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Effects of Amphetamine on Radial Arm Maze Performance in the SHR Model of ADHD versus Age/strain Matched Controls

Kristin Lampley
Connecticut College, kmlampley@gmail.com

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Kristin Lampley

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Abstract

Stimulants have been shown to have a variety of effects on different measures of learning and memory. In general low doses of stimulants like nicotine and caffeine enhance memory acquisition and recall, while high doses can significantly impair performance. Amphetamine, in the form of Adderall, is widely prescribed to improve attention and reduce hyperactivity, which promotes learning in children diagnosed with attention deficit disorder (ADHD). The radial arm maze (RAM) is a test of spatial learning that allows for the tracking of short and long-term memory errors. In the current study, we employed the RAM to examine the effects of amphetamine on spatial learning performance in spontaneously hypertensive rat (SHR), an accepted model of ADHD, animals and age/strain matched controls. An immunohisotchemical analysis of cFos expression in the hippocampus was also utilized to evaluate the effect of amphetamine of neural activation in both groups of animals. Amphetamine (1.0 mg/kg, ip) administered during training significantly increased maze completion time and increased short-term and long-term error rates. The results of this study suggest that chronic amphetamine treatments have hindering effects on learning and memory in control rats. Unlike their age/strain matched controls, amphetamine did not enhance or inhibit radial arm maze performance of SHR animals. This suggests that the neurophysiological mechanisms mediating learning and memory may be different in SHR animals than in humans with ADHD. Therefore, additional studies are needed to evaluate the validity of the SHR model of ADHD.

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Introduction

1.1 Attention-deficit Hyperactivity Disorder: An Overview

George Still gave the first description of ADHD in the Coombs lecture of 1902. Still described ADHD as an "abnormal defect in moral control in children (Still, 1905)." And he defined moral control as "the control in action of conformity with the idea of the good of all… (that) can only exist when there is a cognitive relationship to the environment (Still, 1905)." He believed that moral control required a "consciousness" that informed the capacity of "inhibitory volition (Still, 1905)." Attention-deficit Hyperactivity Disorder (ADHD) is the most common childhood-onset psychiatric disorder affecting an estimated 4.1 percent of adults in a given year. Comparatively to other psychiatric disorders, 3.1 percent of adults have generalized anxiety disorder, 3.5 percent of the adult population has post-traumatic stress disorder, 1.0 percent of people have OCD, and 1.1 percent of the adults have schizophrenia, and 9.5 percent of the U.S. adult population has a mood disorder in a given year (Merikangas, 2010). Therefore, ADHD is a very prevalent disorder among adults and children. The resulting behavioral hindrances often lead to impaired social and academic functioning. ADHD results in a high degree of inattention with or without hyperactivity and impulsivity behavior (Sharp, McQuillin, & Gurling, 2009).

ADHD is a multifactorial disorder with complex etiology and strong genetic underpinnings. Most cases of ADHD are comorbid with other behavioral syndromes or neurodevelopmental patterns, and it is very unusual to find it in pure form (Hill, 2005). There does not seem to be any one cause, and ADHD appears to be more a case of risk factors and the major risk factor being genetics. Also, several predictors of the disorder that have been identified which include family history of ADHD, psychiatric comorbidity and psychosocial adversity (Biederman et al, 1995). The risk of having ADHD increases as the number of risk factors

increase. ADHD is worldwide and possibly affects five to ten percent of children and four percent of adults (Faraone et al., 2003). It is a heterogeneous disorder; however, ADHD is four times more common in boys than girls which is similar to other neurodevelopmental disorders such as autism, specific reading disorders, and specific developmental disorders of speech and language (Hill, 2005). The disorder affects not only the afflicted but puts huge financial strain on society, stress to families, and adverse academic and vocational outcomes (Biederman, 2005). According to Faraone et al. (2005), the inattentive component of ADHD is manifested by daydreaming, distractibility, and difficulty focusing on a single task for a prolonged period, whereas the hyperactivity component is expressed as fidgeting, excessive talking, and restlessness (Biederman, 2005). Those that have ADHD symptoms are more prone to accidents, mood disorders, anxiety, delinquency, have strained interpersonal relationships, and are disruptive to those around them through interruptions and inappropriate behavior. Childhood ADHD is especially worrisome because it predicts adult alcoholism and substance dependence (Clure et al, 1999). Throughout development the overt symptoms of hyperactivity tend to wane in the early part of life but the more covert symptoms of inattention seem to persist over time (Biederman, et al 1996).

1.2 Diagnostic Criteria

The diagnosis in the 1930s for a child that exhibited hyperkinesis, impulsivity, learning disability, and short attention span was described as minimal brain damage and then as minimal brain dysfunction since his or her symptoms were very much like patients that had central nervous system injuries. In the 1950s the label was adjusted to hyperactive child syndrome and then the Diagnostic Statistical Manual of Mental Disorders (DSM)-II adjusted the label to

hyperkinetic reaction of childhood (Spencer et al., 2007). The labels presented focused on motoric hyperactivity and overt impulsivity as the traits of the ADHD. In the DSM-III the focus shifted to inattention as one of the main traits of ADHD. In addition, the DSM-III addressed the varying degrees of the presentation of the disorder at different ages, as well as, recognizing that there is a form of ADHD that persists past childhood. The DSM-IV now defines ADHD as having three subtypes including predominately inattentive, predominately hyperactive-impulsive, and a combined subtype. For a diagnosis of a specific subtype the child must exhibit at least six or greater of the nine symptoms in each subtype classification. There are four additional criteria that include age of onset by seven, ADHD-specific adaptive impairments, and separation from other existing conditions (Spencer et al, 2007). The diagnostic assessment of children includes interviewing the parent, individual assessment of the child, interviewing the child, and information from the school. The parents are interviewed to obtain developmental history as well as information about the child's current behavior. The child is assessed by giving them complex cognitive tasks, such as reading, model-building, and writing and drawing. As the child performs the assigned task the clinician observes the child's behaviors. This step is especially important so that the clinician can make sure that the child's symptoms are not from some other type of impairment, such as deafness. The child is interviewed to understand the child's school environment, peer group functioning, self-esteem and illicit habits (Hill, 2005). The child's teachers are asked to fill out the Connor's teacher rating scale, which includes ratings of oppositional, cognitive problems and inattention, hyperactivity, anxious, perfectionism, and social problems, and teachers are asked to add their comments on the child's behaviors, academic achievement, and social relationships. All the information collected is assessed to see if ADHD is likely, to see if there are comorbid conditions, to create a baseline of functioning, to

determine the fitness of the child for medication, to assess the families attitude toward the child, and to estimate the child's intellectual functioning (Hill, 2005). A thorough evaluation of all the previous assessments allows for the professional diagnosis of ADHD.

1.3 Causes of ADHD

Although the cause of ADHD is not entirely known, many studies have suggested that the disorder has genetic components. In order to determine if a disorder is heritable, genetic linkage studies are performed. Linkage analysis is a method used for identifying the presence of susceptibility genes for a genetic disorder within regions of chromosome. The presence of linkage is usually expressed as log10 of the odds (lod) score for the probability of observing marker alleles co-segregating with the disorder in multiple affected families compared to the null hypothesis of no co-segregation or 50% recombination between marker alleles and the disease. There is also another approach to determining if a disorder is genetic called the sib pair linkage method. In this method marker alleles are observed in affected siblings to test the hypothesis that they are shared in affected cases more than by chance. In heterogeneous disorders, such as ADHD, small or medium sized families have the power to detect different genetic subtypes, as defined by positive lods at linkage hotspots, whereas the affected sib pair linkage method has little or no power to detect heterogeneity. In order to identify which gene is involved in ADHD once a linked region has been confirmed using the lod or sib pair method, the evolutionarily determined patterns of allelic association between disease mutations and closely linked genetic markers are used to narrow down the actual susceptibility gene. Methods combining tests of linkage and allelic association to do both linkage and fine mapping in the same family sample have been popular because they are immune to population stratification effects (Sharp,

McQuillin, & Gurling, 2009). Many studies have been performed using these techniques to identify genes associated with ADHD. The first systematic genome-wide linkage scan for loci influencing ADHD identified chromosomal regions on 2q24, 5p12, 10q26, 12p13, 12q23, and 16p as possibly harboring genes increasing susceptibility of ADHD (Fisher et al., 2002). A second genome-wide linkage study confirmed linkage to chromosome 16p13 (Smalley et al., 2002). A recent meta-analysis of seven ADHD linkage studies (Arcos-Burgos et al., 2004; Asherson et al., 2008; Bakker et al., 2003; Faraone et al., 2008; Hebebrand et al., 2006; Ogdie et al., 2003; Romanos et al., 2008) confirms genome-wide significance for a region on chromosome 16, between 16q21–16q24, that is linked to ADHD (Zhou et al., 2008). Using this compellation of multiple studies, 10 regions on chromosomes 5, 6, 7, 8, 9, 15, 16, and 17 with evidence for linkage with ADHD were identified (Zhou et al., 2008). Thus, many studies have provided evidence supporting the idea that ADHD has genetic components.

Since ADHD is believed to be inherited there have been studies done with twins to determine heritability. In these studies, which varied in their methods and definitions of ADHD, the mean heritability for ADHD was found to be 77% (Biederman, 2005). Other studies point to the possibility that maternal smoking and alcohol exposure during pregnancy, low birth weight and psychological adversity are independent, non-maternally heritable risk factors (Spencer et al., 2007). ADHD is thought to be caused by catecholamine dysfunction (Gillberg, 2001) in brain regions involved with attention and reward, including the nucleus accumbens (Podet et al., 2010; Russell, 2000) and striatum (Krause et al., 2003), so a current theory of the cause of ADHD posits that risk factors, combined with genetic linkages, could lead to catecholamine dysfunction.

Since catecholamine dysfunction is hypothesized to play a role in ADHD, molecular genetic studies have been performed to look at gene mutations potentially leading to ADHD.

Cook et al.'s study (1995) demonstrated a relationship between ADHD and the 480-bp allele of the dopamine transporter gene using a family-based association study. Research regarding the DRD4 gene has also yielded significant results suggesting its involvement in ADHD. LaHoste et al. (1996) observed that the DRD4 7-repeat allele has functional implications that are relevant for ADHD. This variant of DRD4 mediates a blunted response to dopamine, and the distribution of DRD4 mRNA in the brain suggests it plays a role in cognitive and emotional functioning. The study also involved a population study and found higher rates of the 7-repeat allele among ADHD children compared to control children carefully matched for ethnicity and gender. Furthermore, while the etiology of the disorder is unknown, it is likely that non-genetic factors interact with genetic predisposition, leading to neurochemical changes that, unless compensated for neurochemically or behaviourally, present as ADHD (Faraone & Doyle, 2000; Sagvolden et al., 2005; Muller et al., 2008).

1.4 Neurophysiology

Catecholamines are a class of organic compounds that have a catechol group and a side-chain amine. The most abundant catecholamines in the body are epinephrine, norepinephrine, and dopamine, all of which are produced from phenylalanine and tyrosine. Studies have demonstrated that patients with ADHD have depleted levels of dopamine and norepinephrine in the frontal regions of the brain, particularly the prefrontal cortex and associated subcortical structures and circuits, and these depleted levels are thought to be the result of dysfunction of their respective transporter systems (Prince, 2008).

