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An in vivo study of the effects of perinatal caffeine exposure on synaptic efficacy in the hippocampus of freely moving adult rats

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TRINITY COLLEGE

AN *IN VIVO* STUDY OF THE EFFECTS OF PERINATAL CAFFEINE EXPOSURE ON SYNAPTIC EFFICACY IN THE HIPPOCAMPUS OF FREELY MOVING ADULT RATS

BY

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ABSTRACT

The synapse from the perforant path to the dentate gyrus has been widely used successfully to demonstrate long-term potentiation, a cellular model underlying learning and memory. Caffeine is one of the most widely consumed psychoactive stimulants in the world. Caffeine consumption increases in alertness, improvements in motor skills, and neurological functions, and these effects have promoted its use throughout history. Although the many short term cognitive benefits of caffeine intake are well understood, the long term effects of caffeine exposure have been widely disputed. Despite this, it is estimated that over 80% of women continue to consume caffeine throughout pregnancy.

The objective of this experiment was to determine the effects of perinatal caffeine exposure on long term potentiation in adult, freely behaving male rats. Caffeine water (1.0g/L) was administered to pregnant dams and continued until 21 days after the birth of the pups, at which time the pups reverted to normal water. Once the pups reached 70-120 days, stereotaxic surgery was performed on males to implant electrodes into the dentate gyrus and perforant path. After a week of recovery, the population spike amplitude was measured before and after high frequency stimulation to determine LTP levels. LTP level was significantly lower for the early caffeine exposed rats (n=11, p value <0.01). Interestingly, the paired pulse test showed statistically lower levels of local circuit inhibition.These results may be representative of the upregulation of adenosine 1 receptors. Early caffeine exposed rats showed lower body weight at 21 d of age. Our results indicate that caffeine may have long term consequences for normative neuronal function in adult individual exposed to caffeine in early life. Future studies may include spatial memory behavioral tests to further clarify these electrophysiological results.

INTRODUCTION

Although much progress has been made in understanding the brain, it is still the most mysterious organ in the human body. The brain is divided into various functional regions, one of which is the hippocampus. The hippocampus is the "gateway to memory" (Kempermann, 2002). Damage to the hippocampus causes changes in learning and memory as well as behavior (Jarrard, 1993). Although hippocampal involvement in memory formation has been known throughout the 20th century, the exact mechanism underlying this function remains poorly understood. To study the function of the hippocampus, behavioral tests, such as maze runs, are often used to investigate the subject's ability to learn and form long term memories (Jarrard, 1993; Schenk &Morris, 1985). In the early 1970s, researchers found that repetitive activation of the excitatory synapses in the hippocampus led to an increase in synaptic activity that lasted for days (Malenka & Nicoll, 1999; Bliss & Collingridge, 1993). This synaptic plasticity after repeated stimuli became known as long term potentiation (LTP), and over years it has become the most viable neurological model for learning and memory (Malenka & Nicoll, 1999).

Numerous environmental and endogenous factors play a role in formation of long term memories, as seen in both behavioral and electrophysiological studies. For example, neonatal stress lowers LTP in male adult rats (Blaise, *et al.* 2008). In addition, short term caffeine exposure alters synaptic plasticity and LTP in rodents (Schmidt, *et al.* 2012; Hughes & Beveridge, 1991; Nehlig & Debry, 1994). However, the effects of chronic caffeine exposure on learning and memory remain yet to be determined. An *in vitro* study by Greene, *et al.* (1985) showed that caffeine increased neuronal excitation in the hippocampal CA 1 regions. By contrast, other studies observed that chronic caffeine ingestion decreased synaptic activity in the hippocampus (Constenla, *et al.* 2010).

Nevertheless, in today's fast moving and highly competitive society, people have become dependent on caffeine to increase productivity at the workplace and in academic settings. Moreover, children are exposed to caffeine at an earlier age because it is readily available in forms of chocolate and carbonated drinks (Fredholm, *et al.* 1999). Furthermore, caffeine intake during pregnancy and early development has been a highly debated subject. Despite its widespread use, we still do not understand the full effects of caffeine. The aim of my thesis research is to determine the long lasting effects of perinatal caffeine exposure on synaptic plasticity, and ultimately to understand how caffeine affects learning and memory formation in the hippocampus. In this study, free behaving rat model will be used to demonstrate the effects on caffeine on LTP of the dentate gyrus of the hippocampus. Previous studies have observed caffeine and LTP under *in vitro* conditions. However, *in vitro* methods lack the complexity of the interconnectivity of the normally functioning brain. To minimize these effects, the present study uses *in vivo* approach using the intact brain of the freely moving animal model to assess the long-term impact of caffeine exposure in early life.

I. Caffeine Consumption and Metabolism

Caffeine, (1,3,7 trimethylxanthine) is the most widely consumed psychoactive drug in the world. Found in various forms of beverage and food, ranging from coffee to tea to chocolate, it is nearly impossible to avoid caffeine consumption (Fredholm, *et al.* 1999). Throughout the world, it is estimated that the average person consumes 70-76 mg of caffeine per day, but up to 210 to 280 mg per day in US and Canada, and even over 400 mg per day by people in Finland and Sweden (Fredholm, *et al.* 1999). The activity of caffeine depends on its intake and clearance. About 15 to 45 minutes after consumption, 99% of caffeine is absorbed in the gastrointestinal

track in humans (Nehlig, 1997; Grosso& Bracken, 2005). Once absorbed, caffeine and its metabolites, dimetylxanthine, paraxanthine, theobromine, and theophylline, freely cross the blood-brain and placental barriers, as well as umbilical cords (Grosso & Bracken, 2005). In humans, the predominant metabolite of caffeine is theophylline while for rats it is dimetylxanthine. Caffeine plays an important role in synaptic activity because of its high solubility and absorption rates in the nervous system (Fredholm, *et al.* 1999). For example, in the hippocampus, moderate caffeine consumption leads to a caffeine concentration of 5-70 uM (Constenla, *et al.* 2010). In the present study, caffeine treatment was through ingested water (1g/L) to model caffeine intake in humans. In Constenla *et al.*'s (2010) study, rats drinking caffeine water (1g/L) showed a blood plasma concentration of 22 uM in the hippocampus.

In terms of its neurochemistry, caffeine is a nonselective adenosine receptor antagonist. The nitrogenous base of the compound resembles that of adenosine, an inhibitory neurotransmitter. As a result, the presence of caffeine is expected to increase synaptic activity by competitively blocking adenosine receptors and increasing excitability. This increase in neural activity cause heightened arousal and attention, and mild anxiety after caffeine consumption (Mendonca & Ribeiro, 2001; Stavric, 1988). Nonetheless, the effects of chronic caffeine use remain highly debatable because while some studies showed an up-regulation A1 receptor (Guillet & Kellogg, 1991; Han *et al.,* 2007), other studies reported decreased in A1 receptor levels (Leon, *et al.*,2002).

Moreover, in adult humans, only about 2% of ingested caffeine is excreted in its pure form through the urinary system (Stavric, 1988). The primary enzymatic activity for the metabolic clearance of caffeine is dependent on the expression of the CYP1A2 gene, which is responsible for a series of demethylation reaction (Stavric 1988; Grosso & Bracken, 2005).

However, the mode of caffeine removal depends on various endogenous and exogenous factors. When plasma caffeine concentration increases, metabolic clearance decreases, and renal clearance is more effective in removing caffeine. In addition, variation in the CYP1A2 gene influences the half-life of caffeine breakdown (Stavric, 1988). Similarly, exogenous factors, such as cigarette smoking, increase caffeine metabolism by nearly two times. Due to an increase in liver enzyme activity *via* induction of hepatic microsomal oxidative enzymes, exogenous factors increases the first two demethylation processes of caffeine metabolism. On the other hand, people diagnosed with liver disorders have a decreased caffeine metabolic rate (Stavric 1988; Grosso & Bracken, 2005).

