, tethered to the commutator, and placed into the behavior box. A saline injection (s.c.) was administered 1 hour into the start of the experiment, after a stable baseline had been achieved. 1 hour later, MPH was given (2 mg/kg, s.c.). Finally, the animal was left in the behavior box for another 3 hours for behavior and glutamate recording, for a total of 5 recording hours. The animal was then removed from the recording apparatus and returned to its home cage. On the second day, the same process, minus the tethering to the commutator, was performed. This was to reduce the stress on the area around the head-cap and to evaluate the effects of the tether on the longevity of the MEA implant as well as the animal’s behavior. This procedure was repeated for up to 2 weeks (Table 4.3).

**Histology**

Following either the head-cap being dislodged by the animal or completion of the 2 week drug administration, the animals were euthanized and brains were removed and processed (frozen) for histological evaluation of microelectrode recording tracks. No animals were excluded due to an error in electrode placement.
Data Analyses

Collected glutamate data were analyzed using a custom Matlab®-based analysis package. Tonic glutamate and behavior data were averaged to 5 minute bins. Behavior data was analyzed within subject using Prism (GraphPad 6.0), with significance set to p=0.05.

Results

Experiment 1: Simultaneous Glutamate Recordings in Multiple Sub-Regions within the Frontal Cortex: Daily Recordings

Figures 4.3 and 4.4 represent each recording region within the FC and the average tonic levels over the course of the experiment. Each point is a 5 minute tonic average, with the MPH (2 mg/kg) administered half-way through the recordings. The SHR has higher tonic glutamate in the cingulate cortex compared to the WKY (SHR, 50 µM; WKY, 10 µM), regardless of treatment day (Figures 4.3A, 4.4A). Tonic levels were similar between the SHR model of ADHD ad the WKY control strain in the prelimbic region (SHR, 15 µM; WKY, 15 µM; Figures 4.3B, 4.4B). The WKY has higher tonic glutamate levels in the infralimbic cortex compared to the SHR (SHR, 20 µM; WKY, 50 µM), but treatment with MPH may reduce those levels (Figures 4.3C, 4.4C). Finally, there appears to be similar tonic dynamics in the dorsal peduncle between strains regardless of MPH treatment (SHR, 20 µM; WKY, 20 µM; Figures 4.3D, 4.3D). Animal drop-out became an issue as the study progressed, likely due to excessive grooming around the head-cap,
weakening the connection to the skull and causing it to fall off. See Table 4.3 for a reference to animal drop-out.

**Experiment 1: Behavioral Recordings in the Spontaneously Hypertensive Rat**

As expected, chronic methylphenidate (MPH, 2 mg/kg, s.c.) treatment increased the total distance travelled in both the SHR (1 hour pre- vs post-MPH, $t_{(22)}=11.54, p<0.0001$) and WKY (1 hour pre- vs post-MPH, $t_{(22)}=12.31, p<0.0001$, Figure 4.5).

**Experiment 2: Simultaneous Glutamate Recordings in Multiple Sub-Regions within the Frontal Cortex: Alternating Days**

Figure 4.6 represents each recording region of the FC and the average tonic levels over the course of the second experiment during baseline and post-saline administration, while Figure 4.7 shows post-MPH administration. Figures 4.8 and 4.9 more accurately represent the acute and chronic effects of treatment on tonic glutamate. This study differed from experiment 1 because the animals were tethered on alternate days instead of daily. The animals received a saline injection, in addition to the MPH, to examine the effects of the injection itself. The SHR was found to have higher tonic glutamate in the cingulate cortex (SHR, 50 µM; WKY, 10 µM; Figures 4.6A, 4.7A, 4.8A, 4.9A) the prelimbic cortex (SHR, 100 µM; WKY, 50 µM; Figures 4.6B, 4.7B, 4.8B, 4.9B) and the infralimbic cortex (SHR, 50 µM; WKY, 25 µM; Figures 4.6C, 4.7C, 4.8C, 4.9C) compared to the WKY control strain during baseline, post-saline, and post-MPH, regardless of day. The WKY was
observed to have higher tonic glutamate in the dorsal peduncle compared to the SHR model of ADHD, regardless of day (SHR, 10 µM; WKY, 40 µM; Figures 4.6D, 4.7D, 4.8D, 4.9D). Moreover, there was no animal drop-out due to head-cap failings – see Table 4.3. Likely, this was because tethering caused stress to the vulnerable head-cap area in experiment 1. Excessive grooming to that area caused irritation and eventually the demise of the head-cap. By limiting the stress to that area, we were able to preserve the head-cap for the entire 2 week study during experiment 2.

Experiment 2: Behavioral Recordings in the Spontaneously Hypertensive Rat and the Effects of Chronic Methylphenidate Treatment

Experiment 1 demonstrated that tethering each day was stressful to the animal and resulted in loss of head-cap before the experiment was able to be completed. For experiment 2, the animals were tethered on alternate days to examine tether effects (Figure 4.10). The untethered SHR had significantly more activity than the tethered SHR during baseline, and more activity than both of the WKY groups ($F_{(3,84)}=11.59$, $p<0.0001$); however, all animals had similar baseline behavior following 11 days of behavior ($F_{(3,81)}=0.718$, $p=0.54$, Figure 4.11). Post-saline administration (s.c.), the SHR had significantly more activity than the WKY strain, regardless of tethering ($F_{(3,89)}=9.56$, $p<0.0001$) and all animals had similar behavior after the saline injection following 11 days of behavior ($F_{(3,92)}=2.49$, $p=0.065$, Figure 4.12). Finally, the SHR had significantly more activity than the WKY strain, regardless of tethering, after acute MPH (2 mg/kg, s.c.) administration.
The untethered SHR had significantly more behavioral activity than the tethered SHR after chronic MPH, and more activity than both of the WKY groups (F(3,292)=24.29, p<0.0001, Figure 4.13). Additionally, the tethered SHR displayed more activity than the tethered WKY post-chronic MPH.

Discussion

Glutamate has been implicated in a number of psychiatric diseases and disorders, including attention-deficit/hyperactivity disorder (ADHD). There is general agreement that ADHD involves weakened FC function (Arnsten 2009) and medications may work to strengthen the function of the FC and its connections to other brain regions (Barkley, Grodzinsky et al. 1992, Arnsten and Dudley 2005, Arnsten and Li 2005). The role of the rodent medial FC, consisting of the cingulate, prelimbic and infralimbic regions in humans, has been linked to behavior and attention, inhibiting inappropriate responses, and sustaining attention over long delays (Goldman-Rakic 1996, Robbins 1996). Deficits in the FC lead to poor impulse control, distractibility, hyperactivity, forgetfulness and poor organization and planning (Stuss and Levine 2002). The FC receives modulatory dopaminergic projections from the ventral tegmental area in the rodent brain. Additionally, the thalamus, amygdala and hippocampus each have glutamate connections to the FC, resulting in a region that is heavy with both dopaminergic and glutamatergic innervations. By understanding the dysfunction within these systems, it may be possible to better treat ADHD by more accurately targeting the specific dysfunction occurring.
Exploring glutamate dynamics in the FC is crucial to fully understanding the etiology of ADHD (Arnsten and Li 2005, Arnsten 2009). It has previously been shown in the anesthetized spontaneously hypertensive rat (SHR) model of ADHD using a depth profile to characterize each sub-region within the FC that these regions are not identical in their tonic measurements (Miller, Pomerleau et al. 2014) and as previously reported, these sub-regions are different from each other using the DSPR8 MEA. Additionally, in line with previous work from our laboratory, the levels of glutamate observed were within a range of 5 to 50 µM, suggesting that these novel DSPR8 MEAs are measuring levels of glutamate similar to MEAs used in the past (Rutherford, Pomerleau et al. 2007). This is the first time that glutamate recordings have been completed that allowed for multiple region exploration in a freely-moving rodent.

We observed that the total distance traveled increased in the SHR acutely after treatment with a clinically relevant dose of the stimulant methylphenidate (MPH) and in both the SHR and WKY control after chronic exposure to MPH. This increase in activity is consistent with behavioral sensitization in response to chronic MPH treatment (Crawford et al. 1998). The increase in activity assured that the dose of MPH was appropriate to cause behavior effects and would likely cause neurochemical effects as well.

Using the novel DSPR8 MEA, we discovered that the SHR model of ADHD displayed variable glutamate dynamics within the separate sub-regions compared to the WKY control strain. In the cingulate cortex, in both experiments, the SHR had increased levels of tonic glutamate regardless of day compared to the WKY control
strain. These data are supported by a previous study from our laboratory using anesthetized SHR and WKY rodents (Miller, Pomerleau et al. 2014). The SHR has been found to self-administer cocaine more readily than the WKY control strain following chronic exposure to MPH and it’s likely that the increase in tonic glutamate in the cingulate region may potentiate the response for self-administration in the SHR (Harvey, Sen et al. 2011). Moreover, the prelimbic, infralimbic and dorsal peduncle regions exhibited different results in the two separate experiments, though the levels were comparable to each other. The time to reach a stable baseline in the DSPR8 MEAs appeared to be similar to other glutamate-selective MEAs used in the past, taking roughly 30 minutes to be considered stable. To minimize the effects of baseline time on tonic measurements, it’s important to maximize the recording period in order to obtain the most accurate measurements possible. Recordings over 6 hours in the first experiment and 5 hours in the second were sufficient to obtain plenty of data during baseline and following MPH administration.