1.4.1 Dopamine

Neurotransmission is an essential component of brain functioning. The level and function of particular neurotransmitters are controlled by a variety of factors. One of the most common and widely studied neurotransmitters is dopamine. Dopamine is synthesized by the enzyme tyrosine hydroxylase (TH), which catalyzes the conversion of tyrosine to dihydroxyphenylalanine (DOPA). After this process dopa decarboxylase (DDC) catalyzes the conversion of DOPA to dopamine. Once produced and released into the synaptic cleft, dopamine interacts with five major dopamine receptors and is removed from the synaptic cleft by a specific dopamine transporter. Dopamine has a variety of projections and plays a major role in many processes. For example, dopaminergic projections from the midbrain ventral tegmental area (VTA) to the striatal and prefrontal cortical areas play a major role in motor control, attention, and impulsivity (Li et al., 2007). Therefore, mutations in genes involved in translation, synthesis, signaling or metabolism of dopamine, could affect the efficiency of this neurotransmission pathway causing significant deficits in functioning and possibly disorders, such as ADHD.

Many studies have investigated the potential role of dopamine system genes with ADHD. Two genes, DAT1 and DRD4, coding for a dopamine transporter (DAT1) and dopamine receptor four (DRD4) have been reported to be associated with ADHD in different samples. The association of these dopamine genes with ADHD suggests that the two attentional networks that include brain regions rich in dopamine receptors, such as the basal ganglia and anterior cingulate gyrus, may be involved in the attentional deficit component of ADHD (Swanson et al., 2000).

Similarly, depletion of dopamine in the brain is associated with impairment of not only motor but also of memory and cognitive function, which are intimately related to the

hippocampus. Many physiological, pharmacological, and behavioral studies support the idea that dopamine acts as a neurotransmitter in the hippocampus and modulates the activities of hippocampal neurons (Yokoyama, Okamura, & Ibata, 1995). Therefore, the connection between dopamine and learning and memory has been investigated. Studies have shown that mesolimbocortical dopamine plays a role in learning and memory, and many dopamine D1, D2, and D3 receptors are expressed in the hippocampus (Schwegler et al., 1981). The interaction between each dopamine receptor type with respect to learning and memory has also been investigated. In one study, Sigala, Missale, and Spano (1997) suggested that the effects of dopamine on memory consolidation are the result of a balance between dopamine D2 receptor-mediated facilitation and dopamine D3 receptor-mediated inhibition.

1.4.1 Norepinephrine

Another common neurotransmitter that is involved in a variety of brain functioning is norepinphrine or noradrenaline, and areas of the body that produce or are affected by norepinephrine are described as noradrenergic. Executive function and noradrenergic activation is known to profoundly affect the performance of attention, especially the maintenance of arousal, and the ability to sustain attention on a subject. Similarly, attention depends on adequate modulation by catecholamine neurotransmitters of prefrontal, cingulate and parietal cortices, thalamus, striatum, and hippocampus, and these brain networks all have a high distribution of noradrenergic neurons. Arnsten (1999) and colleagues have evidence supporting that adequate levels of noradrenaline are necessary for optimal function of the prefrontal cortex, that very high levels of catecholamine release disrupt cognitive functions of the prefrontal cortex, and that these alterations can improve with α_2-adrenergic agonists.

There is increasing evidence that the locus-coeruleus norepinephrine (LC–NE) system plays a role in the pathophysiology of ADHD. The LC is a small nucleus in the pons of the brainstem, composed of noradrenergic neurons that project widely throughout the neocortex. It is the sole source of norepinephrine in the hippocampus, and several genetic studies have suggested associations between ADHD and various genes involved in norephinephrine transmission. Some of the candidate genes thought to be involved in this association are genes encoding for the enzyme dopamine beta-hydroxylase, which is responsible for converting dopamine to norephinephrine, the norepinephrine adrenoceptors, and the norepinephrine transporter (NET). Also, some treatments that have been proven effective in ADHD patients, such as atomoxetine, a NET blocker, and guanfacine and clonidine, α_2-adrenoceptor agonists, work on norephinephrine transmission, which presents another piece of evidence supporting norephinephrine's role in ADHD (Sterley, Howells, & Russell, 2013). Despite their chemical differences, the various medications with documented therapeutic benefits on ADHD symptoms share a common noradrenergic/dopaminergic activity.

1.5 Neuroanatomy of ADHD

With the improvements in neuroimaging, the knowledge of the details of brain anatomy in children with ADHD has been greatly increased. Using structural brain MRI, evidence of structural abnormalities in children with ADHD has been gathered. Initially, the focus of structural MRIs concerning ADHD was on the frontostriatal circuitry in children with ADHD. Studies focusing on this region pinpointed significant differences in this brain circuitry. As researchers have broadened their scope, other brain regions have been witnessed to exhibit

morphological alterations in areas such as the cerebellum and temporoparietal lobes, basal ganglia, and corpus callosum (Cortese, 2012).

A recent voxel-based morphometric (VBM) meta-analysis found that individuals with ADHD had a significant global reduction in grey matter volumes, most prominently in the right lentiform nucleus and extending to the caudate nucleus (Nakao, et al., 2011). Another recent VBM study in young adults found that individuals with ADHD had less grey matter in the right inferior frontal gyrus, which correlated with poorer outcomes in measures of processing speed, response inhibition and response variability, compared with matched controls (Depue, et al., 2010). Although there is a significant global reduction of grey matter in individuals with ADHD it has been highlighted that the morphological alterations found in children with ADHD are unlikely to be leading to the behavioral symptoms. This is because unaffected first-degree relatives also exhibit similar changes in cortical grey and white matter (Durston et al., 2004).

Furthermore, the imagining studies point to the idea that there is dysfunction in the fronto-subcortical pathways. These findings can help explain why stimulants have a positive effect on the symptoms of ADHD. The areas implicated are high in catecholamines, which are involved in the mechanism of action of stimulant medications (Biederman, 2005). Stimulants work by inhibiting the dopamine transporter and blocking dopamine and norepinephrine reuptake in to the presynaptic neuron. This causes an increase in the monoamine concentration in the extraneuronal space (Elia et al, 1990). In order for a drug to improve ADHD symptoms, the drug needs to cause changes in the dopaminergic and nonadrenergic function. Those changes in dopaminergic and nonadrenergic function lead to the theory that through the dopaminergic and/or the noradrenergic pathways the stimulants increase the inhibitory influences of frontal cortical activity on subcortical structures (Zametkin & Rapoport, 1987). There are three

subcortical structures that are implicated by the imaging studies: caudate, putamen, and globus pallidus. These structures are part of the neural circuitry, underlying motor control, executive functions, inhibition of behavior, and modulation of reward pathways (Biederman, 2005). According to Pontius (1973) the caudate nucleus and its associated circuits have been found to be implicated in ADHD. The caudate nucleus and the putamen are the entry points of the basal ganglia and many studies have documented abnormalities of both structures in ADHD (Krain & Castellanos, 2006). The caudate is generally asymmetrical with the left volume being larger than the right in normal functioning children. Children with ADHD have a reduced volume of the left caudate making the caudate more symmetrical (Krain & Castellanos, 2006). In some studies of the putamen, the volume of this area was not significantly different in those with ADHD or those without ADHD. However, there has been functional imaging studies that show a decreased blood flow to the putamen area of boys with ADHD compared to the control group without ADHD (Teicher et al, 2000). The globus pallidus receives input from the caudate and putamen and was found to be significantly smaller in children with ADHD compared to their control counterparts (Krain & Castellanos, 2006).

In addition, the cerebellum has also been examined in children with ADHD. The cerebellum is involved in coordination of motor movements and is also involved in non-motor functions such as timing and attention shifting through connections in frontal regions (Allen et al, 1997). Studies have shown that the vernal volume is smaller in children with ADHD and there is a decrease in size of the posterior inferior lobe of the cerebellum (lobes VIII-X) of those with ADHD as compared to the controls (Krain & Castellanos, 2006). Other imaging studies have been done on the correlation of regional brain volumes and the neurophysiological functioning in children with ADHD. These studies have found that larger volumes predicted poorer

performance on the Conners' Continuous Performance Test (CPT), variability, and reaction time standard error scores as compared to their healthy controls (Hill et al, 2003). The conclusions of these results are that the right dorsolateral region might be dysfunctional in ADHD individuals and that the more tissue in a region the greater the disruption in attention (Krain & Castellanos, 2006).

In addition to the observed differences in the volume of specific brain areas, there is also evidence that fibers that connect these areas are affected. The corpus callosum connects the homotypic regions of the two cerebral hemispheres. Studies have shown that there are volume differences in those with ADHD, and that there are also volume differences in the number of cortical neurons in the corpus callosum of individuals with ADHD. This change in volume is believed to degrade communication between the hemispheres, which is believed to be responsible for some of the cognitive and behavioral symptoms of ADHD (Spencer, 2007). There seems to be ample evidence to show that ADHD is involved in the decreased brain volume compared to controls. The decreased brain volume is nonprogressive and likely caused by genetic factors, as well as, environmental factors in the early developmental ages (Krain & Castellanos, 2006).

1.6 Pharmacotherapy of ADHD

For the last 50 years the standard ADHD treatment has been catecholaminergic psychostimulants. In 2004 a multimodial treatment study for ADHD was conducted that showed that behavioral therapy was ineffective in the treatment of ADHD but methylphenidate was highly effective in managing the disorder (MTAS Cooperative Group, 2004). Since this study pharmacotherapy has been the primary choice of treatment. The drugs proven to be the most

effective for the treatment of ADHD are amphetamine, atomoxetine and methylphenidate. Amphetamines are monoamine releasing agents. Methylphenidates are psychostimulant catecholamine reuptake inhibitors. Atomoxetine is a reuptake inhibitor. Atomoxetine is a nonstimulant drug because it acts as a potent inhibitor of the presynaptic norepinephrine transporter, which causes more norepinehrine to be available to increase attention and control hyperactivity and impulsivity for the treatment of ADHD (Kratochvil et al., 2002). This non-stimulant is used for patients who react poorly or cannot tolerate stimulant medication. In addition, atomoxetine may be used if there is a concern with drug misuse (Michelson et al, 2003). The research shows that this drug therapy is more effective in adults than children. The theory behind this is that children tend to have more hyperactive signs and symptoms than adults. The research shows that both the inattentive and hyperactive or impulsive symptom clusters responded to the atomoxetine but the magnitude of change was less for children. The theory behind the mechanism of atomoxetine is that the compound is very specific for the norepinephrine transporter and does not seem to be active at the dopaminergic transporters (Michelson et al, 2003). In rat studies atomoxetine increases dopamine in the prefrontal cortex but not in the nucleus accumbens or striatum (Bymaster et al, 2002) which may account for its efficacy and lack of abuse potential (Heil et al, 2002).

Methylphenidate (MPH) is the most effective psychostimulant in use for the treatment of ADHD. It is a catecholamine reuptake inhibitor with stronger dopamine agonist effects in the basal ganglia and both dopamine and noradrenalin agonist effects in the cortical brain regions (Arnsten, 2006). The research shows that patients with ADHD have elevated levels of brain activation. Research suggests that MPH normalizes effects of the brain activation in the regional inter-connectivity pathways and has a greater efficacy on the inattentive problems and less for

impulsivity problems (Rubia, 2009). In the Rubia 2009 study, children with ADHD displayed inactivation of the cerebellum, precuneous, posterior singulate, premotor, inferior frontal and parietal regions compared to controls. MPH reduced the fronto-stratial deficits and normalized the dysfunction in the right and left superior temporal and inferior parietal cortices (Rubia, 2009). MPH is effective in improving attention performance by a very complex regulatory effect of the dysfunctional brain networks by downregulating hypersensitive paralimbic reward processing regions while upregulating hyposensitive and underconnected task-relevant fronto-stratio-cerebello-aprietal attention networks (Rubia, 2009).