Pregnancy also influences caffeine metabolism. During pregnancy, the half-life caffeine increases from 5 to 10 hours, and continues to increase to 18 hours by the third trimester. This is a result of lower CYP1A2 activity (Grosso & Bracken, 2005). In addition, caffeine inhibits uterine activity, which further prevents its excretion (Soyka, 1979). Throughout pregnancy, caffeine levels in the blood serum rise from 2.35 mg/ml in early pregnancy to 4.12 mg/ mL by the third trimester (Grosso&Bracken, 2005). However, in the US, more than 80-99% of pregnant women consume caffeinated beverages, such as coffee, tea, and soda (Hannigan, 1995). As indicated previously, caffeine intake during pregnancy leads to accumulation of caffeine in the fetus.

II. Caffeine and Neonates

In the last 30 years, neonatal caffeine exposure has become more prevalent. Caffeine easily moves across blood-brain and placental barriers, and as a result, fetuses and neonates are exposed to caffeine through the umbilical cord or during lactation. In neonates, the lack of necessary enzymes, such as cytochrome P-450, and demethylation pathways increase the half-

life to 80± 23 hours. After birth, caffeine half-life decreases exponentially (Nehlig & Debry, 1994; Nehlig, 1999). In addition, Galli, *et al* (1975) found that caffeine accumulated in the brain and liver of the fetus, which may be a result of undeveloped metabolic pathways (Stavric, 1988).

Since the 1970s, caffeine has been widely used to treat sleep apnea in premature infants. These neonates were exposed to a regular dosage of caffeine for up to 8 weeks (Aranda, *et al.* 1977). In a study by Aranda, *et al.* (1977), caffeine increased respiratory rate, improved blood pH, and elevated oxygen levels. During hypoxia, adenosine is released in the brain, which lowers neuronal activity to reduce neural damage including respiration. However, caffeine also suppressed this response by antagonizing A1 receptors and enhancing expression of long term ventilator facilitation (Julien, *et al.* 2009). Consequently, caffeine significantly reduced the number of adverse neonatal outcomes and the effects of apnea (Aranda, *et al.*, 1977; Julien, *et al.* 2009).

Although neonatal exposure is pervasive, it correlates to an increase in the rate of abnormal fetal growth and spontaneous abortion (Hannigan, 1995; Grosso, *et al.* 2006). Caffeine intake alters fetal development by inhibiting phosphodiesterase, thereby preventing the breakdown of cyclic AMP (cAMP), an important second messenger regulating many intracellular processes. High cAMP levels interfere with cell division and cause catecholamine-mediated uterine vasoconstriction (Soyka, 1979). In addition to affecting cell division, caffeine exposure delays skeletal ossification during exposure (Nehlig & Debry, 1994). As a result, fetal development may be impaired and cause decreased body weight. In fact, caffeine has been correlated with low birth weight in newborn humans and other animal models (Dlugosz & Bracken, 1992; Nehlig & Debry, 1994).

The understanding of the long term effects of early caffeine exposure are limited. A long term, follow up study of participants in the international Caffeine for Apnea of Prematurity trial from 2005-2011 showed that the benefits of neonatal caffeine therapy significantly altered learning abilities by age five, showing cognitive impairment in these caffeine treated babies (Schmidt, *et al.* 2012). Similarly, in their review, Nehlig and Gerard (1994) discussed several behavioral changes associated with prenatal caffeine exposure. In rodents, prenatal caffeine exposure was correlated with a decrease in locomotion and an increase in defecation, as well as a decrease in learning capacities as adults (Nehlig & Debry, 1994). In another study, adult male rats, ages 170-190 days of age, exposed to caffeine showed increased emotional timidity (Hughes & Beveridge, 1991; Nehlig & Debry, 1994).

Some researchers suggest that these behavioral changes in rats may be due to an upregulation of adenosine receptors. Guillet and Kellogg (1990) traced the level of A1 receptors in several brain regions in different age groups. They found that perinatal caffeine exposure upregulated A1 receptors as the rat reached young adulthood. Since A1 receptors inhibit synaptic activity, increase in A1 receptors suggests a decrease in LTP, correspondingly to decrease in learning abilities. Similarly, Han *et al. (*2007) showed that prenatal caffeine exposure reduced neurogenesis in the hippocampus, and impaired memory in adult male rats. In contrast, in Leon, *et al.*'s study (2002), the same level of caffeine exposure (1.0g/L) to neonates and the mother decreased A_1 receptor levels. Thus, the long-term effects of early caffeine exposure have yet to be confirmed. In addition, these aforementioned studies were performed using either behavioral tests or chemical analysis of adenosine receptors. As a result, based on these studies, it is difficult to make conclusions on how caffeine affects learning and memory in the whole intact brain.

III. Hippocampal Circuitry

The hippocampus is the primary brain region involved in consolidating new experiences to long-term storage of memories (Lisman, 1999; Kempermann, 2002). Many experiments have demonstrated that hippocampal lesions impair memory (Lisman, 1999; Schenk& Morris, 1999). In fact, Schenk and Morris (1999) found that rats with hippocampal lesions failed to find the hidden platforms in a spatial water-maze task.

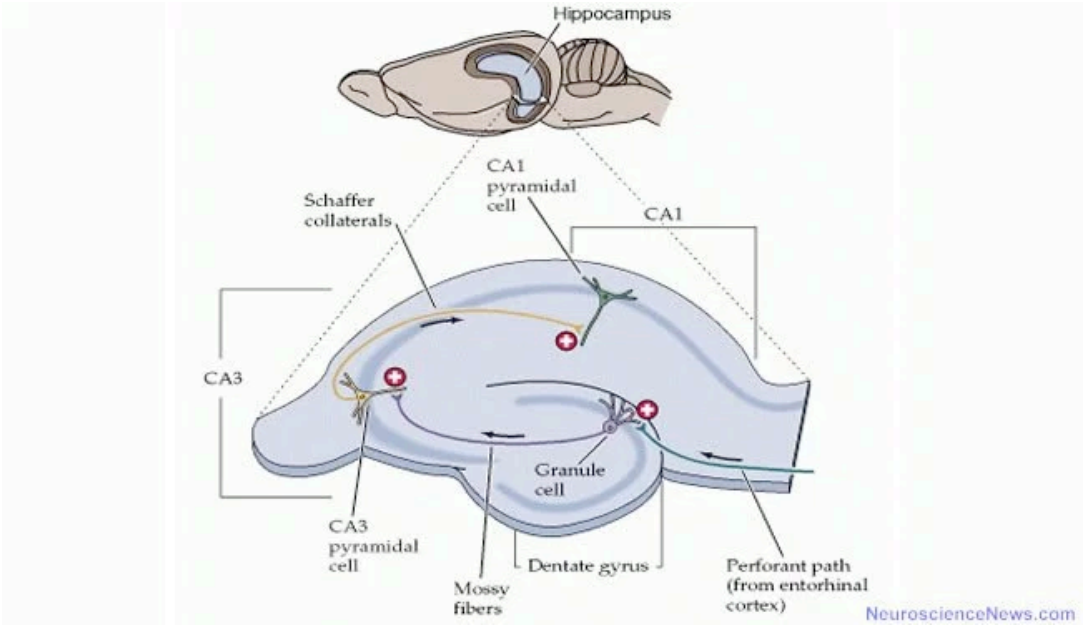


Figure 1. The trisynaptic network of the hippocampal circuitry. Input moves forward from the entorhinal coretex (EC), feeding forward from the perforant path to the dentate gyrus, then to the CA3 then to CA1 pyramidal cells through the mossy fibers, The output signals then leave the hippocampus. (http://neurosciencenews.com/short-term-memories-created-by-researchers-in-vitro/)

The focus of this present study is the dentate gyrus because it is the first region of the hippocampal circuitry and the location for all sensory processing (Bannerman & Sprengel, 2007). During sensory information processing, the dentate gyrus receives signals from the perforant pathway (Figure 1). Next, the dentate gyrus sends output signals through the mossy fibers, reaching pyramidal

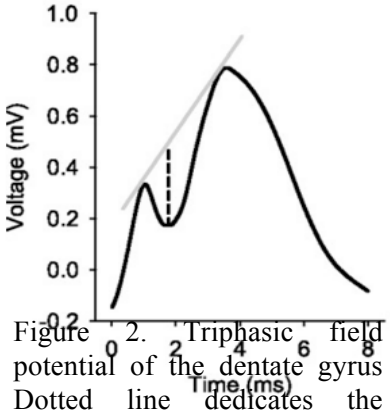


Figure 2. Triphasic field potential of the dentate gyrus Dotted line dedicates the measured population spike amplitude

cells in area CA3. From the CA3, output signals are sent to the CA1 area through Schaffer collateral fibers, and then to the upper cortical regions for "storage" of these memories (Kempermann, 2002). This system is referred to as a feedforward trisynaptic circuit (Bliss & Collingridge, 1993).

