ABSTRACT

Title of Thesis:	THE EFFECT OF DIETARY TARTRAZINE ON BRAIN DOPAMINE AND THE BEHAVIORAL SYMPTOMS OF ATTENTION DEFICIT HYPERACTIVITY DISORDER
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Attention Deficit Hyperactivity Disorder is a neurodevelopmental disorder correlated with a decrease in brain dopamine and an increase in behavioral symptoms of hyperactivity and impulsivity. This experiment explored how tartrazine (Yellow #5) impacts these symptoms. After tartrazine administration to Spontaneously Hypertensive Rats (SHR), dopamine concentrations in regions of brain tissue were measured using Enzyme-Linked Immunosorbent Assay analysis. Behavioral testing with a T-maze and open field test measured impulsivity and hyperactivity, respectively. Results indicate that dietary tartrazine increases hyperactive behaviors in the SHR. However, results do not indicate a relationship between dietary tartrazine and brain dopamine. No conclusions regarding the relationship between dietary tartrazine and impulsivity were drawn.

THE EFFECT OF DIETARY TARTRAZINE ON BRAIN DOPAMINE AND THE BEHAVIORAL SYMPTOMS OF ATTENTION DEFICIT HYPERACTIVITY DISORDER

by

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Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is the most prevalent neurodevelopmental pediatric disorder in the United States, affecting approximately 5% of all school-aged children (Wilens, 2003). ADHD is characterized by symptoms of impulsivity, hyperactivity, and behavioral inhibition (Purdie et al., 2002). Furthermore, individuals with ADHD appear to have abnormal levels of the neurotransmitter dopamine and dopamine-related transporters and receptors (Volkow et al., 2009). In the last decade, there has been a sudden rise in the number of documented cases of ADHD. This is partially due to the appearance of symptoms in varying degrees of severity and modifications in the standards used for diagnosing ADHD. (Purdie et al., 2002)

The increase in the diagnoses of ADHD is attributed in part to its history of misdiagnosed cases. In the past, many children who demonstrated symptoms of excessive impulsivity, hyperactivity, or behavioral inhibition would receive disciplinary action instead of an assessment to test for possible neurodevelopmental disorders such as ADHD (Meschan & Earls, 2005). As parents and teachers have become more aware of ADHD symptoms there has been a trend to seek diagnosis and pre-emptive evaluation sooner. Additionally, the number of diagnoses among boys is significantly higher than those among girls (Purdie et al., 2002). This difference may be due to the lack of the disruptive nature of the behavioral symptoms associated with ADHD among girls. In diagnosed cases of ADHD in males, these disruptive

behavioral symptoms are more prevalent, causing the discrepancy in diagnoses (Meijer et al., 2009).

The diagnosis process is typically very long, tedious, and inconsistent. Despite its high prevalence as a common disorder, ADHD has no cure, and methods of treatment are simply targeted at reducing or temporarily eliminating symptoms (Fan & Hess, 2006). These treatments include behavioral therapy, parental interventions, and educational modification (Purdie et al., 2002). However, the most prevalent and immediate treatment of ADHD symptoms is pharmacotherapy—the use of psychostimulants to reduce excess motor activity and enhance concentration (Fan & Hess, 2006). The most common psychostimulants are methylphenidate (common name Ritalin) and amphetamine (common name Adderall; Fan & Hess, 2006).

Though pharmacotherapy has proven to be effective in the treatment of ADHD, many patients experience negative side effects of these drugs, including severe insomnia, appetite suppression, irritability, anxiety, temporary depression, and dizziness (Efron et al., 1997). Many pediatric patients are not treated with these medications due to the risk of side effects (Meijer et al., 2009). Additionally, pharmacotherapy treatments are ineffective in as many as 25% of patients (Niederhofer, 2010). Consequently, doctors and parents are seeking alternative therapies that provide patients with long-term relief from ADHD symptoms.

The relationship between diet and ADHD symptoms is one possible target of non-pharmacological therapy. Certain food additives, such as artificial food coloring, have caused or heightened the symptoms of behavioral disorders, including ADHD, in some individuals (Tuormaa, 1994). The specific food additive studied in this

experiment is tartrazine, commonly known as Yellow #5. This study explores relationship between dietary tartrazine intake and the biochemical and behavioral symptoms of ADHD.

Team ATTENT completed a multi-phase study to evaluate the effects of dietary tartrazine on ADHD-associated behaviors, hyperactivity and impulsivity, as well as to determine its effect on brain dopamine concentrations in rats. The chosen rats for this study were Spontaneously Hypertensive Rats (SHR), which are characterized by symptoms of inattentiveness, hyperactivity, and impulsivity, making them an ideal rat model for studying ADHD (Pires et al., 2010).

The experiment was initiated with a thirty-day period of daily tartrazine administration through the rats' diets. Rats were divided into four experimental groups—control, low dosage, medium dosage, and high dosage—with different dietary tartrazine concentrations for each group. Beginning on the 26th day of the treatment period, ADHD-associated behaviors were gauged using an open field test and a T-maze for four consecutive days. These tests were conducted to look for behavioral differences between experimental groups.

On the 30th day of tartrazine administration, the rats were euthanized, and their brain tissue underwent biochemical analyses. This phase of the experiment sought to answer the question: How does increasing dietary tartrazine impact the expression of dopamine in the brain? Brain tissue and blood were collected from each rat. An Enzyme-linked Immunosorbent Assay (ELISA) was used to estimate the amount of dopamine found in the various brain quadrants. It was hypothesized that

increased dietary tartrazine increases expression of ADHD symptoms and decreases neural expression of dopamine.

Literature Review

Dopamine

Dopamine is a monoamine neurotransmitter that is released by interneurons and has both regulatory and modulatory effects. In the formation of dopamine, tyrosine hydroxylase (TH) adds a hydroxyl group to tyrosine, forming L-3,4-dihydroxyphenylalanine (L-DOPA). L-DOPA is then converted by DOPA-decarboxylase to form dopamine. Dopamine can then be converted to norepinephrine through the addition of a hydroxyl group on the β-carbon when catalyzed by dopamine β-hydroxylase. Norepinephrine can later be used to synthesize epinephrine when catalyzed with phenylethanoloamine (Kuhar et al., 1999). Dopamine generally acts upon five distinct classes of receptors. D1 receptors activate the enzyme adenylate cyclase. This enzyme forms cAMP, generating a secondary messenger cascade when dopamine acts on the receptor. D5 receptors also increase the concentration of cAMP through other methods (Wu et al., 2012). D2 receptors inhibit adenylate cyclase, thus inhibiting the secondary messenger cascade (Snyder, 2011). D3 and D4 receptors decrease the concentration of cAMP in the cell (Wu et al., 2012). The dopamine transporter, which is also referred to as the dopamine active transporter, is the protein that is responsible for transporting dopamine from the synapse into the cytosol. Therefore, the level of dopamine that is present at the synapse between neurons and at the receptors of postsynaptic neurons is regulated through the dopamine transporter (Ciliax et al., 1995).

Dopamine acts in three distinct systems that have varied effects on an individual's behavior. These systems include the nigrostriatal system associated with motor function, the mesolimbo-cortical system associated with the reward circuit, and lastly the tuberinfundibular system associated with hormone secretion (Keltikangas-Jarvinen & Salo, 2009). Extensive research has been conducted in the nigrostriatal system after it was discovered that a depletion of dopamine is commonly found in the caudate nucleus of Parkinson's disease patients (Snyder, 2011). Research has also been conducted to understand the link between dopamine and the efficacy of anti-psychotic drugs. These drugs generally block dopamine reuptake and have been considered most efficient when only blocking D2 receptors (Snyder, 2011).

Recently, dopamine has been implicated in the pathology of ADHD. Since many ADHD patients have an altered sensitivity to reward and generally do not modify their behavior when rewards change, it has been hypothesized that ADHD is due to a disruption in the reward circuit (Volkow et al., 2009). Dopamine is released in this circuit when the individual receives an unexpected award, thus allowing the individual to learn and modify behavior in order to continue receiving the reward (Schultz, 2002). Furthermore, drug addiction is mediated by an increase in dopamine transmission (Schultz, 2002). For individuals with ADHD, there is a decrease in D2/D3 receptors as well as dopamine availability in the mesolimbo-cortical system – more specifically in the left ventral caudate, accumbens and midbrain regions (Volkow et al., 2009). The decrease in dopamine is correlated with a rise in ADHD related symptoms, including inattention (Volkow et al., 2009). Other dopamine-

related symptoms of ADHD include a decrease in working memory and motor control, as well as an increase in internalization of speech (Levy & Swanson, 2001).

Genetic studies have shown that there might be a link between dopamine associated genes and ADHD. Polymorphisms in at least two dopamine genes, *drd*4 and *dat*1, are highly associated with ADHD (Levy & Swanson, 2001). A highly polymorphic dinucleotide repeat of dopamine gene *drd*5 has also been associated with ADHD, and hyperactivity has been correlated with a certain variation of dopamine gene *drd*2 (Wu et al., 2012). Additionally, a branch of research has determined that there are physical differences between the brain of an individual with ADHD and one without ADHD. This difference affects dopamine transport in specific areas of the brain, including the frontal cortex, basal ganglia, and striatal system (Levy & Swanson, 2001). Furthermore, there is a high comorbidity between Th1- and Th2- mediated disorders. Mice deficient in Stat-6, a transcription factor in Th2 cells, demonstrate an increase in hyperactivity as well as a decrease in striatal dopamine transporter, both of which are common symptoms of ADHD (Verlaet et al., 2014). Dopamine has been heavily implicated in ADHD regarding a variety of factors ranging from an individual's genetics to immune system. Although the extent of how each of these factors affects ADHD is still unknown, it is clear that ADHD is correlated to a decrease in neural dopamine.

Many of the drugs that are currently used to treat ADHD focus on increasing the production of dopamine. ADHD medication inhibits either dopamine or norepinephrine reuptake, thus increasing the amount of available dopamine in the brain (Tripp & Wickens, 2012). Specific norepinephrine reuptake inhibitors can

selectively increase dopamine concentrations in areas such as the prefrontal cortex, which is involved in the reward circuit, without affecting dopamine levels in the entire brain (Tripp & Wickens, 2012).

The link between tartrazine, dopamine, and ADHD is not the only instance in which nutrition has been shown to influence ADHD symptoms. For example, the A1 allele, which codes for D2 dopamine receptor Taq 1A, is associated with Type 2 Diabetes (Barnard et al., 2009). ADHD and binge eating are comorbid, and many of the medications that are used to treat ADHD are also effective in modulating abnormal eating practices (Cortese et al., 2007). Additionally, there have been multiple studies showing high-fat diets have resulted in an increase in expression of many of the genes that regulate dopamine (Lee et al., 2010) such as D2 receptors (South & Huang, 2008).

The process of studying the effects of dopamine on ADHD in human brains requires a combination of neuroimaging and behavioral tests. In one study, researchers took positron emission tomography (PET) scans of participants focusing on dopamine receptors and areas of high dopamine concentrations (Volkow et al., 2009). Functional magnetic resonance imaging (fMRI) and magnetic resonance imaging (MRI) are also common techniques to determine brain function as well as areas of high dopamine concentration (Volkow et al., 2009). In order to rate the behavioral symptoms of ADHD, researchers often score individuals based on DSM-IV guidelines or use questionnaires such as the Strengths and Weaknesses of ADHD-symptoms and Normal-behavior (SWAN) scale (Volkow et al., 2009). Researchers can also observe specific behaviors through a series of tasks including the "stop and

go" paradigm where participants are given a task and then told to stop in order to test the participant's inhibitory control (Levy & Swanson, 2001). By using a combination of measures, researchers correlate differences in dopamine and its receptors to behavioral symptoms of ADHD.

Studying dopamine in animal models is essential for researchers to draw inferences about dopamine and its effects on ADHD. One rat model used to study ADHD is the dopamine transporter knockout mouse (DAT-KO), which exhibits symptoms of ADHD such as hyperactivity. In another study, rats of a different ADHD model were given one of three different dosages of intranasal dopamine and then asked to navigate a radial maze (Ruocco et al., 2009). The results showed that the highest dose of dopamine reduced hyperactivity, while the intermediate dose of dopamine led to increased attention (Ruocco et al., 2009). Rats have also been used to display that a low density of D5 dopamine receptors in the hippocampus are highly correlated to some of the learning difficulties that are observed in ADHD patients (Medin et al. 2013). Common rat models of ADHD, including the SHR and Wistar-Kyoto (WKY) rats, exhibit increased dopamine uptake providing evidence to suggest connections between dopamine and behaviors associated with ADHD (Miller et al., 2012).

Dopamine-rich Areas of the Brain

The quantification of dopamine requires knowledge of the locations of dopamine-rich areas in the brain. These areas will be relatively rich in dopaminergic neurons compared to other areas in the brain, as the neurons release dopamine as the

primary neurotransmitter. Although they correspond to less than one percent of total brain neurons, dopaminergic neurons play a significant role in the normal functions of various organ systems and are most notable for their role in mental and neurological disorders such as schizophrenia and drug addiction (Chinta et al., 2005). In the mammalian central nervous system, the major source of dopamine originates from dopaminergic neurons in the mesencephalon, or midbrain, which contains approximately 90 percent of the total number of brain dopaminergic cells (Chinta et al., 2005).

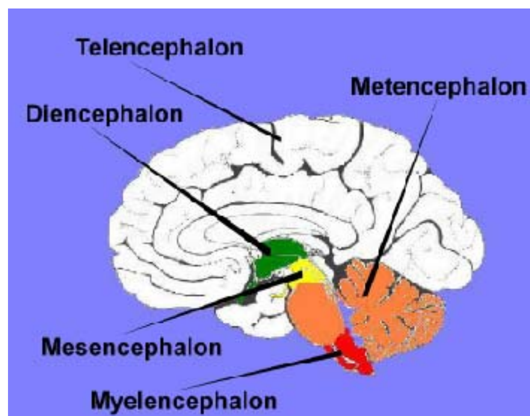


Figure 1: Regions of the brain (Rice University, 2000)

As a result, the study focuses on midbrain structures and their closely related projections to most representatively quantify dopamine in the whole brain. Also, the noted structures may provide the basis for potential hypotheses that attempt to explain the link between ADHD and decreased dopamine in the brain of patients. Structures in two dopaminergic pathways are of interest for this study due to their implication in dopamine-associated ADHD symptoms. The nigrostriatal pathway, which plays a role in controlling voluntary movement, originates in the substantia nigra whose neuronal fibers extend to the striatum (Chinta et al., 2005). The mesolimbic pathway, which plays a role in motivation, reward, and emotion based behavior, originates in

the ventral tegmental area (VTA) whose neuronal fibers project most prominently to the nucleus accumbens (Chinta et al., 2005). The four areas mentioned are of interest in order to quantify dopamine.

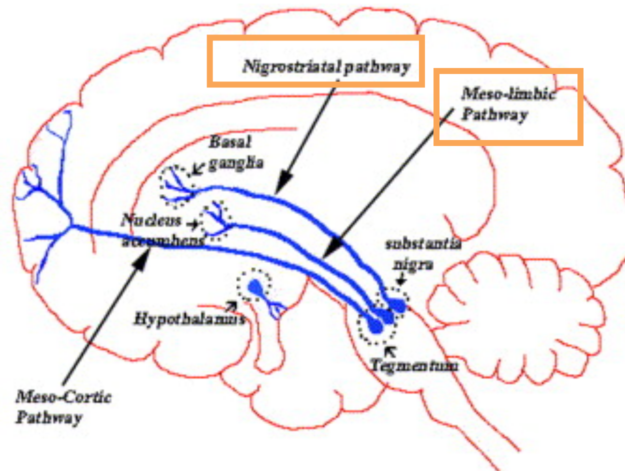


Figure 2: Dopaminergic pathways (Chinta et al., 2005)

Substantia Nigra

The substantia nigra is a large pigmented cluster of neurons located in the anterior midbrain in both hemispheres of the brain (Luijkx et al.). As a component of the extrapyramidal system, the substantia nigra is involved in the modulation and regulation of movement (Luijkx et al.). In the substantia nigra, dopaminergic neurons correspond to approximately three to five percent of the total neurons in the region (Chinta et al., 2005). Consequently, the substantia nigra also plays a role in reward and mood due to the prevalence of dopamine.

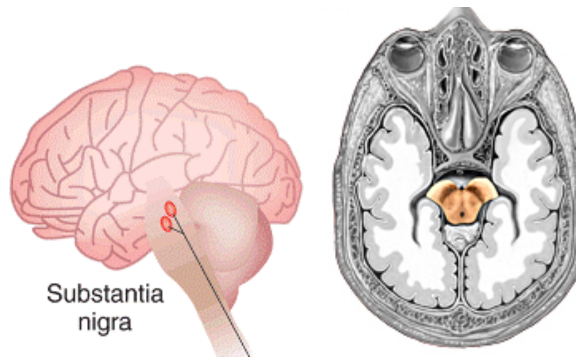


Figure 3: Lateral view (Speert, 2006) and aerial view (Medline Plus, 2015) of substantia nigra

There are two parts that make up the substantia nigra: the pars compacta and pars reticulata. The pars compacta consists of neurons that contain the dark pigment melanin and function primarily to synthesize dopamine and supply this dopamine to either the caudate nucleus or putamen, both of which are structures included in the striatum (Midbrain anatomy). By supplying dopamine to the striatum via the nigrostriatal pathway, the substantia nigra mediates movement and motor coordination. The pars reticulata conveys output signals to various other brain structures such as the thalamus (Midbrain anatomy).