Amphetamines are monoamine releasing agents that work both inside and outside the presynaptic neurons. The drug is actively transported in to the presynaptic terminal where they displace catecholamines from the vesicle storage pools (Sulzer & Rayport, 1990). Two of amphetamine's main targets are cell membrane and vesicular monoamine transporters, such as the neuronal dopamine transporter and the vesicular monoamine transporter-2. The molecular mechanism of amphetamine causes monoamine, particularly dopamine, release, and this monoamine release is caused by amphetamine-induced exchange diffusion, reverse transport, and channel-like transport phenomena as well as the weak base properties of amphetamine. Also, amphetamine analogs may affect monoamine transporters through phosphorylation, transporter trafficking, and the production of reactive oxygen and nitrogen species (Fleckenstein et al., 2007). Treatment of ADHD with amphetamine is especially popular and appealing because it has been shown to be a viable long-term treatment. In children with ADHD, once-daily mixed amphetamine salts such as Adderall were well tolerated, and the children showed significant behavioral improvements that were consistently maintained during 24 months of treatment

(Mcgough et al., 2005). The efficacy of stimulant agents suggests that the neurotransmitter abnormalities seen in ADHD are primarily catecholaminergic in origin.

1.7 Animal Models

Animal models are widely used throughout most fields of neuroscience research, particularly in the examination of psychological disorders. Several criteria need to be met before an animal model can be considered to be a true and effective model of a psychiatric disorder. An animal model for ADHD should include face validity, construct validity, and predictive validity (Davids et al, 2003). Face validity means that the model must mimic fundamental behavioral deficits found in ADHD patients. Construct validity means that the model must conform to a theoretical rationale. The predictive validity means that one must be able to predict the unknown aspects of ADHD in areas like genetics, neurobiology and therapies (Davids et al, 2003). The use of an animal model for a disorder has advantages: data may be easier to interpret than extensive clinical cases, animals are less heterogeneous, and the environment can be controlled. There have been many animal models of ADHD such as rats reared in social isolation, rats exposed to environmental pollutants such as lead or PCBs, rats that have undergone neurotoxic brain lesions, and rats that have undergone hippocampal X-irradiation. The genetic models used for ADHD include the Spontaneously Hypertensive Rat, Naples High/Low Excitability Rat, and Knock Out Mice with disrupted DAT gene, and another category of animals models of ADHD are animals prepared by brain lesioning or exposure to neurotoxins usually early in development (Sagvolden, 2000).

The SHR rat is a rat strain that has genetically inherited hypertension that was developed in Japan by inbreeding rats of the Wistar-Kyoto (WKY) strain (Okamoto, 1963). This rat was

bred for studying hypertension, but it was later found that the rat had unexpectedly high spontaneous motor activity and it was suggested that the strain would be a good animal model for the study of ADHD (Moser et al, 1988). The SHR rat exhibits characteristics that are common to patients with ADHD such as motor hyperactivity in a novel environment, excessive conditioned responses under a fixed interval, and difficulty in acquiring operant tasks. These abnormalities correlate to clinical features of hyperactivity, impulsivity, and learning deficit (Mook, 1993). The SHR rat is also more sensitive to immediate behavior reinforcement and less sensitive to delayed reinforcement than the control rat WKY that is not hypertensive (Sagvolden, 1992). Like children with ADHD, the SHR have also been found to be sensitive to immediate behavioral reinforcement and responsive to stimulant medication. Altered dopaminergic and noradrenergic neurotransmission has also been observed in the SHR as a result of the mutated genes associated with these neurotransmission systems, strongly implicating these systems in the etiology of ADHD. Further sequencing of dopaminergic loci identified polymorphisms in the dopamine transporter gene, the DAT1 gene (Mill, 2007). Therefore, it is possible that DNA sequence changes in the DAT1 gene account for some of the behavioral inconsistencies observed between the SHR and WKY strains, which is also consistent with evidence showing that SHR strains exhibit elevated DAT expression in mesocortical projections (Viggiano et al., 2002; Watanabe et al., 1997).

Li et al. (2007), demonstrated that the SHR have decreased turnover of dopamine in the VTA, striatum, and frontal cortex compared to WKY. Prior studies examining dopamine function in the SHR and WKY rat brains have shown lower dopamine levels in the striatum in the SHR (Linthorst et al., 1991). Striatal uptake of dopamine in the SHR has also been reported to be slower (Leo et al., 2003) compared to the WKY, and a higher concentration of dopamine

transporters in the striatum of the SHR was found (Watanabe et al., 1997). It has been shown that extracellular dopamine levels in the nucleus accumbens are higher in the SHR compared to the WKY (Carboni et al., 2003). Thus, there are several differences in the neurotransmission of dopamine, primarily related to release and uptake, between the WKY and SHR. Since dopamine neurotransmission is related to ADHD, the differences in this neurotransmission system directly relate to the ADHD symptoms in the SHR. Interestingly, this alteration in dopaminergic neurotransmission translates to afflicted humans, since individuals with ADHD have been shown to exhibit increased dopamine transporter density in the brain (Dougherty et al., 1999). Similarly, the norepinehrine system of SHR is over-responsive to acute challenges compared to WKY and other control rat strains (Sagvolden, 2000; Russell et al., 2005)

1.8 Hippocampus

The hippocampus includes the pyramidal cell fields of the hippocampus proper (CA1-CA2) together with the hilar and granular cells in the dentate gyrus (Jarrard, 1993). The hippocampus processes cortical information from the entorhinal cortex and important subcortical projections by way of the fimbria-fornix (Jarrard, 1993). One of the best understood of the cortically related circuits consists of major projections from the entorhinal cortex through the perforant path to dentate gyrus, through the hippocampus and to the subiculum (Amaral & Witter, 1989). The second major set of cortical related connections is the direct projection from entorhinal cortex that bypasses dentate gyrus and terminates in the subiculum and the CA1 cell field (Jarrard, 1993). The intricate nature of the hippocampus makes it especially difficult to pinpoint its exact function. There have been theories over the years that differ in the fact that some believe that the hippocampus is primarily involved in the processing and memory of spatial

information and others believe that the hippocampus is more involved in the more abstract learning and memory processes (Jarrard, 1993). One thing that all theorists agree on is as far as the hippocampus function goes spatial information processing occurs there.

As mentioned, it is well established that the integrity of hippocampal formation is essential for spatial learning. In order to further examine the hippocampus's role in learning and memory, the Morris Water Maze (MWM) has often been utilized. The MWM is as an instrument with particular sensitivity to the effects of hippocampal lesions in rats. Interestingly, from research conducted in the MWM, the spatial learning impairment of hippocampus-lesioned rats has shown to be related to the volume of damaged hippocampal tissue. Also, dorsal hippocampal lesions were reported to have more profound effects than ventral hippocampal lesions. Similarly, evidence indicates that the hippocampus is necessary for acquisition and retrieval of spatial information as well as for consolidation and storage of spatial information (D'Hooge & Deyn, 2001). Riedel et al.'s study demonstrated that reversible hippocampal inactivation using a water-soluble AMPA/kainate antagonist seriously impairs MWM performance in rats. Therefore, since the MWM is a task testing learning and memory, hippocampal activation is essential for normal memory and learning functioning.

1.9 Radial Arm Maze

Since its design 25 years ago (Olton & Samuelson, 1976), the eight-arm radial maze has become very popular and is now widely used to assess spatial memory in rodents. The radial arm maze (RAM) has become a common method for assessing spatial memory in rodents. It has proven to be quite useful in the investigation of the effects of a variety of pharmacological manipulations on spatial memory. The cholinergic system has been found to be crucial for

accurate RAM performance (Levin, 1988). The radial arm maze has been increasingly utilized to examine the psychological and neurobiological substrates of cognitive function (Walsh & Crobak, 1987). In its most basic form the task involves baiting several arms and assessing if the rats visit unbaited arms, commit reference memory errors, or if rats revisit baited arms after retrieving the reward and commit working memory errors. This procedure allows for the quantifiable measure of working and reference memory and separation of working memory from reference memory (Kay, Harper, & Hunt, 2010).

The radial arm maze (RAM) is considered to be one of the most sensitive methods for evaluating spatial learning and memory in rodents. It has been suggested that animals perform it successfully by utilizing a spatial map formed at the start of the maze solution and by using extramaze cues. Studies looking at the hippocampus and learning and memory have been done using a radial maze that permits studying the acquisition of two kinds of information, spatial versus intramaze cues, and two different memory functions, reference memory versus working memory. In the spatial version of the task the eight arms of the radial maze were similar, but differed in their spatial location in the room. There were extramaze cues that remained constant over trials including arrangement of cages, the door, shelves, lighting, location of experimenter, etc. For each animal the same four out of eight arms were consistently baited over trials. In the intramaze cue task different textured floor inserts were moved among the eight arms in a random order, and the rat was rewarded after choosing the same four cues independent of spatial location. Another difference between the tasks was that in the extramaze cue task the normal room lighting was used thus making the extramaze cues readily visible, and in the intramaze cue task a single light bulb with a reflector directly over the maze served to minimize room cues. Correct performance in the extramaze cue task requires the use of distal, spatial cues, and that proximal

cues are important for correct performance in the intramaze cue task. Furthermore, the intramaze and extramaze cue tasks appear to be of equal difficulty for normal rats since the tasks are learned at the same rate. Because only four out of eight arms were baited, reference memory and working memory could be assessed. Reference memory errors were operationally defined as choices of arms that were never baited, and working memory errors were repeated entries into arms that were correct but had already been visited on that trial (Jarrad, 1993). The limited baiting procedure provides a test of the working memory theory proposed by Olton et al. (1979), since the theory would predict that rats with hippocampal damage would be impaired in working memory, but not reference memory, on both the extramaze and intramaze cue versions of the radial maze. In contrast, the spatial mapping theory of O'Keefe and Nadel (1978) would predict that without a hippocampus the animals would be impaired on the extramaze task but not the intramaze task.

The results of these studies yielded very interesting results. Rats with the hippocampus removed showed significant impairment on the intramaze cue task, and they made both reference and working memory errors. Similarly, these rats showed difficulty with the extramaze cue task; however, only working memory errors were made. Therefore, these results supported that the hippocampus plays a role in spatial mapping and working memory. The rats with hippocampal lesions committed reference memory errors in the intramaze cue tasks, and with training these rats learned to make only a few working memory errors. Thus, this evidence also suggests that the hippocampus plays a general role in memory, and it is the memory process that is affected in hippocampal rats, not the type of information that is being learned (Jarrad, 1993). Also, the RAM has been used to examine and show the deficits of spatial learning and working memory in spontaneously hypertensive rats (Nakamura-Palacios, Caldas, Fiorini, Chagas, Chagas, &

Vasquez, 1996). Another study examining the effects of amphetamine on RAM performance have shown that the activation of the dopaminergic system by amphetamine does not compensate for the alteration of the cholinergic activity inducing amnesia; however, the results of the study suggest that amphetamine has an improving effect on locomotor activity but little to no effect on the memory measures (Ennaceur, 1994). Thus, the RAM is a cognitive task that has been proven very useful in the investigation of the neuronal systems and neurotransmitters involved in learning and memory and the influence of drugs on them.