When the perforant pathway is stimulated, it causes a triphasic evoked field potential at the dentate gyrus, as shown in Figure 2 (Blaise, 2001). The dentate granule cell field potential consists of two neural events. First, the field excitatory post-synaptic potential (EPSP) is produced by an influx of ions, which depolarizes the dendritic spine. A recording electrode placed outside of the dentate gyrus records a positive electrode potential. Second, a population spike occurs after the EPSP has reached a threshold. Because the inside of the neuron is positive, the electrode picks up a negative electric potential. In other words, the EPSP is a measure of the strength of synaptic activation. An electrical potential occurs as a result of a summation of all action potentials of dentate neurons. Then, a second peak called a population spike occurs after the drop in electrical potential. Thus, the population spike measures the post synaptic response to the stimulus (Bliss & Lomo, 1973).

The paired pulse index also provides further insight into the dentate gyrus. Application of two consecutive pulses over a range of interpulse intervals (IPI) modulates the excitability of the dentate granule cell population (Blaise & Bronzino, 1998). When a paired pulse stimulus is applied to the perforant path, the second response is compared to the first. The intensity of response to the second stimulus depends on both the stimulus, IPI. And so using the paired pulse index the level of the inhibitory and facilitatory modulation of granule cell excitability can be observed.. At IPI 20 and 50 ms, the second pulse is inhibited and called the early inhibitory phase. At this phase, both feedforward inhibition from inhibitory cells and feedback inhibition

directly from the spontaneous firing of the principal cells causes a $GABA_A$ and presynaptic metabotropic glutamate receptor mediated depression (Blaise & Bronzino, 1998; Buzsaki, 1984). The second phase occurs between IPI of 50-300 ms. At this phase, excitation of granule cells is enhanced, likely due to GABA receptor blockade, preventing suppression of the second response (Blaise & Bronzino, 1998). The third phase is the late-onset inhibition, which occurs at IPI range of 300-1000 ms. This late phase is also $GABA_A$ mediated and possibly caused by a depletion of neurotransmitter (Bronzino, *et al.* 1999). As a result, the paired pulse index provides additional information into how perinatal caffeine exposure affects local and extra hippocampal inputs (Bronzino, *et al.* 1999).

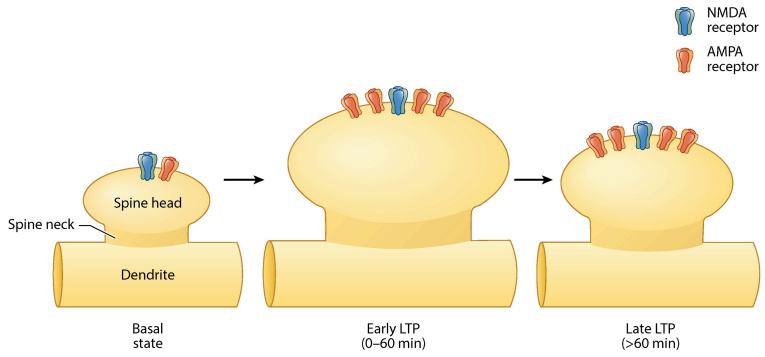
IV. Long Term Potentiation and Learning and Memory

In the early 20th century the neural theory of learning came about which states that:

> "the process of transmission of energy across the synapse leaves its resistance to the passage of the impulse in that direction permanently lowered in some degree, so that the more frequently the discharge of energy has taken place the more readily will it take place in the future. This permanent lowering of resistance, or increase in permeability of synapses seems to be the essential condition of the formation of neural habits, and is therefore an effect of the highest importance. (McDougall 1905).

Over the last century, more evidence supports that learning is associated with an increase in synaptic connections that last for a longer time. In fact, Bliss and Lomo (1973) observed that bursts of high frequency stimulation to the hippocampus greatly increased in synaptic activity. This increase in synaptic activity came to be termed as long term potentiation (LTP).

LTP is induced by high frequency, repetitive, weak stimuli. LTP is input specific. Thus, the increase in synaptic strength is additive, allowing the neuron to reach the threshold for induction (Malenka & Nicoll, 1999). The high frequency excitation of the synapses causes long term structural changes, ranging from physical enlargement of the synapse and formation of more AMPA receptors (Figure 3A) (Cain, *et al.,* 1992; Herring & Nicoll, 2016). Currently, it is believed that structural changes in the postsynaptic cell occur first, which then trigger functional and structural alterations in the presynaptic neuron (Malenka & Nicoll, 1999).



Herring BE, Nicoll RA. 2016.
Annu. Rev. Physiol. 78:351–65

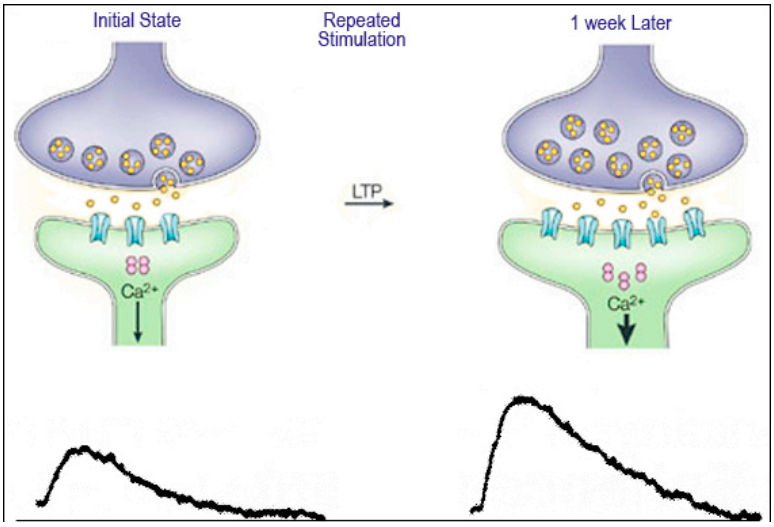


Figure 3. Molecular, cellular, and voltage response of LTP. This is a result of increase in glutamate release as well as synaptic changes that promote depolarization of the neuron. A. Repeated stimulus results in an enlargement of the synapse as well as upregulation of NMDA and AMPA receptors. B. This increase in synaptic response is long lasting
(http://cdn.shopify.com/s/files/1/0269/3025/files/LTP_grande.jpeg?173)
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As a result, the increase in synaptic activity is seen hours or days after the repeated stimuli. These characteristics of LTP provide a cellular model for classical conditioning, in which repeated exposure to a stimulus eventually leads to learned behavioral response (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999).