The most well-known neurodegenerative disease linked to the substantia nigra is Parkinson's disease. Parkinson's disease is caused by the degeneration of dopaminergic neurons localized mostly in the substantia nigra pars compacta (Di Muzio et al.). Significant loss of dopaminergic neurons in the substantia nigra pars compacta will cause subsequent depletion of dopamine in the striatum. Without the relay of dopamine, Parkinson's disease presents as a movement disorder characterized by slowness of movement, tremor, rigidity, and loss of postural control (Chinta et al., 2005).

A lesser-known concept, however, is the role the substantia nigra may play in causing ADHD in children. Scientists have hypothesized that the nigrostriatal dopaminergic system is structurally altered in children with ADHD. In a study conducted in 2010, Romanos et al. investigated the echogenicity of the substantia nigra as a potential marker for the nigrostriatal dysfunction in children with ADHD. Echogenicity is the extent to which a structure is able to give off reflections of ultrasonic waves, or the extent to which a structure is able to appear in an ultrasound image (Dorland's Medical Dictionary, 2007). Using transcranial sonography, researchers used an ultrasound device to view and then manually circle the outer circumference of the substantia nigra in 22 children with ADHD and 22 healthy controls. After quantification of an echogenic area, researchers found that the substantia nigra area was significantly larger in ADHD patients than in healthy controls. Normally, echogenicity of the substantia nigra shows a gradual postnatal decline; thus, the increased echogenicity in children with ADHD implicates a developmental delay in this region. In addition, the increased echogenicity of the substantia nigra has been associated with impaired uptake of dopamine. Overall, this evidence supports the notion that in children with ADHD, there are structural and functional maturational delays in specific brain regions related to dopamine activity.

Striatum

The striatum is a cluster of structures that lies deep in the subcortical region of the forebrain and is a component of the interconnected grey matter in the brain called the basal ganglia. Functionally, the striatum serves as an integration center for dopaminergic and glutamatergic inputs coming from various regions of the brain such

as the midbrain (where the substantia nigra lies), the thalamus, and the cortex (Baez-Mendoza et al., 2013). After integrating multiple inputs, the striatum then relays this information to other structures of the basal ganglia where actions such as learning and movement are mediated (Baez-Mendoza et al., 2013). The striatum is a necessary neuronal circuit for voluntary movement control, but it is also a critical component of the reward system, specifically in social situations.

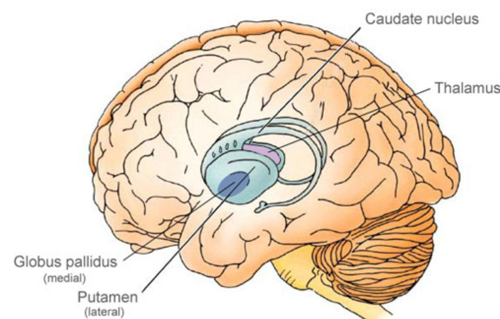


Figure 4: Lateral view of striatum location (Fazzari)

The striatum is further divided into the dorsal and ventral striatum. The dorsal striatum consists of the caudate nucleus and putamen and the ventral striatum consists of the nucleus accumbens (Knierim, 1997). As mentioned before, the caudate nucleus and putamen are primary receivers of dopamine from the substantia nigra; thus, the dorsal striatum is involved in motor function. The ventral striatum is primarily involved in reward cognition (Knierim, 1997).

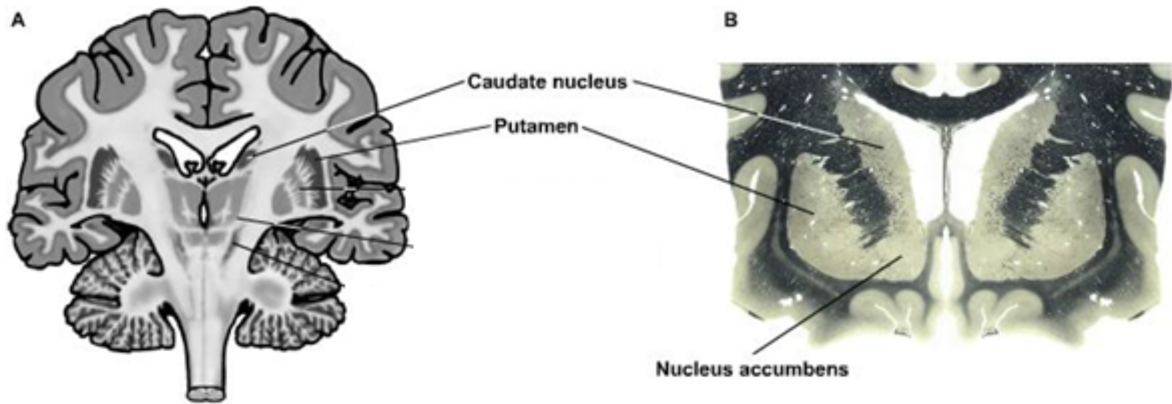


Figure 5: Transverse slice of brain with striatal structures (Knierim, 1997)

Similar to the substantia nigra, the striatum also shows abnormal function in children with ADHD. Few studies exist that address ADHD and its connection to reward centers in the brain. In a study conducted in 1989, Lou et al. researched cerebral blood flow distribution between patients with ADHD and healthy controls. The study concluded that striatal regions were hypoperfused and, by extension, hypofunctional because of the lack of circulating oxygen reaching the region. While hypofunction of the striatum is an accepted aspect of ADHD, a more recent study conducted by Carmona et al. in 2009 investigated the structural aspect of the striatum in patients with ADHD. The researchers compared the volumetric difference between the ventral striatum in 42 children with ADHD and 42 healthy control subjects matched by factors such as age, gender, and handedness. The study revealed that ADHD patients had lower ventral striatum volumes than their control counterparts, showing an average of a 25.28% decrease in the volume of the left and right striatal regions after being corrected for total blood volume. The decrease in ventral striatum volume also correlated with poor symptoms of hyperactivity and impulsivity. Both studies mentioned above give more insight into the pathophysiology of ADHD.

Ventral Tegmental Area

The ventral tegmental area, or VTA, is a dense cluster of structures located close to the substantia nigra within the midbrain. The region is a part of the mesolimbic and mesocortical pathways. The mesocortical pathway connects the VTA to the cortical areas in the brain's frontal lobes. The mesolimbic pathway, of interest to this research, connects the VTA and the nucleus accumbens (Russo & Nestler, 2013). This pathway is part of a reward circuit. Dopamine is released from the VTA and travels to the nucleus accumbens in response to reward or aversion-related stimuli. Pleasurable activities and psychostimulant drugs stimulate this region of the brain and make it especially relevant to the study of addiction (Center for Bioinformatics).

Prior research suggests that the neurons within the VTA play an important role in learning and motivation through their communication with the nucleus accumbens, prefrontal cortex, and amygdala (Saunders & Richard, 2011). A current clinical study at Duke University is examining VTA activation and goal-directed motivation through a series of magnetic resonance imaging (MRI) sessions. The study links low VTA activation with aggravated symptoms of ADHD and attempts to lessen these symptoms and improve motivation through non-medication intervention (Kollins & Itchon-Ramos, 2016).

Nucleus Accumbens

The nucleus accumbens is a cluster of neurons located in the forebrain. This region of the brain plays an important role in reward, addiction, and pleasure. Located at the end of the mesolimbic pathway, the accumbens receives dopamine

from the ventral tegmental area. Other inputs to the nucleus accumbens include the prefrontal cortex, amygdala, and hippocampus (Center for Bioinformatics).

The terminals between the VTA and nucleus accumbens are the action site for drugs such as cocaine and amphetamine. Because addictions to food, sex, and drugs are implicated with dramatic increases in dopamine, the nucleus accumbens is essential to understanding the neurochemical mechanisms behind addiction (Center for Bioinformatics). One study conducted using a rat model discovered that impulsivity significantly increased when the nucleus accumbens was altered or damaged. Impulsivity was induced in the rats by creating lesions in the core of the nucleus accumbens (Cardinal et al., 2001).

Scientists at Universitat Autonoma de Barcelona examined the brains of children with ADHD using magnetic resonance imaging (MRI). The scans revealed anomalies in the brain's reward system in the ADHD brains compared to the normal brains. Children with ADHD exhibited reduced volume in the nucleus accumbens region and these differences were associated with hyperactivity, impulsiveness, and deficiencies in motivation (Nauert, 2015).

Circadian Control of Dopamine

The superchiasmatic nuclei (SCN) located at the base of the hypothalamus is the main circadian pacemaker in mammalian brains (Mendoza & Challet, 2014). The SCN is synchronized to solar time, as light is transmitted by the retina the SCN will regulate the necessary hormonal and nervous pathways. One of these pathways controls the circadian rhythm of dopaminergic activity. The activated SCN will act through the orexinergic (ORX) and medial preoptic nucleus (MPOA) neural pathways

to activate dopamine production in the VTA and substantia nigra. The regulation of dopamine in the brain is thus affected by the light/dark cycles in human environment. Dopamine will then impact motor activity and motivation.

In a study by O'Neill and Fillenz (1985), the pattern of dopamine release in the frontal cortex, striatum, and nucleus accumbens was monitored. It was found that there was no correlation between the amount of dopamine released and time of day in the frontal cortex as there is little feedback regulation of the dopamine release. However, dopamine release in both the nucleus accumbens and striatum is highly regulated by feedback mechanisms. As rats are nocturnal there was a significant rise in dopamine release at night (O'Neill & Fillenz, 1985).

Rat Model

The rat model used in this study is the SHR model, which is frequently used to emulate ADHD in a laboratory setting. This model is helpful in studying ADHD because the rats mirror many of the neurochemical and behavioral symptoms found in human ADHD (Pires et al., 2010). In addition, the SHR often exhibits cognitive difficulties when performing tasks in a manner that is consistent with ADHD. Because of the rat's natural tendency to demonstrate cognitive impairment, the SHR model is of interest in studies involving the reduction of ADHD symptoms to improve mental functioning (Pires et al., 2010).

In one study, the SHR model and a close genetic strain, the Wistar Kyoto (WKY) rat model were both tested to determine if long-term caffeine treatment during prepubescence would improve cognition in later stages of life (Pires et al., 2010). The SHR model exhibited many cognitive deficiencies prior to treatment, but

after caffeine treatments, these symptoms improved. The WKY rat model, however, did not have many cognitive difficulties before treatment, but after caffeine treatment, the WKY rats exhibited impaired ability to discriminate between objects and complete tasks (Pires et al., 2010). The authors suggested that the WKY rat model would be a better indicator of a non-ADHD population, while the SHR model could be used to replicate ADHD in an experiment. A young SHR of around 3-4 weeks of age would also exhibit symptoms found in juvenile ADHD (Pires et al., 2010).

The use of the SHR as a model of ADHD is supported by several other studies, one of which looked at the effects of microdialysis treatment for ADHD on both SHR and Sprague-Dawley (SD) rats (Heal et al., 2008). SD rats were previously used as the basis for ADHD in various studies of pharmacological drugs. The SHR was used in this study due to the fact that hyperactivity and impulsivity develops prior to the hypertension that is observed around ten weeks of age and older. However, it should be noted that ADHD-like symptoms in the SHR rat persist through maturity (Heal et al., 2008). The SHR rats were shown to exhibit the dopaminergic phenotype of ADHD in humans better than the SD rats (Heal et al., 2008). Another study that concluded that the SHR model is the only one that accurately models the behavioral symptoms of ADHD including increased hyperactivity and attention deficits (Sagvolden, 2000). Heal et al.'s (2008) study is just one of many, such as Pires et al.'s (2010), that supports the decision to use the SHR by providing evidence that the ADHD-like symptoms displayed in the SHR phenotype are effective in modeling true ADHD.

The SHR also demonstrates similar neural dopamine patterns as do humans with ADHD. When compared to other ADHD rat models, there seems to be increased levels of dopamine in the synaptic cleft of SHRs (Viggiano, 2004). There is also an increase in D2-mediated inhibition of dopamine release in the striatum (Oades et al., 2005). It has also been discovered that D1 and D2 receptors are overexpressed in the striatal and frontal regions of the SHR (Russel, 2002). Recently, an overexpression of dopamine transporter (DAT) was found in the caudate nucleus and striatum of the SHR than the WKY rat model (Miller et al., 2012). Although there is an increase in DAT, these transporters are hypofunctional, resulting in a decrease in dopamine reuptake (Viggiano, 2004). The reduction of dopamine reuptake by DAT has also been linked to reduction in actual dopamine release from axons terminals (Viggiano, 2004).

There has also been past experimentation on SHR through which researchers have been able to manipulate brain dopamine in the rats based on the addition of ADHD treatment options. Researchers have given SHRs amphetamines and methylphenidates, both drugs commonly prescribed as ADHD medication, and they discovered an increase in dopamine in the rats' brains (Carboni et al., 2003). Specifically, there was a significant increase in extracellular dopamine in the nucleus accumbens (Carboni et al., 2003). Since amphetamines increase dopamine release while methylphenidates block dopamine reuptake, it is evident that both drugs increase extracellular dopamine concentrations (Carboni et al., 2003). As a result, the SHR's reaction to the drugs is consistent with how human ADHD patients would react. On the other hand, when SHR were dosed with caffeine, the rats showed an

improvement in memory and attention deficits and displayed normalized dopamine levels (Pandolfo et al., 2013). There was a decrease in DAT function in the frontal cortex, which led to higher extracellular dopamine concentrations (Pandolfo et al., 2013). Although caffeine is still not a treatment used in humans, the inverse relationship between ADHD symptoms and neural dopamine concentration found in the rats is similar to the relationship evident in humans. The rat models that were used in this study were rats that modeled ADHD behaviors as opposed to a typical wildtype lab rat, or one that behaved "normally". This is because the study focused on how the ADHD symptoms worsened in the rat, rather than inducing the ADHD symptoms in the rats.

Food Additives

This experiment sets out to test the hypothesis that certain food additives will negatively affect the symptoms of ADHD, thus helping identify a specific connection between the diet and neurobiological and behavioral symptoms of ADHD. As a result, specifically looking at the effects of food additives on the symptoms of ADHD can help determine the potential benefits of a restriction diet.

Currently, there are about 3,794 different types of food additives used in our food, and on average, 200,000 tons of these food additives are used per year (Tuormaa, 1994). The increased reliance on food additives to preserve, color, and improve the taste of food leads to the estimate that "each person is now consuming an average 8-10 lbs. of food additives per year, with some possible eating considerably more" (Tuormaa, 1994).

A fundamental concept that supports the need for understanding the effects of food additives on ADHD is the Feingold Hypothesis. In 1973, Dr. Benjamin Feingold of the Department of Allergy at the Kaiser Permanente Foundation Hospital and Permanente Medical Group in San Francisco, proposed that hyperactivity in children stemmed from the use of food additives (Stevens et al., 2011). Feingold proposed, "low molecular weight compounds, like artificial food dyes, can produce behavioral disorders in susceptible individuals" (Tuormaa, 1994). This striking conclusion came from his studies on over 1,200 cases where he found that children who consumed certain chemicals, including particular food additives and natural salicylates, showed signs of hyperactivity and other neurophysiological disturbances. Approximately 30% to 50% of children who exhibited ADHD symptoms could be treated by restricting foods that contain certain food additives and natural salicylates (Tuormaa, 1994). Although Dr. Feingold's findings appear to show a very simple and straightforward correlation, there is still much to be learned and explored when it comes to the relationship between food additives and their effect on ADHD symptoms. Since Feingold's discovery, a multitude of diets have been developed, many of which, like the Kaiser-Permanente Diet (K-P Diet), recommend that those diagnosed with ADHD refrain from consuming natural salicylates, artificial food colorings (AFCs), and artificial flavors (Stevens et al., 2011). Even with its widespread use, there has been little evidence to show the definitive effectiveness of the K-P Diet. Approximately 11% to 33% of children responded to this with changes in ADHD symptoms, yet the biochemical cause of these changes is not understood (Stevens et al., 2011). Various countries have started to put sanctions on the types of

artificial food dyes that are permitted in food production. For example, the European Parliament banned the usage of tartrazine in food production in 2009 (European Food Safety Authority). Due to the increasing reliance on food additives, it is important to further explore the connection between food additives and behavioral disorders.

Tartrazine

Of the total 3,764 food additives, the food additive that will be the focus of this research is tartrazine, whose structure is shown in Figure 6. Tartrazine is a yellow food coloring that is common in the American diet. It is popular in processed foods such as candy, sports drinks, and soft drinks.