1.10 c-Fos

In the mammalian nervous system, induction of an immediate early gene (IEG) is one of the first signs of a genomic response to a stimulus (Sheng & Greenberg, 1990). The best known of the IEGs are the proto-oncogene transcription factors, and a prime example of a proto-oncogene transcription factor is c-Fos (Beckmann & Wilce, 1997). IEG induction can be demonstrated in cells or regions of tissue sections by immunohistochemistry (IHC), which show protein expression in precise locations. Microscopic evaluation of c-Fos expression has been extensively studied in the brain and has become widely regarded as a mapping tool for sites of cell activation (Herrera & Robertson, 1996; Hoffman & Lyo, 2002; Kaczmarek & Robertson, 2002). Many stimuli induce genomic responses. One of the more common stimuli used are pharmacological stimuli. Acute injection of a drug elicits responses, including induction or downregulation of an IEG in the brain (Herrera & Robertson 1996). Drugs that increase dopamine release and favor transmission through D1 receptors, such as amphetamine and cocaine, are effective inducers of c-Fos. Therefore, evidence has shown that the psychostimulant

amphetamine activated the most brain regions, including the most cortical regions (Sumner, 2004).

c-Fos expression is also induced after some forms of learning. It has been shown that increased c-fos expression is linked to increased neural activity, such as learning and memory. Thus, learning and memory formation is also associated with c-Fos expression, and the greater the memory loading, the greater the c-Fos expression expected. Since learning and memory is a major concern in everyday life, learning and memory and more specifically the hippocampal expression pattern of Fos has been studied. One study evaluated the Fos expression in rats exposed to one of two different memory tasks in an eight-arm radial maze. The radial arm maze (RAM) can be used to assess working and reference memory simultaneously in the fixed position of reward task (FPRT). The FPRT consists of baiting half of the arms and having their positions fixed throughout the training trails. Another task can be used to assess memory called the variable position of reward task (VPRT), in which four out of eight arms were baited, but the positions were varied in every training trial. In the VPRT, the rats learned to choose all arms without any discrimination between baited and non-baited arms and the memory retention was time-dependent. After comparing Fos immunohistochemistry between rats that completed FPRT and the rats that completed VPRT, the results revealed that there was more c-Fos expression in the hippocampus in the VPRT than the FPRT. This result demonstrated that the hippocampus may play a more important role in the acquisition of memory because acquisition is known to be more involved in the VPRT than in the FPRT. Thus the mapping of c-Fos expression is a valuable tool in learning about brain regions associated with different stimuli (He, Yamada, Nakajima, Kamei, & Nabeshima, 2002).

1.11 Experimental Goals and Objectives

The diagnosis of childhood ADHD is becoming increasingly prevalent in the United States, and therefore, the prescription of psychostimulants is also becoming more prevalent. Since many children have access to psychotimulants, the abuse of these prescribed psychostimulants, especially Adderall, is becoming very popular in places where increased focus is desired, such as college campuses. The mechanism of action of psychostimulants to produce beneficial effects in individuals with ADHD and what effect these drugs may have in individuals without ADHD is not well known. The cognitive effects of chronic use of low dose psychostimulant treatment on individuals with and without ADHD are also not well understood.

The current study is aimed to examine the effects of exposure to chronic, low dose amphetamine treatments on an ADHD rat model and age/strain matched controls on learning and memory and the underlying neurobiological relationship between the drug conditions and animal models. Understanding this relationship between chronic, low dose amphetamine administration, cFos expression, and learning and memory could have immense practical and theoretical implications in ADHD research and it could shed light on biological causes of the effects of amphetamine on learning and memory.

In order to investigate this relationship, the present study was focused on the SHR model, a validated rat model of ADHD. Using the behavioral results of the SHR animals compared to the age/strain matched control animals the effects of amphetamine on learning and memory in both the age/strain matched control and SHR animals can be assessed. The immunohistochemical analysis of cFos expresson will also allow for a histological analysis of the effects of amphetamine in the hippocampus, a major brain region associated with learning and memory, in the two animal groups.

1.12 Hypotheses

Overall it is hypothesized that chronic, low dose amphetamine administration will enhance RAM performance in both the SHR animals and their age/strain matched controls. More specifically, SHR animals and their age/strain controls given chronic, low dose amphetamine administration are expected to commit fewer reference and working memory errors than animals receiving saline administration throughout the course of the study. Also, SHR animals and their age/strain controls given chronic, low dose amphetamine administration are expected to complete the maze faster than animals receiving saline administration.

It is also hypothesized that the age/strain matched control animals with saline administration would perform better on learning and memory tasks than SHR animals with saline administration. Thus, the age/strain matched control animals given saline administration are expected to commit fewer reference and working memory errors than SHR animals receiving saline administration throughout the course of the study. Similarly, the age/strain matched control animals given saline administration are expected to complete the maze faster than SHR animals receiving saline administration throughout the course of the study.

In terms of immunohistochemistry, it is hypothesized that the greatest hippocampal c-Fos expression will be seen in age/strain matched control with chronic, low dose amphetamine administration. It is also hypothesized that age/strain matched controls receiving saline administration will have greater c-Fos expression than SHR animals receiving saline administration. Additionally, it is hypothesized that age/strain matched controls receiving amphetamine administration will have greater c-Fos expression than age/strain matched controls

receiving saline administration and that SHR animals receiving amphetamine administration will have greater c-Fos expression than SHR animals receiving saline administration.

Method

2.1 Animals and Environment

Fourteen male Wistar-Kyoto rats and sixteen Wistar-Kyoto SHRs were obtained from Charles River Labs and all weighed between 270-375 grams during the time of experimentation. The rats were housed in pairs and kept in the Connecticut College animal facility where they were maintained under standard temperature and humidity conditions. The rats were kept on a 12-hour light dark cycle and had access to food for one hour daily during experimentation and unlimited access to water throughout the experiment. All experimental procedures were in agreement with the NIH Guide for Care and Use of Laboratory Animals (National Research Council of the National Academies, 2011). This experiment was approved by the Connecticut College Institutional Animal Care and Use Committee (IACUC).

2.2 Drugs and Drug Administration

The stimulant D-amphetamine (Sigma-Aldrich) was dissolved in sterile saline (0.9% NaCl) and administered intraperitoneally (i.p.) at 1 mg/kg in a volume of 1mL/kg.

2.2 Radial Arm Maze

The RAM consisted of eight arms (48 cm × 12 cm) radiating from a central area (32 cm in a diameter). Before training, rats were shaped to run to the ends of the radiating arms. Chocolate flavored rice cereal was used as a food reward (bait). In order for the animals to be motivated to complete the maze, they were food deprived one day prior to the beginning of trained. The animals were allowed to eat for one hour per day after the completion of the RAM. The baits were initially available throughout the maze, but were gradually restricted to the end of

the arms. Following this shaping period, rats were trained by performing one trial per day. Four arms of the maze were baited with a single reward, while the remaining four arms were left unbaited. Baited arms varied from animal to animal but remained the same for each animal throughout the experiment. Each trial continued until all four baits had been consumed or until 10 min had elapsed. The number of reference memory errors (entering an arm that was not baited), number of working memory errors (re-entering a bait-containing arm where the bait had been consumed), and total latency to complete the maze were recorded.

In this study, rats received a single injection of amphetamine or saline (1 mg/kg, i.p.) 10 minutes prior to maze exposure daily for 23 days. The rats were sacrificed 30 minutes after they satisfied the following criteria: either committing zero working memory errors and one or less reference memory errors for three consecutive days or the completion of their 23 training day.

2.3 Immunohistochemistry

Tissue Preparation.

On day 23, all animals were euthanized by exposure to carbon dioxide within fifteen minutes of testing. Each animal was transcardially perfused with physiological saline (400-500 ml), followed by 4% paraformaldehyde solution (400-500 mL). The brain of each animal was removed and post-fixed in 4% paraformaldehyde overnight before being transferred to 30% sucrose solution for storage until sectioning. Sections were obtained in a -20°C cryostat and were stored at 4°C in cryoprotectant. Tissue sections (40 μm) were taken from the hippocampus in each rat brain. For each brain, five to six slices of tissue were obtained and stored for examination of c-Fos expression.

Immunohistochemistry.

Immunohistochemistry was conducted to examine levels of c-Fos in the hippocampus of each rat. The staining method utilized was the avidin-biotin-horseradish peroxidase (ABC) method. First, the tissue was washed three times, for ten minutes each, in 0.01M Phosphate Buffer Saline (PBS) before being incubated for 24 hours with a 1:8000 Fos primary antibody dilution. This dilution was made using 6.3 µl of rabbit anti-Fos polyclonal IgG (Santa Cruz) and 50 mL of a blocking solution that contained 98.75 mL 0.01M PBS, 1 mL 1% normal goat serum, 1g 1% bovine serum albumin and 0.25 mL 30% Titron-X100. Following incubation, the tissue was washed three times in 0.01M PBS for ten minutes each and sections were incubated in 1 mL biotinylated goat anti- rabbit polyclongal IgG antibody (Jackson Laboratories) diluted 1:200 in 50 mL of the blocking solution for two hours. A third series of PBS tissue washes were conducted, and sections were incubated for one hour with avidin-biotin-horseradish peroxidase complexes (ABC kit by Vector Laboratories) in 50 mL 0.01M PBS. After this incubation period, sections were washed three times for ten minutes each with 0.1M Phosphate Buffer (PB). For ten minutes sections were placed in a solution composed of glucose oxidase, cobalt, chloride, nickel ammonium sulfate, ammonium chloride and diaminobenzidine (DAB) in 50 mL of 0.1M PB. The presence of glucose started the peroxidase reaction, which lasted approximately fifteen minutes. The reaction was monitored and terminated when the appropriate color was reached by placing sections into 0.01M PBS. The tissue was washed three more times with 0.01M PBS for ten minutes each, and then tissue samples were then stored in 0.01M PBS solution until mounting (Grahn, et al, 1999).

Slide Preparation.

Representative slices of each brain were mounted onto pre-treated slides, and underwent a dehydration sequence before cover slips were glued onto each slide. This dehydration sequence consisted of placing each slide in 50% ethanol for 2 minutes, 70% ethanol for 2 minutes, 95% ethanol for 10 minutes, 100% ethanol for 2 minutes, and then Neoclear for a minimum of 5 minutes. Once slides were fully dehydrated they were coverslipped using mounting glue and underwent microscopic image analysis for examination of c-Fos expression.

2.4 Statistical Analysis

The analysis of data was conducted independently for each experimental group as well as each behavioral measure (reference memory errors, working memory errors, and maze latency). Statistical analyses (one-way analysis of variance) were conducted to examine the relationship between drug and behavioral parameters using the Statistical Package for the Social Sciences (SPSS).

Results

3.1 Radial Arm Maze

To investigate the effect of amphetamine on Wistar-Kyoto rats and SHRs, three parameters were examined for each drug condition: the time to maze completion, the frequency of reference memory errors, and the frequency of working memory errors. Averages for each variable were calculated for week 1 (days 2-8), week 2 (days 9-15) and week 3 (days 17-22) and one-way ANOVAs were performed on the data for each week.

3.1a Time to maze completion. To examine if the time to maze completion differed between control and SHR animals in each drug condition, a one-way ANOVA was performed for each week of the experiment. In week one (days 2-8), significant main effects of drug and

strain were observed between the Control/Amphetamine (M=81.18, SD=19.45) Control/Saline (M=142.23, SD=48.10), SHR/Amphetamine (M=141.94, SD=22.13) and SHR/Saline groups (M=62.42, SD=22.14), ($F(3,31) = 4.482$, $p<0.01$) (Figure 1). Post hoc multiple comparison tests (Tukey's HSD test) determined that the Control/Amphetamine group completed the maze significantly faster than the Control/Saline group, and both SHR groups (Figure 1). In week two (days 9-15), one-way ANOVA revealed a significant main effect between the Control/Amphetamine (M=73.69, SD=15.34) Control/Saline (M=66.71, SD=15.23), SHR/Amphetamine (M=99.01, SD=19.97) and SHR/Amphetamine groups (M=102.21, SD=26.70), ($F(3,31) = 6.450$, $p<0.01$), and Tukey post hoc multiple comparison tests determined that the Control/Amphetamine group completed the maze significantly faster than the SHR/saline group and the Control/Saline group completed the maze significantly faster than both SHR groups (Figure 1). In week three (days 16-22), one-way ANOVA revealed a significant main effect between the Control/Amphetamine (M=106.44, SD=26.75) Control/Saline (M=55.85, SD=12.14), SHR/Amphetamine (M=69.19, SD=23.99) and SHR/Amphetamine groups (M=47.30, SD=8.29), ($F(3,31) = 14.48$, $p<0.01$) (Figure 1). Post hoc multiple comparison tests (Tukey's HSD test) determined that unlike in week one the Control/Saline group completed the maze significantly faster than the Control/Amphetamine group, and again unlike in week one both SHR groups completed the maze significantly faster than the Control/Amphetamine group (Figure 2). In summary, amphetamine appeared to improve latency to complete the maze in control animals during week one but with repeated administration amphetamine hindered maze latency by week three. This effect was not observed in the SHR groups as no significant differences in time to maze completion were observed between the SHR drug conditions during any week (Figure 3).