The initial experimental design was aimed at tonic and phasic glutamate recordings in multiple FC sub-regions during two weeks of drug treatment; however, this was not possible due to technical issues. The animals, with the exception of one control rodent, dislodged their implanted head-caps resulting in immediate euthanasia. We theorized that the stress of the tether that allows for the glutamate recordings increased grooming to the vulnerable area surrounding the head-cap. We then designed experiment 2 to allow for glutamate recordings on alternate days. The animals still received behavior recordings every day in the
open-field box because research has shown that spatial cues are important when administering a stimulant for the development of sensitization (Vezina and Leyton 2009). Experiment 2 was successful in eliminating animal drop-out, as each animal was able to complete 11 days of drug treatment before they were euthanized for histology purposes. Additionally, in experiment 2, we discovered differences in the behavior of tethered and untethered days during the beginning of the experiment, or acute recordings. However, by the time the animals had habituated to the task, these differences were no longer evident and the tethering did not significantly affect behavior in either strain. As a result of these studies, our laboratory is revising the head cap and preamplifier assembly (‘rat-hat’) to have a smaller, more robust and tolerable configuration for long-term chronic recordings.

These experiments sought to characterize glutamate signaling in the freely-moving SHR model of ADHD compared to the WKY control following exposure to a clinically relevant dose of MPH. Furthermore, these studies used a novel double-sided MEA, the DSPR8, to examine differences in multiple brain regions simultaneously. The results from these studies demonstrate that it’s possible to record from multiple brain regions simultaneously in freely-moving rodents, though more studies need to be completed on characterizing the DSPR8 in vivo.

Conclusions

These experiments sought to characterize glutamate signaling in the awake freely-moving SHR model of ADHD compared to the WKY control following exposure to a clinically relevant dose of MPH. Furthermore, these studies used a
novel double-sided MEA, the DSPR8, to examine differences in multiple brain regions simultaneously. The results from these studies demonstrate that it’s possible to record from multiple brain regions in awake, freely-moving rodents, though more studies need to be completed on characterizing the DSPR8 to attempt to eliminate front/back differences causing negative glutamate levels in vivo. Furthermore, the results from these studies demonstrate that acute treatment with a low-dose of MPH increases activity in the SHR model of ADHD, in accordance with previous studies. As expected, chronic MPH treatment caused increased behavior in both the SHR model of ADHD and the WKY control, representing behavioral sensitization to the stimulant. Moving forward, two weeks of stimulant treatment and glutamate measurements in the hyperactive SHR and the normal WKY control are possible with a chronic implant if the animal is tethered on alternate days.
Chapter Four: Figures

Figure 4.1 DSPR8 Microelectrode Array, Implanted DSPR8, and Behavior Set-Up

A) A 100 µm space separated the tip of the MEA from the first Pt sites, 1 mm separated the 1st and 2nd pairs as well as the 2nd and 3rd pairs. Finally, 2 mm separated the 3rd and 4th pairs. B) The entire assembly was finished with a heavy layer of marine-quality epoxy to ensure a water-proof product. C) The rodent tethered to the commutator inside of the behavior box. Inset: Placement of the DSPR8 Pt sites (red) in the rodent frontal cortex. D) The recording apparatus consisted of a large wooden box with an open-field activity box inside, as well as a clear insert, to allow the animals to have unrestricted movement.
Figure 4.2 DSPR8 In Vitro Calibration and In Vivo Trace

The DSPR8 was allowed to baseline for 20 minutes before an addition of 250 µM ascorbic acid (AA) was applied to determine any interfering signal. Next, three additions of 20 µM glutamate (Glu) were used to determine the limit of detection and slope of the MEA. 2 µM dopamine (DA) was used to evaluate its interferent effects. Finally, 8.8 µM of the reporter molecule, H₂O₂, was applied to ensure all sites were working. The front 4 Pt sites (black, red, green, and blue solid lines) all responded only to glutamate and H₂O₂, whereas the back sentinel sites (black, red, green, and blue dashed lines) only responded to the reporter molecule H₂O₂. As expected, no site responded to either interferent. B) In vivo, tonic glutamate recordings were able to be completed on each background-subtracted trace.
Figure 4.3 Daily Tonic Glutamate Levels in the Separate FC Sub-Regions of the SHR and WKY: Experiment 1

Multiple day recordings in the FC over the course of 6 hours, with the MPH (2 mg/kg) administered half-way (green dashed line). Darker colors, earlier in the experiment. Light colors, later in the experiment. Blues, WKY. Reds, SHR. Each point is a 5 minute tonic average. A) The SHR appears to have higher tonic glutamate in the cingulate cortex compared to the WKY, regardless of treatment day. B) Tonic levels appear to be similar in the prelimbic region. C) The WKY appears to have higher tonic glutamate levels in the Infralimbic cortex, but treatment with MPH may reduce those levels. D) There appears to be similar tonic dynamics in the dorsal peduncle between strains regardless of MPH treatment. WKY n=3, SHR n=4. Mean represented.
Multiple day recordings in the FC over the course of 6 hours, with the MPH (2 mg/kg) administered half-way (green dashed line). Blue, WKY. Red, SHR. Each point is a 5 minute tonic average. A) The SHR displays increased tonic glutamate following chronic MPH but not the WKY in the cingulate. B) MPH appears to elevate glutamate chronically in both the SHR and WKY prelimbic cortices. C) The WKY appears to have higher tonic glutamate levels in the infralimbic cortex, but treatment with MPH may reduce those levels. D) MPH appears to elevate glutamate chronically in both the SHR and WKY dorsal peduncle. WKY n=3, SHR n=4. Mean represented.
Figure 4.5 Chronic Effect of Methylphenidate on Total Distance Travelled between the SHR and WKY: Experiment 1

MPH (2 mg/kg, s.c., blue arrow) was found to increase the total distance traveled in both the SHR (red) and WKY (black) strains chronically, but to a much greater extent in the SHR. 5 minute average behavior bins. ****p<0.0001. SHR n=4, WKY n=3. Mean ± SEM.
Figure 4.6 Daily Tonic Glutamate Levels in the Separate FC Sub-Regions of the SHR and WKY: Experiment 2

Baseline and saline (s.c., black dashed line) effects on multiple day recordings in the prefrontal cortex. Dark colors, earlier in the experiment. Light colors, later in the experiment. Blues, WKY. Reds, SHR. Each point is a 5 minute tonic average.

A) The SHR appears to have higher tonic glutamate in the cingulate cortex compared to the WKY, regardless of MPH treatment day. B) The SHR appears to have higher tonic glutamate in the prelimbic cortex compared to the WKY. C) The SHR appears to have higher tonic glutamate in the infralimbic cortex compared to the WKY after continuous treatment with MPH. D) The WKY appears to have higher tonic glutamate in the dorsal peduncle compared to the SHR, regardless of day. WKY n=2, SHR n=2. Mean represented.
Figure 4.7 Daily Tonic Glutamate Levels Using the DSPR8 MEA Post-Methylphenidate Administration in the Separate FC Sub-Regions of the SHR and WKY: Experiment 2

Baseline and saline (s.c., black dashed line) effects on multiple day recordings in the prefrontal cortex. Dark colors, earlier in the experiment. Light colors, later in the experiment. Blues, WKY. Reds, SHR. Each point is a 5 minute tonic average.