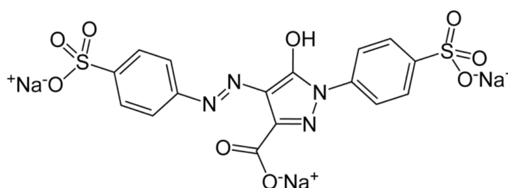


Figure 6: Chemical structure of tartrazine (Tartrazine)

Officially called E-102, Yellow #5 dye, tartrazine is a synthetic organic chemical that serves as a lemon yellow azo dye. This water soluble substance has the official chemical name: trisodium-5- hydroxy-1-(4-sulfonatophenyl)-4-(4-sulfonatophenylazo)-H-pyrazole-3-carboxylate (Gao et al., 2011). Due to the presence of an azo group—two nitrogen atoms double bonded to each other—tartrazine can be detrimental in high quantities (Gao et al., 2011). This nitrous compound is highly sensitizing in the body, causing hypersensitivity, allergic reactions, and mutagenesis (Moutinho et al., 2007). The acceptable daily intake (ADI) of tartrazine is between 0 - 7.5 mg/kg/day (Moutinho et al., 2007). However, it

is highly recommended that children only consume 37.2% of the maximum theoretical intake of 7.5 mg/kg/day (Elhkim et al., 2007).

The accepted daily intake (ADI) level of 7.5 mg/kg/day was established by performing dose-response studies in animal models. By administering varying doses of the substance to laboratory animals, scientists look for the smallest dose that causes any detectable effect on organs, behavior, or body chemicals (Foundation for American Communications). This level, called the Lowest Observable Effect Level (LOEL), is specific to the toxicity imposed on laboratory animals. Another measure scientists obtain from dose-response studies is the No Observable Effect Level (NOEL); this is the highest dose at which no effects occur (Foundation for American Communications). The NOEL is the "safe level" for a chemical in the specific species studied. The NOEL is the value of interest when determining the "safe level" for humans. In order to apply this value to humans, public health officials usually divide the NOEL by a safety factor, usually 100, to determine a "safe level" for humans (Foundation for American Communications). This factor accounts for differences humans may experience with regards to a human's sensitivity to the substance and variability in the genetics, health, and age of humans that may affect the response to the substance being studied. For example, the NOEL determined for tartrazine in dose-response studies in a laboratory animal would be 750 mg/kg/day; thus the "safe level" for humans translates to an ADI of 7.5 mg/kg/day. In general, it is thought that determining "safe levels" in this manner yields a level that is likely lower than the true NOEL for humans, but risk managers agree to use this value in regulatory policy.

While about only 2% of ingested tartrazine is directly absorbed by the body, most tartrazine is broken down into smaller metabolites in the colon (Elhkim et al., 2007). Tartrazine reduction is facilitated by intestinal bacteria that release electron carriers in the anaerobic environment of the colon (Elhkim et al., 2007). Extracellular electron acceptors in these conditions allow for azo dye reduction into nitrous metabolites such as aminopyrazolone and 4-hydrazinobenzenesulfonic acid which can be further reduced to sulfanilic acid (Elhkim et al., 2007). While parent tartrazine is absorbed at a much lower incidence, the body readily absorbs these metabolites. Consequently, it is believed that the metabolic byproducts of tartrazine are the substances causing sensitivity and adverse reactions in the body.

The harmful nature of tartrazine most notably stems from the chemical's transformation into an aromatic sulfanilic acid after being digested by gastrointestinal microflora (Moutinho et al., 2007). Sulfanilic acid is the main metabolite of tartrazine and its structure is also a nitrous derivative, meaning this byproduct equally contributes to the risks associated with consuming food colorings.

Most sulfanilic acid travels through the gastrointestinal system and is released in fecal matter. Smaller amounts of tartrazine, the parent molecule of sulfanilic acid, are released in feces. Electron receptors found in the body aid in the reduction of tartrazine into sulfanilic acid, facilitating improved metabolite excretion (Elhkim et al., 2007). In one study, sulfanilic acid was administered to postnatal rats via an intraperitoneal injection to examine its impact on behavior (Goldenring et al., 1982). Researchers observed hyperactivity and impairment of performance in shock escape activities in the sulfanilic acid rats. The research team refrained from extrapolating

the data to children or adult humans due to differences in the blood-brain barrier and absorption between the two species (Goldenring et al., 1982).

While the Food and Drug Administration (FDA) allows tartrazine in the United States, the European Parliament has banned tartrazine from all food production (Jacobson, 2010). Additionally, The Center for Science in the Public Interest (CSPI) and European food safety officials have warranted studies linking food dyes to childhood hyperactivity and behavioral problems as sufficient evidence to ban certain food dyes. The CSPI has urged the FDA to follow their ban on certain food dyes (Jacobson, 2010). Before the banning of tartrazine in European food, studies that compared the rates of ADHD diagnosis in the United States versus European countries concluded that the prevalence of ADHD in the US is not significantly higher than those in Europe (Faraone et al., 2003; Polanczyk et al., 2007). There has been no comparison test published after the ban.

Tartrazine may be relevant to ADHD studies, as it appears to have a zinc wasting effect in some hyperactive children (Stevens et al., 2005). In one notable study, scientists found that tartrazine increased urinary zinc excretion in children that were hyperactive when compared to a control group (Tuormaa, 1994). Interestingly, it has been proposed that zinc depletion is a cause of childhood hyperactivity since zinc can act as a cofactor in the metabolism of neurotransmitters (Tuormaa, 1994; Stevens et al., 2013). Zinc is also needed for the production of melatonin, which helps to regulate dopamine function (Arnold & DiSilvestro, 2005). This suggests that tartrazine consumption and dopamine expression may be linked.

There appears to be a gap in research regarding the biochemical or physiological mechanisms by which food additives affect ADHD symptoms. As a result, this study aims to track biochemical changes in the body in response to tartrazine ingestion and pinpoint these changes as possible causes of ADHD-like symptoms. While there is much speculation that food colorants such as tartrazine contribute to ADHD-like symptoms, the exact biochemical mechanism is not known. To reason with this ambiguity, this study took accepted premises about ADHD and food colorings to form the basis for research. As indicated before, it is suggested that dopamine levels are lower than expected in patients with ADHD. Since tartrazine may exacerbate ADHD symptoms, this study aims to study the relationship between tartrazine and dopamine.

Project Aims

This mixed methods study aims to assess a dietary aspect that may affect ADHD symptoms. The SHR is used to address the relationship between a nutritional factor, such as food additives, and neurochemicals in the brain that lead to an increase in ADHD symptoms. This information was compiled to perform a qualitative and quantitative experiment on rats. The experiment utilizes quantitative data in the measurement of biochemical results and qualitative data for behavioral and survey-based observations. This qualitative aspect aims to support calculated quantitative data by showing the effect of the biochemical aspect of the experiment on observed behavioral changes.

The study of ADHD and its associated treatments currently displays inconsistencies and aspects requiring increased research. The use of pharmacotherapy has demonstrated effectiveness in treatment for some patients, while others remain unaffected or negatively affected by these drugs. Furthermore, there is an exhibited desire among patients and parents of patients for alternative treatments to ADHD. In one study conducted, 68% of participants investigated complementary and alternative methods (CAM) to pharmacological treatments (Sinha & Efron, 2005). Many medical professionals also concur that alternative treatments would be best in supplementation to pharmacological treatments (Sinha & Efron, 2005). The research completed by this project can impact these missing components of ADHD treatment and management. Furthermore, the potential work with food additives of this project can contribute to methodologies used in the study of their

biochemical effects since not much work has been done on animal models. In summary, both the conceptual results of this project and the methodological techniques utilized can make an impact in the aspects of ADHD treatment and nutritional research that are lacking a strong database and foundation of knowledge.

Methodology

The review of literature pertaining to ADHD and nutrition led to Team ATTENT's research question regarding the biochemical and behavioral impacts of tartrazine consumption. The following methodology was used to support the hypothesis that dopamine levels will decrease in the SHRs, which will cause an increase in expression of the ADHD symptoms of hyperactivity and impulsivity with increasing concentrations of dietary tartrazine.

Housing and Care of Animals

The rats were housed individually in stainless steel grated metal cages. The room had 100% ventilation, receiving a constant supply of fresh air, and was kept at a constant 72 degrees Fahrenheit and 45% humidity. The room was kept at a standard 12 hour light/dark cycle with lights on at 0800 hours. Standard husbandry procedures such as changing bedding, collecting bodily waste as well as spillage, recording body weight and room temperature, and giving general health examinations occurred on a regular basis (Abou-Ismail et al., 2007). While these practices were necessary to keep the animals healthy, they could be stressful for rats and induce short-term changes in behaviors (Abou-Ismail et al., 2007). In order to mitigate these behavioral changes, and thus limit possible stressful effects that general care can have on experimental results, husbandry procedures were done during the light phase of the light/dark cycle (Abou-Ismail et al., 2007). Additionally, all procedures were

completed as quickly as possible to help mitigate the amount of stress to the animal. (Balcombe, Barnard, & Sandusky, 2004).

A total of forty six-week-old male SHRs were purchased from Hilltop Labs (Smalltown, PA). As shown in Table 1, a group of twenty rats (Group Alpha) was purchased first. After 7 days of acclimation, these rats were strategically separated into dosage groups in order to have a similar average weight among the groups. Each dosage group (control, low, medium, and high) consisted of five rats. The rats drank tap water and ate a standard diet *ad libitum*. Food intake and body weight was recorded daily throughout the duration of the study. Tartrazine administration began on the 8th day, and after thirty days of tartrazine administration, this group of twenty rats was euthanized.

A second group of twenty rats was obtained in one round of four rats (Group Beta) and two rounds of eight rats (Group Gamma and Group Delta). These rats were allowed to acclimate for seven days. Group Beta began training for impulsivity testing immediately following acclimation. Tartrazine administration was conducted for 30 days after the completion of training for Group Beta, and behavioral testing for both hyperactivity and impulsivity began on day 26, lasting for the final four days. At the conclusion of 30 days, these rats were euthanized to undergo biochemical testing. Groups Gamma and Delta were also allowed to acclimate for seven days. Then, tartrazine administration was conducted for 30 days, and hyperactivity testing began on day 26, lasting for the final four days. These rats were also euthanized to undergo biochemical testing. Table 2 details the individual identities of each rat test subject and their corresponding tartrazine dosage group in Groups Alpha-Delta.

Group	Acclimation	Tartrazine Administration	Behavioral Training	Behavioral Testing	Sacrifice
Alpha	11/04/2014-11/10/2014	11/11/2014-12/10/2014	n/a	n/a	12/10/2014
Beta	05/05/15-05/11/2015	06/08/2015-07/07/2015	05/12/2015-06/22/2015	07/03/2015-07/07/2015	7/7/2015
Gamma	09/29/2015-10/12/2015	10/13/2015-11/11/2015	n/a	11/08/2015-11/11/2015	11/11/2015
Delta	10/06/2015-10/19/2015	10/20/2015-11/18/2015	n/a	11/16/2015-11/19/2015	11/19/2015

Table 1: Experimental timeline for rat Groups Alpha-Delta

	Rat IDs			
Group	**Control**	**Low Dosage**	**Medium Dosage**	**High Dosage**
Alpha	1, 2, 3, 4, 5	6, 7, 8, 9, 10	11, 12, 13, 14, 15	16, 17, 18, 19, 20
Beta	As	Bs	Cs	Ds
Gamma	A, B	C, D	E, F	G, H
Delta	I, J	K, L	M, N	O, P

Table 2: Identities of rat test subject in each experimental group

Tartrazine Administration

Standard tartrazine with a dye content concentration of ≥85 % was obtained from Sigma-Aldrich. The tartrazine was in a solid, fine powdered state upon arrival. Tartrazine was the same consistency as the standard powdered rat food. Therefore, it was mixed by hand with 1 kilogram of solid food powder for each dosage group to create the correct dosage amounts for each group.

The maximum amount of tartrazine acceptable for administration is 250 mg/kg body weight for a rat, as this corresponds to a human dosage of 40 mg/kg body weight-the maximum amount of tartrazine a human would consume in a day (Yonglin et al., 2011). In a study conducted by G. M. Hassan, a tartrazine dosage of 7.5 mg/kg body weight was used on Wistar rats to test for DNA damage (2009). Drawing from studies by Yonglin et al. and Hassan, Team ATTENT utilized the following dosages of tartrazine: the low dosage was 0.05% tartrazine, the middle dosage was 0.15% tartrazine, and the high dosage was 0.45% tartrazine. The control group received a dosage of 0% tartrazine. The appropriate dosage was administered to each rat in food bowls, at the same time of day, for 30 days. The food was weighed when given to the rats and weighed again the next day after 24 hours in order to track consumption. This procedure was repeated for 30 days.

Behavioral Testing

Behavioral testing of the second group of twenty rats was divided into two separate phases to test hyperactivity and impulsivity. Group Beta rats underwent testing for impulsivity using a T-maze. The approximate dimensions of the maze are 61 cm x 10 cm x 39 cm, and the maze is fitted with two separate arms. The T-maze

also had removable doors on each of the arms to control a rat's access to a particular section of the maze (Mariano et al., 2009). Hyperactivity was tested for Groups Beta, Gamma, and Delta with the use of an open field chamber. The chamber measured 40 cm x 40 cm x 30 cm (Fox et al., 2013).

Testing for Impulsivity

Impulsivity of Group Beta rats was tested using the T-maze impulsivity test. The T-maze had dimensions of approximately 61 cm x 10 cm x 39 cm (Mariano et al., 2009). Each arm had a moveable door that closed behind the rat once the rat selected that arm. Another door then opened to allow the rat access to the sugar reward placed at the end of the arm (Mariano et al., 2009). After each trial, the T-maze was wiped clean in order to reduce any scent that may affect arm selection in subsequent trials. Impulsivity was measured in the times the rat opted for the smaller, more immediate reward versus the larger, delayed one (Mariano et al., 2009).

Once the first seven days of acclimation were completed, rats began receiving training for the T-maze impulsivity test. In this apparatus, one arm contained a small reward of one pellet, and the other arm contained a large reward of five pellets (Mariano et al., 2009). On each day of training, the rats were initially forced to choose each arm at least once, completing what is called a "forced trial". Only one of the arms of the T-maze was open at a time. For example, if the rat was being guided to go into the small reward arm, then the door to the large reward arm was closed while the door to the small reward arm was open. The rats then underwent 5 trials in which they could choose between the maze's two arms. These trials in which the rats had freedom of choice are called "choice trials". Training concluded for a rat when it

selected the arm with the greater reward at least four out of five times over two consecutive days (Mariano et al., 2009). Rats were trained before tartrazine treatment in order to ensure that the treatment would not interfere with T-maze performance.

Starting on day 26 of tartrazine treatment, the rats underwent 12 trials for four consecutive days (Erickson et al., 2014). Before each trial, the rats were allowed to eat and drink ad libitum. At the beginning of each day, the rats underwent a forced trial to each arm of the maze and subsequently participated in ten choice trials (Mariano et al., 2009). On the first day of testing, there was a five second delay until the rat received the larger reward. The time that the rat needed to wait before receiving the larger reward increased by five seconds every day the experiment progressed. Therefore, there was a ten second delay on the second day, a 15 second delay on the third day, and a 20 second delay on the final day. After each choice trial, the arm the rat was recorded. When impulsivity testing was complete, the average impulsivity for each dosage group was compared between groups. Since we had a control group of rats that had not been treated with any tartrazine, we used this group as a baseline measurement, rendering it unnecessary to conduct impulsivity testing before tartrazine treatment. A flowchart of impulsivity training and testing can be found in the Appendix C.

Testing for Hyperactivity

Beginning on day 26 of tartrazine administration each rat in Groups Beta, Gamma, and Delta was subjected to an open field test. Each rat was individually placed into an open field chamber measuring 40 cm x 40 cm x 30 cm (Fox et al., 2013). The rat remained in the chamber for a trial period of 15 minutes, free to move

throughout the chamber during the trial. Rats did not have access to food or water during this time (Fox et al., 2013). Each rat underwent one trial of the open field test each day for four consecutive days. This gives a total of four hyperactivity trials per rat. A high definition camera was used to film each rat during each trial. All film was analyzed and processed using an EthoVision XT 11 program by Noldus Information Technology. Hyperactivity was evaluated utilizing video analysis to measure distance moved and velocity of movements (Fox et al., 2013). The test subjects not receiving tartrazine treatment were used for baseline data to compare to the activity of the other subjects.

Behavioral Video Analysis

We used the Noldus EthoVision XT version 11.5 analysis to quantify the hyperactivity of the rats (Fox et al., 2013). Noldus EthoVision is an automated program that has been previously used to analyze video data for certain parameters of SHRs in ADHD studies (Kim et al., 2012), including the parameter of the distance a rat moves in an open field test (Van den Bergh et al., 2006). Once all the videos of our trials were collected, the program tracked the center of each rat's body as the rat moved around, thus calculating the distance traveled and average velocity of each rat throughout the 15-minute period of each trial. These values quantified the hyperactivity of a tartrazine receiving rat compared to a control rat. The arena and tracking settings were reset specifically for each day of recording in order to ensure that each rat and their subsequent trials were tracked correctly and with the greatest specificity.