3.1b Reference Memory Errors. To examine if the frequency of reference memory errors differed between control and SHR animals in each drug condition, a one-way ANOVA was performed for each week of the experiment. In week one no main effect was observed between conditions ($F(3,31)=1.579$, $p>0.05$), (Figure 4). In week two, a main effect of drug condition was observed between Control/Amphetamine ($M=4.76$, $SD=0.92$) Control/Saline ($M=2.65$, $SD=0.62$), SHR/Amphetamine ($M=4.30$, $SD=0.88$) and SHR/Amphetamine groups ($M=4.46$, $SD=1.01$) ($F(3,31) = 9.556$, $p<0.01$). Post hoc multiple comparison determined that the Control/Saline group committed significantly fewer reference memory errors than all the other groups (Figure 4). In week three, a main effect of drug condition was observed Control/Amphetamine ($M=4.90$, $SD=1.20$) Control/Saline ($M=2.33$, $SD=0.57$), SHR/Amphetamine ($M=4.00$, $SD=1.17$) and SHR/Amphetamine groups ($M=3.75$, $SD=0.81$) ($F(3,31) = 10.19$, $p<0.01$). Tukey post hoc multiple comparison tests determined that like in week two the Control/Saline group committed significantly fewer reference memory errors than all other groups (Figure 5). No significant differences in frequency of reference memory errors were observed between the SHR drug conditions during any week (Figure 6).

3.1c Working Memory Errors. To examine if the frequency of working memory errors differed between conditions, a one-way ANOVA was performed for each week of the experiment. In week one no main effect was observed between conditions ($F(3,31)=1.869$, $p>0.05$), (Figure 7). In week two, a main effect of behavioral condition was observed between Control/Amphetamine ($M=1.93$, $SD=0.85$) Control/Saline ($M=0.88$, $SD=0.43$), SHR/Amphetamine ($M=2.59$, $SD=1.00$) and SHR/Amphetamine groups ($M=3.13$, $SD=1.13$) ($F(3,31) = 9.486$, $p<0.01$), and post hoc multiple comparison tests determined that the Control/Saline group committed significantly fewer working memory errors than both SHR

groups. In week three, a main effect was observed between the Control/Amphetamine (M=2.14, SD=0.80) Control/Saline (M=0.46, SD=0.32) (Figure 8), SHR/Amphetamine (M=1.42, SD=0.64) and SHR/Amphetamine groups (M=1.30, SD=0.44) ($F(3,31) = 10.19$, $p<0.01$). Post hoc multiple comparison tests determined that the Control/Saline group committed significantly fewer working memory errors than all other groups (Figure 7). No significant differences in frequency of working memory were observed between the SHR drug conditions during any week (Figure 9).

3.2 Immunohistochemistry

No c-Fos positive cells were detected after the immunohistochemical procedure; therefore, no results were gathered.

Discussion

Results of this study do not support the theory that amphetamine would promote learning and memory in both control rats and SHRs. Findings suggest that chronic amphetamine treatments have hindering effects on learning and memory in control rats, and chronic amphetamine treatments have no effect on learning and memory of SHRs compared to SHRs receiving saline injections. When maze completion latency is used as a measure of learning and memory, amphetamine appears to enhance maze performance during the first week of learning and hinder learning during the third week. The results were consistent with the hypothesis that control animals treated with saline would perform better on memory tasks than SHRs treated with saline.

4.1 Radial Arm Maze

Findings of the study were inconsistent with the hypotheses that amphetamine would have beneficial effects on maze performance. Initially control animals receiving daily amphetamine injections completed the maze significantly faster than control animals receiving daily saline injections. However, by the third week of training control animals receiving daily saline injections completed the maze faster than control animals receiving daily amphetamine injections. Also, the control animals treated with saline showed a steady decrease in time to maze completion over the course of training. Whereas, the control animals receiving amphetamine treatments showed no decrease in time to maze completion throughout the study. These results suggest that chronic amphetamine treatment in control animals hindered the animals' ability to learn the maze and quickly retrieve the food rewards. In the SHR animals, throughout the entire experiment no significant differences in time to maze completion were seen between the animals receiving daily amphetamine injections and animals receiving daily saline injections. Both SHRs treated with saline and SHRs treated with amphetamine showed steady decreases in time to maze completion throughout the course of the experiment. Therefore, unlike in the control animals, chronic amphetamine treatments did not hinder time to maze completion in SHRs.

The results of reference memory errors also showed inconsistencies from the hypotheses. In week one no significant differences were observed in frequency of reference memory errors between control animals receiving daily saline injections and control animals receiving daily amphetamine injections. However, by week two of the experiment, control animals treated with saline committed significantly fewer reference memory errors than control animals treated with amphetamine. This trend continued through week three because control animals that received daily saline injections again made significantly fewer reference memory errors than control

animals receiving amphetamine injections. Also, control animals treated with saline showed a continuous decrease in reference memory errors each week of the experiment, while control animals treated with amphetamine showed no decrease in reference memory errors over the course of the study. Thus, the results suggest that in control animals chronic amphetamine treatment hindered their learning and memory of the maze.

Conversely, amphetamine did not appear to have the same hindering effect on reference memory in SHR animals. Both the SHRs treated with saline and the SHRs treated with amphetamine showed a slight learning curve illustrated by the slight decrease in frequency of reference memory errors over the course of the experiment. There were also no significant differences between the frequencies of reference memory errors during either of the three weeks between the two SHR conditions. This is inconsistent with the hypothesis that amphetamine would aid in learning and memory in SHRs. Overall, chronic amphetamine treatments showed no hindering or enhancing effects on learning memory which is inconsistent with the known positive effects of amphetamine treatment in children with ADHD. Therefore, further research should be performed to confirm the validity of the model.

One finding that was consistent with the hypothesis was in the comparison of reference memory errors in SHRs treated with saline and control animals treated with saline. Initially in week one of training, there was no significant difference between the SHRs receiving saline injections and the control animals receiving saline injections. However, by the second week of training control animals treated with saline made fewer reference memory errors than SHRs treated with saline. This trend continued in the third week of training, and again control animals treated with saline made fewer reference memory errors than SHRs treated with saline. Therefore, these results suggest that SHRs have more difficulty with learning and memory tasks

than control animals, which is consistent with the results shown in Nakamura-Palacios, Caldas, Fiorini, Chagas, Chagas, and Vasquez's (1996) study that concluded that the SHR has a deficiency in the performance of the radial maze, suggestive of impairment of learning and working memory, mainly for a long-term memory. This suggests that deficits in learning and memory are possible behavioral consequences of neural alterations associated with catecholamine regulation.

Similar to the analysis of reference memory errors, the analysis of the frequency of working memory errors did not support the hypotheses. In week one and two no significant differences were observed in frequency of working memory errors between control animals receiving daily saline injections and control animals receiving daily amphetamine injections. However, by week three of the experiment, control animals treated with saline committed significantly fewer working memory errors than control animals treated with amphetamine. This trend suggests that chronic amphetamine treatment has a hindering effect on working memory in control animals. Also, control animals treated with saline showed a continuous decrease in working memory errors each week of the experiment, while control animals treated with amphetamine showed little to no decrease in working memory errors over the course of the study. Thus, the results suggest that inconsistent with the hypothesis, in control animals chronic amphetamine treatment did not have beneficial effects on their learning and memory of the maze. Chronic exposure to amphetamine could have caused addiction in animals receiving daily amphetamine administration. Since the animals exposed to daily amphetamine treatments showed decreased performance in the maze, it could be implicated that amphetamine addiction played a role in the animals' decreased performance. This is consistent with studies suggesting that chronic psychostimulant abuse leads to significant cognitive impairments, especially in

attention, working memory, and response inhibition functions (Sofuoglu, 2010). Robinson and Kolb's study (1999) extended the evidence for structural change after repeated injections of psychostimulants, by showing morphological changes in the dendritic branching in the ventral striatum and frontal cortex, which further highlighted the possibility that chronic psychostimulant abuse may produce brain changes that lead to cognitive deficits. Another rationale behind the decreased maze performance in animals receiving chronic amphetamine administration could be amphetamine sensitization. Repeated amphetamine exposure can produce reverse tolerance or sensitization to the psychological or locomotor-stimulating effects of the drug. Sensitization refers to a progressive and persistent increase in a drug effect produced by repeated drug administration (Anagnostaras & Robinson, 1996). Amphetamine sensitization is suggested to lead to a reduction in prefrontal dopamine turnover, which is associated with profound deficits in spatial working memory (Castner, Goldman-Rakic, & Williams, 2004). In Hooks, Jones, Neill, and Justice's (1992) study examining dose dependent effects on amphetamine sensitization there was a pronounced sensitization to the locomotor-stimulating properties of repeated 1.0 mg/kg amphetamine administration. Therefore, since the rats in this study were also given repeated 1.0 mg/kg amphetamine administration, the decreased maze performance could be a result of drug sensitization.

Conversely, amphetamine did not appear to have the same hindering effect on working memory in SHR animals. There were also no significant differences between the frequencies of working memory errors during either of the three weeks between the two SHR conditions. This is inconsistent with the hypothesis that amphetamine would aid in learning and memory in SHRs. Another finding that was consistent with the hypothesis was in the comparison of working memory errors in SHRs treated with saline and control animals treated with saline. Initially in

week one of training, there was no significant difference between the SHRs receiving saline injections and the control animals receiving saline injections. However, by the second week of training control animals treated with saline made fewer working memory errors than SHRs treated with saline. This trend continued in the third week of training, and again control animals treated with saline made fewer working memory errors than SHRs treated with saline. Therefore, these results also suggest that SHRs have more difficulty with learning and memory tasks than control animals.

As demonstrated in all three components of the RAM, chronic amphetamine administration appeared to be more inhibiting in RAM performance in age/strain matched control animals; whereas, chronic amphetamine administration did not enhance or inhibit RAM performance in SHR animals. Since in humans amphetamine has been shown to have a beneficial effect on learning and memory in individuals with ADHD, this suggests that the neurophysiological mechanisms mediating learning and memory may be different in SHR animals than humans with ADHD. Therefore, additional studies are needed to evaluate the validity of the SHR as a model of ADHD.

On a behavioral level SHRs showed many different characteristics than control animals. Although no quantifiable data was able to be gathered regarding behavioral differences, many observable differences were witnessed through handling and exploration of the maze. In their home cages, SHR animals appeared to be more social compared to control animals. The animals would explore the cage together at most times, and when the cage was open both animals would climb on one another to peak over the ledge of the cage. Similarly, during acclimation of the maze the SHR animals explored the maze together and rarely separated; whereas, the control animals were much more likely to separate and explore the maze on their own. Another

observable difference was the SHRs overall hyperactivity and inattentiveness. Again, during acclimation to the maze almost all of the SHR animals attempted to climb over the maze walls rather than simply explore the maze. SHR animals also showed less interest in food rewards. Conversely, during acclimation very few of the control animals attempted to climb over the walls of the maze, and the control animals were overall more interested in food rewards during the course of the study. This pattern of attempting to climb out of the maze persisted in the SHR animals during training, while few control animals attempted to climb out of the maze at any time of experimentation. These behavioral observations are consistent with other studies, such as Mook's (1993) study that concluded that SHR animals exhibit motor hyperactivity in a novel environment, excessive responses under a fixed interval, and difficulty in acquiring operant tasks. These abnormalities correlate to clinical features of hyperactivity, impulsivity, and learning deficit seen in individuals with ADHD.