A) The SHR appears to have higher tonic glutamate in the cingulate cortex compared to the WKY, regardless of MPH treatment day. B) The SHR appears to have higher tonic glutamate in the prelimbic cortex compared to the WKY. C) The SHR appears to have higher tonic glutamate in the infralimbic cortex compared to the WKY after continuous treatment with MPH. D) The WKY appears to have
higher tonic glutamate in the dorsal peduncle compared to the SHR, regardless of day. WKY n=2, SHR n=2. Mean represented.
Figure 4.8 Acute and Chronic Effects of Methylphenidate on Tonic Glutamate Levels Using the DSPR8 MEA in the Separate FC Sub-Regions of the SHR and WKY: Experiment 2

Baseline and saline (s.c., black dashed line) effects on multiple day recordings in the FC. Blue, WKY. Red, SHR. Each point is a 5 minute tonic average. A) The SHR appears to have higher tonic glutamate in the cingulate cortex compared to the WKY, regardless of MPH treatment day. B) The SHR appears to have higher tonic glutamate in the prelimbic cortex compared to the WKY. C) The SHR appears to have higher tonic glutamate in the infralimbic cortex compared to the WKY after chronic treatment with MPH. D) The WKY appears to have higher tonic glutamate in the dorsal peduncle compared to the SHR, regardless of day. WKY n=2, SHR n=2. Mean represented.
Figure 4.9 Acute and Chronic Effects of Methylphenidate on Tonic Glutamate Levels Using the DSPR8 MEA Post-Methylphenidate Administration in the Separate FC Sub-Regions of the SHR and WKY: Experiment 2

Baseline and saline (s.c., black dashed line) effects on multiple day recordings in the FC. Blue, WKY. Red, SHR. Each point is a 5 minute tonic average. A) The SHR appears to have higher tonic glutamate in the cingulate cortex compared to the WKY, regardless of MPH treatment day. B) The SHR appears to have higher tonic glutamate in the prelimbic cortex compared to the WKY before MPH, which reduces these levels. C) The SHR appears to have higher tonic glutamate in the infralimbic cortex compared to the WKY after chronic treatment with MPH. D) The WKY appears to have higher tonic glutamate in the dorsal peduncle compared to the SHR, regardless of day. WKY n=2, SHR n=2. Mean represented.
Figure 4.10 Tethering

A) During tethered days (glutamate and behavioral recordings) in experiment 2, the animal was attached to the commutator via a tethered cord that allowed the animal to reach all areas of the activity box. B) During untethered days (behavioral recording only), the animal was not attached to the tether.
Figure 4.11 Effects of Tethering on Baseline Behavior Activity

A) Baseline behavior activity on the first day of tethering (black, day 1) and untethering (color, day 2) in the WKY (green squares) and SHR (red circles). The untethered SHR had significantly more activity than the tethered SHR during baseline, and more activity than both of the WKY groups acutely. B) Baseline behavior during the chronic recordings. All animals had similar baseline behavior following 11 days of behavior. WKY n=2, SHR n=2, *p<0.05, ***p<0.001. Mean represented on left. Mean ± SEM on right.
Figure 4.12 Effects of Tethering on Post-Saline Behavior Activity

A) Behavior activity post-saline administration (s.c.) on the first day of tethering (black, day 1) and untethering (color, day 2) in the WKY (green squares) and SHR (red circles). The SHR had significantly more activity than the WKY strain, regardless of tethering. B) Behavior activity post-saline administration during the chronic recordings. All animals had similar behavior after the saline injection following 11 days of behavior. WKY n=2, SHR n=2, **p<0.01, ***p<0.001. Mean represented on left. Mean ± SEM on right.
Figure 4.13 Effects of Tethering on Post-Methylphenidate Behavior Activity

A) Behavior activity post-methylphenidate administration (MPH, 2 mg/kg, s.c.) on the first day of tethering (black, day 1) and untethering (color, day 2) in the WKY (green squares) and SHR (red circles). The SHR had significantly more activity than the WKY strain, regardless of tethering. B) Behavior post-MPH administration during the chronic recordings. The untethered SHR had significantly more behavior activity than the tethered SHR after chronic MPH, and more activity than both of the WKY groups. Additionally, the tethered SHR displayed more activity than the tethered WKY post-chronic MPH. WKY n=2, SHR n=2, ***p<0.001. Mean represented on left. Mean ± SEM on right.
Table 4.1 DSPR8 Performance in the WKY and SHR: Experiment 1

<table>
<thead>
<tr>
<th>Site</th>
<th>WKY Dorsal Peduncle</th>
<th>WKY Infalimbic</th>
<th>WKY Prelimbic</th>
<th>WKY Cingulate</th>
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<th>SHR Infalimbic</th>
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<td>0.23</td>
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<td>280</td>
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No significant differences were observed in the performance of the DSPR8 MEAs used between strains. Sites 1, 2, 3, and 4 were the front, glutamate-selective sites. Sites 5, 6, 7 and 8 were the back, background recording sites.
Table 4.2 DSPR8 Performance in the WKY and SHR: Experiment 2

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<td></td>
<td>Slope (pA/µM)</td>
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No significant differences were observed in the performance of the DSPR8 MEAs used between strains. Sites 1, 2, 3, and 4 were the front, glutamate-selective sites. Sites 5, 6, 7 and 8 were the back, background recording sites.
Table 4.3 Animal Drop-Out

<table>
<thead>
<tr>
<th>Animal</th>
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<tr>
<td>WKY 3</td>
<td>14 days post-implant</td>
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<td>SHR 1</td>
<td>8 days post-implant</td>
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<td>SHR 3</td>
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<tr>
<td>SHR 4</td>
<td>10 days post-implant</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal</th>
<th>Dislodged Head-Cap &amp; Euthanized</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY A</td>
<td>Lasted full 2 weeks</td>
</tr>
<tr>
<td>WKY B</td>
<td>Lasted full 2 weeks</td>
</tr>
<tr>
<td>SHR A</td>
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</tr>
<tr>
<td>SHR B</td>
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Introduction

Attention-deficit/hyperactivity disorder (ADHD) is estimated to affect 6% of children worldwide and 50% of those diagnosed will continue to suffer into adulthood (Kessler, Adler et al. 2006, Polanczyk, de Lima et al. 2007, Biederman, Petty et al. 2011). Adolescents and adults diagnosed with ADHD are more likely to suffer from mood and anxiety disorders and to develop drug addiction (Biederman, Petty et al. 2010). Methylphenidate (MPH), a dopamine reuptake inhibitor, is one of the most commonly prescribed treatments for ADHD (De Sousa and Kalra 2012). Animal studies have established the reinforcing effects of MPH (Collins, Weeks et al. 1984) and MPH has also been found to serve as a reinforcer for children diagnosed with ADHD (Fredericks and Kollins 2004). Debate has been ongoing whether stimulant treatment for ADHD makes one more susceptible to drug abuse later in life. A meta-analysis of the literature concluded that when stimulant treatment for ADHD is initiated during childhood, there is a decreased risk for developing a substance use disorder during adulthood (Wilens, Faraone et al. 2003). Conversely, when the initiation of stimulant treatment for ADHD occurs during adolescence, there is an increased risk of developing a substance use disorder (Kollins, MacDonald et al. 2001, Kollins 2008). In rodents, exposure to chronic administration of a clinically relevant dose of MPH during adolescence...
caused the animals to self-administer cocaine more quickly as adults (Harvey, Sen et al. 2011). Furthermore, MPH is not always successful and occasionally needs to be stopped due to negative side effects (Cascade, Kalali et al. 2010). Together, these results suggest that adolescents and adults with ADHD would benefit from novel medications for the treatment of ADHD, likely drugs that don’t specifically target the catecholaminergic systems.

Glutamate dysfunction has been implicated in ADHD in an array of studies, including both preclinical and clinical studies. Our laboratory has previously shown that the spontaneously hypertensive rat (SHR), a rodent model of ADHD, has been discovered to have aberrant glutamate signaling (Russell 2001, Lehohla, Kellaway et al. 2004, Warton, Howells et al. 2009) in the frontal cortex compared to its progenitor strain, the Wistar Kyoto (WKY) (Miller, Pomerleau et al. 2014). Better understanding the role of glutamate in ADHD and discovering drugs that can modulate it properly without direct involvement of the catecholamine pathways should reveal novel treatment options for ADHD. The purpose of this study was to examine the role of tonic and phasic glutamate signaling in the prelimbic cortex of the freely-moving SHR during an open-field behavior task to measure hyperactivity, a key symptom of ADHD. Additionally, the effects of MPH versus saline were examined in an effort to discover any exertion of the dopaminergic-altering stimulant over the glutamatergic system in order to better characterize novel pharmacotherapies for the treatment of ADHD.
Materials and Methods

Animals

Male, 7 week old spontaneously hypertensive rats (SHR) were obtained from Charles River Laboratories (NCrl, Wilmington, MA). Male, 7 week old Wistar Kyoto (WKY) rats were obtained from Harlan Laboratories (NHsd, Indianapolis, IN). Animals were given access to food and water *ad libitum* and housed in a 12 hour light/dark cycle. All animals remained in a quarantine period for 1 week before experimental procedures to ensure no travel-related stress confounds were possible. During this time, each animal was handled daily to eliminate handling stress during the experiment.