Biochemical Testing

Brain Tissue Homogenization

Whole brain samples taken from the test subjects were removed from storage in a -80˚ Celsius freezer and set to thaw for 15 minutes. After thawing, the brain samples were prepared to be cut into quadrants. Using a sharp blade, the following cuts were made to create four separate quadrants of the brain (Figure 7):

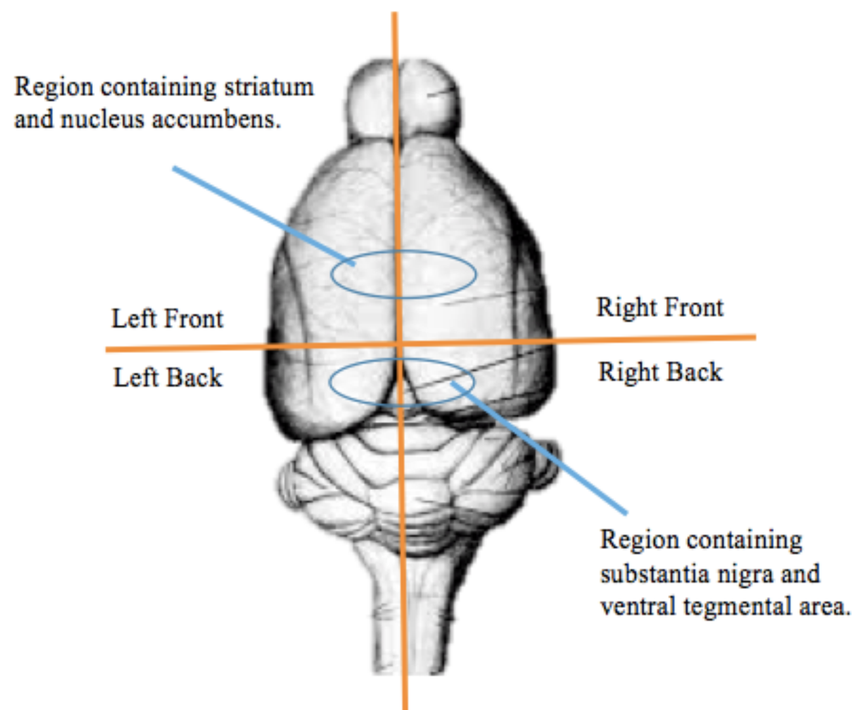


Figure 7: On the rat brain pictured above (Kiernan, 2007), orange lines indicate the vertical and horizontal cuts that were made during brain sectioning to separate the brain sample into four quadrants. The circled blue regions indicate the general areas where the aforementioned dopamine rich areas of the brain are located.

Each quadrant was labeled by its right or left and front or back location. The right and left front quadrants are expected to encompass the striatum and nucleus accumbens while the right and left back regions are expected to encompass the substantia nigra and ventral tegmental area. The weight of each individual quadrant

was measured and recorded. Before the homogenization step, the brain quadrants needed to be suspended in a 1X Phosphate Buffered Saline (PBS) buffer. Prior to beginning brain tissue homogenization, 1X PBS buffer was prepared and brought to a pH of 7.4—physiological pH—according to the following procedure:

1. Dissolve 8 grams (g) of NaCl, 0.2g of KCl, 1.44g of $Na2PO_4$ and 0.24g of KH_2PO_4 in 800 milliliters of distilled water.
2. Adjust the pH to 7.4 with HCl if needed.
3. Add water to bring total volume to 1 Liter.

The recorded weight of each quadrant was multiplied by three, and the resulting number determined the milliliters of PBS buffer the quadrant was suspended in. Homogenization was performed using a Dounce homogenizer, also known as a tissue grinder. The calculated volume of PBS buffer specific to each quadrant sample was transferred via pipette to the shaft of the homogenizer. The sample brain quadrant was then added to the shaft. The pestle of the homogenizer was used to homogenize the sample thoroughly. The resulting liquefied sample was transferred to a microcentrifuge tube and then kept in storage in a -80˚ Celsius freezer.

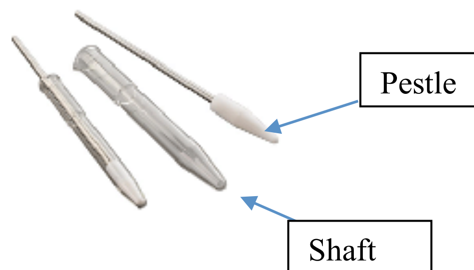


Figure 8: Dounce homogenizer and parts (Wheaton)

Biochemical Assay

Samples were assayed for total protein concentration with a Bio-Rad spectrophotometric protein assay (Catalog number 5000001). Dopamine concentration was subsequently assessed with an Enzyme-linked Immunosorbent Assay (ELISA) for rat dopamine purchased from My Biosource (Catalog number MBS725908). This kit utilized the binding to anti-rat dopamine antibodies, which have a high affinity for rat dopamine, to determine the concentration of dopamine present in samples. In order to assess and compare dopamine concentration between specimens and groups, dopamine concentration was normalized to total protein concentration.

Statistical Analysis

A power calculation was performed using predicted means and standard deviations, even sample sizes across test groups, and three test groups in addition to the control group. The power calculation yielded a test group sample size of 5 rats given a power level of 0.80 and a Type I error rate of 5%.

For each of the two replicate experiments performed, three major sets of biochemical data were analyzed. These sets included the cumulative food intake, total measured brain dopamine, and total measured brain dopamine proportional to brain protein concentration. Analysis of variance (ANOVA) was used to analyze all sets of data. Independence, normality, and homoscedasticity were all assumed true. Only for Groups Gamma and Delta, behavioral analysis was also conducted. Both distance travelled and average velocity were analyzed using ANOVA.

Body Weight

It is important to eliminate or minimize the presence and effect of confounding variables in any research. For the purposes of this project, examining body weights can provide insight into whether different dosages of tartrazine administration can have unanticipated but significant effects on food consumption behaviors or rat body metabolism, among other characteristics. A lack of significant results would support the proposition that any observed significant results in measured brain dopamine are a direct consequence of tartrazine administration. Body weights were analyzed using ANOVA with four groups (n=5) assigned to control and test groups, with group assignments based on tartrazine concentration administered.

Food Intake

The purpose of analyzing cumulative food intake was to check for unexpected abnormalities and deviations that would suggest a confounding variable. For instance, significant differences in food intake between test groups would suggest a relationship between the quantity of tartrazine consumption and behaviors associated with eating. Lack of significance in analyses would indicate that any observed changes in brain dopamine should be solely a consequence of the tartrazine concentration administered.

Cumulative food intake was analyzed using ANOVA with four groups (n=5) assigned to control and test groups in the same way as for body weights. One data value for one of the rats from the experimental control group and one data value for one of the rats from the high dosage group were missing from data collection.

Subsequently, these two data points were omitted from the corresponding data set prior to ANOVA, and analysis for those given test groups and days using data from four SHRs. Data was entered as a table with twenty rows and thirty columns, with each data point as food intake for one rat for one particular day during the thirty-day treatment period. ANOVA was conducted using SAS software. Means and standard errors within each group were collected in addition to the F-value, p-value, and R-squared value. Duncan's multiple range test, which controls for type I error, was used to analyze across groups ($\alpha = 0.05$).

Total Brain Dopamine

As previously stated, dopamine can be used as a biochemical measure for ADHD. Thus, analysis of the quantification of brain dopamine provides insight into potential biochemical effects of tartrazine on ADHD. Total brain dopamine measurements were taken from ELISA results and entered into SAS for an ANOVA. Four groups (n=5) were assigned based on administered tartrazine dosage. Means, standard errors, F-value, p-value, and R-squared value were returned from the ANOVA in addition to the results from a Duncan's multiple range test.

Dopamine Proportional to Protein

Individual SHRs had brains that yielded different total quantities of brain dopamine and protein. To account for discrepancies and differences between SHRs in brain protein, analysis was performed on the quantity of brain dopamine proportional to brain protein concentration, which will henceforth be denoted dopamine per protein. Total brain protein concentration was obtained from the Bio-Rad Bradford Protein Assay. Total brain dopamine values for each SHR were

divided by the corresponding protein concentration obtain the dopamine per protein values, and these values were then entered into SAS as the data set for the ANOVA performed. This ANOVA was also conducted with the same group assignments as the other ANOVAs. As with cumulative food intake and total measured brain dopamine, means, standard errors, F-value, p-value, and R-squared value were computed, and a Duncan's multiple range test was performed as well.

Replicate Testing

In addition to analyzing cumulative food intake, total measured brain dopamine, and total measured brain dopamine proportional to brain protein concentration for each of the two replicates, an ANOVA was performed to test for significance across replicate data. The limited test group size of five rats for each dosage for both experiments may not be sufficient to yield significant results during analysis of the data. Provided the comparison across replicates demonstrates consistency in results across both experiments, the comparison may yield significance given the larger combined test group size (n=10). Each of the three aforementioned ANOVAs was conducted in SAS with combined data sets from both experiments.

Hyperactivity

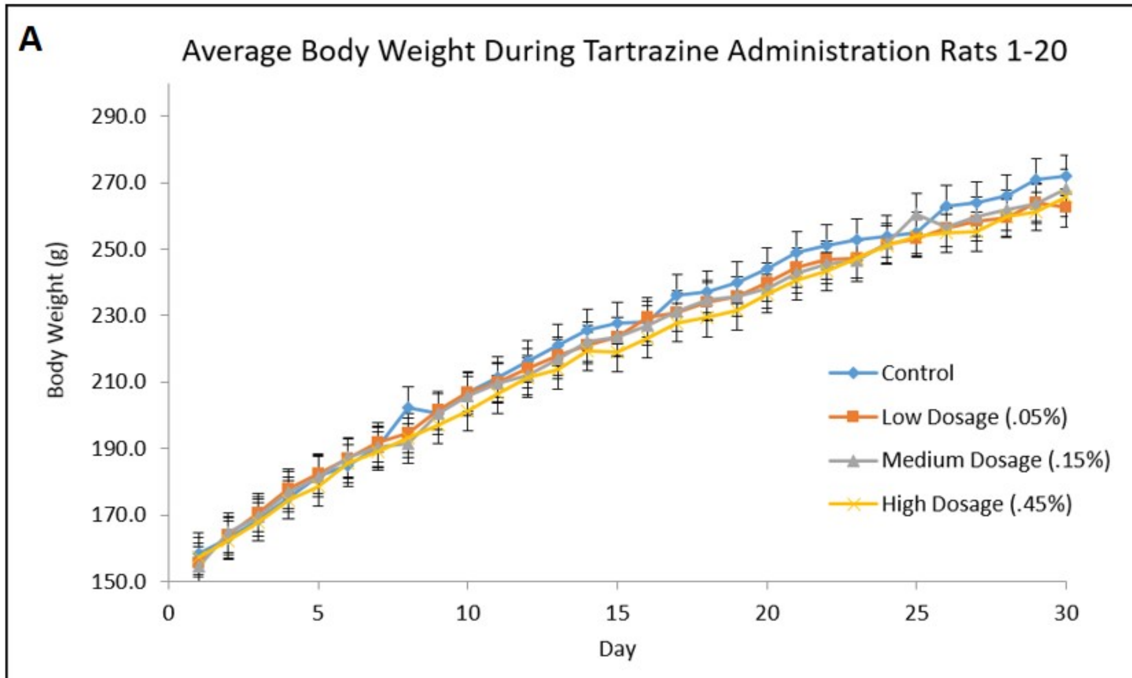
Increased distance travelled and average velocity indicate increased levels of hyperactivity in the rat model. Both distance travelled and average velocity were both obtained from the Noldus EthoVision software. Averages of distance travelled and average velocity over the four days of testing were entered into SAS as the data set for the ANOVA performed. Four groups (n=4) were assigned based on administered tartrazine dosage. Means, standard errors, F-value, p-value, and R-

squared value were returned from the ANOVA in addition to the results from a Duncan's multiple range test.

Results

Body Weight and Food Intake

The body weight and food intake was recorded for each rat during the 30 day period of tartrazine administration. Figure 9 displays the results of the compiled body weight data for rats 1-20 and rats As-P over a duration of 30 days. Figure 9a displays the average body weight for rats 1-20 and Figure 9b displays the average body weight for rats As-P. For each group of 20 rats, body weights were divided into four experimental groups in order to assess average body weights across groups. In Figure 9a, rats 1-5 are in the control group, rats 6-10 are in the low dosage group, rats 11-15 are in the medium dosage group, and rats 16-20 are in the high dosage group. In Figure 9b, rats As, A, B, I, and J are in the control group, rats Bs, C, D, K, and L are in the low dosage group, rats Cs E, F, M, and N are in the medium dosage group, and rats Ds, G, H, O, and P are in the high dosage group. To determine the effects of tartrazine on body weight, a one-way ANOVA followed by Duncan's multiple range Test was used to test for statistical significance. A probability level of less than 0.05 percent was considered to be significant. After these tests, no significant effects of tartrazine on body weight for rats 1-20 [$F(3,4)=0.06$, $P=0.9813$] and for rats As-P [$F(3,4)=0.02$, $P=0.9957$] were observed. Duncan's multiple range test also shows no significance between test groups.



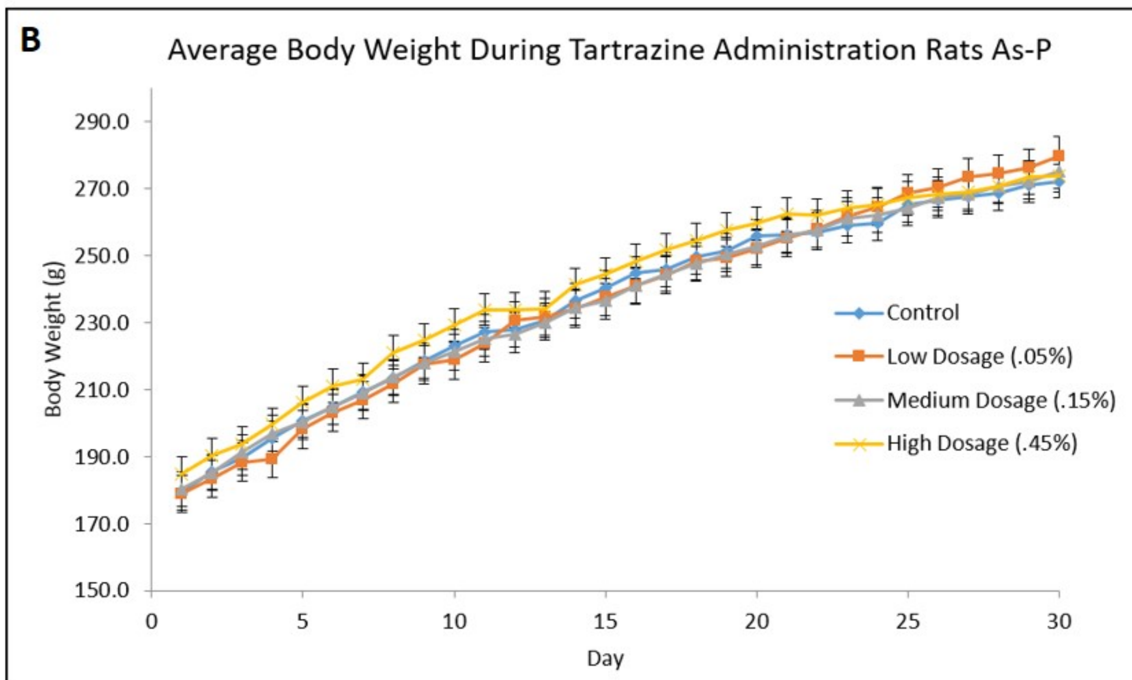


Figure 9: Average body weight of rats 1-20 and As-P. Rats 1-20 and As-P were weighed each day for 30 days through the duration of tatrazine administration. Figure 9a displays the average body weight for each experimental group of rats 1-20—control, low dosage, medium dosage, and high dosage—over a duration of 30 days. Figure 9b displays the average body weight for each experimental group of rats As-P—control, low dosage, medium dosage, and high dosage—over a duration of 30 days.

Cumulative food intake was also measured for rats 1-20 and As-P through the duration of 30 days of tartrazine administration. The food intake was measured for each rat daily and then compiled for a total food intake value at the conclusion of 30 days. These results are shown in Figure 10. Figure 10a displays the average cumulative food intake for rats 1-20 and Figure 10b displays the average cumulative food intake for rats As-P. Food intake is represented as an average for each experimental group. Rats were divided into the experimental groups described above. In order to determine if tartrazine impacted the average cumulative food intake a one-way ANOVA followed by Duncan's multiple range test was used to test for statistical significance. A probability level of less than 0.05 was considered to be significant. After the ANOVA analysis, no significant effects of tartrazine on cumulative food intake over the duration of the 30-day tartrazine administration period was observed for rats 1-20 [F(3,4)=1.65, P=0.2182] and for rats As-P [F(3,4)=1.54, P=0.2418]. Duncan's multiple range test also showed no significant difference between test groups.