4.2 Immunohistochemistry

Although the immunohistochemistry process yielded no results, after witnessing the behavioral results new hypotheses for c-Fos expression were formed. Prior to the study, the greatest level in c-Fos expression in the hippocampus was thought to be observed in the control animals receiving amphetamine treatments. However, initially the acute dose amphetamine administration was thought to aid in learning and memory, yet the results of the study demonstrated that acute dose amphetamine administration caused learning and memory deficits in the control animals. Since a depletion of dopamine in the brain is associated with impairment of memory and cognitive function and many physiological, pharmacological, and behavioral

studies support the idea that dopamine acts as a neurotransmitter in the hippocampus and modulate the activities of hippocampal neurons, the increased levels of dopamine released in the brain as a result of acute dose amphetamine administration would theoretically cause an increase in c-Fos expression in the hippocampus (Yokoyama, Okamura, & Ibata, 1995). Thus, the hypothesis remains that the greatest c-Fos expression would be seen in the control animals treated with amphetamine, yet unlike previously this level of neural activity or c-Fos expression is no longer hypothesized to be helpful in learning and memory. In fact, hyperexpression of c-Fos might be the cause of the deficits in learning and memory witnessed in the control animals given acute dose amphetamine administration.

Another hypothesis pertaining to c-Fos expression was that greater c-Fos expression would be witnessed in SHR animals with amphetamine administration versus SHR animals with saline administration. In retrospect, this hypothesis may be inconsistent with the behavioral results. The behavioral results showed no significant differences between the two SHR treatment groups; therefore, the SHR animals' learning and memory was not benefited from chronic, acute dose amphetamine administration. Since the learning and memory is associated with hippocampal activity and the learning and memory of SHR animals were not inhibited or enhanced by acute dose amphetamine administration, little to no difference in c-Fos expression would be expected to be seen between the two SHR conditions.

The hypothesis that greater c-Fos expression would be witnessed in control animals with saline administration compared to SHR animals with saline administration is consistent with the behavioral results. The basis of this hypothesis stems from evidence that SHRs have deficits in learning and memory (Nakamura-Palacios, et al., 1996). Thus SHR animals would be expected to have less neural activity in the hippocampus since the hippocampus is critical in learning and

memory. The behavioral results showed that control animals treated with saline showed better learning and memory in the RAM than SHR animals treated with saline. Therefore, the behavioral results suggest that greater neural activity in the hippocampus, expressed as c-Fos, would be observed in control animals with saline administration rather than SHR animals with saline administration.

Unfortunately, none of the previous hypotheses were able to be examined because of the lack of c-Fos expression in the brain tissue. There are many potential explanations as to why the immunohistochemistry procedure was unsuccessful. One of main potential reasons that no c-Fos was detected could have been from the malfunction of the refrigerator. Approximately one week after the completion of tissue cutting, the temperature of the refrigerator that is used to store the cut tissue raised significantly. Other potential explanations as to why the immunohistochemistry process was unsuccessful could be errors in any of the steps of the immunohistochemistry or problems with one of the reagents. The immunohisochemistry procedure is a two day long process with a number of integral steps. Therefore, if any of the incorrect reagents were used or one of the steps was not properly performed then the immunohistochemistry process could have been compromised.

In conclusion, the results of this study suggest that chronic amphetamine treatments have detrimental effects on learning and memory in control rats. Unlike their age/strain matched controls, amphetamine did not enhance or inhibit radial arm maze performance of SHR animals. Since amphetamine is known to cause beneficial effects on learning and memory in children with ADHD, this suggests that the neurophysiological mechanisms mediating learning and memory may be different in SHR animals than in humans with ADHD. Therefore, additional studies are needed to evaluate the validity of the SHR model of ADHD.

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Figure 1. The effects of amphetamine on radial arm maze performance in the SHR model of ADHD vs. age/strain matched controls: Latency to maze completion

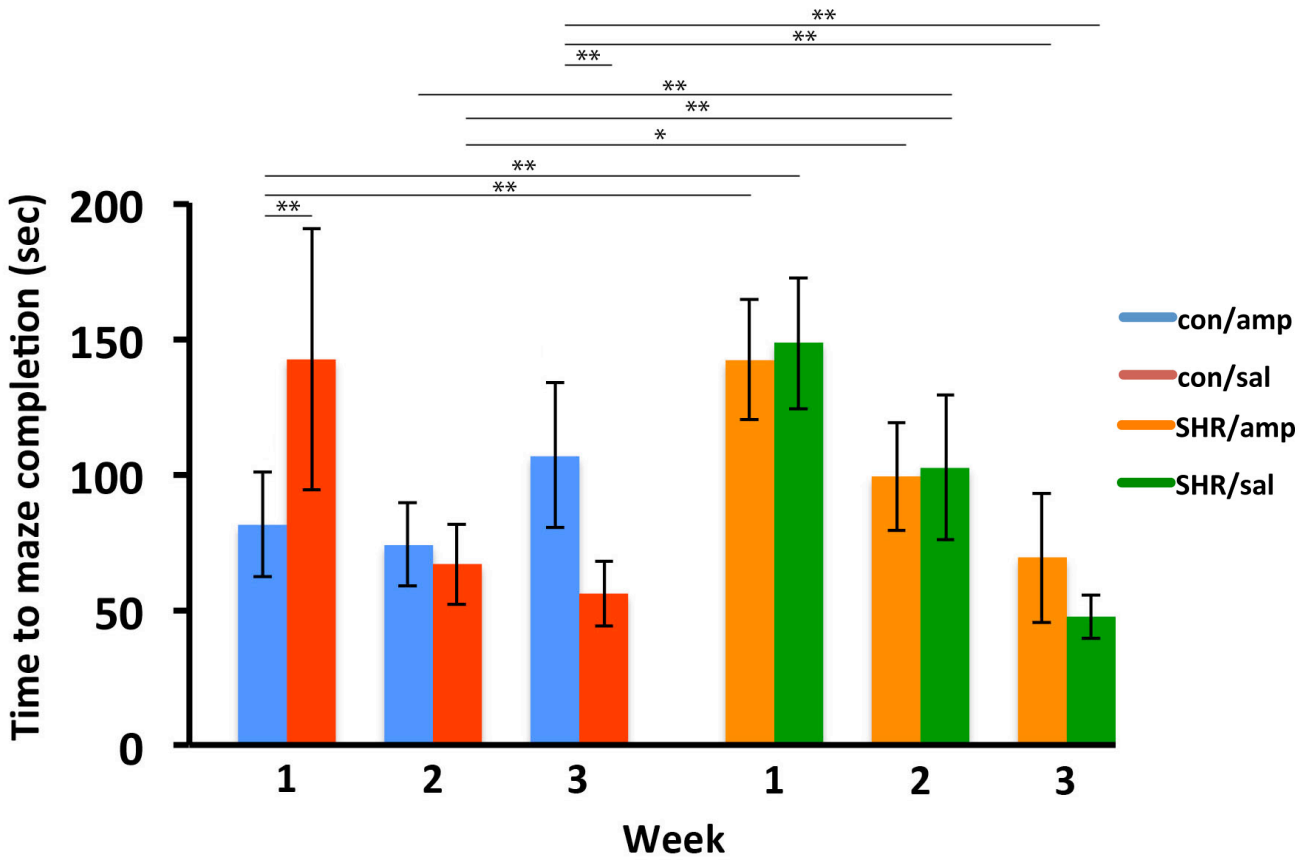

Figure 1. Average time to maze completion was quantified for each group during each week of training. One-way ANOVAs revealed significant differences between groups on each week, $F(3,31)=4.482$, $p<0.01$, $F(3,31)=6.450$, $p<0.01$, $F(3,31)=14.48$, $p<0.01$. A post hoc multiple comparison Tukey HSD test suggested that acute dose amphetamine administration in age/strain matched control animals initially enhanced maze performance; however, by week three acute dose amphetamine administration appeared to hinder maze performance in age/strain matched controls. Age/strain matched controls receiving saline administration also appeared to complete the maze significantly faster than SHR animals receiving saline administration by the second and third weeks.

Figure 2. The effects of amphetamine on radial arm maze performance in age/strain matched controls: Latency to maze completion

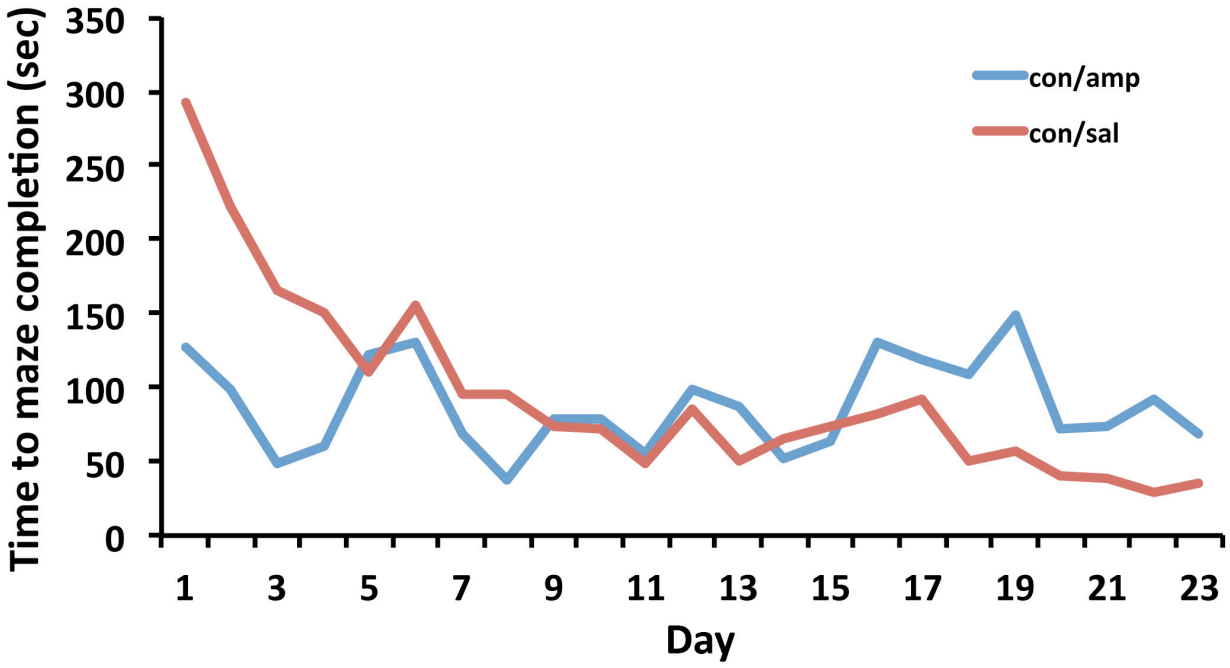

Figure 2. Performance of age/strain matched control animals in the radial arm maze was quantified by the time it took the rats to retrieve all of the food rewards.