Electrode Preparation

Glutamate-oxidase (GluOx) coated microelectrode arrays (MEAs) consisting of 4 Pt sites measuring 15 x 333 µm arranged vertically in dual pairs (S2 conformation) were used as previously described (Burmeister, Davis et al. 2013, Miller, Pomerleau et al. 2014). After the S2 MEAs were made sensitive for glutamate using 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), 0.125% glutaraldehyde (Glut; Sigma-Aldrich), and 1% GluOx (US biological, Salem, MA), they were built into an implantable head-cap. Both ends of a piece of 1 inch long 30 American wire gauge varnished copper wire (Radioshack, Fort Worth, TX) were scraped (~0.25 cm) and fluxed (#186 Rosin flux type RMA, Kester). One end of the copper wire was soldered using eutectic solder and a low heat soldering iron (200°C) to a gold plated-socket (Ginder Scientific, Nepean,
ON). The other end of the copper wire was soldered to holes within the stubbed paddle portion of the MEA. The 4 gold pins (one for each Pt recording site) were inserted into a 9 pin ABS plug (Ginder Scientific, Nepean, ON) and the copper wires were wrapped around the body of the ABS plug. A reference electrode, consisting of a 1 inch Teflon-coated silver wire (200 µm bare, 275 µm coated; A-M Systems, Carlsberg, WA), was scraped similar to the copper wires and soldered into a gold plated-socket and then placed into the ABS plug. The entire assembly was then finished with a heavy layer of marine-quality epoxy (Loctite Quick Set Epoxy, Home Depot, Atlanta, GA) and allowed to cure for at least 24 hours to ensure a water-proof product (Rutherford, Pomerleau et al. 2007, Hascup, Hascup et al. 2011). Finally, at least 2 days before the MEAs were surgically implanted, 1,3–phenylenediamine (mPD, Acros, Fisher Scientific, Waltham, MA), a molecular size exclusion layer, was electroplated onto both the GluOx and sentinel sites to eliminate interferent molecules such as ascorbic acid and dopamine from reaching the Pt sites (Hinzman, Thomas et al. 2010).

In Vitro Calibration

High-speed amperometric recordings, displayed at a frequency of 10 Hz, were performed using the FAST16mkIII electrochemical recording system (Fast Analytical Sensing Technology, Quanteon, LLC, Nicholasville, KY). Immediately before in vivo implantation, an in vitro calibration, as described previously (Hinzman, Thomas et al. 2010), was used to determine selectivity (glutamate vs. ascorbic acid), limit of detection (in µM, S/N=3), and sensitivity (slope, pA/µM). The
mean slope, limit of detection and selectivity for electrodes used are presented in Tables 5.1. No significant differences in the performance of the MEAs used between strains were observed.

**Implantation Surgery**

After calibration and selection of MEAs, animals were prepared for surgery. Immediately before surgery, rats were given injections of carprofen (Rimadyl®, Pfizer, Inc., New York City, NY; 10 mg/kg, s.c.) and 1 ml of 0.9% NaCl (s.c.). Isoflurane (Isothesia, Butler Schein, Dublin, OH) was used as the anesthetic for all survival surgeries. Initial anesthesia was induced with 4% isoflurane, and once the rat was secured in the stereotaxic frame (David Kopf Instruments, Tujunga, California), the isoflurane level was reduced to 2.5%, but the levels of isoflurane ranged between 3% and 1% during surgery dependent upon the breathing style of each animal. 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 Povidone scrub (The Butler Co., Columbus, OH) and ethanol (70%) were used to disinfect the surgery site. A craniotomy was performed, and 5 holes were drilled: 1 for the MEA, 1 for the reference electrode, and 3 for anchoring skull screws (Amazon, part #B00FN0K02). After the skull screws were placed in the skull, dura was reflected and the MEA was stereotaxically placed in the prelimbic PFC (from bregma: AP +3.2 mm and ML -0.8 mm; from the surface of the brain: DV -3.5 mm) (Paxinos and Watson 2009). The reference electrode was then positioned into its placement hole, sliding along the underside of the skull. Multiple applications of dental cement
(Ortho Jet Powder and Jet Acrylic Liquid, Lang Dental Manufacturing Co., Wheeling, IL) were used to secure the MEA assembly to the rat’s skull. The animal was then monitored for the next 2 days and given daily injections of 10 mg/kg carprofen and 1 ml 0.9% NaCl (both administered s.c.). On the third day post-surgery, behavior and glutamate recordings began. Protocols for animal use were approved by the Institutional Animal Care and Use Committee, which is Association for Assessment and Accreditation of Laboratory Animal Care International approved. All procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

**Recording Apparatus**

The recording apparatus consisted of a large wooden box with an open-field activity box (Omnitech Electronics Inc., Columbus, OH) inside and was used for all behavioral measures. Additionally, a clear insert was used to allow the animals to have unrestricted movement. The recording head-stage consisted of a miniature connector with 5 connector pins (one connecting each of the 4 channels and 1 connecting the reference electrode). The recording assembly hung from the top center of the box from an 18 lead commutator (Plastics1, Roanoke, VA). This allowed the animal to freely move to all areas of the box (Rutherford, Pomerleau et al. 2007).
Methylphenidate Administration

Methylphenidate (MPH) HCl [threo-methyl-α-phenyl-α-(2-piperidyl)acetate hydrochloride; M2892; Sigma-Aldrich, St. Louis, MO] was dissolved in 0.9% saline (2 mg/ml solution). Clinically, stimulants are administered orally and exert therapeutic actions at low doses that result in peak plasma concentrations within the range of 8 to 40 ng/ml (Swanson and Volkow 2002). Although MPH is administered orally for the treatment of ADHD, the majority of studies that have examined the behavioral, neurochemical, and electrophysiological actions of moderate and high dose stimulants in rodents used intraperitoneal (i.p.) or subcutaneous (s.c.) administration (Kuczenski and Segal 2005). Moreover, s.c. administration has more rapid pharmacokinetics compared to oral administration (Wargin, Patrick et al. 1983). This method of administration was easiest to administer while the animal was tethered and in the open-field box.

Behavior and Amperometry

Behavior and glutamate recordings began on the third day post-MEA implant. Recordings occurred during the animal’s light cycle to reduce any effects of foraging (Jensen, Collier et al. 1983). The majority of studies focused on SHR behavior have been completed during the light cycle (Kuczenski and Segal 2001, Mc Fie, Sterley et al. 2012) and studies have shown that MPH administration during the dark cycle failed to produce sensitization (Kuczenski and Segal 2002). The animal was removed from the home cage, tethered to the commutator, and placed into the behavior box. The total distance traveled was measured in 1 minute.
time bins over the course of the session using Oasis software. A saline injection (s.c.) was administered at an hour after the start of the experiment. MPH was given (2 mg/kg, s.c.) 1 hour later. The animal was then left in the behavior box for another 3 hours for behavior and glutamate recording (Figure 5.1A). During the final 10 minutes, the voltage applied was changed to +0.2 V vs. the Ag/AgCl reference electrode, which removed the oxidation of $\text{H}_2\text{O}_2$ on the MEA Pt recording sites and thereby further confirming peroxide-mediated measurements. The animal was then removed from the recording apparatus and returned to the home cage. On the second day, the same process, minus tethering to the commutator, was performed. Previous experiments from our laboratory have shown that this process reduces the stress on the area around the head-cap and ensures 2 weeks of drug treatment was possible. Alternate days of tethering then continued for a total of 11 days (Figure 5.1B). After each recording session, the recording chamber was cleaned with Roccal disinfectant (Pfizer, Inc., New York City, NY). Additionally, the experimenter occasionally entered the room to monitor the animal and check to ensure that the tether was not tangled.

**Histology**

Following completion of the 2 week drug administration, the animals were euthanized and brains were removed and processed (frozen) for histological evaluation of microelectrode recording tracks. 2 animals knocked-off their head-caps on the last day of recordings and the histology was inconclusive.
Data Analyses

Collected glutamate data were analyzed using a custom Matlab®-based analysis package. Figure 5.1 explains the experiment timeline and how data was picked for analyses. Data for each animal was organized into 3 blocks to examine drug effects: 1) acute, day 1 or day 3; 2) intermediate, day 5 or day 7; and 3) chronic, day 9 or day 11. Next, the chosen full glutamate data file had a boxcar filter, or moving average, of 20 points applied before being separated into 4 separate 10 minute files per animal per block: 1) saline (90 minutes after the start of experiment), 2) treatment 1 (30 minutes post-treatment or 150 minutes after the start of experiment), 3) treatment 2 (80 minutes post-treatment or 200 minutes after the start of experiment), and 4) treatment 3 (130 minutes post-treatment or 250 minutes after start of experiment). This created 12 separate data files for each animal. Each file was analyzed for the following:

- Phasic peak criteria (for the subtracted signal): event offset: -2; threshold points: 5; peak S/N threshold: 1.5; minimum event spacing: 10; $T_{\text{rise}}$ min: 0.25
- Phasic peak exclusion criteria (for the subtracted signal): control $R^2$: >0.80; $T_{\text{rise}}$: <0.25; peak S/N: <2.0; peak area: negative; peak amplitude: negative or >5.0
- Tonic glutamate criterion (for the subtracted signal): first positive phasic peak baseline was used as tonic glutamate
- Tonic glutamate exclusion criteria (for the subtracted signal): negative or below the in vitro calibrated LOD
Using the above criteria, 8 out of 30 total animals were excluded from intermediate treatment tonic glutamate levels. 10 out of 30 animals were excluded from chronic treatment tonic glutamate levels. The primary reason for exclusion of these animals was because of negative tonic levels, which occurred when the sentinel recording sites were measuring a higher current than the glutamate-selective recording sites, though phasic signaling was still observed. All data were analyzed using two-way repeated measures ANOVAs followed by Bonferroni corrections for multiple comparisons (GraphPad Prism 6.0). For all data, significance was set to $p=0.05$.