LTP develops in all three synapses in the hippocampus, but differs in induction among the synapses. In the dentate gyrus, the two main neurotransmitters are aspartate and glutamate. Glutamate acts upon N-methyl-D-aspartate (NMDA) receptors. When antagonists such as D-2-amino-5-phosphonovalerate (APV) block NMDA receptors, LTP is not formed in the dentate gyrus, but APV has little to no effect on normal low frequency responses (Collingridge & Bliss, 1987). When glutamate is released in response to high frequency stimulation, it binds to the second NMDA subunit (N2RA) eventually leading to LTP (Liu, *et al.* 2004).

Another receptor in LTP formation is the adenosine receptor (Mendonca & Ribeiro, 2001). Although there are four subtypes, the A1 receptor subtype is the most prevalent in the central nervous system (CNS), concentrated in the hippocampus and cerebral cortex (Fredholm, *et al.* 1999). A1 receptors play a neuroprotective function in the CNS because they decrease the firing rate of neurons (Fredholm, *et al.* 1999; Mendonca & Ribeiro, 2001). Constenla *et al.* (2011) found that A1 receptors modulated the magnitude of LTP in young rats. Adenosine receptors are G-protein coupled receptors which inhibit synaptic activity, preventing overstimulation of the neuron. When adenosine binds to A1 receptors, G-protein is inactivated, resulting in inactivation of adenylyl cyclase. This results in a decrease in cyclic AMP levels, leading to inactivation of calcium channels. Accordingly, hyperpolarization of the presynaptic neuron prevents the glutamate release. Due to the inhibition of neurotransmitter release in the

presynaptic neuron, the post synaptic neuron fails to form an action potential (Wu & Saggau, 1994; Nehlig; 1999). In the postsynaptic neuron, adenosine binding leads to Cl^- and K^+ influx that inhibits depolarization (Cunha, 2004). In my study, adenosine receptors are of interest because caffeine is a nonselective antagonist of adenosine receptors (Mendonca & Ribeiro, 2001).

V. *In Vivo* vs *In Vitro*

As suggested previously, the effect of perinatal caffeine exposure on LTP formation in the hippocampus remains yet to be determined. Therefore, the aim of this study is to determine the long term effects of perinatal caffeine exposure on synaptic plasticity in freely moving rats. Many studies involving caffeine exposure and LTP use *in vitro* or anesthetized *in vivo* models (Constenla *et al.* 2010, Greene, *et al.* 1985, Simons, *et al.* 2012). *In vitro* slice preparations isolate the brain region of interest from the rest of the specimen. These slices are then placed in bathing solutions to form an artificial environment to keep neurons viable (Anderson, *et al.* 1970; Schwartzkroin& Wester, 1974). The brain is a highly complex, interconnected organ. Thus, when these connections are removed, the end results is a simplified, less than complete reflection of what actually occurs in the brain. In addition, slight variations in the artificial environment, such as oxygen level, temperature, can affect the experiment. Similarly, the effects of the anesthetic can introduce confounding factors *in vivo* anesthetized studies. Anesthetics have been found to attenuate LTP expression in the dentate gyrus (Ikegaya *et al.*, 1995). Because anesthetics disrupts normal neuronal activity, the possibility of large variability and fluctuation of results cannot be minimized.

Freely behaving rat models offer the best method of studying synaptic plasticity because it removes these limitations present in *in vitro* and anesthetized *in vivo* studies. The hippocampus receives connections from the medial septum, locus coeruleus, and dorsal and median raphe nuclei, which modulate dentate granule cell excitability (Bronzino, *et al.* 1999). Under *in vivo* conditions, the interconnections throughout the brain are still present and operate in the freely behaving rat. The extrinsic inputs are still intact, providing a more accurate representation of the dentate response (Bronzino, *et al.* 1997). In addition, because the brain remains in the skull, variation in the environmental conditions plays little impact on neuronal activity. Electrophysiological analysis will be used to measure the synaptic activity of the dentate gyrus. The electrodes record the activity of the region of interest and collects brain's response to the eliciting input in time. The evoked response from the targeted stimulus indicates changes in brain activity, providing insight into the formation of learning and memory at the synaptic level (Molfese, *et al.* 2001).

In the present study, the effect of perinatal exposure of caffeine on learning and memory is investigated *in vivo* by measuring synaptic plasticity in freely behaving animal model of learning and memory. Using electrophysiological techniques, LTP is quantified and compared between the control group and the experimental group to determine whether "learning and memory formation" is affected by early caffeine exposure.

MATERIALS AND METHODS

I. Animal Preparation

All experimental protocols were done under the US Public Health Service's Guide for the Care and Use of Laboratory Animals, and were approved by the Trinity College Institutional Animal Care and Use Committee (IACUC). Caffeine water solution (1.0 g/L) was administered to the experimental group to model the average caffeine intake of three cups of coffee a day in humans (Constela, *et al.,* 2010). Caffeine water (1.0g/L) was administered to female dams Sprague-Daley rats prior to mating. Unlimited caffeine water was accessible 24 hours a day. Pregnant mothers received caffeine water throughout gestation and until weaning of pups. Pups were weaned at 21 days old, or until they were large enough to reach the food and water. After 21 days after birth, mothers were given regular tap water. To reenter the breeding cycle, female rats were placed on tap water for a minimum of 1 week to make sure all caffeine metabolites have been broken down and removed from the body. The control group received water without caffeine during gestation and development. All rats were maintained on a 12 hour light and 12 hour dark cycle at 22°C.

After weaning, all rats pups were allowed *ad lib* access to regular tap water and rodent chow pellets (Purina 5000) in accordance to the Trinity College animal care plan.

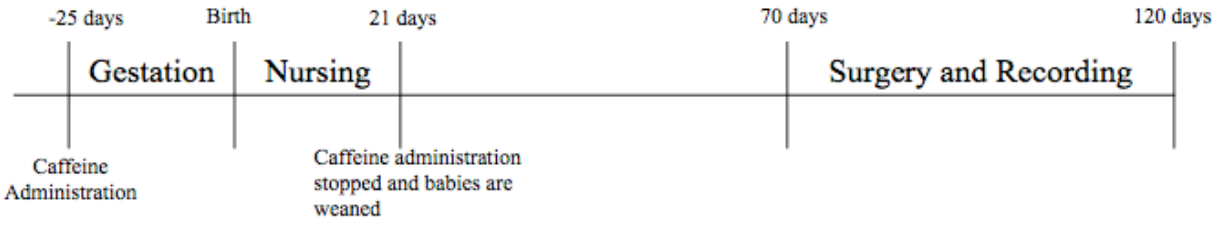


Figure 4. Timeline of caffeine administration of perianal caffeine exposed experimental group.

II. Stereotaxic Surgery and LTP Recording

All surgical and recording procedures were performed between 7:00 and 17:00 to account for the rats' circadian rhythm. Between 70-120 days of age, male rats underwent stereotaxic surgery to implant electrodes into the perforant path and the dentate gyrus of the hippocampus.

The surgical procedure was performed using the same method as previous experiments involving perforant path-dentate gyrus (PP-DG) LTP studies in this lab (Blaise, *et. al*, 2008). Each rat was anesthetized using interperitoneal injection of a mixture of ketamine, xylazine, and acepromazine. The dose of anesthetic was proportional to the body weight. While the rat was under anesthesia, body temperature was stabilized using heat lamps. Once the rat lost all muscle reflex, the head was immobilized in stereotaxic head gear. Using the bregma and lambda as a reference, the head was adjusted to form a flat skull surface. Precise burr holes (1mm in diameter) were made into these regions of the skull using a small, electrically operated dental drill (Figure 5). Bipolar, stainless steel stimulating electrodes were lowered into the perforant pathway (AP: −8.1 mm; LAT: +4.0 mm; relative to the Bregma). Monopolar tungsten recording electrodes were lowered into the dentate gyrus using a single pulse stimulation to determine the depth of the recording electrode (AP: −4.0 mm; LAT: +2.5 mm relative to Bregma). Once the digital oscilloscope indicated the maximum triphasic signal of the PP-DG signal, the electrodes were stabilized using dental cement, forming a crown on the dorsal side of the skull. The

Figure 5. Electrode placement relative to the bregma and lambda.