Figure 3. The effects of amphetamine on radial arm maze performance in SHR model of ADHD animals: Latency to maze completion

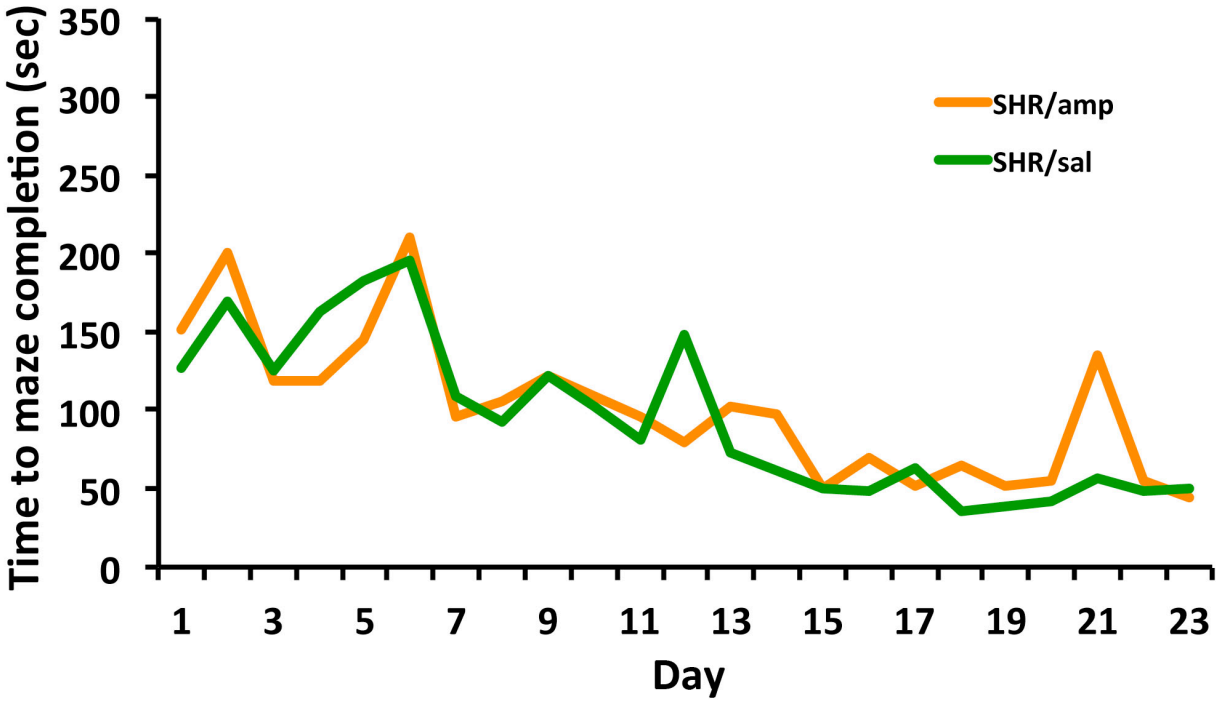

Figure 3. Performance of SHR animals in the radial arm maze was quantified by the time it took the rats to retrieve all of the food rewards.

Figure 4. The effects of amphetamine on radial arm maze performance in the SHR model of ADHD vs. age/strain matched controls: Number of reference memory errors

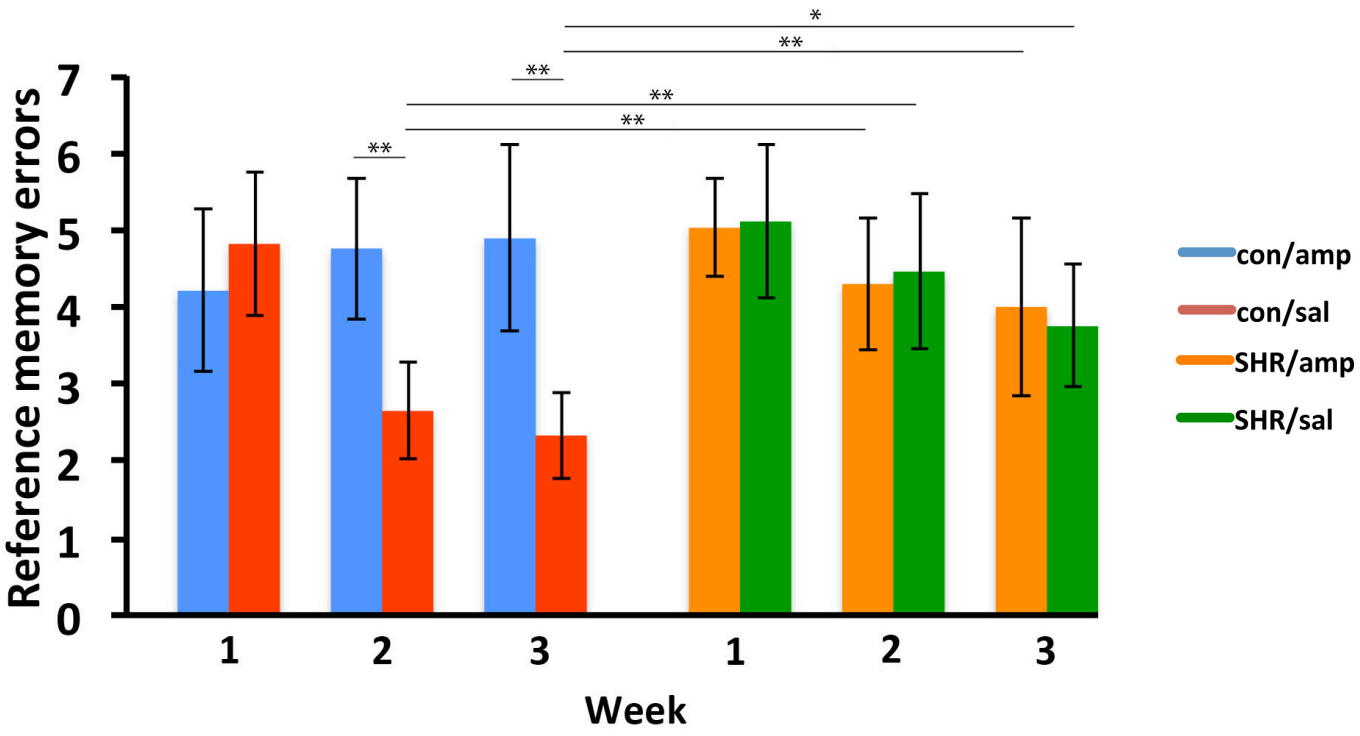

Figure 4. Average number of reference memory errors was quantified for each group during each week of training. One-way ANOVAs revealed significant differences between groups on week one, ($F(3,31)=9.556$, $p<0.01$), and week two ($F(3,31)=10.19$, $p<0.01$). A post hoc multiple comparison Tukey HSD test suggested that acute dose amphetamine administration in age/strain matched control animals hindered maze performance by the second and third week compared to age/strain matched controls receiving saline administration. Age/strain matched controls receiving saline administration also appeared to commit fewer reference memory errors than SHR animals receiving saline administration by the second and third weeks.

Figure 5. The effects of amphetamine on radial arm maze performance in age/strain matched controls: Number of reference memory errors

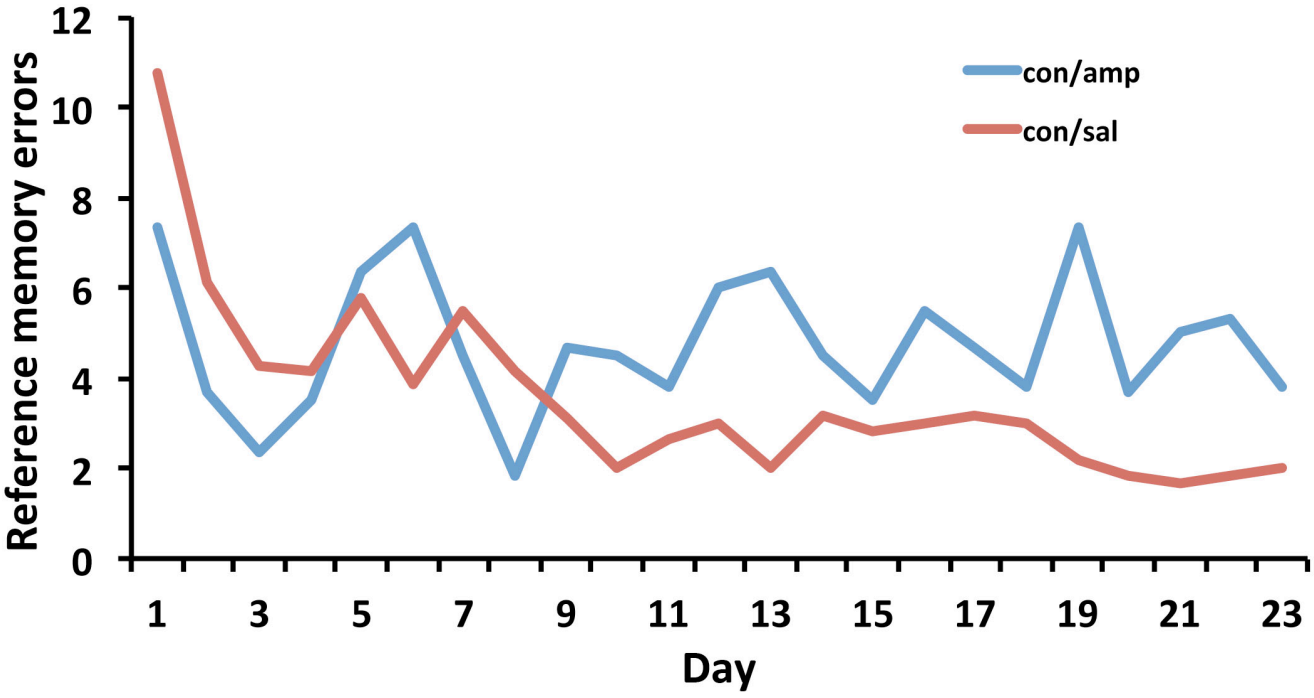

Figure 5. Performance of age/strain matched control animals in the radial arm maze was quantified by the number of reference memory errors made before retrieving all of the food rewards.

Figure 6. The effects of amphetamine on radial arm maze performance in SHR model of ADHD animals: Number of reference memory errors

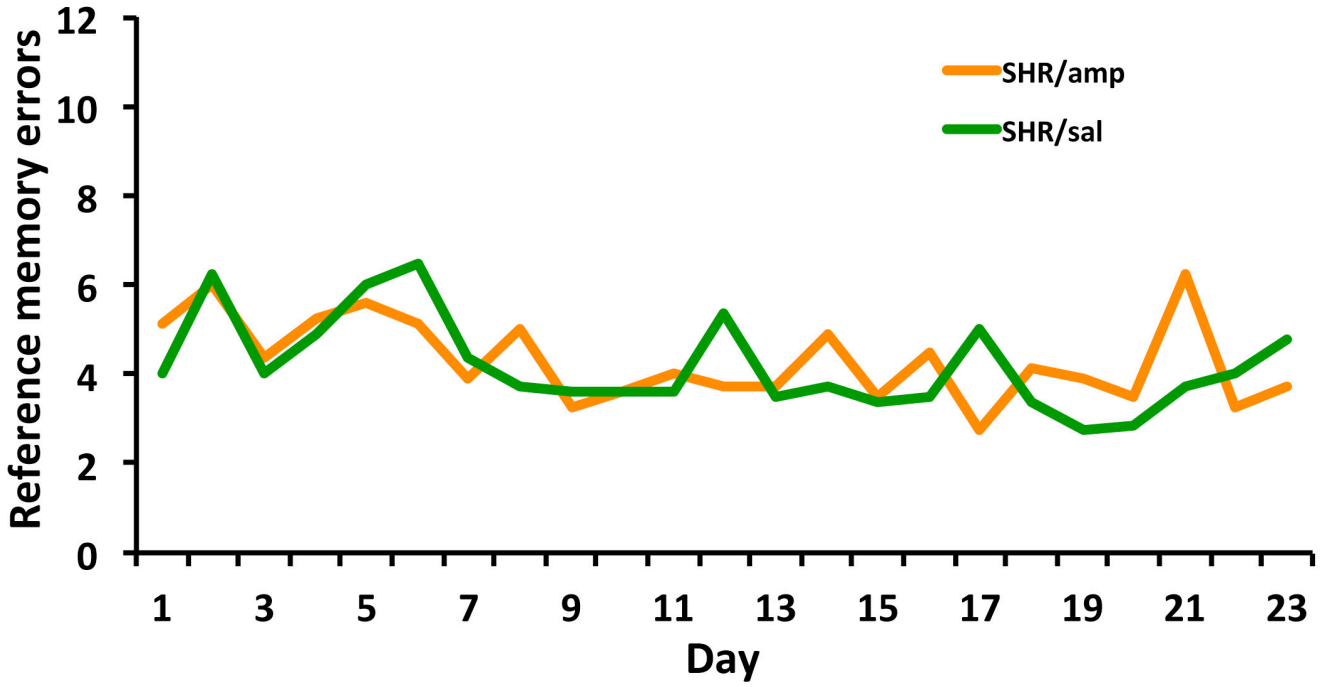

Figure 6. Performance of SHR animals in the radial arm maze was quantified by the number of reference memory errors made before retrieving all of the food rewards.

