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# THE NEURODEVELOPMENTAL EFFECTS OF CHRONIC MODERATE PRENATAL CAFFEINE EXPOSURE IN RATS

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# THE NEURODEVELOPMENTAL EFFECTS OF CHRONIC MODERATE PRENATAL CAFFEINE EXPOSURE IN RATS

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## ABSTRACT

THE NEURODEVELOPMENTAL EFFECTS OF CHRONIC MODERATE PRENATAL CAFFEINE EXPOSURE IN RATS

By

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Caffeine is the most widely used psychostimulant in the world. Although women are currently advised to reduce caffeine intake throughout pregnancy, it is estimated that 70-95% of pregnant women continue to consume the drug. The widespread use of caffeine is of concern because it crosses both the placental and blood brain barriers to accumulate in the developing brain. Furthermore, the half-life of caffeine is greatly prolonged in the developing fetus, being twenty fold that of an adult. Caffeine is an adenosine receptor antagonist and adenosine is a well-known critical neuromodulator during brain development. Adenosine functions to reduce neuronal activity thus providing protection against excitotoxicity. When adenosine receptors are inhibited by caffeine, there is an overall increase in glutamate neurotransmission particularly in brain regions with an abundance of A1 adenosine receptors such as the hippocampus. Glutamate receptors play a role in numerous developmental processes including activity-dependent migration, synapse formation, and synaptic plasticity. Therefore, increased glutamate neurotransmission in the developing brain as a result of prenatal caffeine exposure could have numerous neurodevelopmental consequences. In order to elucidate the effects of prenatal caffeine exposure, pregnant rats were exposed to chronic moderate doses of caffeine (75, 150, or 300 mg/L) *ad libitum* in their drinking water throughout

gestation. Moderate doses and oral administration were chosen to maintain human relevance of the studies.

The objectives of this dissertation were to determine whether caffeine and its primary metabolites accumulate in the fetal brain and the effects of prenatal exposure to these drugs on hippocampal glutamate receptor expression, glutamate-mediated calcium transients, learning and memory behaviors, and anatomy. The findings presented in this dissertation show that chronic prenatal exposure to moderate doses of caffeine results in an accumulation of caffeine and its primary metabolites in the fetal brain. Prenatal caffeine exposure also alters protein expression of the NR1, NR2A, and NR2B subunits of the NMDA receptor and reduces glutamate-induced peak calcium transients in caffeine-exposed hippocampal neurons. Further, caffeine-exposed offspring show impaired learning and memory behavior in both the novel object recognition task and radial arm maze, but not in the Morris water maze. Lastly, prenatal caffeine exposure results in a significant increase in total hippocampal volume in caffeine-exposed juvenile rats, however this effect does not persist into adulthood. In summary, the studies in this dissertation present novel findings of cellular, behavioral, and anatomical neurodevelopmental effects following moderate exposure to prenatal caffeine.

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## Chapter 1. Introduction and Specific Aims

### A. Caffeine Consumption, Metabolism, and Distribution

Caffeine is the most widely consumed psychoactive drug in the world due to its presence in coffee, tea, chocolate, soft drinks and numerous medications. The caffeine content in food and beverages varies considerably, depending on the food or beverage and its method of preparation. For instance, an 8 oz. cup of coffee contains approximately 40-160 mg caffeine depending on whether it is prepared by brew or drip. A single cup of tea contains approximately 30-70 mg depending on the type of tea leave used. Soft drinks contain between 30-45 mg caffeine per 8 oz. The daily intake of caffeine in the United States is estimated to be 4.0 mg/kg in a 70 kg person (approximately 280 mg/day) (Barone & Roberts, 1996).

Once consumed, caffeine is rapidly absorbed by the stomach and intestines to reach the blood (Fredholm *et al.*, 1999). Caffeine's hydrophobic chemical composition allows it to cross all biological membranes, including the blood brain barrier, to be distributed throughout the body (McCall *et al.*, 1982). Peak plasma concentrations are reached within 15-120 minutes of intake, with consumption of a single cup of coffee resulting in plasma concentrations of 1-10 μM caffeine (or 0.25-2 mg/L) (Fredholm *et al.*, 1999). Excessive consumption of caffeine can produce toxic effects. In rats, caffeine is toxic at doses greater than 200 mg/kg, resulting in seizures, which may lead to death (Bonati *et al.*, 1984-1985). In humans and animals, the effects of caffeine are biphasic. At low doses, such as those commonly consumed in a few cups of coffee, caffeine produces stimulatory effects such as increased locomotor activity, enhanced attention,

and reduced fatigue. However, at high doses, caffeine leads to reduced locomotor activity, inability to concentrate, increased anxiety, and other negative side effects.

Caffeine is a methylxanthine that is metabolized by the cytochrome P-450 enzyme CYP1A2 in the liver to three primary dimethylxanthines: theophylline, theobromine, and paraxanthine. These metabolites, like caffeine, are pharmacologically active within the body and brain. The metabolism of caffeine is similar in humans and rodents, however the primary metabolites differ. For instance, in humans the primary metabolite of caffeine is paraxanthine (Nehlig & Derby, 1994), whereas in rats, the primary metabolite of caffeine is theobromine although the levels of each metabolite have been found to be comparable (Eteng *et al.*, 1997). The half-life of caffeine is approximately 2-4 hours in the adult human and 1-2 hours in the adult rat (Bonati *et al.*, 1984-1985). In the adult rat, the half-life of each metabolite is approximately 5-6 hours for theobromine, 4-5 hours for theophylline, and 1-2 hours for paraxanthine. For humans, the half-life of each metabolite is approximately double the half-life in rats. The half-life of caffeine and its metabolites can be prolonged by various factors including pregnancy and the use of oral contraceptives. During the third trimester of pregnancy, the half-life of caffeine is increased to 10-20 hours (Aldridge *et al.*, 1981). The use of caffeine during pregnancy is also of concern because it is estimated that the half-life of caffeine in a newborn ranges from 50 to 100 hours with a more prolonged half-life in the fetus (Arnaud, 1993). The slower clearance of caffeine in the newborn and fetus is due to a deficiency in cytochrome P-450 activity (Aranda *et al.*, 1979a). Cytochrome P-450 enzymes develop during early postnatal life and clearance of caffeine does not attain adult rates until 6 months of age (Aranda *et al.*, 1979a; Parsons & Neims, 1981).

The psychostimulatory effects of caffeine are a result of its actions within the central nervous system. Caffeine enters the brain by both passive diffusion as well as a carrier-mediated adenine transport system (McCall *et al.*, 1982). Caffeine enters the brain more rapidly than its metabolites due to its increased lipid solubility and decreased plasma protein binding. In adult rats, a single oral dose of 5 or 25 mg/kg results in caffeine levels that exceed metabolite levels in both the brain and plasma (Wilkinson & Pollard, 1993). However, each metabolite attained measurable levels within the plasma after 5 min and within the brain by 30-60 minutes. In mice, brain levels of caffeine exceeded levels of each metabolite as quickly as 15 minutes following exposure (Shi & Daly, 1999). Therefore, caffeine enters the brain rapidly to produce its effects, but is more quickly metabolized allowing its active metabolites to then exert their effects within the nervous system.

**B. Actions of Caffeine and its Metabolites**

Caffeine is known to have multiple dose-dependent biological actions. The four best characterized effects of caffeine are 1) inhibition of cyclic nucleotide phosphodiesterase activity leading to reduced cyclic AMP degradation, 2) ryanodine receptor mediated stimulation of intracellular calcium release, 3) antagonism of GABA(A) receptors, and 4) antagonism of adenosine receptors (Daly, 2007). At the concentrations attained following normal consumption of caffeine, it is unlikely that caffeine has any effects through the first three mechanisms **(Figure 1-1)**. For instance, it has been shown that caffeine and theophylline are relatively weak phosphodiesterase inhibitors and levels of these compounds must reach concentrations of 500 μM and 100

μM, respectively, in order to exert inhibitory effects (Daly, 2000). Caffeine increases the sensitivity of ryanodine receptors to intracellular calcium causing opening of calcium release channels (Daly, 2000), however millimolar concentrations of caffeine must be used in order to elicit ryanodine receptor mediated intracellular calcium release (McPherson *et al.*, 1991). Similar to the ryanodine receptor, caffeine influences GABA(A) receptors only at extremely high doses. Caffeine inhibits the binding of both GABA and the benzodiazepine diazepam to GABA(A) receptors (Marangos *et al.*, 1979). However, the affinity of caffeine for GABA receptors is low and it is estimated that concentrations needed to effectively block these receptors would have to be approximately 40 times greater than concentrations acquired following normal coffee consumption (Fredholm *et al.*, 1999). Further, caffeine may act primarily on glycine receptors. Caffeine significantly inhibits glycine receptors at concentrations of 500 μM, whereas concentrations of 4mM are needed to produce similar inhibition of GABA receptors (Daly, 2007). Although not well studied, millimolar concentrations of caffeine may also have direct effects on numerous ion channels, including voltage sensitive L-type calcium channels, potassium channels, and sodium channels (Daly, 2000). Given the millimolar concentrations required for caffeine activate these mechanisms, the primary effects of caffeine within the nervous system are likely mediated through antagonism of adenosine receptors.

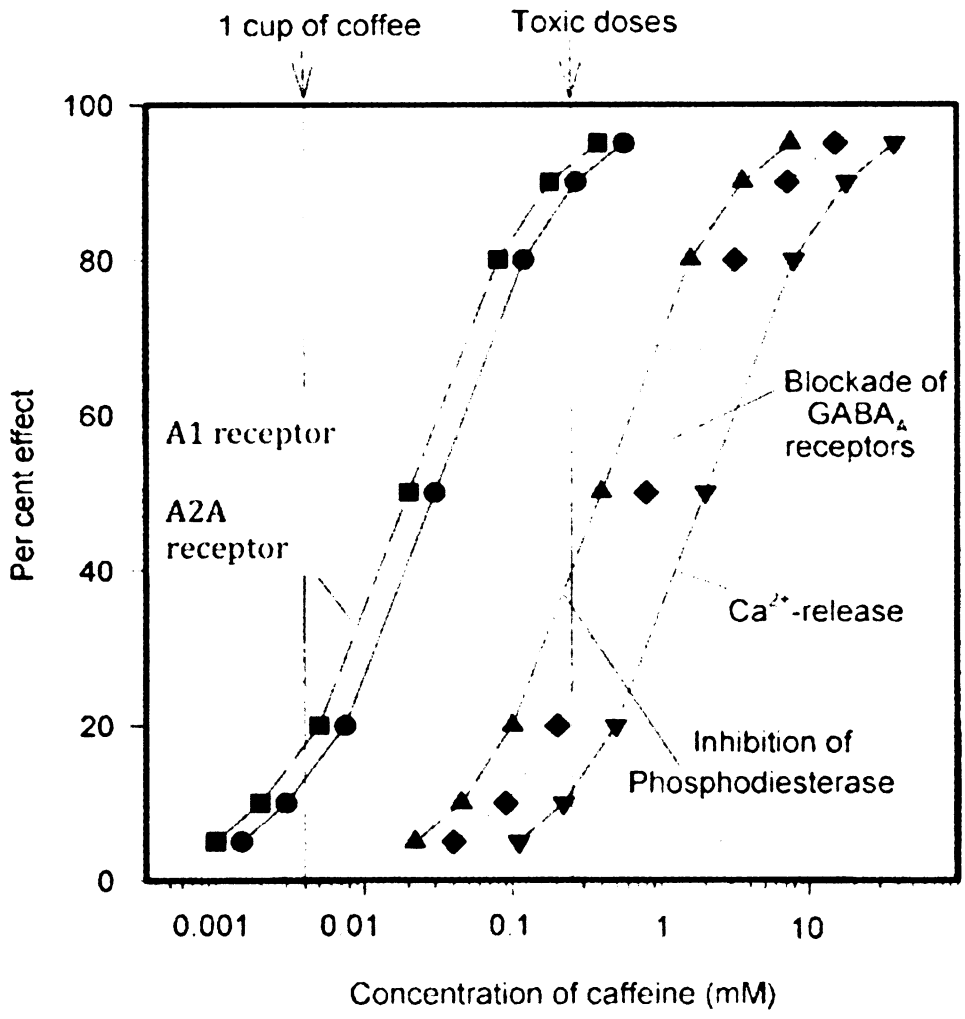


**Figure 1-1**. Caffeine elicits differential concentration-dependent effects within the brain. After consumption of a single cup of coffee, the concentrations of caffeine attained are likely acting exclusively at the A1 and A2A adenosine receptors. Supraphysiological concentrations of caffeine, which approach toxic levels, are needed to induce inhibition of phosphodiesterase activity, blockade of GABA(A) receptors, and activation of intracellular calcium release. Reproduced from Fredholm et al., 1999.