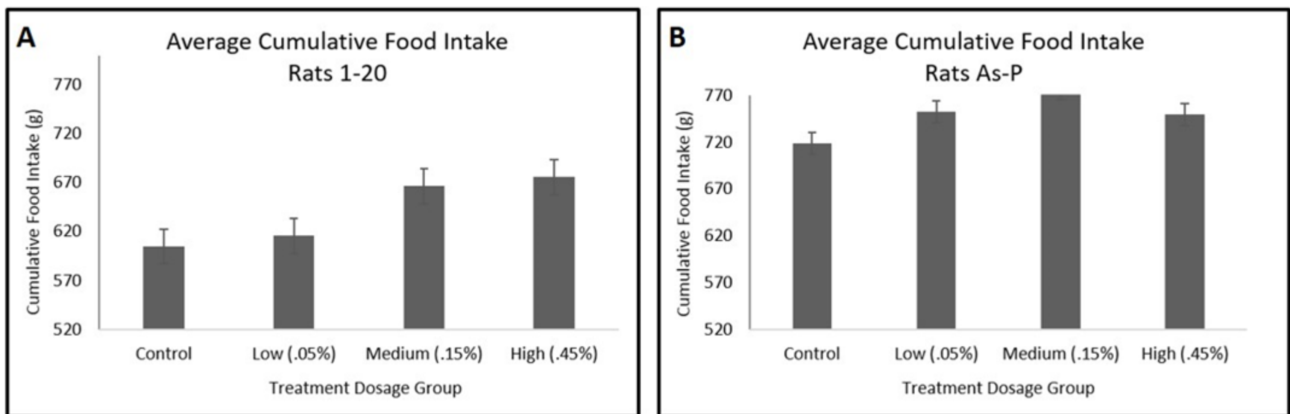


Figure 10: Average cumulative food intake of rats 1-20 and As-P. Food intake for rats 1-20 and As-P was measured each day for 30 days through the duration of tatrazine administration. Figure 10a displays the average cumulative food intake for each experimental group of rats 1-20—control, low dosage, medium dosage, and high dosage—over a duration of 30 days. Figure 10b displays the average cumulative food intake for each experimental group of rats As-P—control, low dosage, medium dosage, and high dosage—over a duration of 30 days.

Behavioral Analysis

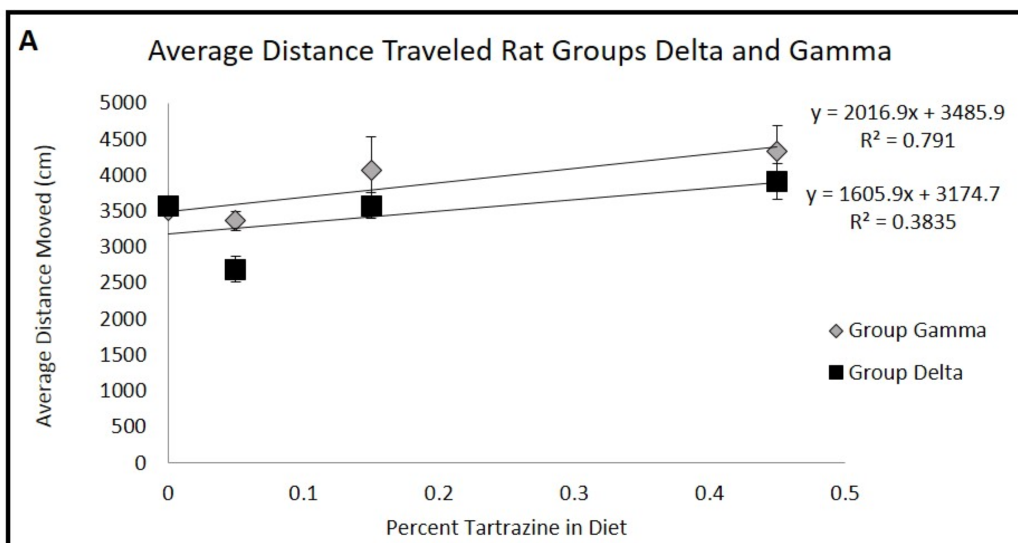
Following administration of tartrazine, rats in Group Beta were subjected to a T-maze test to assess impulsivity. This test yielded inconclusive results as rats As, Bs, Cs, and Ds were unable to successfully complete the training phase of the T-maze test.

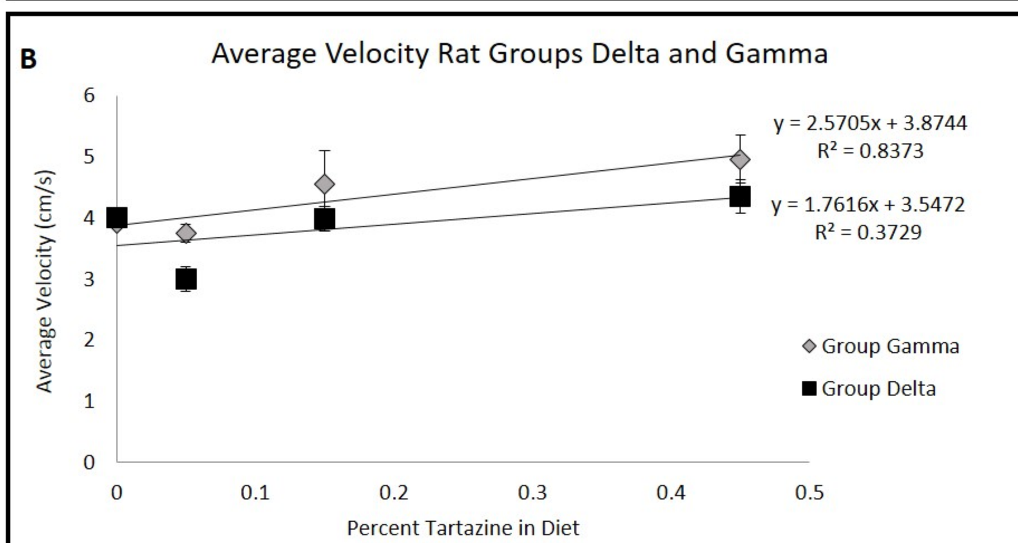
Rats in Groups Beta, Delta and Gamma were subjected to an open-field test to assess hyperactivity. Hyperactivity is being quantified by the average distance traveled and average velocity during the open-field test where an increase in distance traveled and average velocity indicates an increase in hyperactivity. The results from Group Beta were inconclusive as all rats jumped out of the open field box during trials. The results of the successful trials for Groups Gamma and Delta are displayed in Figure 11. Figure 11a displays the results of average distance traveled. This value was calculated by averaging the distance each rat traveled during all four of its 15-minute trials in the open-field. Then, rats in Groups Delta and Gamma were clustered together by their experimental groups (Table 2)—control, low dosage, medium dosage, and high dosage—and the average distance traveled for each experimental group was calculated, and plotted versus the percent of tartrazine present in each group's diet. The data sets for Group Gamma and Delta were fitted with a linear trend. As seen in Figure 11a, the linear trend for Group Gamma has equation $y=2016.9x+3485.9$ with an R^2 value of 0.791. The linear trend for Group Delta has equation $y=1605.9x+3174.7$ with an R^2 value of 0.3835. To determine the effects of tartrazine on average distance traveled a one-way ANOVA followed by Duncan's

multiple range test was used for statistical significance. A probability level of less than 0.05 was considered to be significant. After a one-way ANOVA analysis, significant effects of tartrazine on total distance traveled in the open field test for rats in Groups Gamma and Delta [F(3,15)=6.97, P=0.0004] were observed. Duncan's multiple range test shows significance between test groups, as well, placing rats from the medium (0.15%) and high (0.45%) dosage groups in Group A, rats from the control and medium dosage groups in Group B, and rats from the low (0.05%) dosage group in Group C.

Figure 11b displays the results of the average velocity of rat Groups Gamma and Delta during the open-field test. This value was calculated by tracking the average velocity for each rat during all four of its 15-minute trials in the open-field. The rats in Groups Gamma and Delta were clustered together by their experimental group (Table 2) and the average velocity for each group was calculated and plotted versus the percent of tartrazine present in each group's diet. The data sets for Group Gamma and Delta were fitted with a linear trend in a similar fashion to the data sets displayed in Figure 11a. As seen in Figure 11b, the linear trend for Group Gamma has equation y=2.5705x+3.8744 with an R^2 value of 0.8873 and the linear trend for Group Delta has equation y=1.7616x+3.5472 with an R^2 value of .3729. To determine the effects of tartrazine on the average velocity a one-way ANOVA followed by Duncan's multiple range test was used for statistical significance. A probability level of less than 0.05 was considered to be significant. After one-way ANOVA analysis, significant effects of tartrazine on average velocity in the open field test for Gamma/Delta rats [F(3,15)=6.83, P=0.0006] were observed. Similarly

to the statistical results from total distance traveled, Duncan's multiple range test shows significance between test groups as well, placing rats from the medium (0.15%) and high (0.45%) dosage groups in Group A, rats from the control and medium dosage groups in Group B, and rats from the low (0.05%) dosage group in Group C.





Treatment Group	Percent Tatrazine in Diet	Average Velocity	Standard Error	Average Distance Traveled	Standard Error
Control	0	3.95	0.09	3538.64	78.16
Low	0.05	3.52	0.16	3152.77	142.09
Medium	0.15	4.27	0.26	3818.39	222.70
High	0.45	4.66	0.24	4117.37	215.74

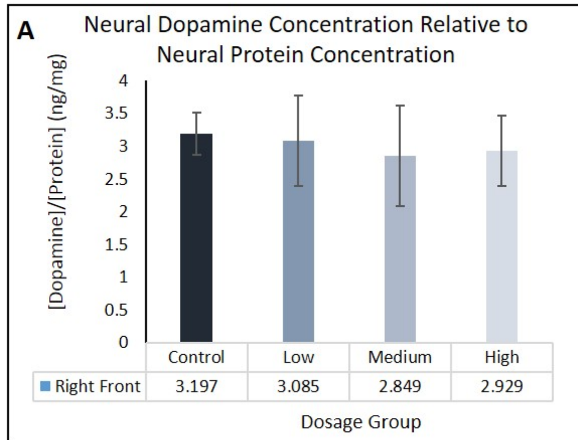
Figure 11: Average distance traveled and average velocity of rat groups delta and gamma during hyperactivity testing. Rats in groups delta and gamma were subjected to an open-field test. Each subject was tested in the open field for 15 minutes, once per day for a total of four days. Noldus Ethovision technology was used to track the total distance traveled and average velocity of each rat during each trial. Figure 11a displays the average distance traveled versus the percent of tartrazine in diet for rat groups delta and gamma. Each data set was fitted with a linear trend with the equation and R value displayed with each linear trend. Figure 11b displays the average velocity versus percent of tartrazine in diet for rat groups delta and gamma. Each data set was fitted with a linear trend with the equation and R value displayed with each linear trend.

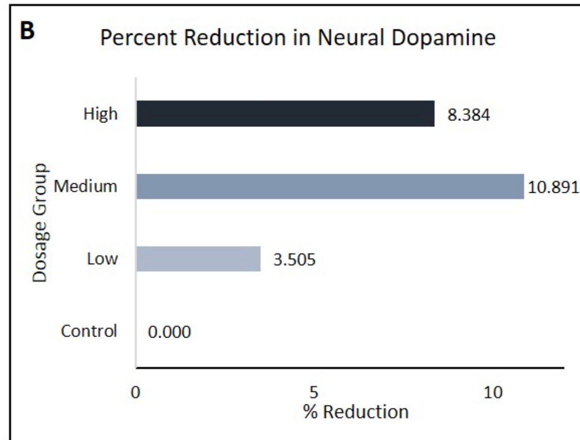
Biochemical Analysis

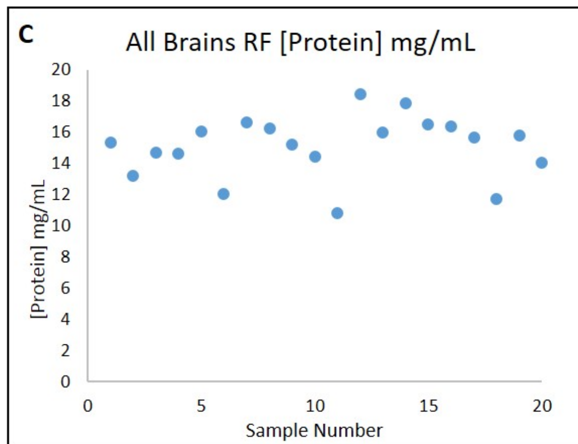
After the 30th day of tartrazine administration, rats in all groups were euthanized and their brain tissue was collected and assayed for brain dopamine. The whole brains were frozen in isopentane and dry ice upon dissection. The right-front and left-hind region of brains from rats 1-20 and As-P were assayed for brain dopamine with an ELISA assay. Each brain region was also weighed and assayed for total brain protein with a BioRad assay. The results of these assays are displayed in figures 12-15. In order to assess the effects of tartrazine on brain dopamine across regions and experimental groups, the dopamine and whole protein results for brains 1-20 and As-P were clustered by experimental dosage group (Table 2). The dopamine concentration for each sample was normalized to the whole protein concentration (dopamine/protein) and those values were averaged in each experimental group as seen in Figures 12a, 13a, 14a, and 15a. These normalized values were used to calculate the percent change in neural dopamine compared to the control group samples. This data is represented in figures 12a, 13a, 14a, and 15a.

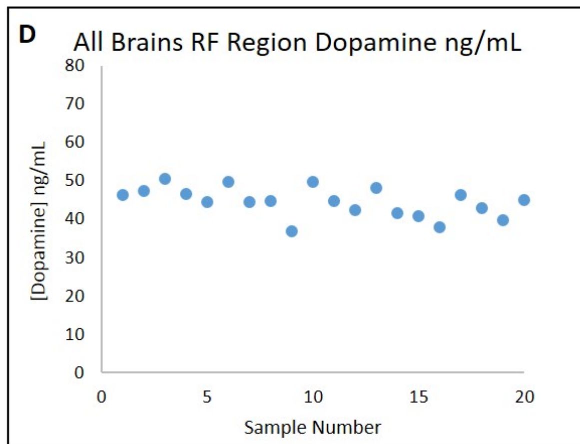
In order to determine if tartrazine consumption had an effect of brain dopamine a one-way ANOVA followed by a Duncan's multiple range test was used to test for statistical significance. A probability level of less than 0.05 was considered to be significant. After one-way ANOVA analysis, no significant effects of tartrazine on brain dopamine from the right front brain region for brains 1-20 [$F(3,4)=0.33$, $P=0.8016$] and for brains As-P [$F(3,4)=2.96$, $P=0.0717$] were observed. Duncan's multiple range test also shows no significance between test groups for brains 1-20.

However, it shows a difference between test groups for brains As-P, placing rats from low (0.05%), medium (0.15%), and high (0.45%) dosage groups in Group A and rats from control and low dosage groups in Group B. After one-way ANOVA analysis, no significant effects of tartrazine on brain dopamine from the left hind brain region for brains 1-20 [$F(3,4)=0.06$, $P=0.9821$] and for brains As-P [$F(3,4)=2.46$, $P=0.1028$] were observed. Duncan's multiple range test also shows no significance between test groups for brains 1-20. However, it shows a difference between test groups for brains As-P, placing rats from low (0.05%), medium (0.15%), and high (0.45%) dosage groups in Group A and rats from control, low, and medium dosage groups in Group B.









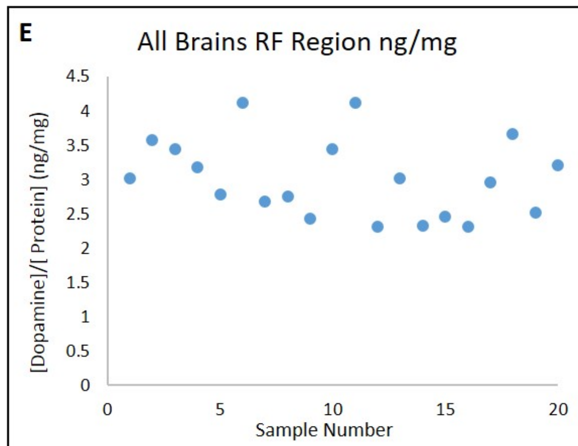


Figure 12: Brains 1-20, right-front region. The right front region of brains 1-20 were analyzed for neural dopamine concentration via an ELISA assay and whole protein concentration with a BioRad assay. Figure 12a displays the average normalized neural dopamine concentration data, figure 12b displays the percent change in normalized dopamine concentration, figure 12c displays the raw whole protein concentration data, figure 12d displays the raw neural dopamine concentration data, and figure 12e displays the raw normalized neural dopamine concentration data.