**Results**

*Weight Change Post-Implant*

A significant interaction between treatment (saline or MPH) and day (days 1-13) ($F_{13,91}=3.254$, $p=0.005$), a significant effect of treatment ($F_{1,7}=7.169$, $p=0.03$), and a significant effect of day ($F_{13,91}=37.60$, $p<0.0001$) existed in the WKY control group on the amount of weight gained following MEA implantation. The WKY strain treated with MPH was found to weigh significantly more than the WKY group treated with saline alone during the last four days of the experiment (Figure 5.2A). When examining the weights of the SHR group, no interaction was found between treatment (saline or MPH) and day (days 1-13), which is consistent with clinical data (Vincent, Varley et al. 1990). A two-way repeated measures ANOVA found a significant effect of day ($F_{13,78}=28.91$, $p<0.0001$), which was expected as the rodents were gaining weight as they were reaching young adulthood (Figure 5.2B).
For acute behavioral measurements, a significant interaction between strain/treatment (WKY saline, WKY MPH, SHR saline, SHR MPH) and time (300 1 minute bins) \(F_{897,5083}=1.717, p<0.0001\) as well as significant effects of strain/treatment \(F_{3,17}=23.57, p<0.0001\) and time \(F_{299,5083}=4.471, p<0.0001\) were found. Baseline activity during the first day of behavioral recordings was similar between the SHR and WKY, consistent with previous reports that the hyperactive behaviors of the SHR model of ADHD are absent in a novel environment (Johansen, Sagvolden et al. 2005, Howells, Bindewald et al. 2009). Administration of saline (s.c.) also did not produce any significant differences between strains. The difference in activity started 30 minutes following treatment. The SHR treated with MPH had significantly higher total distance traveled than the SHR treated with saline. Additionally, the SHR MPH group had greater activity than the WKY MPH group. These differences lasted for about 90 minutes before the groups again displayed similar activity (Figure 5.3). These results are consistent with previous studies reporting that a low, clinically relevant dose of MPH increased activity in the SHR (Yang, Amini et al. 2003, Amini, Yang et al. 2004).

For intermediate behavioral measurements, a significant interaction between strain/treatment and time \(F_{897,5083}=3.110, p<0.0001\) as well as significant effects of strain/treatment \(F_{3,17}=9.153, p=0.0008\) and time \(F_{299,5083}=4.628, p<0.0001\) were found. Baseline activity was found to be similar.
between the SHR and WKY and saline (s.c.) did not produce any significant differences between strains. The difference in activity started 30 minutes after treatment, similar to the acute recording. The SHR treated with MPH had significantly higher total distance traveled than the SHR treated with saline. Additionally, the SHR: MPH group displayed increased activity compared to the WKY: MPH group (Figure 5.4). These differences lasted longer than they did for the acute recordings. It took nearly 120 minutes before the groups again displayed similar activity.

For chronic behavioral measurements, a significant interaction between strain/treatment and time (F\text{897,5083}=1.805, p<0.0001) as well as significant effects of strain/treatment (F\text{3,17}=7.293, p=0.003) and time (F\text{299,5083}=3.637, p<0.0001) were found. The SHR, regardless of treatment, displayed increased activity during the first 5 minutes of the experiment compared to both groups of the WKY, consistent with reports that the hyperactive behaviors of the SHR model of ADHD become evident after multiple exposures to an environment (Knardahl and Sagvolden 1981, Sagvolden, Pettersen et al. 1993, Howells, Bindewald et al. 2009). Administration of saline (s.c.) did not produce any significant differences between strains. The difference in activity started 30 minutes after treatment, when the SHR treated with MPH had significantly more total distance traveled than the SHR treated with saline (Figure 5.5). These differences lasted for 120 minutes before the groups again displayed similar activity. The SHR MPH group also had increased activity than the WKY treated with
MPH group, beginning 90 minutes post-MPH and this effect only lasted for approximately 30 minutes.

*Examination of Tonic and Phasic Glutamate Signaling*

Figure 5.6A shows an example of tonic and phasic glutamate signaling over the course of the 5 hour experiment in one animal. Three major types of phasic signaling are apparent: 1) fast single peaks (5-10 seconds long), 2) fast multi-peaks (5-10 seconds long), and 3) slower phasic signaling (1-3 minutes long) which consisted of many fast multi- and single-peaks (Figure 5.6B). Figure 5.7 shows the differences in tonic and phasic glutamate between strains and treatments. Additionally, Figure 5.7 shows the change in applied potential from +0.7 V to +0.2 V vs. Ag/AgCl reference electrode, which is below the H₂O₂ oxidation potential.

*Intermediate Methylphenidate Treatment Increases Tonic Glutamate Levels in the SHR but not the WKY*

A significant effect of time (30 minutes after saline, and 30, 80, and 130 minutes after treatment) (F₃,₃₆=2.866, p<0.05) and an effect of strain/treatment (WKY saline, WKY MPH, SHR saline, and SHR MPH) (F₃,₁₂=3.710, p<0.05) after intermediate treatment with MPH/saline were found, though no interaction between the two existed. This data demonstrates that the SHR treated with MPH (6.2 ± 1.4 µM) for 1 week has higher tonic levels than the SHR treated with saline alone (1.3 ± 1.4 µM). Additionally, the WKY saline group (6.4 ± 1.4 µM) had higher
tonic glutamate than the SHR saline group. The WKY MPH group (5.0 ± 1.3 µM) was not significantly different than the other groups (Figure 5.8A).

Chronic Methylphenidate Treatment Increases Tonic Glutamate Levels in both the SHR and WKY

Following chronic treatment, a significant interaction between time and strain/treatment \( (F_{9,39}=2.433, \ p<0.05) \) and an effect of strain/treatment \( (F_{3,13}=4.009, \ p<0.05) \) were found. These data demonstrate that in both the SHR model of ADHD and the WKY control, chronic MPH treatment with a clinically relevant dose (SHR MPH 9.9 ± 2.1 µM, WKY MPH 10 ± 1.9 µM) increases tonic levels of glutamate compared to animals treated with saline alone (SHR saline 3.2 ± 2.1 µM, WKY saline 3.3 ± 2.1 µM) (Figure 5.8B).

Neither Intermediate nor Chronic Methylphenidate Treatment Changes the Frequency of Phasic Glutamate Events

Figures 5.9A and 5.9B reveal that the number of phasic events per 10 minute sample did not differ between strain, treatment, nor time.

Chronic, but not Intermediate, Methylphenidate Treatment Reduces Phasic Glutamate Signaling in the SHR

After intermediate drug treatment, no differences in the amplitude of phasic glutamate signals were found. Average (± SEM) phasic amplitudes during
intermediate treatment were as follows: WKY saline 0.40 ± 0.033 µM, WKY MPH 0.21 ± 0.018 µM, SHR saline 0.13 ± 0.016 µM, SHR MPH 0.22 ± 0.044 µM.

Following chronic treatment, a significant effect of strain/treatment (WKY saline, WKY MPH, SHR saline, SHR MPH) on phasic glutamate amplitudes (F_{3,22}=3.316, \ p<0.05) was discovered. Also, there was a trend towards an effect of time (F_{3,66}=2.413, \ p=0.07). These data demonstrate that in the SHR, a significant difference existed between the MPH (SHR MPH 0.11 ± 0.0056 µM) and saline treated animals (SHR saline 0.53 ± 0.0072 µM). No effects of chronic MPH treatment on phasic glutamate amplitude were found in the WKY groups (WKY saline 0.13 ± 0.011 µM, WKY MPH 0.22 ± 0.011 µM) (Figure 5.10B).

*Types of Phasic Signaling Changes with Methylphenidate Treatment*

Following intermediate treatment, (30 minutes after treatment), a significant interaction between strain/treatment (WKY saline, WKY MPH, SHR saline, SHR MPH) and type of event (rapid single, rapid multi, slow) was discovered (F_{6,52}=7.311, \ p<0.0001). Significant effects of strain/treatment (F_{3,26}=10.55, \ p=0.001) and type of event (F_{2,52}=46.71, \ p<0.0001) were also found to exist. The WKY saline group had less of the rapid single peaks compared to the WKY MPH and SHR saline groups. The SHR MPH had significantly more of the rapid multi peaks compared to the WKY MPH and significantly more rapid multi and slow phasic events compared to the SHR saline group (Figure 5.11A).