**C. Adenosine and its Receptors**

The main target of caffeine and its metabolites is antagonism of adenosine receptors. Four heptahelical G-protein-coupled adenosine receptors have been classified and named the A1, A2A, A2B, and A3 receptors (Fredholm *et al.*, 2000). Although adenosine is not classified as a neurotransmitter, it has considerable neuromodulatory effects, including general inhibition of neuronal activity (Fisone *et al.*, 2004). Adenosine is found ubiquitously throughout the brain and is synthesized both intracellularly and extracellularly. Adenosine can be formed via two main pathways. The first pathway involves the breakdown of adenine nucleotides, such as ATP. When this occurs, ATP is dephosphorylated to produce AMP, which then forms adenosine (Fredholm *et al.*, 1999; Fisone *et al.*, 2004). Thus, adenosine levels produced via this pathway are highly dependent on the utilization of ATP. A second pathway involves the hydrolysis of S-adenosyl-homocysteine, although this pathway is not thought to be the primary pathway involved in adenosine formation (Fisone *et al.*, 2004; Fredholm *et al.*, 2005). Levels of intracellular adenosine are in the 25-250 nM range and increase with cellular activity, oxygen utilization, and neurotransmitter release (Fredholm, 2007). Adenosine is broken down by adenosine kinase and adenosine deaminase (Fredholm, 2007). Adenosine kinase functions to regulate physiological levels of adenosine and is the primary enzyme responsible for maintaining low levels of intracellular adenosine (Fredholm *et al.*, 2005). Adenosine deaminase functions to reduce supraphysiological levels of adenosine produced during pathological conditions such as hypoxia or ischemia. During these conditions, adenosine levels rise rapidly due to the massive release of ATP, which is rapidly converted to adenosine (Fredholm, 2007). Because adenosine generally functions

within the brain to reduce the level of neuronal activity, it plays a prominent role in protecting the brain from excitotoxic cell death caused by hypoxia, seizures, and inflammation (Fredholm, 2007).

Of the four adenosine receptors, the A1 and A2A adenosine receptors bind caffeine with the highest affinity. These receptors are primary receptors antagonized by caffeine and therefore are responsible for caffeine's effects within the nervous system (Fredholm *et al.*, 1999). The A2B receptor, although blocked by caffeine, is only functionally active when endogenous adenosine reaches extremely high levels. These levels are generally only attained during pathological conditions, such as hypoxia-ischemia, and thus caffeine has little effect at these receptors during normal circumstances (Fredholm, 2007). Further, caffeine has been shown to have little action at A3 receptors (Fredholm *et al.*, 1999). Theophylline, paraxanthine, and theobromine also target the A1 and A2A adenosine receptors. Theophylline and paraxanthine are more potent than caffeine at non-selectively inhibiting both the A1 and A2A adenosine receptors (Shi & Daly, 1999). Theobromine has a lower affinity than caffeine at both A1 and A2A receptors, but it is selective for the A1 receptor (Shi & Daly, 1999). Therefore, to understand the effects of caffeine in the brain, the combined actions of caffeine and its metabolites must be considered since they all exert effects on adenosine receptor function.

### D. Developmental Expression of A1 and A2A Receptors

The A1 and A2A receptors are both expressed during gestational development. In the developing rat brain, the A1 receptor is one of the earliest receptors expressed

(Rivkees *et al.*, 2001) with mRNA detectable by gestational day 14 and adult patterns of distribution established by gestational day 20 (Weaver, 1996). By gestational day 14, functional binding of the A1 receptor can be detected in low levels throughout the brain using radioligand binding with the A1 receptor antagonist DPCPX (Rivkees, 1995). A2A receptors are also present prenatally (Svenningsson *et al.*, 1999) with mRNA expression detectable in the striatum by gestational day 14 in rats (Weaver, 1993). In humans, adenosine receptors are believed to be the earliest receptor system developed (Herlenius & Lagercrantz, 2001) and as a result may be susceptible to the effects of prenatal caffeine exposure. Developmentally, adenosine plays a key role in the inhibition of neuronal activity (Fisone *et al.*, 2004), formation of axons (Turner *et al.*, 2002), and neuroprotection (Rivkees *et al.*, 2001).

**E. A1 and A2A Adenosine Receptors**

The A1 and A2A adenosine receptors are G-protein-coupled and are characterized by their differential effects on adenylyl cyclase, with A1 receptors being inhibitory and A2A receptors being stimulatory to adenylyl cyclase (Londos & Wolff, 1977). The A1 receptors are linked to both Gi and Go proteins, whereas A2A receptors are linked to Gs and $G_{olf}$ proteins. A1 receptor expression is highest in the cortex, hippocampus and cerebellum, although the receptor is ubiquitously expressed throughout the brain. In each region, A1 receptors are primarily located on presynaptic nerve terminals (Fredholm & Dunwiddie, 1988). Activation of Gi protein-coupled A1 receptors leads to inhibition of adenylyl cyclase activity, decreases in cAMP concentrations, and consequently inhibition of N, P, and Q-type voltage gated calcium channels (Fredholm *et al.*, 2000). Activation

of Go-protein-coupled A1 receptors leads to an activation of G-protein-dependent inward rectifying potassium channels causing neuronal hyperpolarization (**Figure 1-2**; Schubert *et al.*, 1997). These two actions 1) activation of potassium channels and 2) inhibition of calcium channels, allows adenosine via the A1 receptor to inhibit neuronal activity and diminish transmitter release, providing protection against excitotoxicity (Schubert *et al.*, 1997). In the hippocampus, adenosine acting at the A1 receptor can decrease presynaptic calcium influx via the N, P, and Q-type voltage gated calcium channels. This decrease in calcium influx leads to an inhibition of synaptic transmission at mossy fibre synapses (Gundlfinger *et al.*, 2007).

A2A receptors play a stimulatory rather than inhibitory role. Thus, activation of Gs-protein-coupled A2A receptors leads to stimulation of adenylyl cyclase and activation of L-type calcium channels (Fredholm *et al.*, 2003). A2A receptor expression is highest in dopamine-containing regions of the brain, such as the striatum, nucleus accumbens, and olfactory tubercle (Fredholm *et al.*, 2007). In the striatum, A2A receptors are located postsynaptically on GABAergic medium spiny neurons. Adenosine increases adenylyl cyclase activity, which results in increased activity of GABAergic medium spiny neurons, which project to the basal ganglia. Increased activity of the GABAergic neurons results in greater inhibition of cells in the basal ganglia, which reduces overall motor activity (**Figure 1-3**). Recently, it has been found that A1 and A2A receptors are sometimes co-expressed. For instance, these receptors have been shown to form heteromers in glutamatergic nerve terminals in the striatum (Ciruela *et al.*, 2006). Current understanding of the neuromodulatory actions of adenosine may be broadened if

it is found that the A1 and A2A receptors produce opposing actions within the same regions of the brain.

A1 receptor mediated inhibition of calcium channels and activation of potassium channels results in decreased transmitter release. A1 receptor activation decreases transmission of almost every major neurotransmitter within the brain, including glutamate, GABA, dopamine, and serotonin (Dunwiddie & Masino, 2001). Glutamatergic transmission is the most effected in that adenosine is able to almost completely abolish release of glutamate. For instance, in hypothalamic cultures, in the absence of glutamate transmission, adenosine has little effect on intracellular calcium, however when glutamate transmission is not blocked, adenosine can decrease glutamate transmission resulting in decreased postsynaptic calcium influx (Obrietan *et al.*, 1995). These results suggest that the effects of adenosine at the A1 receptor are glutamate dependent.

Understanding the actions of adenosine at the A1 and A2A receptors makes it possible to understand how antagonism of these receptors by caffeine produces its stimulatory effects. Antagonism of A1 receptors by caffeine reduces the inhibition of adenylyl cyclase, which leads to an increase in neurotransmitter release and an overall increase in neuronal excitability (**Figure 1-2**). Increased activity of neurons in the cortex and hippocampus is thought to mediate the ability of caffeine to increase attention and information processing. Antagonism of A2A receptors leads to a reduction in adenylyl cyclase activity, which in turn reduces the activity of the GABAergic medium spiny neurons projecting to the basal ganglia. This allows neurons in the basal ganglia to

become more active and is believed to account for the ability of caffeine to increase locomotor activity **(Figure 1-3)**.

Recently, it has been shown that caffeine acts via A1 receptors on cortical nerve terminals to enhance glutamate transmission (Wang, 2007). Application of caffeine (3 μM) or the A1 receptor antagonist DPCPX facilitates glutamate release. However, when both antagonists were applied at the same time, there was no additional facilitation of glutamate release suggesting a common mechanism of action (Wang, 2007). Further, A1 receptors play an important role in limiting excitotoxicity following seizures or hypoxic conditions, with antagonism of these receptors exacerbating damage (Fredholm, 2007).

Although both the A1 and A2A receptors are present and affected by caffeine, the primary focus of the studies presented in this dissertation will be on the A1 receptor due to its widespread distribution throughout the brain and its interactions with glutamate neurotransmission. The A1 receptor is the most abundant of the four adenosine receptors within the brain and is distributed throughout the brain and spinal cord, with the highest levels of expression in the hippocampus, cerebellum, and cerebral cortex (Fastbom *et al.*, 1986; Jarvis *et al.*, 1987; Weaver, 1996; Svenningsson *et al.*, 1997; Dunwiddie & Masino, 2001). Although A2A receptors are also expressed in the hippocampus, their most prominent levels of expression are in dopamine rich brain regions such as the striatum (Svenningsson *et al.*, 1999). The role of the A2A receptors in the hippocampus has begun to be understood through the use of knockout animals. Currently, it is hypothesized that the actions of the A2A receptor in the hippocampus are the result of its ability to modulate the A1 receptor (Johansson *et al.*, 2001). Thus, the role of the A2A receptors in the hippocampus will be discussed further with regards to its effects on A1

receptor function. Currently, the cellular consequences of blocking the regulatory functions of either adenosine receptor during development are unknown, however receptor knockout studies have provided some insight.