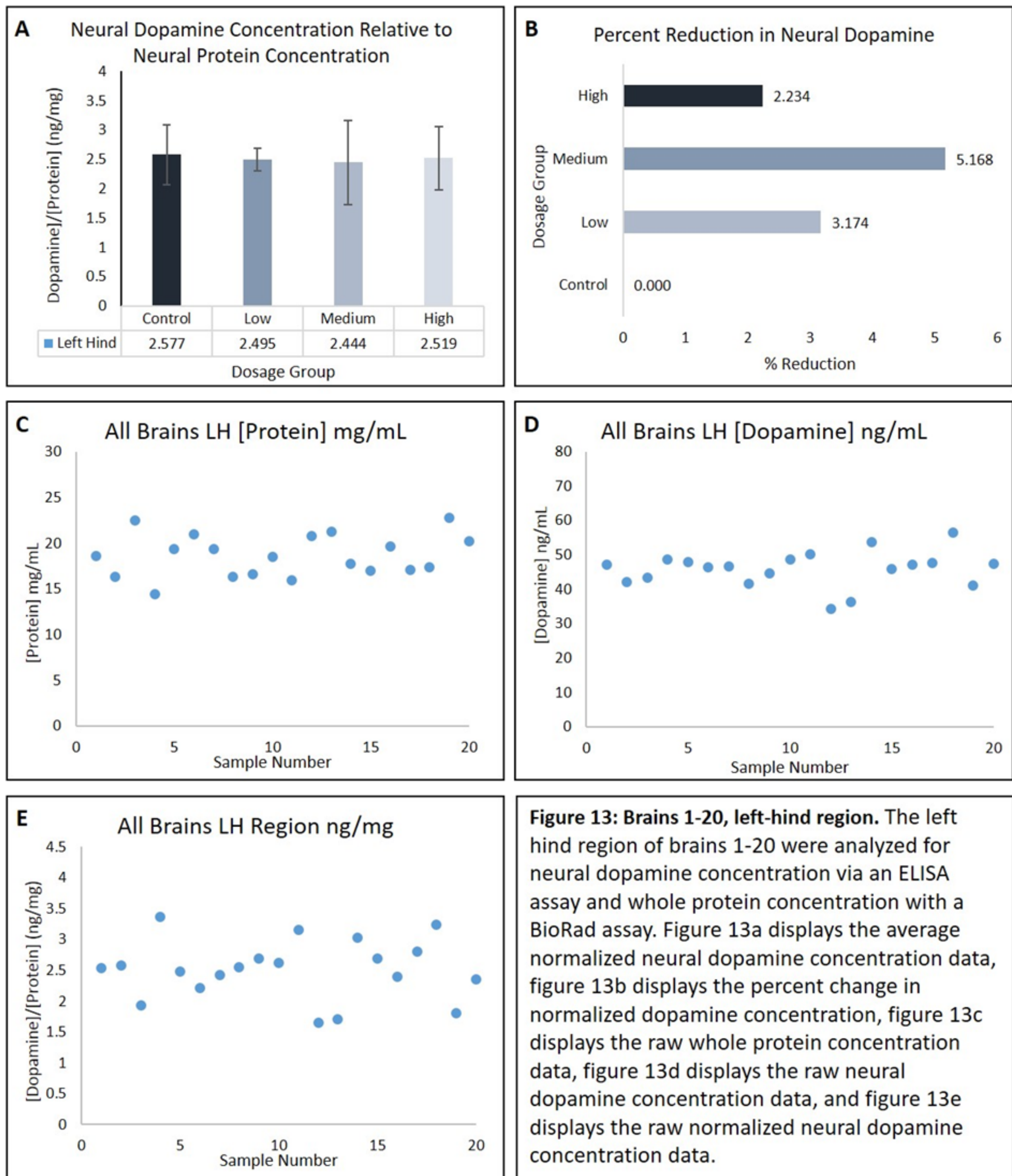
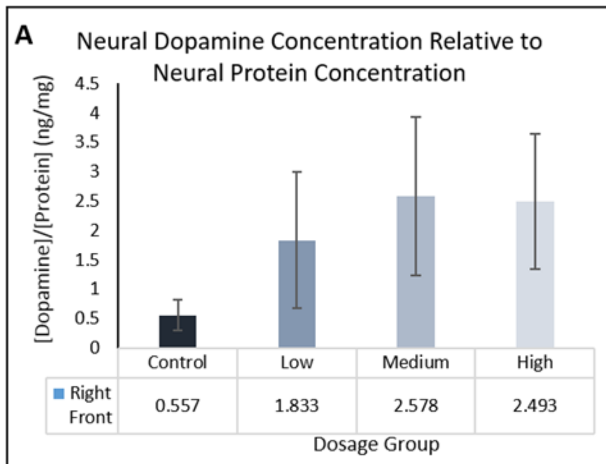
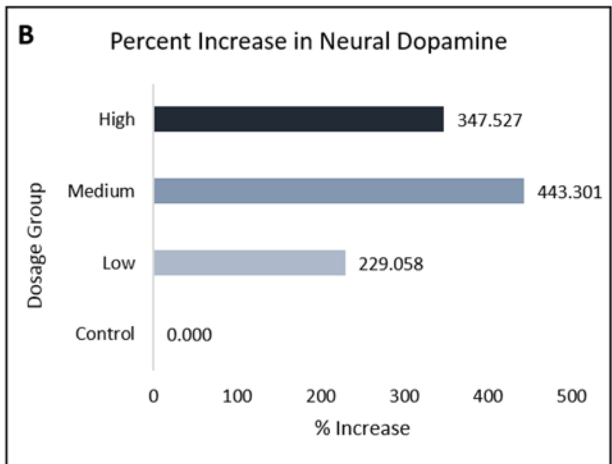
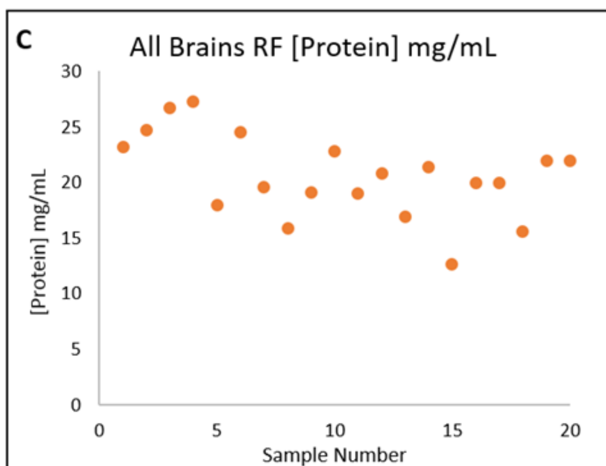
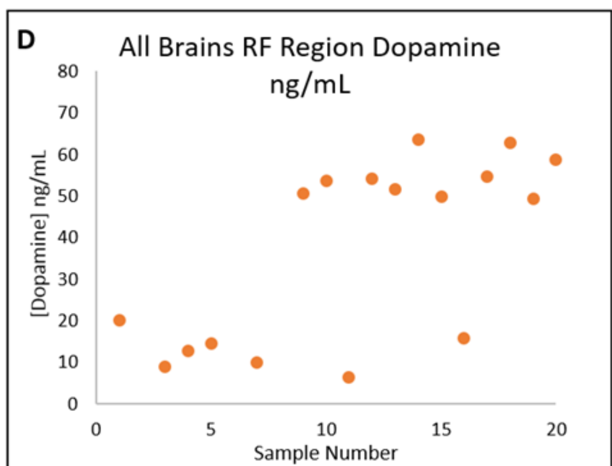


Figure 13: Brains 1-20, left-hind region. The left hind region of brains 1-20 were analyzed for neural dopamine concentration via an ELISA assay and whole protein concentration with a BioRad assay. Figure 13a displays the average normalized neural dopamine concentration data, figure 13b displays the percent change in normalized dopamine concentration, figure 13c displays the raw whole protein concentration data, figure 13d displays the raw neural dopamine concentration data, and figure 13e displays the raw normalized neural dopamine concentration data.









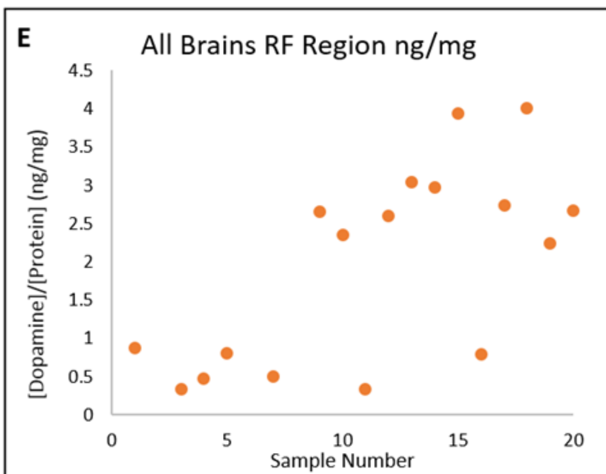
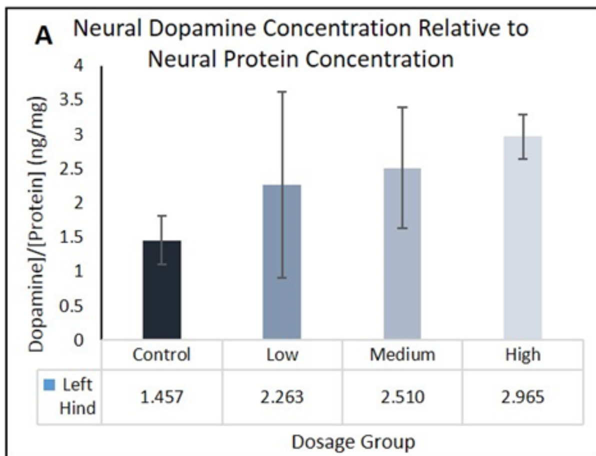
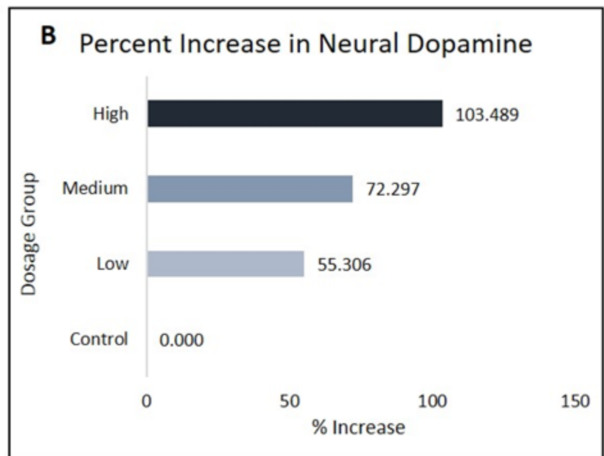
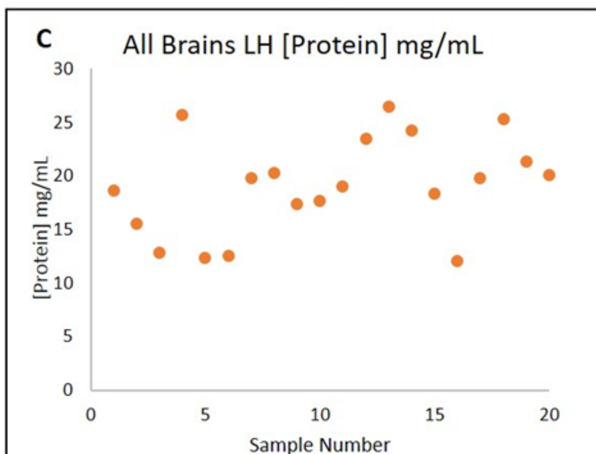
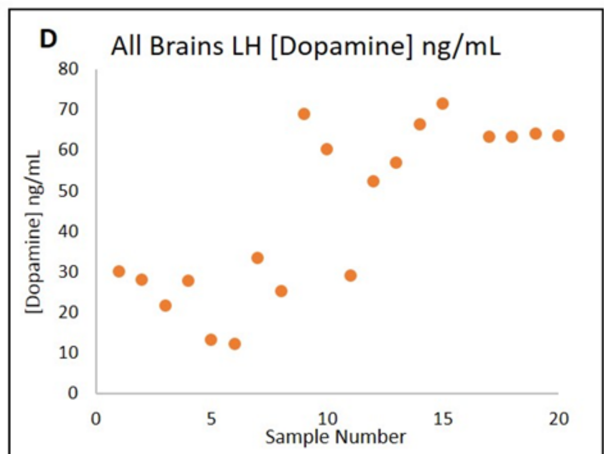


Figure 14: Brains As-P, right-front region. The right front region of brains As-P were analyzed for neural dopamine concentration via an ELISA assay and whole protein concentration with a BioRad assay. Figure 14a displays the average normalized neural dopamine concentration data, figure 14b displays the percent change in normalized dopamine concentration, figure 14c displays the raw whole protein concentration data, figure 14d displays the raw neural dopamine concentration data, and figure 14e displays the raw normalized neural dopamine concentration data.









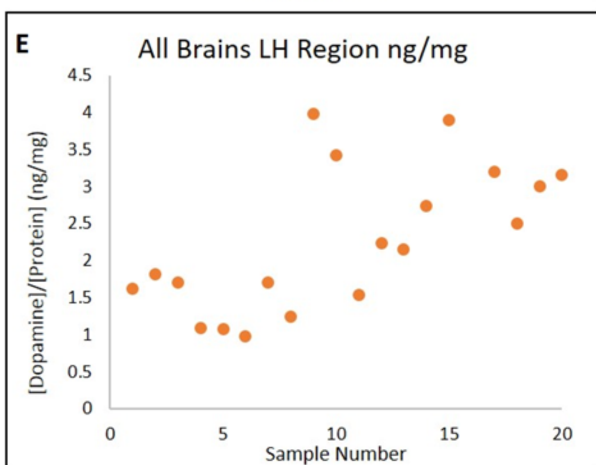


Figure 15: Brains As-P, left-hind region. The left hind region of brains As-P were analyzed for neural dopamine concentration via an ELISA assay and whole protein concentration with a BioRad assay. Figure 15a displays the average normalized neural dopamine concentration data, figure 15b displays the percent change in normalized dopamine concentration, figure 15c displays the raw whole protein concentration data, figure 15d displays the raw neural dopamine concentration data, and figure 15e displays the raw normalized neural dopamine concentration data.

Discussion

Body Weight and Food Intake

The original hypothesis of this study was that increasing the concentration of dietary tartrazine in the daily diet of the SHR model would result in a decrease in brain dopamine and increase in ADHD behavioral symptoms of impulsivity and hyperactivity. During the 30 days of tartrazine administration, food consumption and body weight was recorded for each test subject. This was done to assess if dietary tartrazine impacted either of these variables. Statistical analysis of the average body weight and average cumulative food intake indicated that there is no statistically significant difference between experimental groups as determined by a one-way ANOVA and Duncan's multiple range test. This suggests that, provided all other experimental factors are controlled and consistent across test groups, any variation in measured brain dopamine and behavioral phenotypes can be attributed to the change in dietary tartrazine, and not changes in food intake or body weight. Several factors that could have confounded these results include food that was spilled by the rat instead of orally ingested. This variable was accounted for by daily measurements of each rat's spillage on a collection platform placed beneath each cage; however, it is possible that residual food spillage could be unaccounted for in these measurements. The high p-value between experimental groups for average cumulative food intake

(p=0.2181 and p=0.2418) suggests that it is unlikely that marginal changes in cumulative food intake would result in statistical significance.

Behavioral Analysis

Following administration of tartrazine rats As-P were subjected to behavioral analyses. Rats in the high dosage group showed significantly increased levels of hyperactivity in the open field maze when compared to the control group. Generally, rats in the high dosage group travelled longer distances and exhibited an increase in average velocities. The medium dosage group displayed no significant difference in distance travelled or averaged velocity from the control group. The low dosage groups exhibited a statistically significant decrease in velocity and distance travelled when compared to the control group. The results of the behavioral analysis shows that rats fed the highest concentration of tartrazine, (0.45%) exhibited higher hyperactive levels. As a result, one can conclude that there is a relationship between amount of tartrazine consumed and hyperactivity levels. There also seems to be a threshold in which tartrazine does affect hyperactivity, which explains why there was no significant difference in the medium group. The most puzzling aspect of the results was that rats fed with the lowest dosage of tartrazine (0.05%) exhibited decreased levels of hyperactivity compared to the normal.

The results from this experiment seem to be consistent with past findings in the field. When rats were administered tartrazine in doses of 250 mg/kg and 500 mg/kg the rats exhibited significantly increased levels of hyperactivity, quantified by the distance the rat traveled (Gao et. al., 2011). Rats who were only given a 125 mg/kg dose of tartrazine did not exhibit a significant difference (Gao et al., 2011).

Although the exact dosages of tartrazine in this experiment differed from those of Gao et al., researchers could only see significant changes in hyperactivity levels at higher dosages of tartrazine. This does seem to support the idea that there is a threshold level in which tartrazine affects hyperactivity in an animal. Other researchers have also examined the effect tartrazine has upon children's hyperactivity. When children with ADHD modified their diets to remove artificial food colors (AFCs), including tartrazine, there was a decrease in their hyperactivity (Arnold et al., 2012). When these children resumed consumption of the AFCs they generally returned to their usual hyperactivity levels (Arnold et al., 2012). Furthermore, children who were given 6 doses of tartrazine reacted with an increase in irritability (Arnold et al., 2012). By replicating what has been seen in humans, this experiment not only lends more validity of using SHR rat to model ADHD but also provides more evidence indicating a relationship between tartrazine and hyperactivity.

Other research in the field has indicated that tartrazine may not have had an effect on hyperactivity. Rats treated with a 4% concentration of a combination of artificial food dyes, including tartrazine, did not experience significant differences in mean distance traveled (Erickson et al., 2014). Rats then underwent the open field test four times, each session lasting ten minutes (Erickson et al., 2014). Yet, interactions between the different food dyes may have made it impossible to determine the true relationship between just tartrazine and hyperactivity. Despite these conflicting results, the current experiment remains consistent with most of the information in the field.