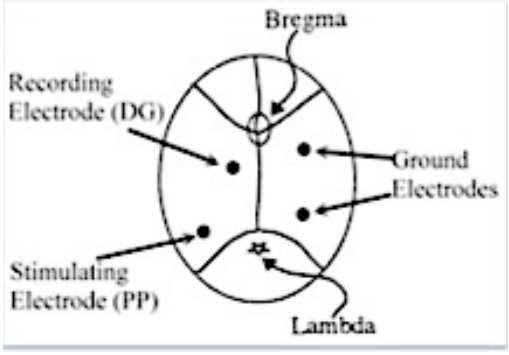
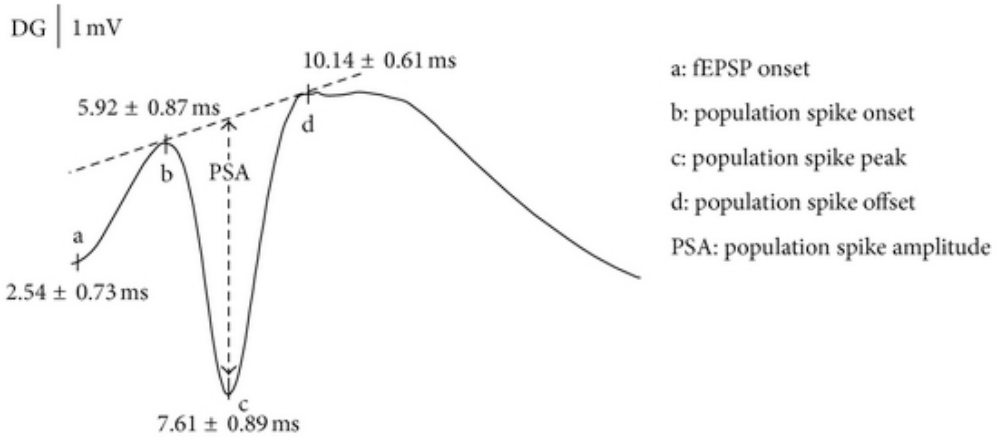


Figure 6: Trace of evoked dentate gyrus field potential stimulated by the perforant path.

incision was sutured using violet microfilament polydioxanone synthetic absorbable sterile suture. Once all anesthetics wore off, rats were returned to the colony room for five days to recover from the surgery.

III. Electrophysiological recording



After a minimum of five days post-surgery, rats were placed overnight inside the recording chamber to acclimate prior to recording. Rats were connected to the recording instruments using long, low noise wires to allow for free movement. Biphasic square wave pulses were sent using the DAM 50 differential amplifier and Grass S-88 stimulator (pulse width = 0.25 ms, 50% duty-cycle). The response was amplified (100 or 1000 fold), bandpass filtered (1Hz-3kHz) and displayed on the BK precision digital storage oscilloscope. The signal was recorded using LabView analysis software to acquire and record the triphasic PP-DG signal, which was quantified using population spike amplitude (PSA) and excitatory post synaptic potential (EPSP) measurements (Figure 6). The Input/Output curve was constructed by recording the PSA and EPSP at current intensities from the lowest current that produced the PP-DG

response to 1500 uA. Most rats began recording at 400 uA, and ten recordings were each made at, 600, 800, 1000, 1200, 1400, and 1500 uA. The current with 50% maximum response was used for further recording processes.

Using the 50% maximum response current, ten responses were recorded at each interpulse intervals (20,30,50,70,150,300,500,1000 ms) (Blaise & Bronzino, 2000). To determine the paired pulse index, the percent change between the first and second PSA, was calculated.

Baseline response level was determined using an average of five evoked responses at 50% current intensity every minute for 15 minutes. To induce LTP, rats were tetanized with 5 Hz burst stimulation (10 bursts of 10 pulses at 400 Hz) at a current of 800 uA. After tetanization, an average of five PSA and EPSP values of PP-DG signal was measured each minute for 15 minutes. At 0.5, 1, 2, 3, 24, 48 hours post tetanization, 10 recordings of PP-DG response were measured, and averaged for each time interval. The percent change of PSA and EPSP between the pre- and post-tetanization was calculated for each time interval post tetanization.

Throughout the recording process, the experimental group was given access to unlimited caffeine water and food. Electrophysiological measurements were performed on control (N=10) and early caffeine treated (N=11) rats. T-test assuming equal variance was used to compare the percent change in PSA post-tetanization between the experimental and control group to determine significant difference in LTP.

RESULTS

The objective of this experiment was to determine the effects of early caffeine exposure on learning and memory in freely behaving, adult rats. LTP was quantified by using the population spike amplitude, a measure of post-synaptic neuronal discharge. After electrode placement and a 5-7 days post-surgical recovery period, no behavioral differences were observed between the experimental (N=11) and control (N=10) groups. When measuring LTP data, EPSP and PSA were recorded. However, in this study, EPSP was not used to quantify LTP because it has been shown that the EPSP waveform is occluded by the PSA waveform in dentate gyrus recordings. For this reason, the PSA which has been traditionally used in *in vivo* electrophysiology was used to determine statistical significance between the experimental groups due to its robustness (Bliss and Lomo, 1973).

Additionally, it must be noted that several physical characteristics were observed between the control and experimental groups. According Charles River® Laboratories (a lab animal vendor), the mean weight of normal rat pups at 21 days is 50.0g (http://www.criver.com/products-services/basic-research/find-a-model/sprague-dawley-rat), compared to to an average 39.6g for rat pups exposed to caffeine in our lab. However, we did not observe any statistical difference in weight between control and caffeine exposed rats within the age tested (70-120 days). We also noted anecdotally that the early caffeine exposed group showed more behavioral hyperactivity as measured by observations of physical movements. However, no systematic quantifiable measurements were made to determine whether these behaviors were significantly different from the control group.

Prior to measuring LTP levels, an input/output curve was measured for the caffeine exposed rats.