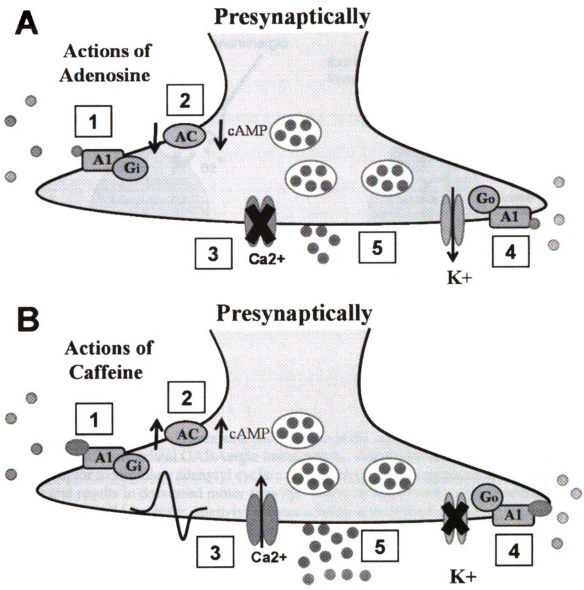


**Figure 1-2**. The actions of (A) adenosine and (B) caffeine at the A1 receptor. (A) When adenosine binds the A1 receptor (1) it decreases adenylyl cyclase activity (AC) and cAMP formation (2). Adenosine also reduces calcium entry via voltage-gated calcium channels (3) and increases potassium efflux via potassium channels, which results in hyperpolarization of neuron and decreased neurotransmitter release (5). (B) When caffeine antagonizes the A1 receptor (1), adenosine can no longer inhibit adenylyl cyclase activity resulting in an increase in both adenylyl cyclase activity and cAMP formation (2). Further, voltage-gated calcium channels are open which allows calcium entry (3) and potassium channels are blocked (4). The accumulation of positive calcium and potassium ions within the neuron allows the cell to be more easily depolarized, thereby increasing neurotransmitter release (5).

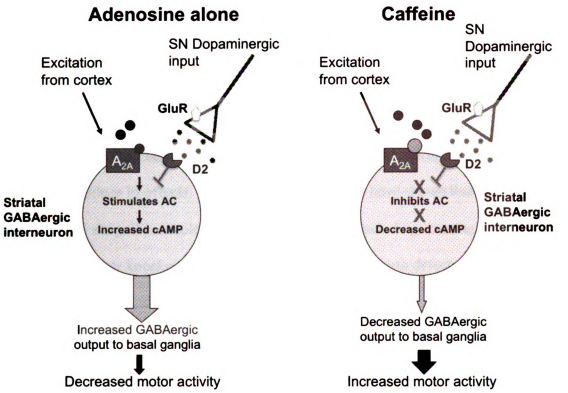


**Figure 1-3**. The actions of adenosine and caffeine at the A2A receptor located postsynaptically on striatal GABAergic interneurons. Adenosine normally binds to the A2A receptor to stimulate adenylyl cyclase (AC) activity, which increases GABAergic output and results in decreased motor activity. When caffeine blocks adenosine from binding to the A2A receptor, adenylyl cylcase activity is decreased resulting in decreased GABAergic output to the basal ganglia which increases overall motor activity. SN= substantia nigra.

## F. A1 and A2A Receptor Knockout and Transgenic Studies

The developmental effects of chronic antagonism of adenosine receptors by caffeine may be similar to the lack of functional A1 and A2A receptors in knockout studies. Knockout studies have provided valuable insight into the neuromodulatory roles of adenosine and have been instrumental in understanding how caffeine is acting at each receptor to produce its effects. Importantly, these studies have shown that adenosine receptors play a significant role in numerous behaviors, including learning and memory.

A1 receptor knockout mice (A1R-/-) do not exhibit birth defects or decreased birth weights, but do differ at the cellular level when compared to wildtype mice (A1R +/+) (Johansson *et al.*, 2001). In hippocampal slices from A1R-/- mice, application of adenosine does not inhibit glutamate transmission or induce potassium mediated postsynaptic hyperpolarization as it does in wildtype mice (Masino *et al.*, 2002). Fast excitatory postsynaptic potentials (fEPSPs) are increased in hippocampal slices in A1R wildtype mice after the application of A1R antagonists, however this effect is abolished in A1R knockout mice (Johansson *et al.*, 2001). Together, these results show that adenosine does indeed act at the A1 receptor to modulate glutamatergic neurotransmission. Further, A1R knockout mice lack paired pulse facilitation in hippocampal slices but do not show any differences in long-term potentiation (LTP) or long-term depression (LTD) when compared to wildtype mice (Gimenez-Llort *et al.*, 2005).

Behaviorally, A1 receptor knockout animals have similar performance to wildtype animals in sensorimotor reflex tests, including tests of grasping ability, visual placing, and locomotor activity (Johansson *et al.*, 2001). However, A1R knockout mice show

impairments in anxiety and habituation tests. A1R knockout and A1R heterozygous mice show increased measures of anxiety, with knockout mice entering less and spending less total time in the lighted area during dark-light testing (Johansson *et al.*, 2001). Similar results were found using an elevated plus maze to test anxiety (Gimenez-Llort *et al.*, 2002). In a six-arm radial tunnel maze, A1R knockout mice habituated significantly less to the maze, and exhibited greater total activity and arm explorations than wildtype mice (Gimenez-Llort *et al.*, 2005). However, in open-field testing A1R knockout mice show decreased exploratory behavior (Gimenez-Llort *et al.*, 2002). Strikingly, A1R knockout mice do not show any deficits in various paradigms of the water maze, indicating no differences in spatial or working memory in these mice (Gimenez-Llort *et al.*, 2005).

Genetic manipulation of the A2A receptor also results in changes in learning and memory behaviors. A2A receptor knockout mice show improved spatial recognition memory in a Y-maze task, which is a spatial two-trial recognition task (Wang *et al.*, 2006). A2A knockout mice also exhibit higher anxiety and decreased exploratory behavior (Ledent *et al.*, 1997). Interestingly, transgenic rats with an over-expression of A2A receptors in the brain exhibit working memory deficits. Specifically, these rats show impaired working memory in a novel object recognition task, a repeated acquisition Morris water maze task, and a six-arm radial tunnel maze task when compared to wildtype rats (Gimenez-Llort *et al.*, 2007). These findings suggest that activation of adenosine receptors decreases memory abilities, while reduced receptor function or a lack of receptors results in memory improvements.

Mice over-expressing adenosine kinase, a key enzyme involved in adenosine degradation, also exhibit alterations in learning and memory behaviors (Yee *et al.*, 2007).

An increase in adenosine kinase activity functions to reduce the overall levels of adenosine available within the brain, thereby reducing adenosine receptor activity. Transgenic mice over-expressing adenosine kinase exhibit deficits in both working and reference memory Morris water maze tasks, as well as deficits in acquisition of a conditioned response to a conditioned stimulus (Yee *et al.*, 2007). These findings are in contrast to studies using knockout mice and suggest that involvement of adenosine in learning and memory behaviors may not be as simple as originally believed, i.e. reduced receptor activity enhances memory, while increased receptor activity impairs memory.

In knockout and transgenic studies, compensatory mechanisms must be taken into account when considering the behavioral changes that occur in the brain due to the lack or altered expression of a functional receptor. In A2A heterozygous and homozygous mice A1 receptor expression is altered such that in A2A heterozygous mice there is a decrease in A1 receptor radioligand binding in the hippocampus, whereas in A2A knockout mice there is an increase in A1 receptor binding (Snell *et al.*, 2000). Up- or down-regulation of the A1 receptors in the hippocampus may be a factor in the learning and memory related changes observed in A2A receptor knockout mice. Unfortunately, there are not many studies available discussing the compensatory mechanisms that may occur following loss of the A1 or A2A adenosine receptors in these animals.

## G. Pharmacological Manipulation of A1 and A2A Receptor Function

Pharmacological manipulation of A1 and A2A receptors with selective agonists and antagonists has also provided insight into the function of these receptors and their role in learning and memory. A1 receptor agonists disrupt learning and memory in rats

(Zarrindast & Shafaghi, 1994; Ohno & Watanabe, 1996). Administration of the A1 receptor agonist N6-cyclohexyladenosine (CHA) into the rat dorsal hippocampus significantly impairs working memory, whereas an A2A agonist fails to induce working memory deficits (Ohno & Watanabe, 1996). Infusion of an adenosine agonist to the posterior cingulate cortex also impairs memory function in an inhibitory avoidance task (Pereira *et al.*, 2005). The posterior cingulate cortex is an area known to contain projections to the hippocampus and is currently believed to modulate hippocampal function. In summary, increased A1 receptor activity in the hippocampus and related cortical areas impairs learning and memory.

On the other hand, blockade of A1 or A2A receptors with specific antagonists facilitates learning and memory behaviors (Hauber & Bareiss, 2001). In adult rats, caffeine facilitates learning and memory behaviors in paradigms such as the Morris water maze (Angelucci *et al.*, 2002). The facilitative effects of caffeine on learning and memory are thought to result from its non-selective antagonism of A1 and A2A receptors. Similarly, antagonism of A1 receptors by DPCPX (8-cyclopentyl-1,3-dipropylxanthine) infusion to the posterior cingulate cortex facilitates memory retention in a inhibitory avoidance task (Pereira *et al.*, 2002). Therefore, blockade of adenosine receptors and the concurrent reduction of receptor activity in the hippocampus and related cortical areas results in enhanced learning and memory behaviors.

The behavioral changes that result from altering adenosine receptor function align well to the effects of adenosine agonists and antagonists on long-term potentiation (LTP) and long-term depression (LTD). A1 receptor agonists impair LTP in the hippocampus while A1 receptor antagonists enhance LTP (de Mendonca & Ribeiro, 1990). The A1

receptor antagonist DPCPX greatly enhances the effects of LTP, likely due to an increase in excitatory neurotransmitter release which occurs after adenosine antagonism in the hippocampus (de Mendonca & Ribeiro, 2000). A link between adenosine receptor function and learning memory is beginning to be established, however, it is unknown whether chronic prenatal caffeine exposure can cause long-term changes in adenosine receptor function that may alter learning and memory.

## H. Perinatal Caffeine Exposure and Adenosine Receptor Regulation

Determination of the effects of perinatal caffeine exposure on adenosine receptor expression has proved difficult due the effects of dose and timing of caffeine exposure. For instance, A1 receptor expression is up-regulated following neonatal caffeine exposure (Guillet & Kellogg, 1991) but down-regulated following fetal caffeine exposure (Leon *et al.*, 2002). In animals treated with caffeine during postnatal days 2-6, a significant increase in A1 receptor binding is detectable in the cortex, hippocampus, and cerebellum, indicating an increase in receptor expression that persists into adulthood (Guillet & Kellogg, 1991).

In the fetal brain, A1 receptors are significantly decreased following chronic exposure to caffeine (by 49%) or theophylline (by 41%) (Leon *et al.*, 2002). However, the same prenatal treatment results in increased A1 receptor mRNA (Leon *et al.*, 2002). Levels of the Gi proteins, to which A1 receptors are coupled, are also found to decrease in the fetal brain following caffeine (by 15.2 %) or theophylline (by 18.4 %) treatment, along with an increase in Gi mRNA (Leon *et al.*, 2005a; Leon *et al.*, 2005b). The levels of mGluRs are also decreased with caffeine (by 27 %) or theophylline (by 15 %)

treatment in fetal brain tissue (Leon *et al.*, 2005a). It is hypothesized that the decrease in mGluRs may be due to increased glutamate release resulting in over-stimulation of glutamate receptors and consequently receptor down-regulation.