Although the effect tartrazine has upon hyperactivity was not statistically significant in the lower dosages in this experiment, this does not rule out a relationship between the two. One limitation of the behavioral analysis is the small sample size. With a greater sample size, smaller and more nuanced differences in hyperactivity in the lower doses would be detected. Another major factor that may have deterred the ability to properly examine the correlation between tartrazine consumption and hyperactivity of the SHR was faulty equipment. While analyzing the videos using the Noldus EthoVision software, the tracking points would occasionally experience difficulties accurately tracking the rat. This was due to a variety of reasons including an unfocused camera, poor camera quality, inconsistencies in the open field maze surface due to the glare of the lights in the room, which caused the software to lose track of the rat, and the edges of the box being cut out of some videos. If the software had been able to track the rats' movements consistently and without noise, the analysis would have taken approximately an hour. However, the analysis took upwards of 13 hours, which confirmed that the software had difficulty tracking the rat.

One confounding variable that may have affected our data is the order in which we performed the hyperactivity test on the rats. Instead of randomly selecting the order of rats to be administered the task each day, we consistently ran every trial of hyperactivity experimentation with rats being tested in the same order. At the onset of the experiment, rats in Groups Gamma and Delta were labeled A through P, and were thus tested in that order. In short, all the rats in the control group were tested first, then all the rats in the low dosage group, then all the rats in the medium

dosage group, and finally all the rats in the high dosage group. Since experimenters would be coming in and out of the rat housing room during this time and rats would be more agitated when they were brought back from the hyperactivity chamber, rats who were tested later may have sensed the increased disturbance in the room and became more agitated. As a result, the increased stimulation and agitation of the rats tested later may have contributed to detecting higher hyperactivity levels than what was truly there. As a result, the order in which we assessed the hyperactivity of the rats may have affected our data, resulting in higher than actual hyperactivity levels in the medium and high dosage groups.

In the future, further research should be conducted to determine if even greater dosage of tartrazine – greater than 0.45% of the diet – affects hyperactivity. One could use this data to determine if increasing tartrazine will continue to cause an increase in hyperactivity or if there is a limit to the amount tartrazine can affect hyperactivity. Using a finer range of doses around the high dosage may lead to the discovery of the exact threshold of tartrazine's effects upon hyperactivity. It is also important for future studies to determine whether a dosage above the NOEL of tartrazine would lead to significant measurable affects in hyperactivity. Conducting future studies on the effect of very low levels of tartrazine on hyperactivity may also lead to new insights regarding the relationship between the food dye and hyperactivity. Since the results indicated significantly lower levels of hyperactivity when the rats were fed the smallest tartrazine dosages, it would be noteworthy to conduct more studies to understand why this phenomenon occurred. By conducting the experiment with a wider tartrazine dosage range, we would be able to determine

whether there could be a dosage dependent curve, such as a sigmoidal curve. Finally, it would be valuable to study the effects tartrazine has upon other symptoms of ADHD, including impulsivity. By looking at a range of different characteristics, researchers would also gain a better understanding between the food additive and ADHD.

It is also important to note that neural dopamine in SHR has been manipulated in the past using different chemicals relating to ADHD, including amphetamine and methylphenidate (Carboni et al., 2003). Although there were no consistent changes in neural dopamine in this experiment, the ability to even change dopamine levels in the SHR further validates the usage of SHR to model dopamine. Compared to other ADHD models, the WKY rat model in particular, the SHR has one of the most dynamic dopamine responses and most accurately mimics neural ADHD symptoms in humans.

Biochemical Analysis

As described previously, a decrease in neural dopamine levels has been implicated in human ADHD patients. Increasing dopamine levels is a common target of pharmacotherapy. Consequently, each rat test subject was assessed for brain dopamine levels following 30 days of tartrazine administration. Brain tissue was harvested from each rat, sectioned into four regions, and assayed for brain dopamine and whole brain protein utilizing an ELISA and BioRad assay, respectively.

Interestingly, in rat brains 1-20 there was a net decrease in brain dopamine between the control and high dosage groups in both the right-front and left-hind brain regions. However in rat brains As-P, the opposite trend was observed with a net

increase in brain dopamine between the control and high dosage groups. A one-way ANOVA and Duncan's multiple range test were employed to determine if tartrazine had a statistically significant effect on brain dopamine. The results of the ANOVA test indicated that there was no statistically significant change in brain dopamine in a dose-dependent manner in the right-front and left-hind regions of rat brains 1-20, and the right-front region of rat brains As-P. The Duncan's multiple range test also indicates that for rat brains 1-20 there is no statistically significant change in brain dopamine in the right-front region.

Despite the lack of statistically significant changes in the previously described regions, the Duncan's multiple range test indicated that in the left-hind region of rat brains As-P there was as statistically significant difference in brain dopamine between the control and high dosage groups. This indicates that dietary tartrazine could have had a measurable effect on brain dopamine in the highest dosage group. The Duncan's multiple range test also indicated a statistically significant difference in brain dopamine between the medium and high dosage groups in the right-front region of brains As-P. Both of these results suggest that in high doses, dietary tartrazine could have a measurable effect of brain dopamine in the SHR. The discrepancy between the ANOVA and Duncan's multiple range test suggests that there may be a threshold of dietary tartrazine needed to observe measureable effects in the rat model.

As mentioned previously, in brains As-P there was net increase in brain dopamine between the control and high dosage groups. This significantly contradicts the results of rat brains 1-20 as the expected hypothesis, and the literature (Volkow et al., 2009). While these results could suggest a new hypothesis, there are several other

factors that may be able to explain this discrepancy. Rats As-P were subjected to behavioral analysis prior to euthanasia, while rats 1-20 were not. This could contribute to the difference in biochemical results. Additionally, there were inconsistencies in the ELISA results for brains As-P, where some replicate samples were outside of the measurable range of the assay. This could have been due to experimental error during the preparation of the samples or contamination in the kit. The ELISA kit used in this assay has a sensitivity limitation, which prevents it from detecting more subtle differences in brain dopamine concentration between samples. In future analysis, brain tissue could be individually sectioned with a cryostat and key regions of the brain described in the literature could be dissected and probed for dopamine with a Western Blot. This experimental method would allow for more precise and sensitive detection of brain dopamine, which could resolve the discrepancies seen in these data sets.

In the future, extensions of this research could include examining the kinetics of sulfanilic acid absorption as well as the rate of tartrazine absorption. While this research studied the amount of tartrazine ingested, it is unclear how much of that tartrazine was actually metabolized by the test subjects. To see how much of the tartrazine was absorbed or excreted, future studies could use biomarkers, such as radioactive sulfur or carbon, to visualize where the tartrazine or its metabolites traveled in the rat's body.

Lastly, previous literature has shown that dopamine regulation follows a circadian rhythm in humans and rats, and because rats are nocturnal, dopamine release is the highest at night. Therefore, by switching the light and dark cycles in the

animal care room, we would have been able to dissect the SHR brains during the time of the highest possible dopamine release. However, the animal care facility did not allow for control of the lights in the room, and the procedure room used to euthanize the SHR was unavailable at night. In future projects, it would be best if the animal care facility being used permitted the switching of the light and dark cycles so that when euthanized, the SHR brains would be most active and releasing the amount of dopamine that would affect the brain, just as the dopamine in a human brain would be most affected during its peak release times during the day.

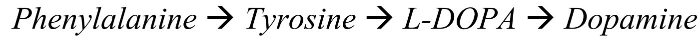
Conclusion

The results of the behavioral analysis indicate that dietary tartrazine increased hyperactivity in the SHR for certain dosage groups. This is consistent with the original hypothesis of this study that predicted that increasing dietary tartrazine would result in an increase in hyperactivity. The results of the impulsivity test were inconclusive due to incomplete analysis. In order to test this portion of the hypothesis, the study could be completed with a more efficacious method of impulsivity testing, such as the operant condition chamber, which correlates reward-uptake behavior with impulsivity. The biochemical data suggests that dietary tartrazine does impact brain dopamine. However, the data does not support the original hypothesis that there would be a net decrease in brain dopamine in response to dietary tartrazine. Due to discrepancies in these data sets, though, further analysis is necessary to verify or nullify this hypothesis. Additionally, there are other methods of statistical analysis that may be chosen for similar future studies. For instance, the Duncan's multiple range test may be used with the restriction of comparing experimental values to the standard zero value as opposed to simply comparing group means. This could yield dose-dependent responses that may not otherwise be observed. Ultimately, the results of this study do suggest that dietary tartrazine can impact ADHD-associated symptoms and behaviors.

While this study was conducted in a rat model of ADHD, a similar experiment must be conducted in human patients to determine if the results of this study are

relevant in human ADHD. ADHD is a behavioral disorder that negatively impacts that lives of patients, especially school-aged patients. Understanding the role that dietary chemicals, such as tartrazine, have on the presentation of ADHD-like symptoms is important for improving the treatment options available for patients. Traditional pharmacotherapies have proven successful for managing ADHD in some patients, yet fail to work in other patient subset, and generally induce detrimental side effects regardless of the success of these drugs. If patients can alleviate symptoms by employing less harmful measures, such as dietary modifications then efficacy of ADHD treatment may be improved. Ultimately, additional research is necessary to verify the biochemical origin of ADHD and to identify key factors, dietary or otherwise, that may be able to mitigate the cause of ADHD and improve the quality of life for patients.

Appendix A



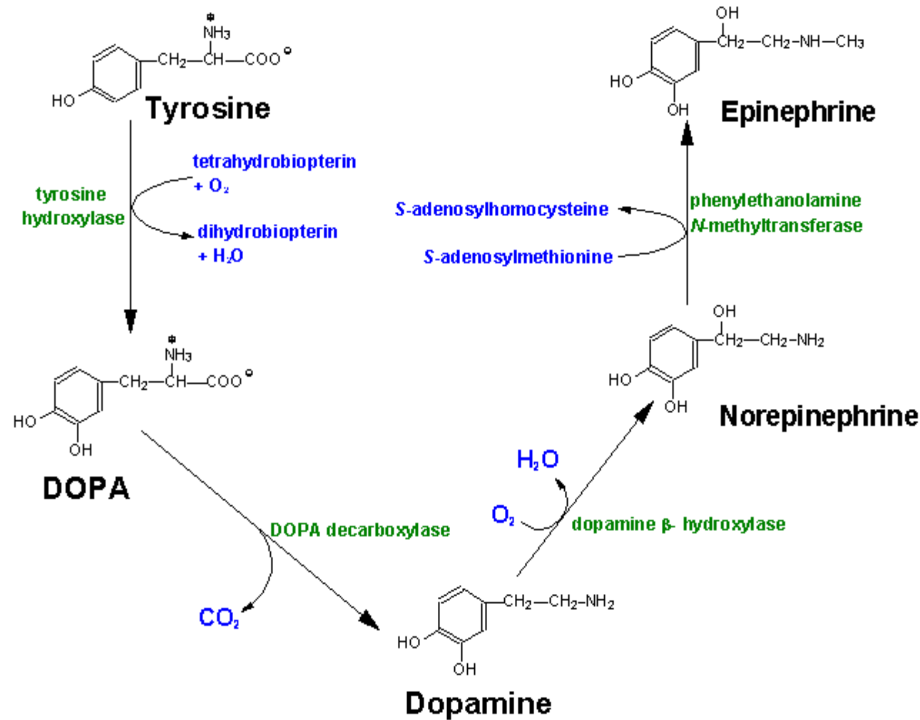


Figure 16: Synthesis of Tyrosine (The Medical Biochemistry Page, 1996)

Phenylalanine is the essential amino acid, which is used to synthesize Tyrosine. The metabolic pathway (Figure 16) for this synthesis involves the use of *phenylalanine hydroxylase* to add an alcohol (-OH) group in the *para* position and synthesize tyrosine (Elsworth & Roth, 1997). Tyrosine is also ingested directly in the diet because it is a non-essential amino acid. The enzyme tyrosine hydroxylase is responsible for the conversion of tyrosine into L-3,4-dihydroxyphenylalanine (L-

DOPA). Along with O_2, Fe^{2+}, and tetrahydrobiopterin, tyrosine hydroxylase adds an alcohol group to the carbon adjacent to the already existent alcohol group. In the final step of the pathway, DOPA decarboxylase, also known as aromatic amino acid decarboxylase (AADC decarboxylase; Elsworth et al., 1997), causes the carboxylic acid group (CO_2^-) to break its bond with the ethylamine group, forming dopamine as the final product.