Following chronic treatment (30 minutes after treatment), a significant interaction between strain/treatment and type of event was found (F_{6,52}=8.120,
A significant effect of type of event (F_{2,52}=97.79, p<0.0001) was also found. The SHR saline group was observed to have more of the rapid single peaks compared to the WKY saline and the SHR MPH groups. The SHR MPH had more slow phasic events than the SHR saline group (Figure 5.11B). An example of the three major types of phasic signaling are represented in Figure 5.11C-E.

**Discussion**

Methylphenidate (MPH) administration has been found to increase dopamine levels by blocking the dopamine transporter (DAT) (Kuczenski and Segal 1997, Gerasimov, Franceschi et al. 2000, Gerasimov, Franceschi et al. 2000, Kuczenski and Segal 2001, Volkow, Wang et al. 2001, Huff and Davies 2002, Marsteller, Gerasimov et al. 2002), as well as the norepinephrine transporter (NET) in the PFC (Gatley, Pan et al. 1996). The increase in dopamine is thought to be responsible for increased motor activity (Rebec 2006). Studies of signaling interactions between the dopaminergic and glutamatergic systems demonstrate that stimulation of D2 dopamine receptors is involved in the inhibition of the NMDA receptor, weakening the excitatory response to those neurons (Kotecha, Oak et al. 2002). Likewise, activation of D4 receptors decreased AMPA receptors at the synapse (Yuen, Liu et al. 2010). Because of these links between the dopamine and glutamate systems, we sought to use a known dopaminergic-altering drug to examine the effects on the glutamatergic system. Moreover, the behavioral effects of a clinically relevant dose of MPH treatment are predictable and have been well established in the spontaneously hypertensive rat (SHR) model of ADHD (Wultz,
Sagvolden et al. 1990, Sagvolden, Metzger et al. 1992, van den Bergh, Bloemarts et al. 2006, Barron, Yang et al. 2009). We observed that total distance traveled increased in the SHR acutely and chronically following MPH. This increase in activity is consistent with behavioral sensitization in response to chronic MPH treatment (Crawford, McDougall et al. 1998), though the WKY control failed to reach sensitization levels. No challenge dose was used in this study, as has been done in previous work exploring MPH sensitization (Gaytan, al-Rahim et al. 1997, Crawford, McDougall et al. 1998, McDougall, Collins et al. 1999) and studies have shown that chronic administration alone isn’t enough to produce behavioral sensitization following low-dose MPH treatment (Suzuki, Shindo et al. 2007) and explains the lack of increased behavior in the control WKY strain following 11 treatment days with MPH. However, the increase in activity of the hyperactive SHR assured that the low, clinically relevant dose of MPH used was appropriate to cause significant behavior effects in the ADHD model but not the control.

The link between alterations in glutamate neurotransmission and substances of abuse have been well established in such drugs as cocaine (Pulvirenti, Swerdlow et al. 1989, Pulvirenti, Swerdlow et al. 1991, Witkin 1993), amphetamine (Pulvirenti, Swerdlow et al. 1989), and methamphetamine (Witkin 1993). Given the role of glutamate on the addictive properties of these drugs, we sought to investigate the role of glutamate signaling following chronic MPH administration. The SHR model of ADHD self-administers cocaine more readily than the progenitor control strain, the WKY (Harvey, Sen et al. 2011). The results from the present study demonstrate that in the SHR model of ADHD, overall tonic
glutamate levels were higher in the MPH treated group than the saline treated SHRs. These observed increases of glutamate following MPH administration may then potentiate the responses for drug abuse later in life. Supporting this theory, the SHR was discovered to be more likely to self-administer cocaine as an adult than controls following chronic treatment with a clinically relevant dose of MPH (administered p.o.) at a young age (Harvey, Sen et al. 2011). Interestingly, SHRs chronically administered the non-stimulant atomoxetine as adolescents were no more likely to self-administer cocaine in adulthood than controls (Somkuwar, Jordan et al. 2013), demonstrating that non-stimulant medications represent an important alternative for treatment of ADHD. Future studies should examine if increases in glutamate are observed following chronic treatment with atomoxetine within the SHR.

In addition to increases in tonic glutamate following MPH in the SHR, we found that chronic MPH exposure decreased the amplitude of phasic glutamate signaling in the SHR but not the WKY control. The increase of tonic glutamate in the SHR model of ADHD following chronic MPH treatment does not cause an increase in the phasic release of glutamate, but instead decreases the phasic signaling amplitude. It is possible that the rise of tonic levels following MPH causes activation of more auto-receptors, attempting to compensate for the higher glutamate concentrations (Lin, Wang et al. 2000, Johnson, Niswender et al. 2011, Tang, Liu et al. 2013). Research has been focused on group II and III inhibitory metabotropic glutamate auto-receptors, linking them as possible therapeutic targets for anxiety (Swanson, Bures et al. 2005, Riaza Bermudo-Soriano, Perez-
Rodriguez et al. 2012), schizophrenia (Hashimoto, Malchow et al. 2013), depression (Chaki, Ago et al. 2013), alcoholism (Holmes, Spanagel et al. 2013), and cocaine addiction (Li, Xi et al. 2013). The results from this study suggest that ADHD should now be included as a disorder that may benefit from targeting these auto-receptors, potentially as a co-treatment with stimulants if they are deemed effective. A recent study examining attention deficits caused by nicotine exposure during adolescence found reduced group II mGluRs in the PFC and an mGluR2 agonist was found to restore attention upon maturation into adulthood (Counotte, Goriounova et al. 2011). These results suggest that targeting the inhibitory auto-receptor to restore basal glutamate signaling could therapeutically benefit individuals with ADHD. Future research should examine the effects of mGluR group II and III agonists, such as LY354740 and related compounds (Hascup, Hascup et al. 2012, Koltunowska, Gibula-Bruzda et al. 2013, Wang, Li et al. 2013), and antagonists, such as LY341495 (Diraddo, Miller et al. 2014) on glutamate signaling as well as the effects on the ADHD-like behaviors of the SHR.

Along with the discovery that chronic MPH treatment reduced the amplitude of phasic glutamate signals in the SHR model of ADHD, different types of phasic signaling were observed and found to be changed following treatment with MPH. To our knowledge, this is the first time that phasic glutamate signaling has been able to be quantified as having different characteristics, though this is not the first time that phasic signaling has been observed in awake, freely-moving animals (Hascup, Hascup et al. 2011, Wassum, Tolosa et al. 2012). The SHR had more rapid single peaks, lasting about 10 seconds before returning to baseline, than the
WKY control after both intermediate and chronic saline (or vehicle) alone administration, suggesting that the SHR model of ADHD has higher activity of phasic glutamate events. In the SHR, intermediate MPH treatment was found to increase the rapid multi-peaks compared to the SHR saline group, whereas chronic MPH actually decreased these fast multi-peak phasic events in the SHR. Additionally, after chronic treatment with MPH, the SHR had more of the slow phasic type signals, which could take as long as 3 minutes before returning to a stable baseline. These signals seem to contribute more towards the changes in tonic glutamate than the other phasic signals and may explain why the SHR had increased tonic levels following MPH treatment. These data all suggest that targeting tonic and phasic glutamate may be a useful approach for novel ADHD treatments.

**Conclusions**

These studies raise the exciting possibility that targeting group II and III mGluRs may provide a novel approach to treatment of ADHD without the adverse effects associated with currently available drugs. More importantly, these findings illuminate a pathway targeting glutamate dysfunction for investigating novel therapies for the treatment of ADHD. Future studies should examine the effects of current FDA-approved pharmacotherapies that are known to target the glutamate system, such as memantine and ceftriaxone, on the SHR model of ADHD. Using these medications, it may be possible to better treat ADHD in adolescents and adults without the negative side effects and abuse liability of current medications.

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Chapter Five: Figures

Figure 5.1 Experiment Timeline

A) Each day started with the animal being placed into the behavior box to allow for one hour of baseline activity. A saline injection (s.c.) was used to determine any injection effects on behavior or glutamate. Glutamate levels were then assessed 30 minutes post-saline administration. Next, either saline or MPH was given (s.c.) and three glutamate time-points were sampled. The experiment ended three hours after the 2nd injection. B) Experimental paradigm. The S2 MEA was implanted on day 0 and the animal was allowed two days to recover from surgery. On day three, glutamate and behavior recordings began. Behavior was completed each day in the open-field box, whereas glutamate recordings were performed on alternate days.
A) The WKY strain treated with MPH weighed significantly more than the WKY group treated with saline alone during the last 4 days of the experiment, suggesting that MPH may have a protective effect in these animals. B) In the SHR, no differences were observed between the MPH and saline treated animals. *p<0.05. Mean ± SEM.
Baseline locomotion of the SHR and WKY strains was similar, as was the activity following saline (s.c.) administration (orange arrow). Beginning 30 minutes after treatment (green arrow, 2 mg/kg s.c.), the SHR treated with MPH for the first time (pink circles) had significantly increased total distance traveled than the SHR saline group (open black circles, \( p < 0.05 \)) as well as the WKY treated with MPH group (blue triangles, \( x p < 0.05 \)). This effect lasted approximately 90 minutes. 1 minute bins. Mean ± SEM.