Some studies show no changes in A1 and A2A receptor expression following developmental caffeine exposure. Aden et al. (2000) found that a moderate dose of caffeine administered throughout gestation does not significantly alter A1 or A2A receptor levels in fetal and newborn brains.

If caffeine exposure during brain development alters adenosine receptor expression in the hippocampus and cortical areas related to learning and memory, the changes in adenosine receptor expression may alter learning and memory behaviors. Accordingly, it is reasonable to hypothesize that if developmental caffeine exposure increases adenosine receptor expression in the brain this may result in impairments in learning and memory, whereas decreases in adenosine receptor expression may result in enhanced learning and memory.

## I. The Hippocampus and Learning and Memory

The role of the hippocampus in learning and memory behaviors is well known and the integrity of hippocampal circuits is essential for spatial and temporal memory paradigms (Eichenbaum, 2000). Spatial learning, assessed by tasks such as the Morris water maze, is disrupted in rats with hippocampal lesions (Morris *et al.*, 1982). Similarly, both recognition memory and working memory tasks are disrupted following lesions of the temporal lobe (Clark *et al.*, 2000; Laroche *et al.*, 2000). In humans, hippocampal damage is correlated to the extent of a patient's retrograde and anterograde

amnesia, such that damage to only the CA1 regions produces mild memory loss, while widespread damage throughout the hippocampus produces severe memory loss (Squire & Zola, 1996). Administration of selective antagonists directly into the hippocampus prior to analysis of hippocampal-dependent behavior has indicated specific receptors involved in learning. Antagonism of NMDA receptors with AP5 results in impaired spatial memory in the water maze, but does not impair visual discrimination behavior (Morris *et al.*, 1986). This was one of the first studies to identify a role for NMDA receptors in specific learning behaviors. Furthermore, as the dose of AP5 is increased in the hippocampus and presumably more NMDA receptors are blocked, performance in the water maze is increasingly impaired (Davis *et al.*, 1992). Hippocampal infusion of NMDA antagonists also impairs behavior in step-down inhibitory avoidance tasks (Roesler *et al.*, 1998), delayed matching-to-place tasks (Steele & Morris, 1999), and numerous working memory tasks.

Hippocampal neurons are well known for demonstrating LTP. Although a direct link between learning and memory and LTP has yet to be elucidated, there is robust evidence that these processes are integrated. Many studies found that in addition to impairments of learning and memory behavior, NMDA receptor blockade impairs LTP (Davis *et al.*, 1992; Tsien *et al.*, 1996). Following increasing doses of AP5 administered directly into the hippocampus, both spatial learning and LTP are impaired in a correlated, dose-dependent fashion. Thus, doses that do not impair LTP *in vivo* are not associated with spatial learning deficits, while doses that do impair LTP are associated with impaired spatial learning (Davis *et al.*, 1992). Mice lacking NMDA receptors in the CA1 region of the hippocampus exhibit both spatial memory deficits and impaired LTP (Tsien

*et al.*, 1996). Conversely, over-expression of the NR2B subunit of the NMDA receptor in the mouse hippocampus facilitates learning and memory behaviors in a novel object recognition task, a spatial water maze task, and a contextual fear-conditioning task. Hippocampal slices from NR2B subunit over-expressing mice also exhibit enhanced LTP (Tang *et al.*, 1999). The link between NMDA receptor function, LTP, and learning and memory behaviors is widely studied and supported, although the direct relationship between these processes is still not fully elucidated.

Calcium influx through postsynaptic NMDA receptors is an integral step in the signaling cascades that mediate many forms of hippocampal neuroplasticity (Miyamoto, 2006). LTP is the most studied form of synaptic plasticity in the hippocampus. The molecular mechanisms underlying LTP are complex and not completely understood, but a few key components have been elucidated. In a simplified version of this molecular cascade, glutamate is released in the hippocampus, which activates postsynaptic NMDA receptors. Activation of NMDA receptors allows calcium entry into the postsynaptic cell, where calcium activates numerous calcium-dependent kinases. Activation of calcium/calmodulin-dependent protein kinase II (CaMKII) is believed to be necessary for LTP in the hippocampus (Lynch, 2004; Malenka & Bear, 2004). Phosphorylation of CaMKII has been associated with both increased AMPA receptor conductance and insertion of AMPA receptors into the postsynaptic density following LTP (Derkach *et al.*, 1999; Liao *et al.*, 2001). Activation of CaMKIV and mitogen-activated protein kinase/extracellular signal-related kinase pathway (MAPK/ERK) modulates phosphorylation of cAMP response element binding protein (CREB), which is involved in gene transcription (Lynch, 2004; Malenka & Bear, 2004). Importantly, inhibitors of

ERK have been shown to block both LTP and CREB activation (Impey *et al.*, 1998). Furthermore, CREB activation has been shown to be an important transcription factor for long-term protein synthesis, an essential step for maintenance of synaptic plasticity (Lynch, 2004; Malenka & Bear, 2004). CREB-dependent protein synthesis plays a role in development of new spines, a morphological change that occurs following LTP and is believed to account for increased synaptic efficacy. Disruption of ERK or CREB activation completely abolishes LTP, making these signaling molecules an important measure of activation of this signaling cascade (Lynch, 2004).

As discussed previously, the high levels of adenosine receptor expression in the hippocampus play a prominent role in modulating learning and memory as well as LTP, likely through glutamate mediated mechanisms. In the hippocampus, adenosine receptor activation reduces glutamatergic synaptic transmission by decreasing presynaptic calcium influx and thereby reducing vesicle release (Gundlfinger *et al.*, 2007). The inhibition of adenosine by antagonists such as caffeine increases glutamate release thereby activating postsynaptic glutamatergic receptors (Wang, 2007). Because glutamate receptors play an important role in hippocampal plasticity as well as learning and memory, an increase in glutamate receptor activity may alter NMDA receptor activity as well as activation of ERK and CREB. These changes could lead an overall change in LTP as well as learning and memory.

Induction of LTP in the neonatal hippocampus has a developmental profile that varies by age and distinct signaling pathways. It has been demonstrated that LTP cannot be induced in the rat hippocampus prior to postnatal day 8, with LTD being the predominant form of synaptic plasticity during this stage of neonatal development

(Dudek & Bear, 1993). However, other studies show induction of LTP as early as postnatal day 2 in the rat hippocampus if postsynaptic depolarization and presynaptic stimulation occur simultaneously (Durand *et al.*, 1996). Postsynaptic depolarization is likely necessary because during early postnatal development NMDA receptor activity predominates whereas AMPA receptors are functionally silent (Liao *et al.*, 1999).

In the neonatal hippocampus, distinct signaling pathways are associated with induction of LTP. The CaMKII pathway, which is essential during adult LTP, is not needed during neonatal LTP induction (Yasuda *et al.*, 2003). Rather, the cAMP-dependent PKA pathway seems to play a major role in induction of LTP prior to postnatal day 8 (Yasuda *et al.*, 2003). The cAMP-dependent PKA pathway may be directly affected by caffeine in neonates. As discussed previously, blockade of the A1 and A2A adenosine receptors by caffeine results in an increase or decrease, respectively, of adenylyl cyclase and cAMP activity. An alteration in cAMP activity is coupled to activation of the PKA pathway since PKA activation is dependent on cAMP activity. Although the regulation and timing of the developmental switch between the neonatal cAMP-dependent PKA pathway and the CaMKII dependent pathway is unknown, it is possible that developmental caffeine exposure is modulating this switch and interfering with LTP induction in the developing brain.

### J. Perinatal Development of the Hippocampus

The development of the hippocampus and dentate gyrus occurs during gestation and early neonatal periods, making the hippocampus especially susceptible to developmental drug exposure. In the rodent hippocampus, all of the pyramidal cells and

neurons in Ammon's horn are formed prior to birth (Bayer, 1980a, b). Dentate gyrus development takes place between gestational day 14 and the second postnatal week of life, with a peak of activity around postnatal day 5-7 (Schlessinger *et al.*, 1975). Neurogenesis of dentate gyrus granule cells occurs postnatally and continues at a reduced rate throughout life. Granule cells increase their dendritic trees until PN14 after which the branches are sculpted to form adult-like morphological features (Rahimi & Claiborne, 2007).

In humans, hippocampal development occurs over a prolonged period of gestation. By gestational week 15, neurogenesis of pyramidal neurons is visible in Ammon's horn and adult-like morphological features of the CA1-CA3 fields are established by the 23-25th gestational week (Arnold & Trojanowski, 1996a, b). Similar to the findings in rats, neurogenesis in the granule cell layer of the dentate gyrus in humans begins around gestational week 14 (Humphrey, 1967) and continues postnatally (Seress *et al.*, 2001). Prenatal exposure to drugs during these critical stages of hippocampus and dentate gyrus formation can alter the overall morphology of both structures in treated offspring.

Adenosine, acting at the A1 receptors, depresses glutamatergic activity and regulates giant depolarizing potentials within the hippocampus during the first week of postnatal life in rodents (Safiulina *et al.*, 2005). Giant depolarizing potentials are a trait of the developing hippocampus and are characterized by recurrent spontaneous membrane depolarizations (Ben-Ari *et al.*, 1989). Giant depolarizing potentials are instrumental in shaping the synaptic connections of the hippocampal circuitry and are mediated by the actions of GABA and glutamate in the developing brain (Safiulina *et al.*,

2006). Alterations in the frequency of giant depolarizing potentials may effect the development of hippocampal neural networks (Dzhala *et al.*, 1999). If caffeine antagonizes the A1 adenosine receptors in the hippocampus, such that adenosine can no longer regulate or depress the frequency of the giant depolarizing potentials, the development of the hippocampus may be negatively affected. Further, glutamatergic NMDA receptors play a fundamental role in activity-dependent migration, synapse formation, and functional connectivity of the developing hippocampus (Ben-Ari & Holmes, 2006). Caffeine may increase glutamate release in the developing hippocampus thereby disrupting these processes.

**K. Glutamate Receptors and Development**

Glutamate receptors play a critical role in numerous developmental processes including axon migration, synaptogenesis, and synaptic plasticity. Alterations of glutamatergic activity during development may interrupt or postpone critical developmental processes. Thus, it is necessary to understand the normal ontogeny and regulation of glutamate receptors during development in order to determine if the regulation of these critical receptors is altered by prenatal caffeine exposure.

Ionotropic glutamate receptors are categorized into three subclasses based on the ligand specificity of each receptor. The three subclasses include the N-methyl-D-aspartate (NMDA), α-amino-3-hydoxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptors, and are specific for ligands of the same name. The NMDA receptors are comprised of tetrameric assemblies of the obligate NR1 subunit along with NR2 subunits (NR2A-NR2D) and NR3 subunits (NR3A-NR3B) (Dingledine *et al.*,

1999). The majority of NMDA receptors in the hippocampus are NR1/NR2A or NR1/NR2B subunit assemblies. AMPA receptor subunits are classified as GluR1-GluR4 and normally form tetrameric complexes of GluR2 with either GlurR1 or GluR3 (Dingledine *et al.*, 1999). Kainate receptor subunits are classified as GluR5-GluR7 or KA1-KA2 and form either homomeric or heteromeric four unit assemblies. NMDA and AMPA receptors play a predominant role in development and thus the expression and function of these receptors is of primary importance.