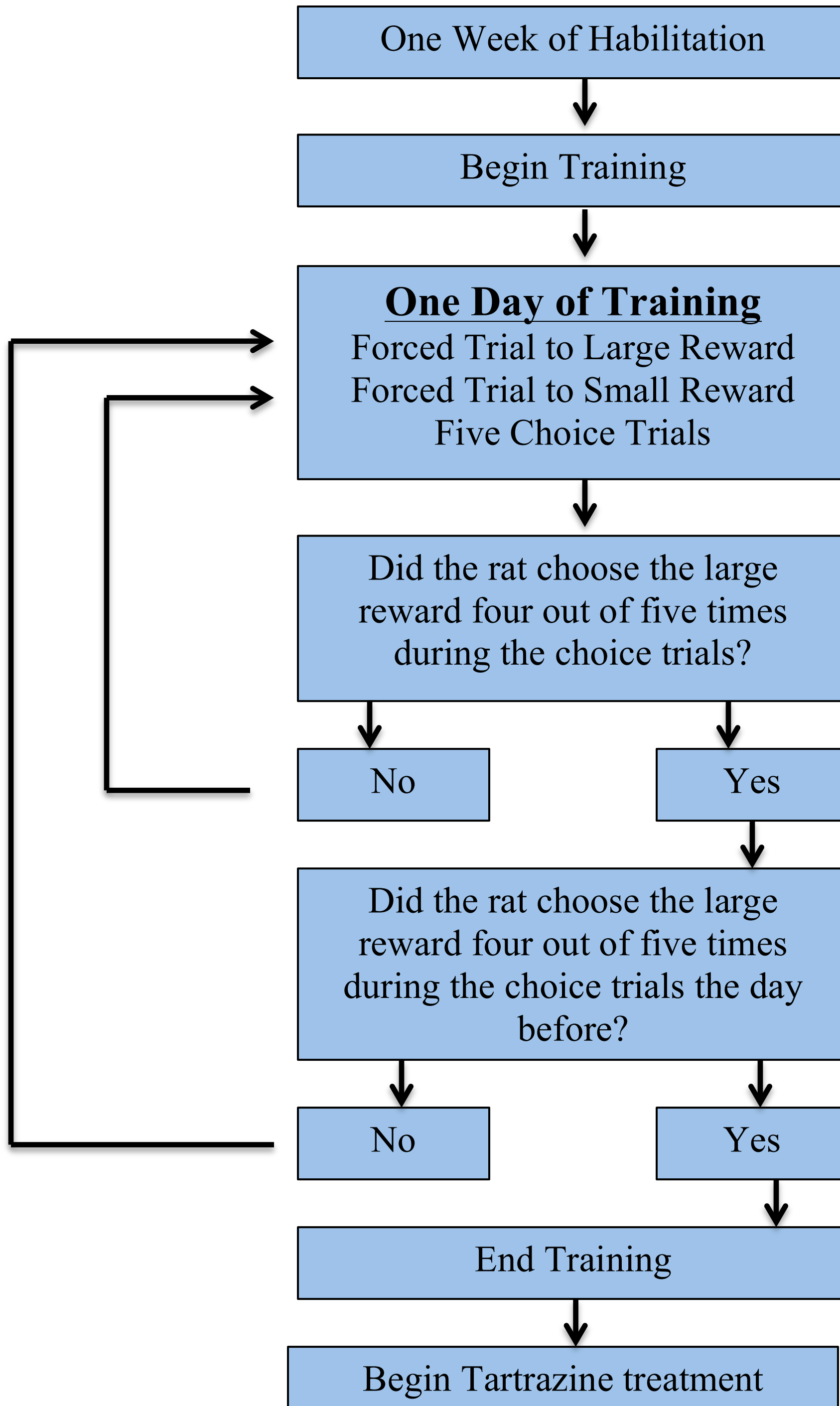
Appendix B

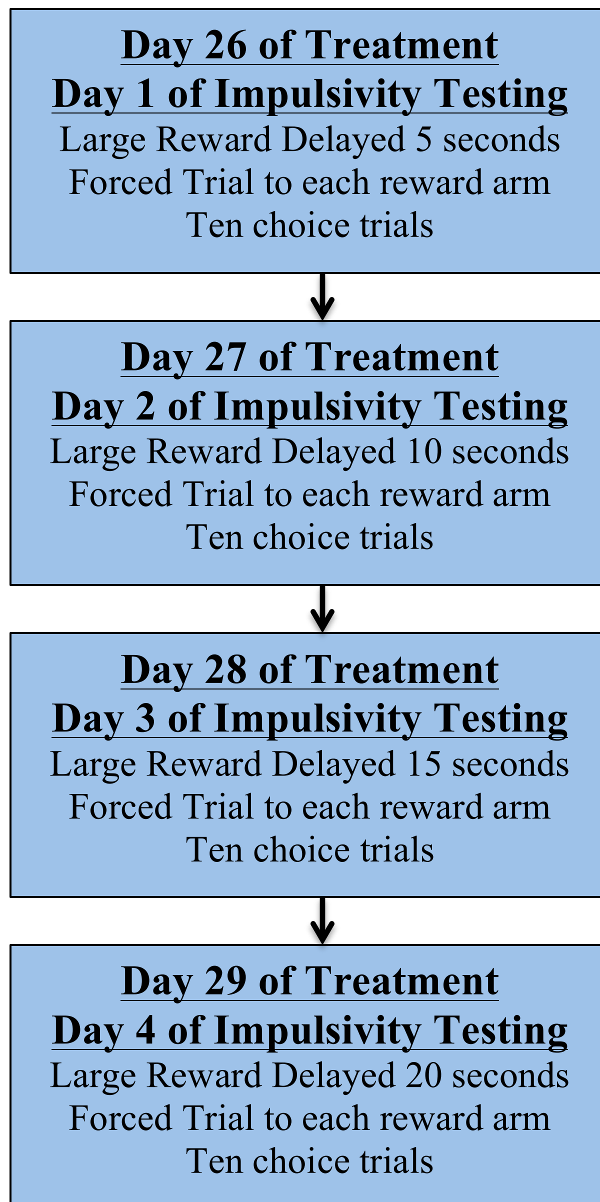
Budget

Date	Transaction	Debit/Credit	Balance	Items
Sep-13	Yearly money from Gemstone	+$600.0	$600.00	
Mar-14	Surgical West Pico Chemiluninescence Kit	-$142.00	$458.00	
Mar-14	beta-actin Antibody	-$279.00	$179.00	
Jul-14	Loss of Remaining Gemstone money	-$179.00		
Aug-14	CFS3 Mini Grant	+$2,500.00	$2,500.00	
Sep-14	Yearly money from Gemstone	+$600.00	$3,100.00	
Oct-14	Home Depot	-$49.92	$3,050.08	Wood and Paint
Oct-14	Sigma Aldrich	-$60.04	$2,990.04	Tartrazine
Dec-14	FisherChem	-$59.25	$2,930.79	Isopentane
Jan-15	AGNR Dean matching	+$1,250.00	$4,180.79	
Jan-15	Gemstone matching	+$1,2500.00	$5,430.79	
Apr-15	CARF	-$22.00	$5,408.79	Diet
Apr-15	CARF	-$22.00	$5,386.79	Diet
Apr-15	CARF	-$85.66	$5,301.13	Animal Care, Cage Changing, Rat 'P' Care
Apr-15	CARF	-$24.80	$5,276.33	Care for Rat 'P'
Apr-15	CARF	-$136.62	$5,139.71	Animal Care and Cage Changing - Dec.
Apr-15	Chemstore	-$15.48	$5,124.23	Dry Ice
Apr-15	CARF	-$1,467.56	$3,656.67	Group Alpha
May-15	MyBioSource	-$662.50	$2,994.17	Elisa Kit
May-15	CARF	-$24.00	$2,970.17	Care for Rat 'P'
May-15	CARF	-$94.86	$2,875.31	Animal Care
Jul-15	Loss of Remaining Gemstone money	-$600.00	$2,275.31	
Jul-15	Yearly money from Gemstone	+$600.00	$2,875.31	
Jul-15	CARF	-$319.35	$2,555.96	Group Beta
Jul-15	CARF	-$22.00	$2,533.96	
Jul-15	CARF	-$24.48	$2,509.48	Animal Care - Jul.
Aug-15	CARF	-$91.80	$2,417.68	Animal Care
Aug-15	CARF	-$ 24.48	$2,393.20	Animal Care - Aug.
Oct-15	MyBioSource	-$662.50	$1,730.70	Elisa Kit
Nov-15	Sara Kreshpanji	-$37.30	$1,693.40	Tripod
Nov-15	MyBioSource	-$662.50	$1,030.90	Elisa Kit
Oct-15	CARF	-$91.80	$939.10	Animal Care - Oct.
Oct-15	CARF	-$22.00	$917.10	Diet
Oct-15	CARF	-$1,177.40	$(260.30)	Groups Gamma and Delta Rats
	Total spent	**$7,060.30**		

Appendix C

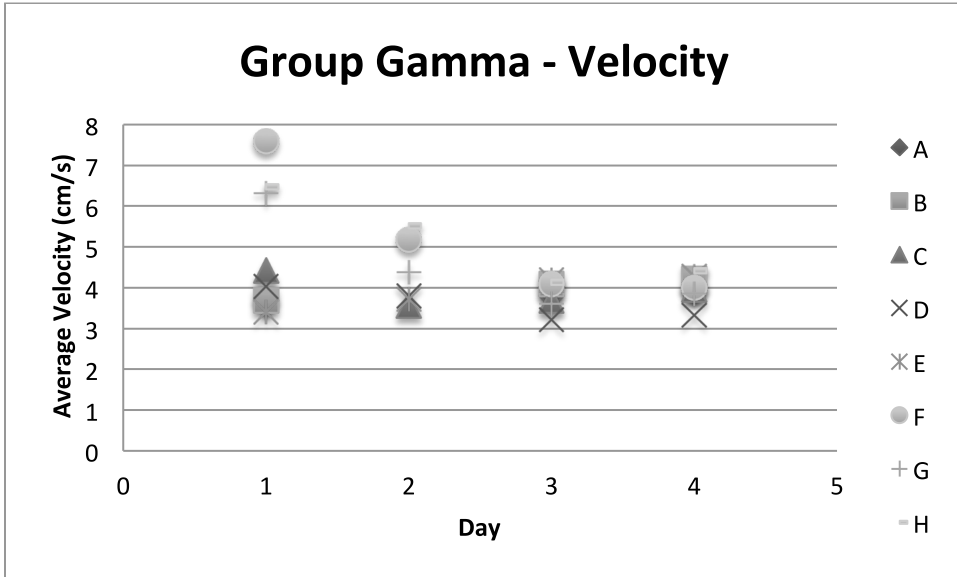
Impulsivity Training Flowchart

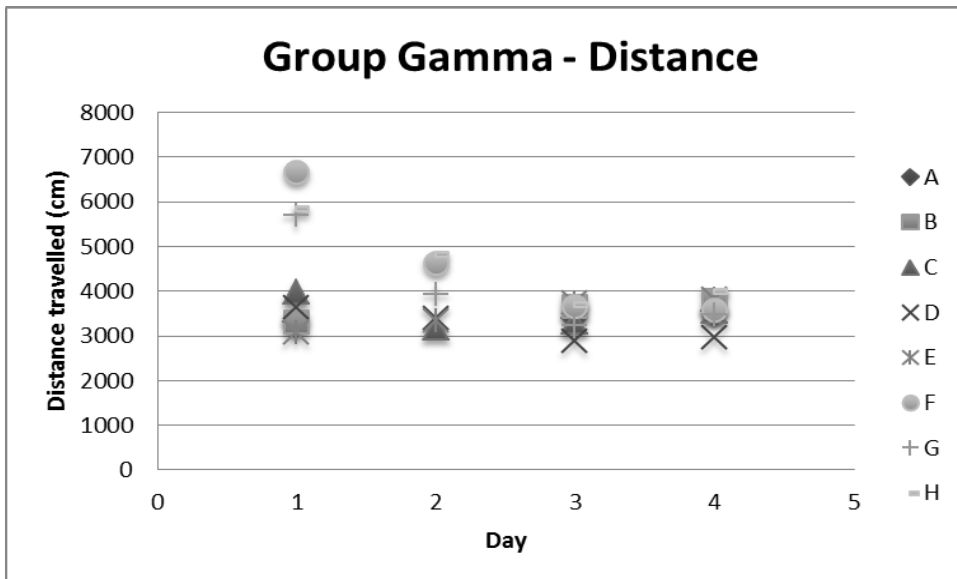


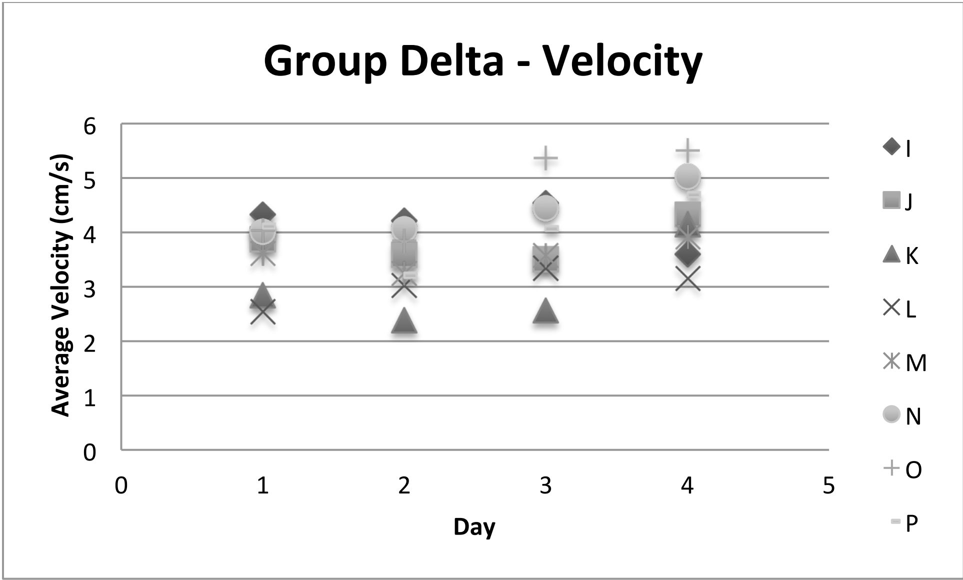

Day 26 of Treatment
Day 1 of Impulsivity Testing
Large Reward Delayed 5 seconds
Forced Trial to each reward arm
Ten choice trials
Day 27 of Treatment
Day 2 of Impulsivity Testing
Large Reward Delayed 10 seconds
Forced Trial to each reward arm
Ten choice trials
Day 28 of Treatment
Day 3 of Impulsivity Testing
Large Reward Delayed 15 seconds
Forced Trial to each reward arm
Ten choice trials
Day 29 of Treatment
Day 4 of Impulsivity Testing
Large Reward Delayed 20 seconds
Forced Trial to each reward arm
Ten choice trials

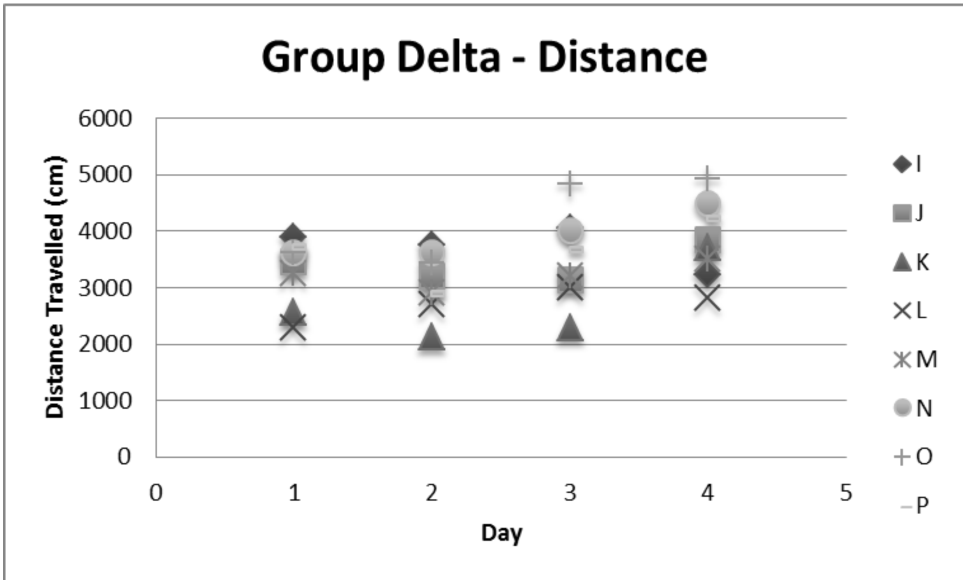
Appendix D

Average velocity and distance travelled of each rat plotted versus the day of hyperactivity testing






Group Delta - Velocity
Average Velocity (cm/s)
6
5
4
3
2
1
0
0
1
2
3
4
5
Day
I
J
K
L
M
N
O
P


Group Delta - Distance
Distance Travelled (cm)
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
Day
I
J
K
L
M
N
O
P

Glossary

Attention Deficit Hyperactivity Disorder: Type of brain disorder; symptoms include difficulty staying focused and paying attention, difficulty controlling behavior, and hyperactivity (over-activity). These symptoms can make it difficult for a child or adult with ADHD to succeed in school, get along with other children or adults, or finish tasks at home.

Allura red AC: A red azo dye

Artificial food coloring: A man-made, digestible substance used to give color to food

Aspartame: A very sweet substance used as an artificial sweetener, chiefly in low-calorie products

Behavioral Assessment (in terms of ADHD): The measurement of behavior through direct observation and applications of the patient; interviews of parents and teachers

Behavioral Inhibition: Refers to the consistent tendency of some children to demonstrate fear and withdrawal in novel situations

Benzoate: A salt or ester of benzoic acid

Carmoisine: Red 3, a synthetic red food dye from the azo dye group

Catecholamine: Any of a class of aromatic amines that includes a number of neurotransmitters such as epinephrine and dopamine

Cliff Avoidance Reaction (CAR): A natural tendency of animals to avoid a potential fall from a height

Comorbid: Existing simultaneously with and usually independently of another medical condition

Dopamine Transporter Knockout Mouse (DAT-KO): A mouse model that is suggested to constitute an animal model of ADHD, produced by transgenic inactivation of the DAT gene

Dopamine: A compound present in the body as a neurotransmitter and a precursor to other neurotransmitters

Epinephrine: A hormone secreted by the adrenal medulla that is released into the bloodstream in response to physical or mental stress, as from fear or injury

Erythrosine: Red 3, an organoiodine compound, specifically a derivative of fluorone, primarily used for food coloring

Feingold Hypothesis: Hypothesis that states that hyperactivity in children may be caused by food additives such as artificial colors, artificial flavorings, and preservatives

Food additive: A substance that becomes part of a food product when added during the processing or making of that food

Functional magnetic resonance imaging (fMRI): A form of magnetic resonance imaging of the brain that registers blood flow to functioning areas of the brain

Gene: A fundamental and functional unit of heredity that determines a characteristic. Also a sequence of nucleotides that is responsible for an organism's phenotype

Hyperactivity: A condition characterized by excessive restlessness and movement

Impulsivity: Proceeding from natural feeling or impulse without external stimulus

Maternal Phenylketonuria (MPKU): A mother with PKU passes on the disorder to her baby while he/she is still in the fetal developmental stage.

Methylphenidate: A prescription drug that is used to treat attention deficit hyperactivity disorder

Monosodium glutamate: A compound that occurs naturally as a breakdown product of proteins and is used as a flavor enhancer in food (although itself tasteless)

Naples High Excitability Rat: A rat model that features the main aspects of attention deficit hyperactivity disorder and display high activity levels in a Làt-maze

Natural Salicylates: Salts, anions, or esters of salicylic acid

Neurite growth: The growth of projections from the cell body of a neuron

Neuro-imaging: Process of producing images of the structure of the structure or activity of the brain of the brain or other part of the nervous system by techniques such as magnetic resonance imaging or computerized tomography

Neurodevelopmental: Impairments of the growth and development of the brain or central nervous system

Neurotoxicity: Poisonous to nerves or nerve cells

Neurotransmitter: A chemical substance that is released at the end of a nerve fiber by the arrival of a nerve impulse and, by diffusing across the synapse or junction, causes the transfer of the impulse to another nerve fiber, a muscle fiber, or some other structure

Pharmacotherapy: Medical treatment by means of drugs.

Pharmacostimulants: Drugs that provide biochemical or mental stimulus

Phenylalanine: An essential amino acid that is a precursor to the non-essential amino acid tyrosine. L-phenylalanine is the most commonly ingested form by humans and is found in common food sources of protein.

Phenylketonuria (PKU): A rare genetic condition in which a baby is born without phenylalanine hydroxylase, the enzyme required to properly metabolize the amino acid phenylalanine. This defect leads to complications including abnormal tyrosine synthesis and dopamine deficiency.

Polymorhphisms: Occurrence of something in several different forms

Ponceau 4R: A synthetic colorant that may be used as a food coloring, from the azo family of dyes, red

Positron emission tomography (PET): A technique for measuring brain function in living human subjects by detecting the location and concentration of tiny amounts of radioactive chemicals

Prepulse Inhibition (PPI): A reduction in the startling and reflex reaction to a startle-eliciting stimulus shortly after a weaker stimulus (prepulse stimulus)

Quinoline: An aromatic organic base, having a pungent tarlike odor, synthesized or obtained from coal tar, and used as a food preservative and in making antiseptics and dyes

Serotonin: A compound present in blood platelets and serum that constricts the blood vessels and acts as a neurotransmitter

"Stop and go" paradigm: A stop task is designed to measure response inhibition. The purpose is to measure the ability to inhibit a response when a go cue is unexpectedly accompanied by a stop clue.

Strengths and Weaknesses of ADHD: Symptoms and Normal-behavior (SWAN) scale: An 18-item parent questionnaire for children 18 years and younger in order to assess symptoms of Attention-Deficit/Hyperactivity Disorder.

Sunset yellow: A synthetic yellow azo dye, manufactured from aromatic hydrocarbons from petroleum

Tartrazine: A brilliant yellow synthetic dye derived from tartaric acid and used to color food, drugs, and cosmetics

Tryptophan: an amino acid that is a constituent of most proteins

Tyrosine: A non-essential amino acid that the human body synthesizes from the essential amino acid. It is the precursor to dopamine synthesis.

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