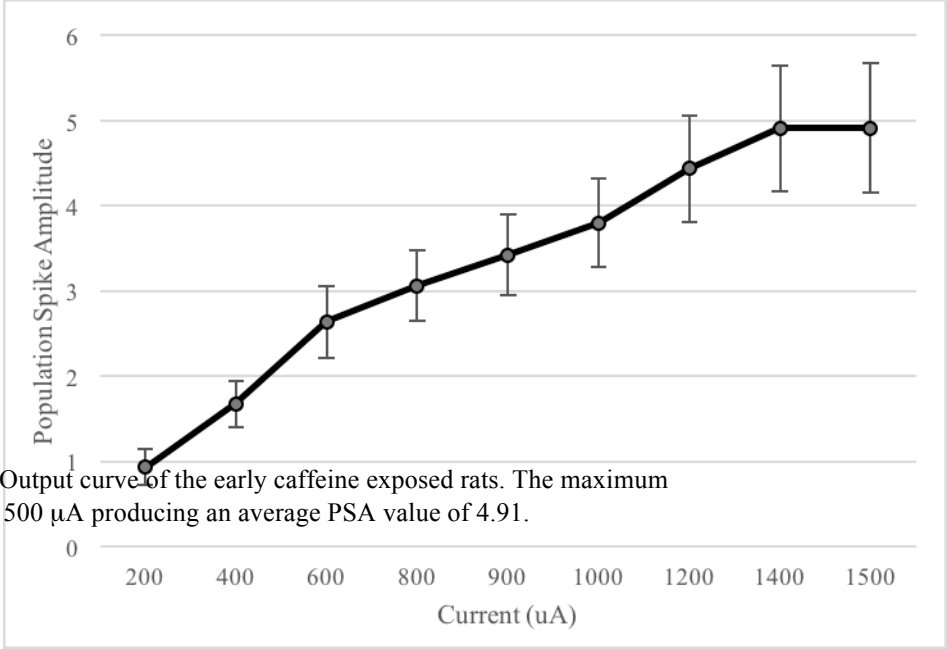
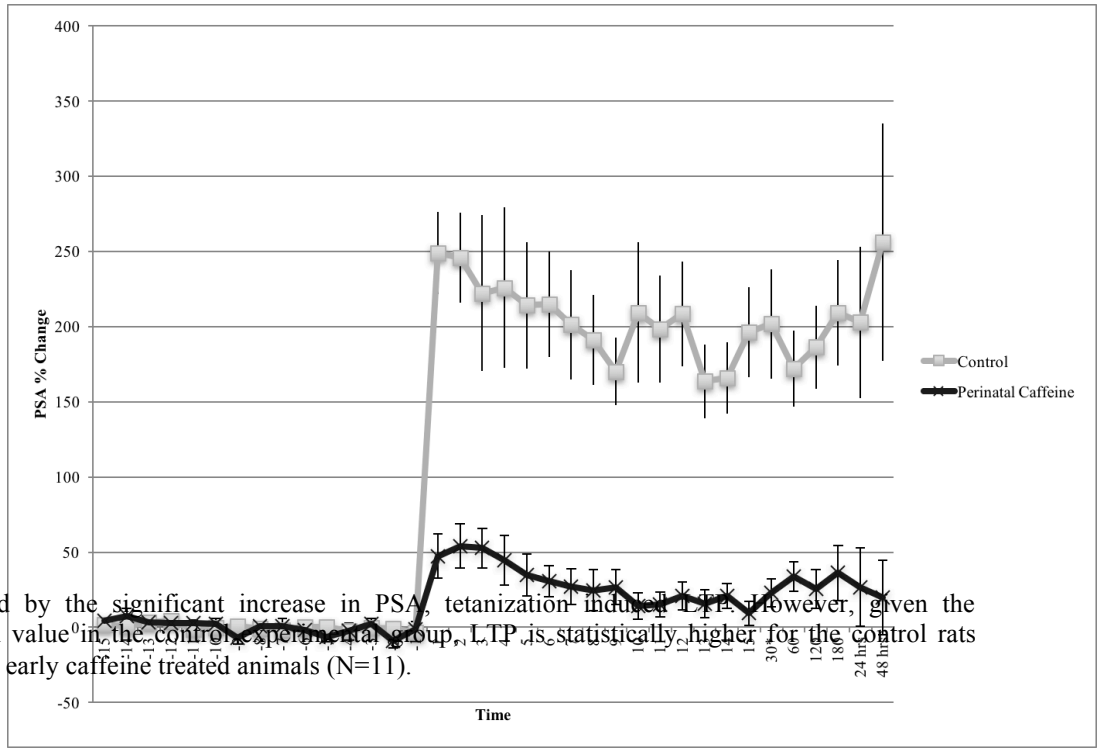


Figure 7. Input/Output curve of the early caffeine exposed rats. The maximum PSA occurs at 1500 μA producing an average PSA value of 4.91.

The IO curve begins to plateau around 1400 μA, and the maximum PSA was on average 4.91 at 1500 μA (Figure 7). On average, the 50% maximum PSA value occurred between 600 μA and 800 μA, and majority of the recordings of PSA were performed using this current. The IO curves were not statistically significant from those of control animals.

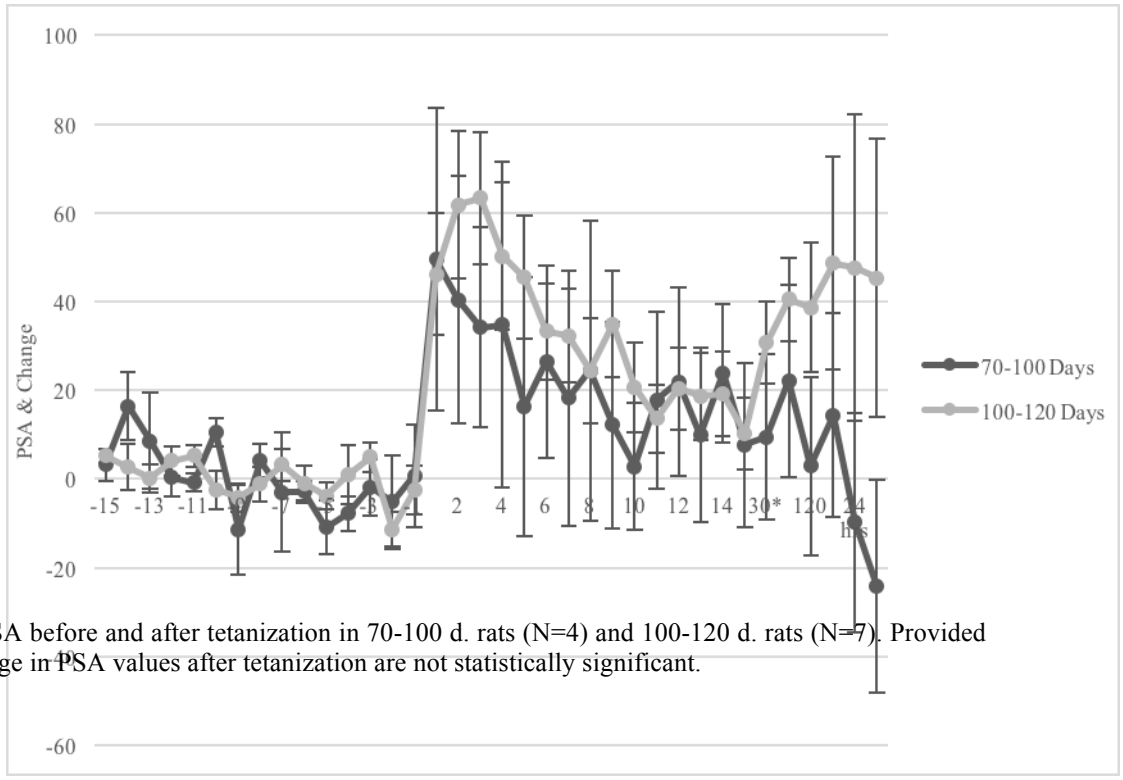
Both control ($t = 2.032$, $p < 0.01$) and caffeine groups ($t = 2.032$, $p < 0.01$) showed potentiation after tetanization.



gure 8. As illustrated by the significant increase in PSA, tetanization induced LTP. However, given the tistically higher PSA value in the control experimental group, LTP is statistically higher for the control rats =10) compared to the early caffeine treated animals (N=11).

The control group (N=10) and caffeine exposed group (N=11) showed significant difference in PSA percent change after tetanization ($t = 1.994$, $p < 0.01$) (Figure 8). The control group showed significantly higher increase in PSA percent change after tetanization.

After noting that two of the four animals under 100 d showed no potentiation, the LTP data were separated by subject age to determine if there was any statistical difference between two age subgroups: 70-100 d (N=4) and 100-120 d. (N=7). The change in PSA is shown in Figure 9.



gure 9. Change in PSA before and after tetanization in 70-100 d. rats (N=4) and 100-120 d. rats (N=7). Provided
e error bars, the change in PSA values after tetanization are not statistically significant.

Although, the error bars suggest that the two caffeine subgroups do not seem statistically significant, further statistical testing using the standard t-test indicates that the 100-120 d. rats showed statically higher PSA percent change after tetanization ($t = 1.994$, $p < 0.05$).

The paired pulse index was also measured in the early caffeine exposed animals in order to assess differences in short-term synaptic plasticity. The paired pulse data of the early caffeine exposed animals were compared to the results of 90 d control animals in a quiet waking state (Blaise and Bronzino's 2000). Using paired two sample means t-test, early caffeine exposed animals showed statistically greater paired pulse index ($p < 0.05$). Additionally, the graphic representation of the control and caffeine treated paired pulse index further supports this statistical analysis (Figure 10).