Figure 5.3 Total Distance Traveled: Acute Effect of Methylphenidate
Figure 5.4 Total Distance Traveled: Intermediate Effect of Methylphenidate

Baseline locomotion of the SHR and WKY strains was similar, as was the activity following saline (s.c.) administration (orange arrow). Beginning 30 minutes after treatment (green arrow, 2 mg/kg s.c.), the SHR treated with MPH (pink circles) had increased total distance traveled than the SHR saline group (open black circles, \( *p<0.05 \)) as well as the WKY treated with MPH group (blue triangles, \( *p<0.05 \)). This effect lasted approximately 120 minutes. 1 minute bins. Mean ± SEM.
Figure 5.5 Total Distance Traveled: Chronic Effect of Methylphenidate

The SHR: MPH (pink) and SHR: saline (black) groups had increased locomotion during the first 5 minutes of baseline compared to both WKY groups (\(^{*}p<0.05\) vs. WKY: MPH; \(^{*}p<0.05\) vs. WKY: saline). The activity following saline (s.c.) administration (orange arrow) was similar between all groups. Beginning 30 minutes after treatment (green arrow, 2 mg/kg s.c.), the SHR treated with MPH (pink circles) had increased total distance traveled than the SHR saline group (open black circles, \(^{*}p<0.05\)). The SHR: MPH group also had increased activity than the WKY treated with MPH group (blue triangles, \(^{*}p<0.05\), beginning 90 minutes post-MPH and this effect only lasted for approximately 30 minutes. 1 minute bins. Mean ± SEM.
Figure 5.6 Glutamate Signal, Sentinel, and Behavior Traces

A) An example of tonic and phasic glutamate signaling over the course of the 5 hour experiment in one animal. The blue trace is the background subtracted glutamate signal which results in tonic glutamate levels. The black trace is the sentinel, or background noise and is included only to compare the signaling to the blue, thus the y-axis location is unimportant. Examining the blue trace compared to the black trace allows for the inspection of changes in phasic glutamate during the experiment. Total distance traveled (in 1 minute bins) is shown in the gray bars on the bottom. Orange arrow, saline (s.c.). Green arrow, methylphenidate (2
mg/kg, s.c.). The blue dashed box is represented in B. B) An example of tonic and phasic glutamate signaling post-MPH administration for 10 minutes. Comparing the blue trace to the black trace allows for changes in phasic glutamate during the experiment to be apparent. 3 major types of phasic signaling are apparent here: 1) the pink oval arrow - fast multi-peaks, 2) the black pointed arrow - fast single peaks, and 3) the black half-circles – slow phasic signaling.
Figure 5.7 Strain Differences in Tonic and Phasic Glutamate Levels

After baseline of the S2 MEA, strain differences were observed in tonic and phasic glutamate levels. Only the raw, background-subtracted glutamate signal is represented here. Black, WKY saline. Blue, WKY MPH. Grey, SHR saline. Pink, SHR MPH. Orange arrow, saline (s.c.). Green arrow, methylphenidate (2 mg/kg, s.c.). Purple arrow, applied voltage changed to +0.2 V vs. Ag/AgCl reference electrode.
Figure 5.8 Intermediate and Chronic Effects of MPH (2 mg/kg) Administration on Tonic Glutamate Levels

A) The SHR MPH group (pink circles) had higher tonic glutamate than the SHR saline (open circles), and suggests that MPH causes the tonic levels of the SHR to be more similar to that of the WKY control (blue triangles), which had similar tonic levels when treated with MPH or saline (black triangles). Additionally, the WKY saline group had higher tonic glutamate than SHR saline. B) Following chronic treatment, both the SHR model of ADHD and the WKY control had increased tonic glutamate than animals treated with saline only. *p<0.05. Mean ± SEM.
Figure 5.9 Intermediate and Chronic Effects of MPH (2 mg/kg) Administration on Phasic Glutamate Signaling Frequency

The number of phasic events per 10 minute sample did not differ between strain, treatment, nor time.
Figure 5.10 Intermediate and Chronic Effects of MPH (2 mg/kg) Administration on Phasic Glutamate Signaling Amplitudes

A) No differences in the size of the phasic glutamate signals were apparent following intermediate treatment with MPH. B) Following chronic treatment, the SHR model of ADHD treated with MPH had lower phasic glutamate amplitudes compared to SHRs treated with saline. *$p<0.05$. Mean ± SEM.
A) Following intermediate treatment, during the first 10 minute sample period (30 minutes post-treatment), it was discovered that the WKY saline group had less rapid single peaks than the WKY MPH and SHR saline groups. The SHR MPH
had more rapid multi peaks compared to the WKY MPH and more rapid multi and slow phasic events than the SHR saline group. B) Following chronic treatment, during the first 10 minute sample period (30 minutes post-treatment), the SHR saline group had more rapid single peaks than the WKY saline and the SHR MPH groups. The SHR MPH had more slow phasic events than the SHR saline group. An example of the three major types of phasic signaling: C) fast single peaks, D) fast multi-peaks, and E) slow phasic signaling. *p<0.05. Mean ± SEM.
### Chapter Five: Tables

Table 5.1 Freely-Moving S2 Microelectrode Array Specifications

<table>
<thead>
<tr>
<th></th>
<th>WKY: Saline</th>
<th>WKY: MPH</th>
<th>SHR: Saline</th>
<th>SHR: MPH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Slope (pA/µM)</strong></td>
<td>7.9 ± 0.70</td>
<td>6.8 ± 0.72</td>
<td>9.0 ± 1.2</td>
<td>8.1 ± 1.4</td>
</tr>
<tr>
<td><strong>Limit of Detection (µM)</strong></td>
<td>0.38 ± 0.046</td>
<td>0.75 ± 0.21</td>
<td>0.23 ± 0.055</td>
<td>0.43 ± 0.15</td>
</tr>
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</table>

No significant differences were observed in the performance of the S2 microelectrode arrays used between strains. Mean ± SEM.
Chapter Six: Final Conclusions

The spontaneously hypertensive rat (SHR) model of attention-deficit/hyperactivity disorder (ADHD) grants us the ability to investigate neurotransmitter system regulation \textit{in vivo} in a diseased state. In this dissertation, we observed dysfunction in multiple brain regions as well as multiple neurotransmitters within these regions in the SHR brain. The ultimate goal of the described studies was to discover novel ways to treat ADHD with minimal side-effects and clear long-term safety and efficacy. Aberrant dopamine (DA) regulation has long been thought to play a large role in the pathophysiology of ADHD, though studies examining DA in the SHR have been equivocal. In the first set of experiments, we demonstrated that the SHR model of ADHD combined type (ADHD-C) and the Wistar Kyoto (WKY) progenitor strain from Charles River Laboratories (WKY/NCrl), which has been suggested as a model of the ADHD predominantly inattentive type, have distinct differences in the regulation of DA in the striatum and nucleus accumbens core compared to the inbred WKY from Harlan Laboratories (WKY/NHsd) and the outbred Sprague Dawley controls. Further data presented in Chapter Two provides evidence for dopaminergic system dysfunction in the frontal cortex (FC). These results suggest that increased surface expression of DA transporters (DAT) may explain the more rapid uptake of DA in the striatum, nucleus accumbens core and FC of these rodent models of ADHD. While these studies are compelling, there is an increasing number of studies that suggest ADHD is not limited to purely DA dysfunction (Carrey, MacMaster et al. 2002, Lehohla, Kellaway et al. 2004, Jensen, Rinholm et al. 2009,
Ludolph, Udvardi et al. 2010, Dramsdahl, Ersland et al. 2011, Hammerness, Biederman et al. 2012). Because of this, we deemed it necessary to study other neurotransmitter systems in the SHR model of ADHD.