During prenatal and postnatal development, the ionotropic glutamate receptors are expressed in both temporally and regionally specific patterns. Glutamate receptor expression is elevated during development when compared to expression in the mature brain, with peak levels of expression corresponding to neonatal periods of synaptogenesis (Ritter *et al.*, 2002). The NR1 subunits are found prenatally throughout the brain with expression detectable in the rat cortex as early as embryonic day 14 (Babb *et al.*, 2005). Peak levels of NR1 expression occur in the neonatal rat hippocampus between postnatal days 3-14 (Ritter *et al.*, 2002). The NR2A and NR2B subunits are differentially expressed, allowing distinct pharmacological properties mediated by each receptor to predominate during different periods of brain development. By embryonic day 14, NR2B expression is detectable in the rat cortex with almost double the level of expression of the NR1 subunit (Babb *et al.*, 2005). High levels of NR2B expression are maintained until postnatal day 7 after which levels begin to decline (Ritter *et al.*, 2002). In contrast, NR2A expression is very low prenatally, but begins to increase around postnatal day 10 in the rat hippocampus and reaches peak levels of expression around postnatal day 21 (Ritter *et al.*, 2002). The NR2A subunit is characterized by fast channel kinetics and

increased channel open probability, whereas the NR2B subunit is characterized by slower channel kinetics and decreased channel open probability (Lau & Zukin, 2007). The developmental switch from NR2B to NR2A receptor expression and the distinct pharmacological properties of each subunit is believed to play an important role in the activation of different intracellular cascades and synaptic plasticity (Lau & Zukin, 2007). However, the exact role and mechanisms behind the switch in subunit expression have remained elusive.

AMPA receptor subunits are also detectable in the embryonic brain. GluR1 subunit expression is detectable in the rat cortex as early as embryonic day 15 (Martin *et al.*, 1998). Unlike the developmental profiles of other subunits, GluR1 expression remains fairly constant in the hippocampus during prenatal and postnatal development (Ritter *et al.*, 2002). Expression of GluR2/3 subunits is also detectable during embryonic development. In the rat hippocampus, GluR2 subunits show a rapid increase in expression immediately following birth, which peaks at postnatal day 10 and then rapidly declines to prenatal levels of expression by postnatal day 18 (Ritter *et al.*, 2002). GluR3 subunit expression in the rat hippocampus is very low around birth and increases to peak levels around postnatal day 14 after which it remains elevated into adulthood (Ritter *et al.*, 2002).

Given the complex and dynamic patterns of NMDA and AMPA receptor expression in the developing brain, it is not surprising that developmental drug exposure during these time periods can interrupt the delicate timing of receptor expression. For instance, gestational and neonatal ethanol exposure significantly increases NR2A subunit expression on postnatal day 10 in both the hippocampus and cortex (Nixon *et al.*, 2002,

2004). However, perinatal alcohol exposure reduces radioligand binding of the NMDA antagonist MK-801 in the hippocampus and cortex of treated rats (Diaz-Granados *et al.*, 1997) and decreases NMDA-receptor mediated calcium transients in cultured neurons (Lee *et al.*, 1994; Gruol *et al.*, 1998) suggesting a decrease in expression or function of NMDA receptors. Neonatal nicotine exposure also alters the expression of NMDA receptor subunits in the developing brain. Exposure to nicotine on postnatal days 8-12 increases NR2A mRNA in the rat forebrain and enhances the duration of NMDA-mediated EPSPs (Hsieh *et al.*, 2002). Similarly, prenatal exposure to morphine alters the kinetic properties of NMDA receptors in neonatal hippocampal pyramidal neurons (Yang *et al.*, 2000). It is currently unknown whether perinatal exposure to caffeine induces alterations in glutamate receptor expression or function. However, as the previous studies elucidate, changes in NMDA receptor expression can lead to changes in intracellular calcium signaling which may disrupt calcium-dependent signaling cascades.

Glutamate receptors also play a critical role in modulating excitotoxic and apoptotic cell death. Increased NMDA receptor activation promotes excitotoxic cell death (Choi, 1987; Chen *et al.*, 1997), whereas prolonged inhibition of activity induces apoptosis (Olney, 2002). Neonatal caffeine exposure (50 mg/kg administered 3 times per day) induces cell death in the rat dentate gyrus, thalamus, hypothalamus, and caudate putamen as early as one day following treatment (Kang *et al.*, 2002). Similarly, cortical (Kang *et al.*, 2002) and cerebellar (Gepdiremen *et al.*, 1998) cultures exposed to 250μM caffeine exhibit signs of apoptosis following one day of continual exposure. Attenuation of cell death occurs after administration of cycloheximide, an anti-apoptotic drug that inhibits protein synthesis, to cortical cultures (Kang *et al.*, 2002) and nimodipine, a

calcium channel blocker, to cerebellar cultures prior to caffeine exposure (Gepdiremen *et al.*, 1998). These findings suggest that developmental caffeine exposure may induce cell death via glutamate and calcium dependent mechanisms.

**L. Prenatal Caffeine and Development**

Given the numerous neuromodulatory roles of adenosine receptors during development and the widespread use of caffeine during pregnancy, it is surprising that there is not an abundance of research on the effects of prenatal exposure to caffeine. It is estimated that 70-95% of women consume caffeine during pregnancy (Fredholm *et al.*, 1999). Caffeine can readily cross the placental and blood-brain barriers to reach the developing fetus and its brain (Arnaud, 1993). The altered half-life of caffeine in the fetus and pregnant women may result in prolonged effects of caffeine in the fetal brain. In non-pregnant adults, the half-life of caffeine varies between 2-6 hours while in pregnant women the half-life is increased to 10-20 hours (Aldridge *et al.*, 1981). The effect of caffeine is further prolonged in the fetus and newborn infants due to a lack of cytochrome P-450 activity (Aranda *et al.*, 1979b) resulting in a half-life of caffeine ranging from 50 to 100 hours (Arnaud, 1993). Thus, once an expectant mother has cleared caffeine from her system, the drug or its metabolites may accumulate and remain in the fetus for days.

The prolonged exposure of the fetus to caffeine can result in an accumulation of caffeine and its metabolites in the developing brain. After a single dose of caffeine (5 or 25 mg/kg) to pregnant rats, the levels of the primary metabolites of caffeine, theophylline, theobromine and paraxanthine, are three times greater in the fetal brain than

in the plasma (Wilkinson & Pollard, 1993). In the brain, the main target of caffeine is antagonism of adenosine receptors. The expression of A1 receptor mRNA is detectable using in situ hybridization by gestational day 14 (GD14) in the developing rat brain (Weaver, 1996), and by GD20 the distribution of A1 receptor mRNA resembles that of an adult rat. Functional binding of the A1 receptor can be detected in low levels throughout the brain by gestational day 14 using radioligand binding with the antagonist DPCPX (Rivkees, 1995). Although adenosine is not classified as a neurotransmitter, it has considerable neuromodulatory effects, including general inhibition of neuronal activity (Fisone *et al.*, 2004). As discussed previously, the A1 and A2A adenosine receptors bind caffeine with high affinity and are considered to be crucial for the stimulatory effects of caffeine (Fredholm *et al.*, 1999). It is currently unknown whether caffeine acts in the developing brain via the same mechanisms known to mediate the actions of caffeine in the adult brain. Further, the effects of chronic moderate prenatal caffeine exposure are not well understood. This area needs further examination given the numerous studies that have indicated that high dose prenatal caffeine exposure is harmful to developing offspring.

High-dose prenatal exposure to caffeine has detrimental effects on development. Mothers who consumed greater than 300 mg/day of caffeine during the third trimester of pregnancy have an increased risk of delivering a small for gestational age infant (Vik *et al.*, 2003). In rodents, decreased birth weight and delayed eye opening results from *in utero* caffeine exposure to as little as 25 mg/kg/day (West *et al.*, 1986). In humans, intake of caffeine decreases birth weight by approximately 28 g per 100 mg of daily caffeine consumption (Bracken *et al.*, 2003). At doses equivalent to one to two cups of

coffee, prenatal caffeine exposure does not alter brain weight, although caffeine at higher doses of between two and twelve cups of coffee does decrease fetal brain weight (Tanaka *et al.*, 1983, 1987; Yazdani *et al.*, 1990). Congenital malformations occur only at supra-pharmacological doses (consumption of 10-14 cups of coffee) and thus, caffeine exposure in humans in not considered teratogenic (Nehlig & Derby, 1994). Overall, the scientific literature has mainly focused on the effects of caffeine on birth weight, brain weight, timing of birth, rates of miscarriage, and congenital malformations and most reports indicate that moderate prenatal caffeine exposure has only mild effects on these gross measures of health.

The developmental effects of low to moderate caffeine exposure are beginning to be studied and understood. Recently it has been shown that daily neonatal exposure to caffeine (50 mg/kg) results in a persistent increase of dendritic length and arborization of neurons located in the prefrontal cortex (Juarez-Mendez *et al.*, 2006). Intact neonatal hippocampal cultures exposed to acute caffeine (50 μM) exhibit seizure-like activity following a brief anoxic episode, whereas in control hippocampi, anoxia only induces a depression of neuronal activity (Dzhala *et al.*, 1999). This epileptogenic activity resulted from blockade of A1 receptors. Furthermore, caffeine withdrawal in newborns chronically exposed to prenatal caffeine has become of interest. Newborns of mothers who chronically consumed high levels of caffeine throughout their pregnancies exhibit increased irritability, jitteriness, and vomiting during the first week after birth (Martin *et al.*, 2007). Prenatal caffeine exposure is also a risk factor for respiratory problems in newborns, with exposed infants exhibiting episodes of apnea (suspended breathing) and tachypnea (abnormally fast breathing) (Saadani-Makki *et al.*, 2004). Lastly, neonatal

exposure to low doses of caffeine significantly decreases the protein and cholesterol concentrations in brains of treated rat pups (Nakamoto *et al.*, 1988). Interestingly, this effect occurs following low dose, but not high dose treatment, suggesting that low dose exposure may have distinct effects from high dose exposure. Further studies are needed to fully understand the long-term effects of chronic developmental caffeine exposure.

Learning and memory behaviors are also altered following perinatal caffeine exposure. Prenatal administration of 150 mg/L caffeine throughout gestation induces hyperactivity in open field testing in adult offspring (Grimm & Frieder, 1988). Higher doses of 300 or 450 mg/L induce learning disabilities. Similarly, prenatal doses of 28 mg/kg, but not 36 mg/kg, induce increased locomotor and rearing activity in open-field testing in adult offspring (Hughes & Beveridge, 1990). Interestingly, young rats exposed prenatally to caffeine have altered locomotor responses to the NMDA receptor antagonist MK-801 (da Silva *et al.*, 2005). This suggests that prenatal caffeine exposure leads to a persistent change in NMDA receptors, perhaps due to increased glutamate release after antagonism of A1 receptors. Neonatal exposure to caffeine also significantly decreases passive avoidance learning and retention. Caffeine exposure to 15-20 mg/kg/day on postnatal days 2-6 results in slowed avoidance learning in juvenile rats (Fisher & Guillet, 1997). In adult rats, retention but not learning is altered following developmental caffeine exposure to 15-20 mg/kg/day on postnatal days 2-6. Adult female rats have significantly improved retention of passive avoidance learning whereas male rats have significantly reduced retention when tested 72 hours after training (Fisher & Guillet, 1997). Recently, it has been shown that caffeine exposure the same administration on

postnatal days 2-6 significantly decreases 24 hour memory retention of passive avoidance in both males and females when tested on postnatal days 35-37 (Pan & Chen, 2007).