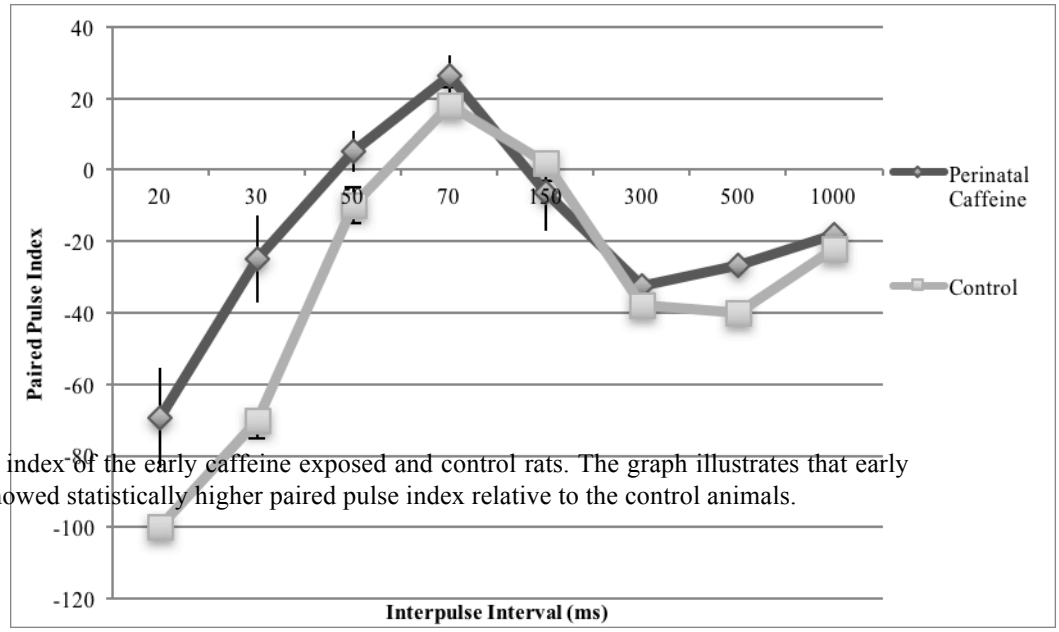


Figure 10. Paired pulse index of the early caffeine exposed and control rats. The graph illustrates that early caffeine exposed rats showed statistically higher paired pulse index relative to the control animals.

Given the greater paired pulse index of the early exposed caffeine animals, perinatal caffeine exposure seems to increase short-term synaptic inhibition relative to the control. However, it is important to note that the control paired pulse index was collected from Blaise and Bronzino's publication done in 2000. Therefore, further research may be necessary for direct comparison from this data.

DISCUSSION

The objective of this study was to determine the effects of early caffeine exposure on learning and memory, in adult, freely-behaving rats. Instead of using behavioral tests or *in vitro* methods to measure learning and memory, I used *in vivo* electrophysiological methods to measure LTP of the dentate gyrus in the hippocampus in freely behaving rats. After modeling caffeine intake during pregnancy and nursing in Sprague-Dawley rats, the experiment showed that perinatal caffeine exposure had lower levels of inhibitory modulation in the dentate gyrus, but had significantly lowered LTP, where potentiation occurred at very low levels in the early caffeine-treated animals.

Inhibition in LTP formation

LTP exemplifies the classic Hebbian Theory in which repeated stimuli causes long lasting changes in the synapse. These changes in the synaptic structure coincide with an increase in post synaptic excitation. Due to the long lasting nature of synaptic efficacy, LTP has become a widely accepted neurological model of adult memory storage (Stanton, 1996). This synaptic activity associated with LTP is affected by several variables such as neonatal stress, sleep deprivation, and pharmacological manipulations such as caffeine exposure(Blaise, *et al.* 2007; Kim *et al.,* 2005; Constenla *et al.,* 2010). There are several ways to inhibit the induction of LTP. LTP requires activation of presynaptic kainite receptors by endogenous glutamate. In addition to increasing neurotransmitter release, the calcium/calmodulin-dependent protein kinase II (CaMKII) causes the presynaptic structural changes in LTP expression. For this increase in synaptic activity to persist, signaling molecules (PKA, CaMKIV, and MAPK) are necessary to activate gene transcription and consequently, protein synthesis. In addition, morphological

changes associated with LTP include growth of dendritic spine (Malenka & Bear, 2004). Many components are necessary for LTP. Therefore, caffeine exposure may disturb any one or more of these steps in synaptic plasticity.

Despite the prevalent use of caffeine, studies on long term caffeine exposure on learning and development presents several discrepancies. While some studies suggest negative impact of caffeine on learning and memory, others show beneficial, neuroprotective characteristics during sleep deprivation or in brain disorders such as Alzheimer's disease, Parkinson's disease, and Attention Deficit Hyperactivity Disorder (Soellner, *et al.* 2009; Alhaider, *et al*. 2010). Even more uncertain is how Perinatal caffeine exposure affects neural activity after maturation. However, the results of my study suggest that caffeine exposure early in life significantly impairs LTP formation in freely-behaving adult male rats as noted by the lower levels of potentiation in the dentate gyrus suggested by the significantly lower PSA values in the early caffeine treated rats.

Alteration in Development During Critical Period

Humans and rats show similar brain development. Overall brain growth rate is the highest between 7-14 days old. Specifically, the granule cells of the dentate gyrus begin growth 7 d prior to birth, reach a maximum growth rate around 7 d after birth, and decrease past 35 d after birth (Morgane, *et al.* 2002).

The developmental sequence is regulated genetically. However, several exogenous factors, such as caffeine, can alter the activity of the enzymes in brain development by preventing proper neuronal formation (Morgane, *et al*. 2002). In this experiment, early caffeine exposed animals were treated with caffeine during these critical periods of brain development. In addition, because embryos lack the caffeine metabolizing proteins, prenatal caffeine exposure

leads to a buildup of the methylxantheine in the brain. In other words, factors that disturb normal development during critical periods can have major lasting consequences.

Maternal caffeine ingestion may cause major consequences in fetal brain development. Kirkinen, *et al.* (1983) observed that caffeine consumption lowered the placental blood flow after caffeine consumption of just 200 mg. Endothelin-1 (ET-1), a vasoactive polypeptide increases placental blood flow, decreased by 51% after caffeine exposure (Liu, *et al.* 2008). Due to the decrease in blood flow, several stress responses, such as release of catecholamines, may interfere with proper neuronal development (Gressens, *et. al*, 2001; Grosso & Bracken, *et al*. 2005).

Adenosine is present early in development. As an antagonist of adenosine, caffeine inhibits phosphodiesterase and breakdown of cAMP. This increase in cAMP may interfere with cell division or lead to uterine vasoconstriction (Souza, *et al.*, 2007; Soyka, 1979). By 18 d after fertilization, adenosine receptor distribution is similar to that of adult levels (da Silva, *et al.*, 2007). Because embryos lack the metabolic enzymes necessary for caffeine breakdown, this increase in cAMP level can be prolonged and have long term detrimental effects on neuronal development.

In addition, because caffeine interferes with proper distribution of blood from the mother, it may slow down normal neuronal development. Decreased oxygen availability may cause stress responses, buildup of metabolic precursors, and hormonal changes. Because caffeine prevents proper development during the critical period, it has long-term consequences that affects synapse formation and plasticity even in adult hood.

Caffeine and Body Weight

Pups from caffeine treated mothers had significantly lower body weight measurements compared to the control rats. In addition, caffeine treated pups were weaned at an older age than the control rats because of its significantly small size. This decrease in body weight has been observed in previous studies. Many cohort studies have identified a negative correlation between caffeine intake and low birth weight in humans (Larroque, *et al.,* 1993; Chen, *et al.* 2014; Rhee, *et al.* 2015). As previously mentioned, the low developmental rate may be due to the high cAMP levels that impacts cell proliferation (Souza, *et al.*, 2007). Due to the inhibition in cell proliferation, caffeine may have lowered developmental rate during gestation, decreasing body weight. However, the exact mechanism of low birth weight is not fully understood.