Recent proton magnetic resonance spectroscopy imaging studies in humans with ADHD are beginning to implicate a dysfunctional glutamate system in ADHD (Moore, Biederman et al. 2006, Moore, Frazier et al. 2007, Hammerness, Biederman et al. 2012) and it is becoming clear that glutamate may be playing some role in the pathophysiology of ADHD. Glutamate regulation in the SHR may be impaired, creating the possibility that targeting dopamine-glutamate interactions in this model of ADHD may prove useful with novel therapeutics. Understanding the role of glutamate in ADHD and drugs that can modulate it should open a new mechanism of action for the treatment of ADHD. Chapter Three characterized glutamate signaling in the striatum, nucleus accumbens core, and FC of the SHR model of ADHD and compared the results to the WKY control. Interestingly, we discovered that the SHR exhibited altered glutamate regulation compared to the WKY control; however, the most impaired region was the FC of the SHR, displaying aberrant glutamate release and uptake, suggesting that fully characterizing this glutamate-rich region could provide us the opportunity to discover new therapeutics to better treat ADHD. These results suggest that the glutamatergic system in the FC of the SHR model of ADHD is hyperfunctional and that targeting glutamate in the FC could lead to the development of novel therapeutics for the treatment of ADHD.
Recording glutamate in anesthetized animals provided information that the SHR had aberrant glutamate signaling compared to the control WKY strain and we wanted to extend these findings into the awake, freely-moving rodent to more accurately define these abnormalities and examine the effects of a common ADHD treatment on glutamate. Chapter Four was able to translate the findings of Chapter Three into the awake, freely-moving rodent. Microelectrode array technology, however improved over technologies used in the past, was limited in that it could only record glutamate dynamics in a vertical height of 333 µm. However, a novel probe became available that allowed for recordings in four separate regions along the dorsal-ventral axis in the brain each having a vertical height of 333 µm, broadening the recording areas available. This new technology was chronically implanted into the FC of the 8 week old SHR and WKY, allowing for glutamate recordings in real-time within the cingulate, prelimbic, infralimbic and dorsal peduncle cortices simultaneously. We were able to record both tonic and phasic glutamate in the SHR and WKY, as well as investigate the effects of chronic methylphenidate treatment using a clinically relevant dose. The results from these studies demonstrate that it is possible to record from multiple brain regions in awake, freely-moving rodents, though more studies need to be completed on characterizing the novel DSPR8 microelectrode array in vivo. As the project developed, we realized that the DSPR8 microelectrode arrays used were in need of improvements, so in order to complete the study to fully characterize glutamate levels in the awake rodent, we employed the glutamate-selective microelectrode array used in Chapter Three, thus limiting us to recording in the prelimbic region.
only. However restrictive this was, the consistency and reliability of the microelectrode array used in the rest of the studies is superior to other commercially available *in vivo* neurochemistry recording devices.

Chapter Five examined tonic and phasic glutamate changes in prelimbic cortex within the FC of the SHR model of ADHD and the WKY control strain. A clinically relevant dose of methylphenidate (2 mg/kg) or the vehicle saline were given for 11 days to evaluate acute, intermediate, and chronic effects on glutamate signaling, while simultaneously recording behavior, including locomotion. We chose locomotion in the open-field behavior task because it has previously been shown that the SHR displays hyperactive behavior in this task, similar to that seen in humans with ADHD. Additionally, we wanted a task that had been characterized using both the SHR and methylphenidate, as we were trying to find novel changes in glutamate. Future directions should include a less characterized but still relevant to ADHD behavior task, such as delayed discounting, while exploring changes in glutamate and the effects of pharmacology. Using the open-field task, we observed that methylphenidate significantly increased the total distance traveled in the SHR acutely but not the WKY. Chronically, only the SHR displayed increased total distance traveled after methylphenidate compared to the saline control group. Moreover, intermediate treatment (5 or 7 days) with methylphenidate increased tonic glutamate levels in the SHR only, whereas chronic methylphenidate (9 or 11 days) increased tonic glutamate in both the SHR and WKY strains. Surprisingly, chronic methylphenidate treatment, at a clinically relevant dose, decreased the amplitude, or size, of the phasic glutamate signals compared to the saline treated
SHR group. This effect did not exist in the control WKY group treated with methylphenidate. These data suggest that methylphenidate, a DA reuptake inhibitor, has an effect on the glutamatergic system via normal dopamine-glutamate interactions, possibly via auto-receptors on both the presynaptic and postsynaptic neurons.

Overall, the SHR model of ADHD was characterized for both dopamine and glutamate dysfunction in multiple brain regions implicated in the etiology of ADHD. Although there is no perfect animal mode of ADHD, the results from the described experiments together demonstrate that the SHR model of ADHD is a great resource for understanding and studying dysfunction of ADHD-like behaviors. In this study, the stimulant medication methylphenidate increased locomotion in the SHR and these were in parallel to differences on the glutamate system. This finding gives us a pathway for investigating novel therapies for the treatment of ADHD targeting glutamate.

Future studies should examine the effects of current FDA-approved pharmacotherapies that are known to target the glutamatergic system, such as memantine and ceftriaxone, on the glutamate and dopamine systems of the SHR. Additionally, the results from Chapter Five suggest that targeting the metabotropic glutamate receptors may be a useful therapeutic avenue for ADHD. These studies raise the exciting possibility that targeting group II and III mGluRs may provide a novel approach to treatment of ADHD without the adverse effects associated with currently available drugs. Because of these studies in the SHR rodent model of
ADHD, it may be possible to better treat ADHD in adolescents and adults without the negative side effects and abuse liability of current medications.
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2011-2014 Competitive NIH T32 Training Grant 5T32AG000242-13

2011 Outstanding Poster Award, Spring Neuroscience Day, Bluegrass Society for Neuroscience (BGSfN), University of Kentucky, Lexington, Kentucky

2008-2009 Competitive Academic Fellowship: Graduate School Academic Year Fellowship. University of Kentucky, Lexington, Kentucky
2008 Competitive Research Grant: Psi Chi Undergraduate Research Grant. Mount Union College, Alliance, Ohio

2007 Competitive Summer Research Opportunities Program: Donna M. Martin, M.D., Ph.D., University of Michigan, Ann Arbor, Michigan

2007 Inducted into Psi Chi, National Psychology Honor Society. Mount Union College, Alliance, Ohio

2004-2008 Annual merit based scholarship: Academic Merit Award. Mount Union College, Alliance, Ohio

2004, 2006, 2007 Dean’s List (three semesters), Mount Union College, Alliance, OH

Published Book Chapter

Published Review Article

Published Peer-Reviewed Manuscripts

Posters and Published Abstracts

   - *Presented at the Society for Neuroscience Conference in San Diego, California, November 2013.*
   - *Presented at the American College of Neuropsychopharmacology in Hollywood, Florida, December 2013.*

2. **Erin M. Miller**, George Quintero, Francois Pomerleau, Peter Huettl, Greg A. Gerhardt & Paul E.A. Glaser. The Effects of Chronic Methylphenidate Treatment on Tonic and Phasic Glutamate in Prefrontal Cortical Regions of a Rodent Model of ADHD.
   - *Presented at the Bluegrass Society for Neuroscience Conference at the University of Kentucky, in Lexington, Kentucky, April 2013.*

3. **Erin M. Miller**, Francois Pomerleau, Peter Huettl, Greg A. Gerhardt & Paul E.A. Glaser. Direct second-by-second measures of tonic and phasic glutamate in the cingulate, prelimbic and infralimbic cortices in an awake rodent model of ADHD.
   - *Presented at the American College of Neuropsychopharmacology Conference in Hollywood, Florida, December 2012.*

4. **Erin M. Miller**, Francois Pomerleau, Peter Huettl, Greg A. Gerhardt & Paul E.A. Glaser. Dysfunctional glutamate regulation in rodent models of ADHD.
   - *Presented at the Bluegrass Society for Neuroscience Conference at the University of Kentucky, in Lexington, Kentucky, March 2012.*

5. **Erin M. Miller**, Francois Pomerleau, Peter Huettl, Greg A. Gerhardt & Paul E.A. Glaser. Increased glutamate levels exist in the striatum and prefrontal cortex of the spontaneously hypertensive and Wistar Kyoto rodent models of ADHD.
   - *Presented at the Society for Neuroscience Conference in Washington DC, November 2011.*
   - *Presented at the American College of Neuropsychopharmacology Conference in Waikoloa Beach, Hawaii, December 2011.*

   - *Presented at the Bluegrass Society for Neuroscience Conference at the University of Kentucky, in Lexington, Kentucky, March 2011.*
   - *Presented at the Center for Clinical and Translational Science Conference in Lexington, Kentucky, April 2011.*

   - *Presented at the Society for Neuroscience Conference in San Diego, California, November 2010.*

   - *Presented at the Center for Clinical and Translational Science Conference in Lexington, Kentucky, March 2010.*

10. Paul E.A. Glaser, **Erin M. Morgart**, & Greg A. Gerhardt. High-Speed Voltammetric Studies of Dopamine Regulation in the Spontaneously Hypertensive Rat Model of ADHD.

11. Brian L. Woodside, Darren M. Miller, Tim J. Teyler, Jon P. Niemi, & **Erin M. Morgart**. Effects of Environment on Spatial Learning and Memory Following Blockade of NMDA and VDCC Receptors.
   - *Presented at the Society for Neuroscience Conference in Washington DC, November 2008.*

   - *Presented at the Faculty for Undergraduate Neuroscience (FUN) session of the Society for Neuroscience Conference in San Diego, California, November 2007.*
- Presented at the Faculty for Undergraduate Neuroscience (FUN) session of the Society for Neuroscience Conference in San Diego, California, November 2007.

- Presented at the Committee on Institutional Cooperation (CIC) Conference at Purdue University, July 2007.


Oral Presentations