**M. Summary**

Caffeine is the most widely used psychostimulant in the world. The use of this drug during pregnancy is common but controversial. Although women are currently advised to reduce caffeine intake throughout pregnancy, it is estimated that 70-95% of pregnant women continue to consume the drug. The widespread use of caffeine is of concern because it crosses both the placental and blood brain barriers to accumulate in the developing brain. Furthermore, the half-life of caffeine is greatly prolonged in the developing fetus, being twenty fold that of an adult. Caffeine primarily acts as an adenosine receptor antagonist and therefore may increase glutamate neurotransmission in the developing brain, specifically in the developing hippocampus due to an abundance of A1 adenosine receptors in this brain region. Increased glutamate neurotransmission during development may alter the expression and function of NMDA receptors, which may induce long-term changes in calcium-dependent intracellular cascades, learning and memory behaviors, and hippocampal anatomy. In order to elucidate the effects of gestational caffeine exposure, pregnant rats were exposed to chronic moderate doses of caffeine. Rats are an appropriate model given the similar pharmacokinetics of caffeine metabolism between rats and humans. Metabolic body weight (=body weight^3/4) calculations were used based on findings that 20 mg/kg in a rat is equivalent to 4-6 cups of coffee in a 70 kg human (Nehlig & Derby, 1994).

**N. Hypotheses**

1. Caffeine and its metabolites accumulate in the developing brain during chronic prenatal exposure.

2. Accumulation of caffeine and its metabolites in the fetal brain antagonizes A1 adenosine receptors, which increases glutamate neurotransmission in the developing hippocampus and alters the expression of hippocampal NMDA receptors and calcium-dependent signaling cascades.

3. Alterations in glutamate-mediated hippocampal signaling results in long-term changes in hippocampal-dependent learning and memory behaviors.

4. Alterations in glutamate-mediated hippocampal signaling results in long-term changes in hippocampal anatomy.

**O. Specific Aims**

**Specific Aim 1: Does chronic prenatal caffeine exposure result in an accumulation of caffeine and its primary metabolites in the fetal brain?**

Chapter 2 presents the results from high-performance liquid chromatography (HPLC) studies that determined the concentrations of caffeine and its primary metabolites in maternal and fetal samples of brain and plasma following chronic administration of caffeine. Maternal rats were provided 75, 150, or 300 mg/L *ad libitum* caffeinated water throughout gestation. On gestational day 21-23, fetal and maternal samples of plasma and brain were collected for

HPLC determination of caffeine, theobromine, theophylline, and paraxanthine concentrations. The results reveal that caffeine and its metabolites significantly accumulate in the fetal brain following chronic maternal caffeine consumption.

**Specific Aim 2: Does chronic prenatal caffeine exposure alter the expression of hippocampal NMDA receptors and calcium-dependent signaling?**

Chapter 3 presents the results of Western blot and calcium imaging studies in which the expression of NMDA receptors and calcium-dependent signaling in the hippocampus were studied. For the Western blot analyses, the protein expression of the NR1, NR2A, and NR2B NMDA receptors were measured in hippocampal tissue collected from control and prenatal caffeine-treated offspring on postnatal days 1, 5, 10, and 20. Western blot analyses were also performed on hippocampal tissue from control and prenatal-caffeine treated offspring acutely treated with caffeine on postnatal days 10 and 20 to determine whether there were any long-term changes in calcium-dependent cascades following prenatal caffeine exposure. For this study, levels of phospho-CREB and phospho-ERK proteins were determined. Lastly, calcium imaging was performed on hippocampal cultures from control and caffeine-treated pups. Glutamate was briefly applied during the live cell recordings to determine the resulting peak calcium transient. The results reveal that total protein expression of the NR1, NR2A, and NR2B subunits are altered by prenatal caffeine at different postnatal ages. Further, peak calcium transients were reduced in caffeine-treated neurons, however pCREB and pERK activation were not found to be significantly different in control and treated animals.

**Specific Aim 3: Does chronic prenatal caffeine exposure alter hippocampal-dependent learning and memory behaviors in adult rats?**

Chapter 4 presents the results of behavioral studies in which the novel object recognition task, radial arm maze, and Morris water maze were used to determine if chronic prenatal caffeine exposure alters learning and memory behaviors in adult rats. Pregnant rats were provided 75 mg/L caffeine throughout gestation and the offspring underwent behavior testing on postnatal days 110-150. The results reveal that novel object recognition and radial arm maze behaviors are impaired in caffeine-treated offspring while Morris water maze behaviors are unimpaired.

**Specific Aim 4: Does chronic prenatal caffeine exposure alter the volume of the hippocampus in juvenile and adult rats?**

Chapter 5 presents the results of stereological studies using the Cavalieri estimator method to determine whether hippocampal volume is altered in caffeine-exposed offspring. Pregnant rats were provided 75 mg/L caffeine throughout gestation and tissue from offspring was collected on postnatal day 30 for juvenile analysis and postnatal day 150 for adult analysis. The results reveal a significant increase in total hippocampal volume in caffeine-treated juvenile rats, which does not persist into adulthood.

## Chapter 2. Caffeine and its Active Metabolites are Elevated in the Fetal Brain after Chronic Gestational Exposure

### A. Introduction

Caffeine, a methylxanthine, is the most widely used psychoactive drug in the world due to its presence in coffee, tea, colas, and chocolate (Fredholm *et al.*, 1999). It is estimated that 70-80% of pregnant women regularly consume caffeine during pregnancy despite ongoing disagreement about the developmental consequences of fetal exposure (Pastore & Savitz, 1995). Human and animals studies have shown that moderate prenatal caffeine consumption does not induce gross malformations of the fetus (Linn *et al.*, 1982; Kurppa *et al.*, 1983). However, in humans and non-human primates, maternal caffeine consumption has been positively correlated with increased risk of fetal death late in gestation (Gilbert *et al.*, 1988; Bech *et al.*, 2005; Weng *et al.*, 2008).

Methylxanthines cross both the placental and blood brain barriers to reach the developing fetal brain (Ikeda *et al.*, 1982; Tanaka *et al.*, 1984; Arnaud, 1993). Within the fetal brain, these substances have a prolonged duration of action due to an increased fetal half-life (Aranda *et al.*, 1979a). The increased half-life of caffeine is due to both a lack of cytochrome P-450 1A2 (CYP1A2) metabolism in the fetus and slowed maternal metabolism of caffeine during the second and third trimesters of pregnancy (Aldridge *et al.*, 1981; Knutti *et al.*, 1981; Brazier *et al.*, 1983). Slowed fetal elimination of caffeine and its metabolites may result in accumulation of these substances to detrimental levels during chronic prenatal exposure.

In humans and rodents, caffeine is demethylated to three primary metabolites: paraxanthine, theobromine, and theophylline. These metabolites are all biologically active, and like caffeine, function as adenosine receptor antagonists at physiological doses (Fredholm *et al.*, 1999). There are four known adenosine receptors, which include the A1, A2A, A2B, and A3 receptors (Fredholm *et al.*, 2000). Of the adenosine receptors, A1 receptors have the highest levels of expression, with mRNA detectable by gestational day 14 in the rat hippocampus and cerebellum (Rivkees, 1995; Weaver, 1996). Adenosine is a critical neuromodulator during brain development playing a major role in axon guidance (Turner *et al.*, 2002), white matter development (Rivkees *et al.*, 2001), and neuronal inhibition (Fisone *et al.*, 2004). Chronic prenatal caffeine exposure has been shown to alter fetal brain development by reducing adenosine receptor expression (Aden *et al.*, 2000; Leon *et al.*, 2002), altering DNA and protein content (Tanaka *et al.*, 1983), and delaying developmental outcomes such as locomotor reflexes (Tchekalarova *et al.*, 2005).

Previous reports in the literature have shown that as little as a single maternal dose of caffeine can lead to elevated levels of methylxanthines in the fetal brain. For example, a single maternal dose of 5 or 25 mg/kg caffeine by gavage on gestational day 20 results in metabolite levels three times higher in the fetal brain than in fetal plasma (Wilkinson & Pollard, 1993). Similarly, an intramuscular injection of radiolabeled caffeine causes significantly higher accumulation of caffeine in the fetal brain as compared to the maternal brain after 8 hours (Galli *et al.*, 1975). Elevated levels of caffeine and its metabolites in the fetal brain following maternal intake are of concern due to the varied actions of high concentrations of caffeine which inhibit phosphodiesterase activity and

induce intracellular calcium release (Fredholm *et al.*, 1999). The use of injections for caffeine administration may result in bypassing first-pass metabolism in the liver, resulting in fetal levels that are not comparable to those reached after oral maternal intake.

The purpose of the present study was to determine the concentrations of caffeine and its metabolites in maternal and fetal samples of brain and plasma following chronic oral *ad libitum* ingestion of caffeine. Maternal rats consumed approximately 30-60 ml/day of 75, 150, or 300 mg/L caffeinated water. On gestational day 21-23, fetal and maternal samples of plasma and brain were collected for high performance liquid chromatographic (HPLC) determination of caffeine and metabolite concentrations. Individual male and female fetal samples were collected to determine if there is a sex difference in prenatal caffeine metabolism. The results reveal that caffeine and its metabolites are elevated in the fetal brain of both male and female rats following chronic maternal caffeine consumption.

## B. Materials and Methods

*Animals*

Fourteen pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were provided either caffeinated tap water (75, 150, 300 mg/L; n=4 per treatment) or tap water alone (n=2) from gestational day 4 until gestational day 21-23 and intake was recorded daily. On gestational day 21-23, the dams were anesthetized and removal of embryonic pups was performed. Plasma and whole brain samples were collected for four pups from each litter (2 males and 2 females) for a total of n=16 pups per dose. Plasma and whole brain samples were also collected from each dam.

Trunk blood from pups and dams was collected in heparinized glass capillary tubes, immediately transferred to centrifuge tubes, and centrifuged at 1000 g for 10 min at 4°C. Plasma was removed and stored at -80°C. Whole brains from pups and dams were removed and immediately flash frozen in 2-methylbutane. Brains were stored at -80°C.

*Chemicals*

Caffeine, theophylline, 7-(β-hydroxyethyl)theophylline, theobromine, and 1,7-dimethylxanthine (paraxanthine) were obtained from Sigma, St. Louis, MO. Methanol and glacial acetic acid (99% HPLC grade) were obtained from Fisher Scientific. Stock solutions of caffeine (1 mg/ml), theobromine (1 mg/ml), theophylline (1 mg/ml), paraxanthine (1 mg/ml), and 7-(β-hydroxyethyl)theophylline (1 mg/ml) were prepared in 0.1% acetic acid. All stock solutions were stored at -80°C and have been previously

shown to be stable for at least 6 months (Hartley *et al.*, 1985). Standards (0-10 μg/ml) were prepared by serial dilution in 0.1% acetic acid from the stock solutions prior to each HPLC run.