Upregulation of Adenosine 1 Receptors

Adenosine plays a role in regulating neurotransmitter release. Adenosine also influences neuron development and death during early development (da Silva, *et al.* 2008). However, several studies have shown that early caffeine exposure directly affects the ontogeny of A1 receptors in various brain regions.

Guillet and Kellogg (1991) administered caffeine to neonatal pups and measured A1 receptor density and binding affinity at various stages of development. A1 receptor expression increased without changes in binding affinity. Interestingly, this upregulation of A1 receptor was significant even after three months after caffeine exposure. Caffeine exposure during critical periods may have accelerated reaching adult A1 receptor density at a faster rate (Guillet & Kellogg, 1991). As a result, significantly high A1 receptors in adults may have caused an inhibitory effect at the dentate gyrus synapse, resulting in lower LTP in the early caffeine

exposed rats. Similarly, Zimmerberg, *et al*. (1991) further demonstrated this up-regulation of A1 receptors. After early caffeine exposure, learning was measured using spatial operant tasks after 69 days of age. Postnatal caffeine exposed rats showed impaired learning abilities.

A1 expression is first detectable at 14 days of gestation, and throughout embryonic development, A1 mRNA is highly expressed. Weaver (1996) carefully measured the A1 gene expression during gestation. Given the role of A1 receptors during embryonic development as well as caffeine's interaction, prenatal exposure may influence neuronal differentiation and migration, causing long-term effects on brain and behavior. Given the alteration of A1 receptor expression, perinatal caffeine exposure may have a long-term effect on LTP formation resulting from inhibitory effects of adenosine receptors in the synapses.

Decrease in inhibitory modulation

The paired pulse test showed that early caffeine exposed animals showed statistically higher paired pulse index values, suggesting less inhibitory modulation on the granule cells of the dentate gyrus (Blaise & Bronzino, 2000). However, the low levels of inhibition exhibited by the early caffeine exposed rats was surprising considering that the LTP data suggests more inhibition in synaptic activity of the perinatal caffeine exposed rats. Inhibitory effect of the interneurons in feed-forward inhibition is mediated by $GABA_A$ and presynaptic metabotropic glutamate receptors (mGluRs). Similarly, these proteins are also necessary in the structural changes associated with LTP formation (Balschun, *et al.*, 1999). Given elevated A1 receptor expression in early caffeine exposed rats, it may have decrease glutamate release. As a result, this decrease in glutamate release may be attributed to less inhibition of the interneurons (Iglesias, *et al.*, 2005). In addition, Iglesias, *et al.* (2005) indentified that chronic intake of

caffeine during gestation decreased mGluR levels in fetal heart. Therefore, could it be that early caffeine exposure have also led to a down-regulation of mGluR in the hippocampus?

However, the paired pulse data was compared to the control paired-pulse index of 90 d rats (Blaise and Bronzino, 2000). Therefore, further research needs to be performed in order to conclude the effects of early caffeine exposure are mediated by interneurons.

Alteration in Synaptic Proteins and Neurotransmitters

In this current study, *in vivo* methods were used to confirm that continuous caffeine exposure during gestation and the critical period impairs LTP in adult rats (Ardais, *et al.* 2016). Prenatal caffeine exposure has been shown to have major consequences in synaptic formation during gestation (Mioranzza, *et al.* 2014). Various proteins are necessary for proper synaptic formation in the brain. The TrkB receptor binds to the brain-derived neurotrophic factor (BDNF) to influence regulation and structural development of the synapse. Specifically, in development, it is involved in growth of dendrites oat excitatory and inhibitory synapses. Secondly, the Growth Associated Protein-43 (GAP-43) is responsible for the proliferation of neural precursors, affecting maturation and outgrowth of neurons and synapse formation in the brain. Mioranzza, *et al.* (2014) administered 1.0g/L caffeine water during gestation and found decreased TrkB and GAP-43 levels. Given the decrease in these synaptic protein levels, caffeine may inhibit proper synapse formation during this critical developmental period.

Interestingly, given the statistically higher LTP data of 100- 120 d rats compared to 70-100 d rats, the effects of caffeine exposure during gestation can be recovered through prolonged caffeine intake after birth. In the study by Ardais, *et al.* (2016), additional caffeine exposure after birth and throughout life reversed the decreased BDNF levels. However, male rats showed no

impairment in memory. Therefore, how further caffeine exposure after birth affects LTP in the adult rats is another question to be studied. If BDNF levels returns to normal levels, it may be expected that LTP levels will reach same potentiation level as control rats.

One of the major neurotransmitter regulated by adenosine receptors is acetylcholine. Caffeine's antagonistic binding to A1 receptors may promote acetylcholine activity. Souza, *et al.* (2015) showed that chronic, maternal caffeine exposure increased acetylcholinesterase function. Firstly, caffeine's antagonistic effect on A1 receptors triggers PKA phosphorylation. Increase in phosphorylation of PKA protein causes an increase in acetylcholinesterase activity. Given that A1 receptors are highly expressed in the hippocampus starting at an early stage of gestation as well as the up-regulation of A1 receptors, caffeine's inhibitory effect on adenosine receptors during gestation as well as after birth may have significantly increased acetylcholine activity in the rats (Souza, *et al.*, 2015). However, in the study by Da Silva, *et al.* (2008), 1.0 g/L caffeine exposure increased hippocampal acetylcholine degrading enzyme activity in pups 21 days old. The increase in acetylcholine degradation then decreases the level of the neurotransmitter. This has significant effects in normal neuronal development because during critical time, monoamines neurotransmitters play an important role in the architecture of the nervous system (Souza, *et al.*, 2015).

Future Studies

Although it has been widely used as a model for learning and memory, LTP may not perfectly correspond to memory performance. Rather than learning and memory, LTP is more accurately a measure of excitability (Hölscher, C., 1997). When injected with mGluR antagonist, LTP formation was completely inhibited. Although LTP formation was inhibited, animals were

still able to perform spatial and nonspatial tasks (Soellner, *et al.* 2009). Therefore, to further confirm the results of this experiment, it may be required to repeat this experiment using behavioral and biochemical analysis, such as adenosine receptor and neurotransmitter levels. Using spatial memory tasks, it would be possible to determine whether early caffeine exposure will inhibit proper task performances (Soellner, *et al.* 2009).

In addition, some studies have suggested that the dentate gyrus is not the ideal hippocampal location to measure long term memory formation (Hölscher, 1999). As mentioned earlier, the dentate gyrus was the focus of the study because it is the first region of processing of sensory information in the hippocampus. Because of this, synaptic activity in the dentate is nonspecific. By contrast, LTP of CA3 and CA1 areas may better represent long term memory formation. This is because, as the sensory information proceeds through the hippocampus, all of the stimuli are filtered. The CA1 encodes specific, processed information, and, consequently, it may be a better region to study for learning-specific changes (Hölscher, 1999).

It would be quite interesting to further study age dependence of caffeine exposure. Although the results of the study did not show statistical significance between the 70-100 d (N=4) and 100-120 d (N=7) rats, it may be that the sample size was not large enough to remove random variance. By having larger sample size, given specific time frame of age, it would be possible to determine whether it would be possible to recover from the effects of early caffeine consumption.

CONCLUSION

In conclusion, early caffeine exposure was observed to decrease LTP in the dentate gyrus of the hippocampus. This suggests that early caffeine exposure may impair the ability to form long term memory in rats. The results of this study may be caused by caffeine's disturbance of proper neuronal and synapse maturation during gestation and critical period, and permanently impact on synaptic plasticity.

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