Figure 7. The effects of amphetamine on radial arm maze performance in the SHR model of ADHD vs. age/strain matched controls: Number of working memory errors

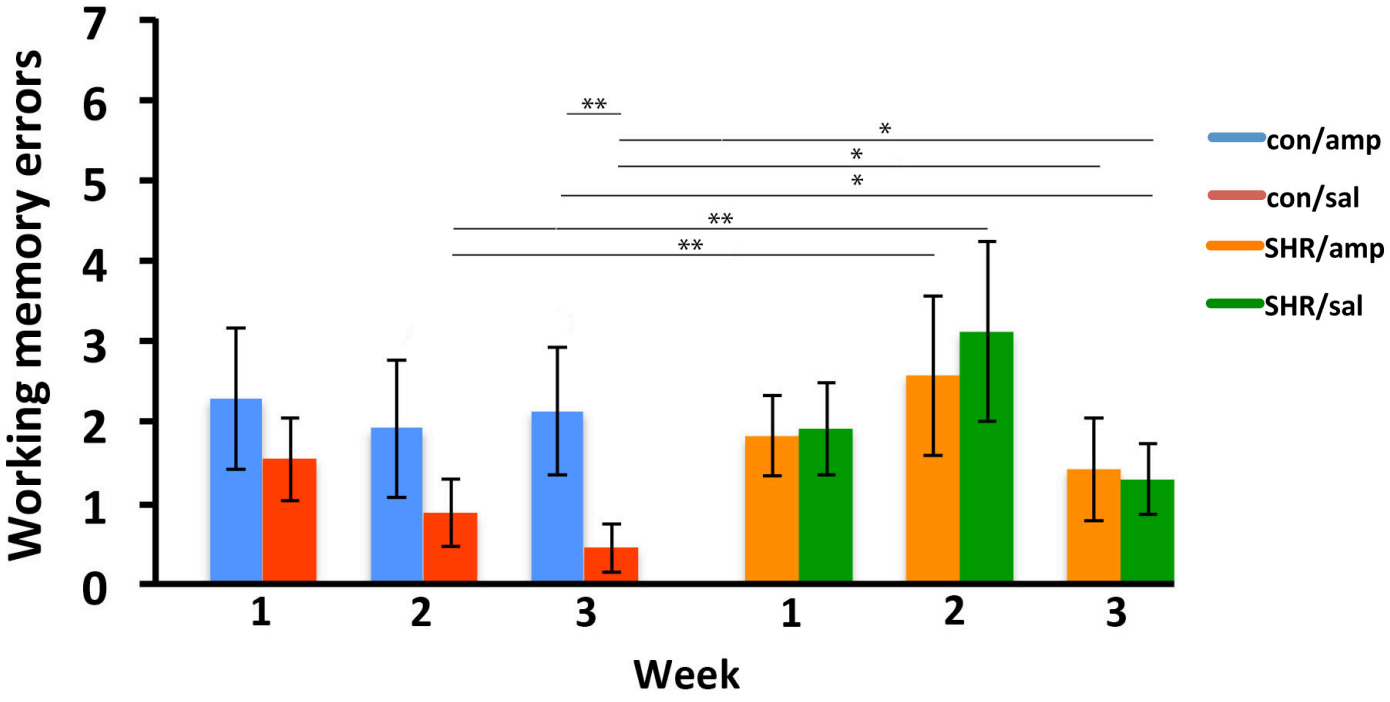

Figure 7. Average number of working memory errors was quantified for each group during each week of training. One-way ANOVAs revealed significant differences between groups on week two, $F(3,31)=9.486$, $p<0.01$, and week three, $F(3,31)=11.63$, $p<0.01$. A post hoc multiple comparison Tukey HSD test suggested that acute dose amphetamine administration in age/strain matched control animals hindered maze performance by the second and third week compared to age/strain matched controls receiving saline administration. Age/strain matched controls receiving saline administration also appeared to commit fewer working memory errors than SHR animals receiving saline administration by the second and third weeks.

Figure 8. The effects of amphetamine on radial arm maze performance in age/strain matched controls: Number of working memory errors

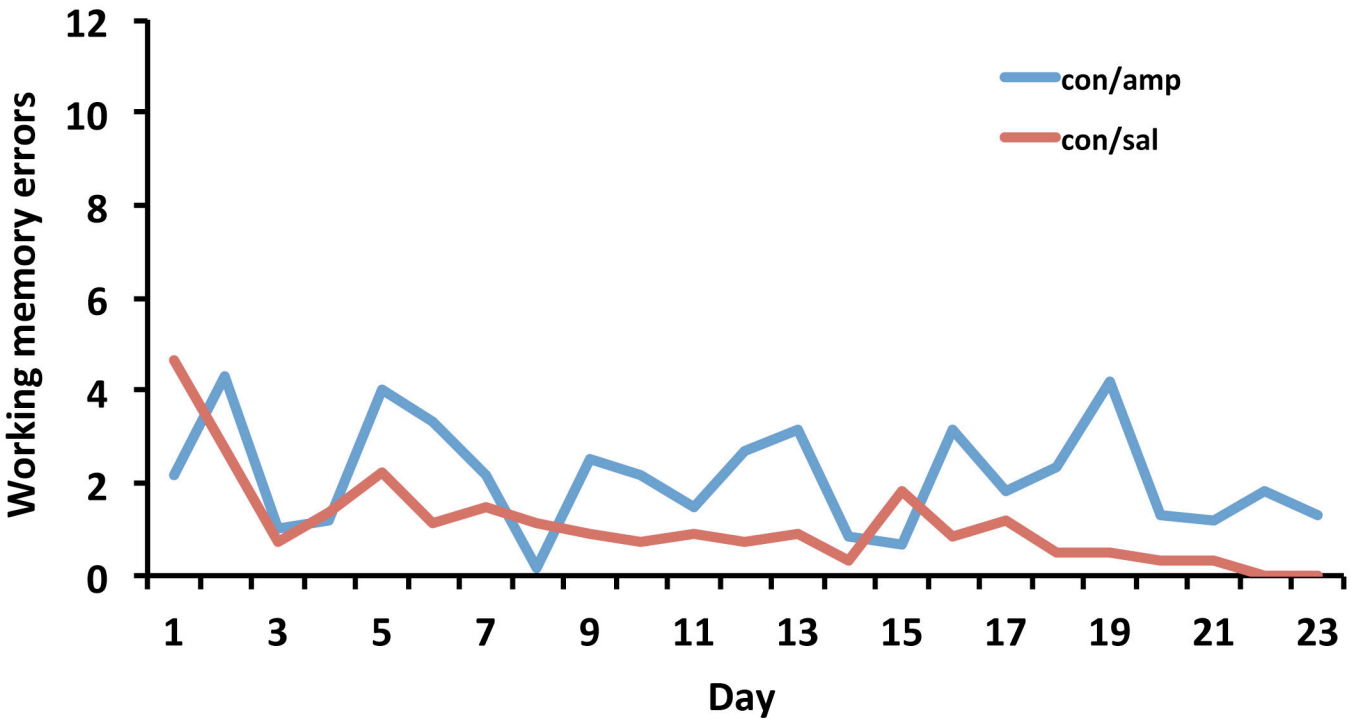

Figure 8. Performance of age/strain matched control animals in the radial arm maze was quantified by the number of working memory errors made before retrieving all of the food rewards.

Figure 9. The effects of amphetamine on radial arm maze performance in SHR model of ADHD animals: Number of working memory errors

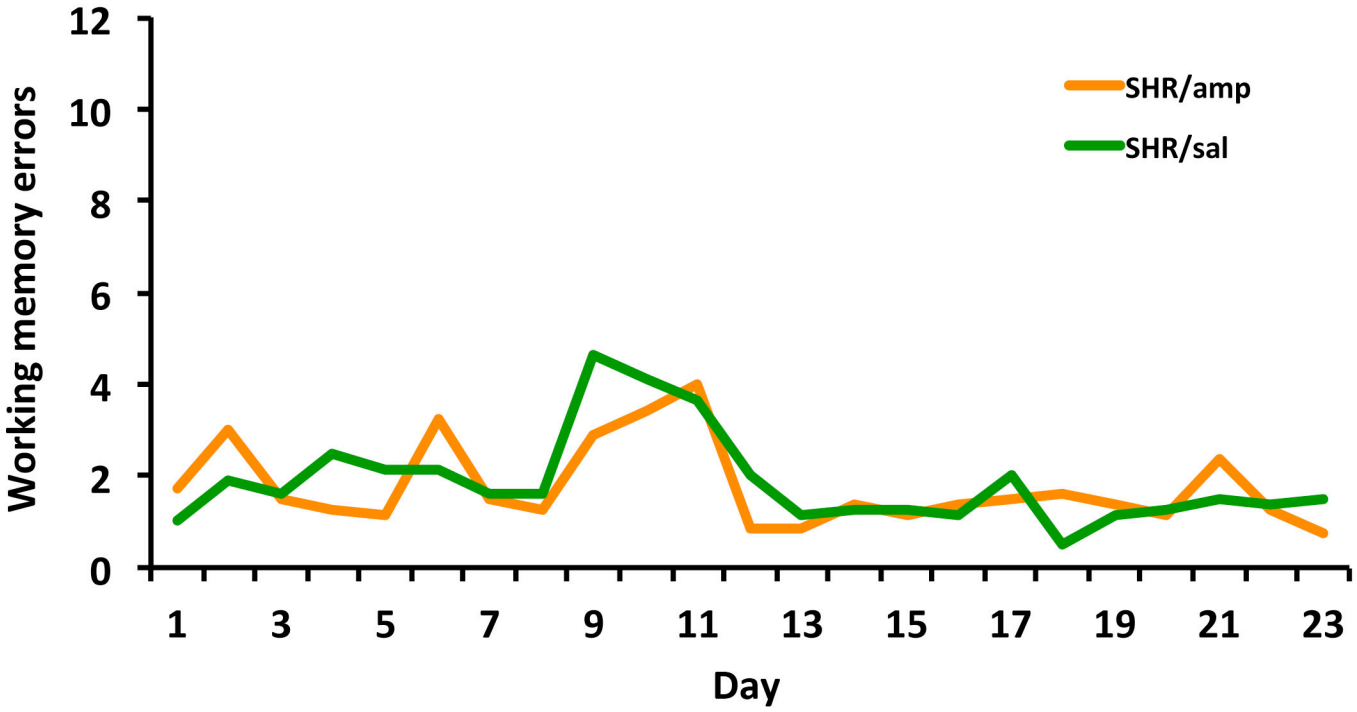

Figure 9. Performance of SHR animals in the radial arm maze was quantified by the number of working memory errors made before retrieving all of the food rewards.