*Plasma Extraction*

Waters C18 solid-phase extraction preparation columns (1.5 ml capacity) were used for rapid sample extraction. The columns were pre-conditioned by drawing 2 x 2 ml methanol followed by 2 x 2 ml water through the column. A single plasma sample (100 μl) and the internal standard 7-(β-hydroxyethyl)theophylline (133 ng/100 μl) were pulled into the column and allowed to equilibrate for 2 min. The column was washed with 2 x 2 ml water followed by 3 x 1 ml acetone. The eluent was evaporated to dryness using a speed-vac (Savant, SVC100) and reconstituted in 150 ul of 1% glacial acetic acid-methanol (83:17) mobile phase by vortexing. Injections of 20 ul were made for each plasma sample.

*Brain Preparation*

Extraction of caffeine and its metabolites in fetal brain was performed by homogenizing each brain in 400 μl tissue buffer (0.1 M phosphate/citrate containing 15% methanol) for 30 sec. Homogenized tissue was sonicated briefly (5 x 1 sec) and centrifuged at 18,000g for 10 min. The supernatant was immediately removed and brought to a final volume of 550 μl using tissue buffer. Injections of 50 μl were made for each brain sample extract.

Extraction of caffeine and its metabolites in maternal brain was performed by homogenizing each brain in 4 ml tissue buffer for 1 min. Homogenized tissue was sonicated briefly (10 x 1 sec) and centrifuged. The supernatant was removed and brought to a final volume of 5.5 ml using tissue buffer to maintain the same ratio used for the fetal brain extraction. Injections of 50 µl were made for each brain sample.

*Protein Determination*

One ml of 1.0N NaOH was added to the tissue pellet for determination of protein levels using a standard Lowry assay (Lowry *et al.*, 1951). Each sample was sonicated and vortexed until dissolved. Five µl of each protein sample was added to 95 µl NaOH. A single ml of Reagent A (50 ml sodium carbonate, 500 µl cupric sulfate, and 500 µl KNA tartrate) was added to each protein sample for 10 min followed by 100µl of Folin-Phenol Reagent for 30 min. Protein standards (12.5, 25, 50 µg/10µl) were run in duplicate. Protein levels were assayed using a spectrophotometer set to a wavelength of 700nm.

*Extraction Recovery*

Normal rat plasma used to determine extraction efficiency was spiked with 100 ug/ml each of internal standard 7-(β-hydroxyethyl)theophylline, caffeine, theophylline, theobromine, and paraxanthine. For extraction, 100 µl of the mix solution was passed through the preparation column using the same procedure described above. The extraction efficiencies of both acetone and methanol were tested. Extraction efficiency was determined as [(peak height of the sample)/(peak height of standard) x 100].

*HPLC Analyses of Caffeine and its Major Metabolites*

A reverse-phase C18 column was used with a Waters 510 HPLC system, Waters 717 autosampler, and Waters 996 Photodiode array for analyses of caffeine and its metabolites. A mobile phase of 1% glacial acetic acid-methanol (83:17) was filtered and degassed prior to use (Hartley *et al.*, 1985). A flow-rate of 1.0 ml/min was used with UV detection wavelength set at 270nm. Waters Millennium32 Version 4.0 software was used to process the data.

Levels of caffeine and its primary metabolites were determined in plasma using 20 µl injections of plasma extracts for each sample. Assays were performed on individual plasma samples from control and caffeine exposed male and female pups. High (1,250 ng/ml) and low (156.3 ng/ml) standards were injected between every four consecutive samples to ensure retention times were maintained. The peak height for caffeine and its metabolites was used and calculated as [(sample peak height/standard peak height)*(dilution factor)*(1/extraction efficiency)*10] to determine the concentration of each peak of interest (ng/ml).

Levels of caffeine and its primary metabolites were determined in brain using 50 µl injections of brain extracts for each sample. Assays were performed on each individual fetal and maternal brain. High (625 ng/ml) and low (312.5 ng/ml) standards were injected between every four consecutive samples. The peak height was used and calculated as [(sample peak height/standard peak height)*(dilution factor)*1000]/[sample protein content].

*Statistics*

Statistical assessment of weekly water intake was performed using two-way repeated measures analysis of variance (week, treatment). Linear regression was used to determine $R^2$ values for standard curves. One-way analysis of variance for sex was used to compare concentrations of each metabolite in male and female offspring. Two-way analysis of variance (dose, age) was used to compare the concentrations of caffeine and each metabolite in maternal and fetal brain and plasma across the three doses. A level of $p<0.05$ was required to obtain statistical significance. All analyses were performed using the SYSTAT program (version 11.0).

## C. Results

*Chromatograms and Standard curve*

A representative chromatogram resulting from a 50 µl injection of a 625 ng/ml standard mixture containing theobromine, paraxanthine, theophylline, β-hydroxyethyltheophylline and caffeine, respectively, is shown in **Figure 2-1A**. Standards of 19.5–10,000 ng/ml concentration were prepared by serial dilution in 0.1% acetic acid prior to each analysis. No methylxanthine peaks were observed in control chromatograms. Further, there were no discernable differences between chromatograms of males and females. There was excellent resolution between peaks with approximate retention times of: 5.8 min for theobromine, 8.9 min for paraxanthine, 10.1 min for theophylline, 11.4 min for the internal standard β-hydroxyethyltheophylline, and 17.4 min for caffeine.

As shown in **Figure 2-2**, standard curves for caffeine and its metabolites were made by comparing the peak height across concentrations ranging from 78.1–5000 ng/ml. The standard curves for caffeine and each metabolite were found to be linear with correlation coefficient ($R^2$) values above 0.9998 for each standard (**Figure 2-2**). The threshold of detection for all metabolites was 39.1 ng/ml.

*Extraction Efficiency*

Using the extraction methods described for plasma, acetone was determined to have higher extraction efficiency with less variance (99-101% ± 0.8; **Table 2-1**) for caffeine, its metabolites, and the internal standard as compared to methanol (88-91% ± 5.4). Thus, acetone was used as our final eluent.

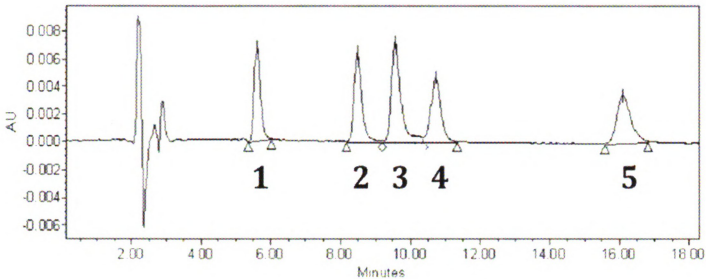


**Figure 2-1.** HPLC-UV detection of caffeine and its major metabolites in standards. Shown is a chromatogram of 625 ng/ml standard solution. Standards of 0-10 μg/ml were prepared by serial dilution in 0.1% acetic acid prior to each analysis. The retention times for each methylxanthine were approximately 1) Theobromine – 5.61, 2) Paraxanthine – 8.49, 3) Theophylline – 9.57, β-Hydroxyethyltheophylline – 10.72, and 5) Caffeine – 16.10. AU=Absorbance Unit.

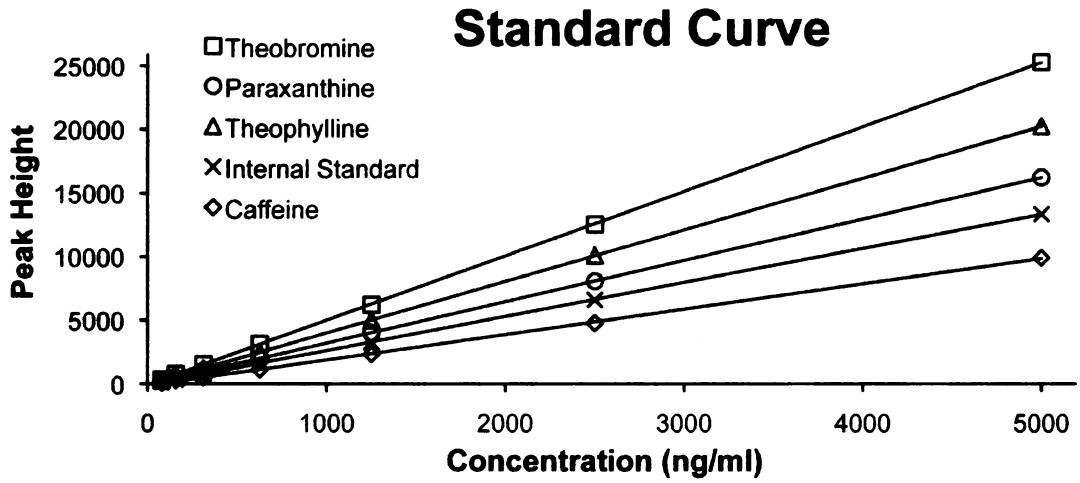


**Figure 2-2.** Standard curves for caffeine, its metabolites, and the internal standard β-hydroxyethyltheophylline. Standards were prepared in 0.1% acetic acid across concentrations of 78.1– 5000 ng/ml. Linear correlation coefficients ($R^2$) above 0.9998 were found for each standard by comparing the peak height across each concentration. The lowest limit of detection was 39.1 ng/ml.

| | Acetone | Methanol |
|---|---|---|
| **Theobromine** | 101.3 ± 0.7 | 91.1 ± 5.3 |
| **Paraxanthine** | 101.1 ± 0.7 | 91.1 ± 5.1 |
| **Theophylline** | 101.0 ± 0.6 | 91.0 ± 5.3 |
| **Internal Standard** | 101.5 ± 0.8 | 91.0 ± 5.4 |
| **Caffeine** | 99.2 ± 0.3 | 88.3 ± 5.4 |

**Table 2-1**. The plasma extraction efficiency of acetone and methanol were compared for each methylxanthine. Data represent [(peak height of the sample/peak height of standard) x 100] ± SEM.

*Gestational Caffeine Consumption*

Pregnant dams were administered *ad libitum* tap water (n=2) or caffeinated water (n=12) from gestational day (GD) 4 through GD 21-23 with intake recorded daily. Daily intake ranged from approximately 40-60 ml/day. The average water intake in control and caffeine-treated dams significantly increased each week (**Figure 2-3**; within-group analyses – [$F(2,124)=27.425$, $p<0.01$]) and showed a significant main effect of treatment [$F(1,62)=4.136$, $p<0.05$], but did not show a week by treatment interaction. To determine whether the main effect of treatment was a result of the different doses of caffeine administered, a two-way repeated measures analysis of variance for week by dose was performed. This analysis also revealed a significant within-group effect of week [$F(2,120)=46.012$, $p<0.01$] as well as a significant main effect of dose [$F(3,60)=10.262$, $p<0.01$] but no week by dose interaction. Interestingly, the dams administered 300 mg/L caffeine had significantly reduced intake each week when compared to the control and lower dose administrations **(Figure 2-3)**. Reduced intake may be a result of the increasingly bitter taste of caffeine in the drinking water at higher doses.

*Methylxanthine Concentrations in Male and Female Offspring*

One-way analysis of variance for sex revealed no differences in brain concentrations between male and female offspring for caffeine, theobromine, paraxanthine, or theophylline levels. Similarly, there were no sex differences in plasma concentrations between male and female offspring for caffeine or any of its metabolites. Based on these findings, data from male and female offspring were combined for all other analyses.

*Methylxanthine Concentrations in Fetal and Maternal Brain and Plasma*

Two-way analysis of varian5ce (dose, age) revealed a significant difference between fetal and maternal brain concentrations for caffeine and each metabolite. Interestingly, two-way analysis of variance (dose, age) for plasma concentrations did not reveal any differences between maternal and fetal levels for caffeine or any metabolite.

Concentrations of caffeine were found to be significantly greater in the fetal brain than the maternal brain [$F(1,54)=4.406$, $p<0.05$] however there was no significant effect of dose or dose by age interaction **(Figure 2-4A)**. Caffeine concentrations were approximately 3 times higher in the fetal brain than the maternal brain with mean fetal concentrations of 6.2, 21.8, and 35.6 ng/mg and mean maternal concentrations of 1.9, 7.9, and 10.3 ng/mg across the three doses of 75, 150, and 300 mg/L caffeine, respectively.

Plasma concentrations of caffeine were found to significantly differ across dose [$F(2,54)=4.623$, $p<0.02$] however there was no difference between maternal and fetal levels nor was there a dose by age interaction **(Figure 2-4B)**. Plasma caffeine levels increased with dose in both dams and their offspring such that mean maternal and fetal levels were 199.9 and 185.1 ng/ml at the 75 mg/L dose, 740.6 and 644.4 ng/ml at the 150mg/L dose, and 1013.4 and 1366.6 ng/ml at the 300 mg/L dose.

As shown in **Figure 2-5A,** concentrations of theobromine were also found to be significantly greater in the fetal brain than the maternal brain [$F(1,54)=47.332$, $p<0.001$]. Furthermore, there was a significant effect of dose [$F(2,54)=16.023$, $p<0.001$] as well as a significant dose by age interaction [$F(2,54)=7.317$, $p<0.002$]. Theobromine concentrations were the highest of any methylxanthine in the fetal brain with mean fetal concentrations of 11.7, 22.6, and 51.7 ng/mg and mean maternal concentrations of 1.1,

2.6, and 8.7 ng/mg across the three doses of 75, 150, and 300 mg/L caffeine, respectively. Concentrations of theobromine are 6-11 times higher in the fetal brain than the maternal brain at the doses used.

Plasma concentrations of theobromine were found to significantly differ across dose [$F(2,54)=40.227$, $p<0.001$], however there was no difference between maternal and fetal levels nor was there a dose by age interaction **(Figure 2-5B)**. Plasma theobromine levels increased by dose in both dams and their offspring such that mean maternal and fetal levels were 525.5 and 491.5 ng/ml at the 75 mg/L dose, 892.1 and 802.3 ng/ml at the 150mg/L dose, and 2616.0 and 2212.3 ng/ml at the 300 mg/L dose. Similar to brain concentrations, plasma concentrations of theobromine were higher than caffeine and the other metabolites.

Paraxanthine concentrations were also found to be significantly higher in the fetal brain when compared with the maternal brain [$F(1,54)=26.243$, $p<0.001$] and there was also a significant main effect of dose [$F(2,54)=7.293$, $p<0.002$] as well as a significant dose by age interaction [$F(2,54)=5.035$, $p<0.01$] **(Figure 2-6A)**. Across the three incremental doses of caffeine, mean fetal brain concentrations of paraxanthine were 5.6, 6.2, and 19.9 ng/mg, whereas mean maternal brain concentrations were 0.3, 0.6, and 1.7 ng/mg. Thus, concentrations of paraxanthine are approximately 11-18 times higher in the fetal brain when compared to the maternal brain depending on the dose despite overall concentrations of paraxanthine being the lowest in the brain compared to the other methylxanthines.

Plasma concentrations for paraxanthine did not differ between maternal and fetal samples, however there was a main effect of dose [$F(2,54)=23.19$, $p<0.001$] but no dose

by age interaction **(Figure 2-6B).** Mean maternal and fetal levels of plasma paraxanthine were 172.5 and 142.6 ng/ml at the 75 mg/L dose, 338.6 and 234.9 ng/ml at the 150 mg/L dose, and 1016.3 and 868.6 ng/ml at the 300 mg/L dose.

Similar to caffeine and the other metabolites, theophylline levels were also found to be significantly elevated in the fetal brain when compared to the maternal brain [$F(1,54)=28.488$, $p<0.001$] **(Figure 2-7A).** A significant main effect of dose [$F(2,54)=16.124$, $p<0.001$] and dose by age interaction [$F(2,54)=6.299$, $p<0.004$] were also found. Theophylline concentrations were lower than caffeine and theobromine levels in the fetal brain with mean fetal concentrations of 7.2, 10.8, and 28.0 ng/mg and mean maternal concentrations of 2.1, 4.7, and 7.6 ng/mg across the three doses of 75, 150, and 300 mg/L caffeine, respectively. Overall, fetal brain concentrations of theophylline are approximately 3 times higher than maternal brain concentrations at each dose.

Plasma concentrations of theophylline did not differ between dams and their offspring however there was a significant effect of dose [$F(2,54)=31.977$, $p<0.001$] but no dose by age interaction **(Figure 2-7B).** Mean maternal and fetal levels of plasma theophylline were 249.5 and 208.4 ng/ml at the 75 mg/L dose, 453.1 and 373.8 ng/ml at the 150 mg/L dose, and 1392.0 and 1071.0 ng/ml at the 300 mg/L dose.

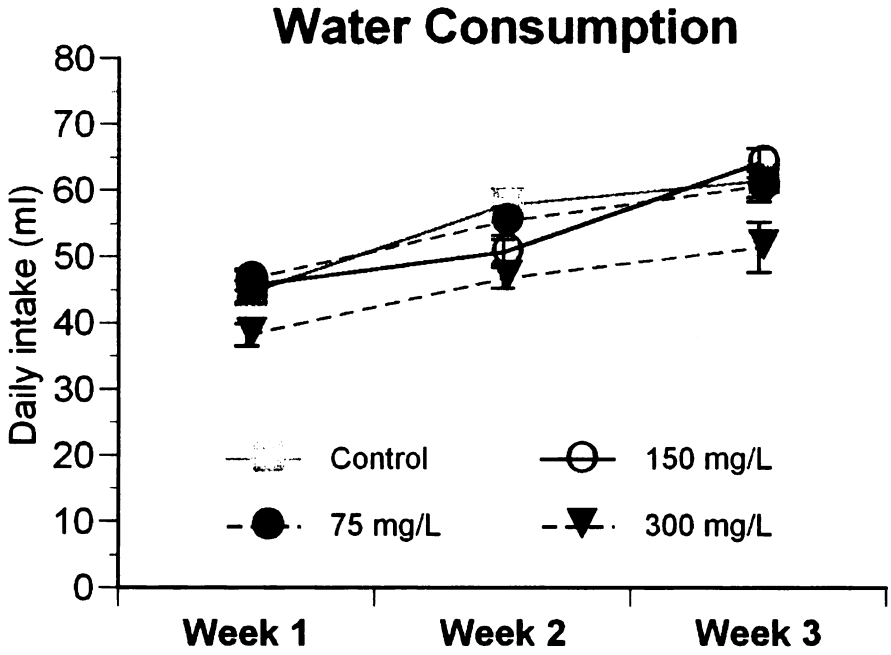


**Figure 2-3**. Maternal intake of either untreated tap water (control) or 75, 150, or 300 mg/L caffeine-treated tap water across each gestational week. Data represent the mean daily intake ± SEM for each week. Water intake increased throughout gestation in all treatment groups, however dams consuming 300 mg/L caffeine-treated water drank significantly less than dams treated with either no or low caffeine doses.

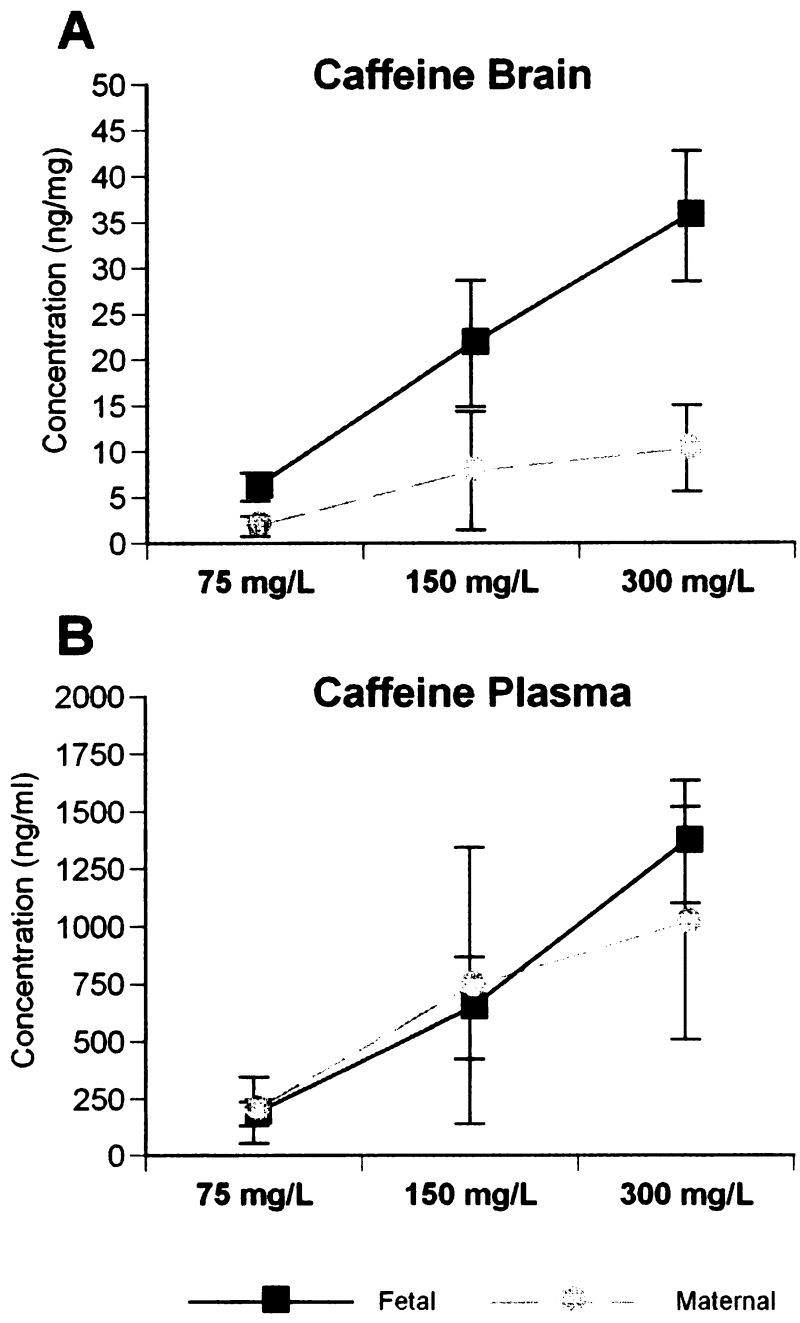


**Figure 2-4.** Concentrations of caffeine in fetal and maternal (A) brain and (B) plasma following prenatal caffeine exposure. Data represent the mean caffeine concentration (± SEM) reached following exposure to each dose. Chronic prenatal exposure to caffeine results in a significant accumulation of caffeine within the fetal brain when compared to the maternal brain, however there is no difference in caffeine concentrations in fetal and maternal plasma.

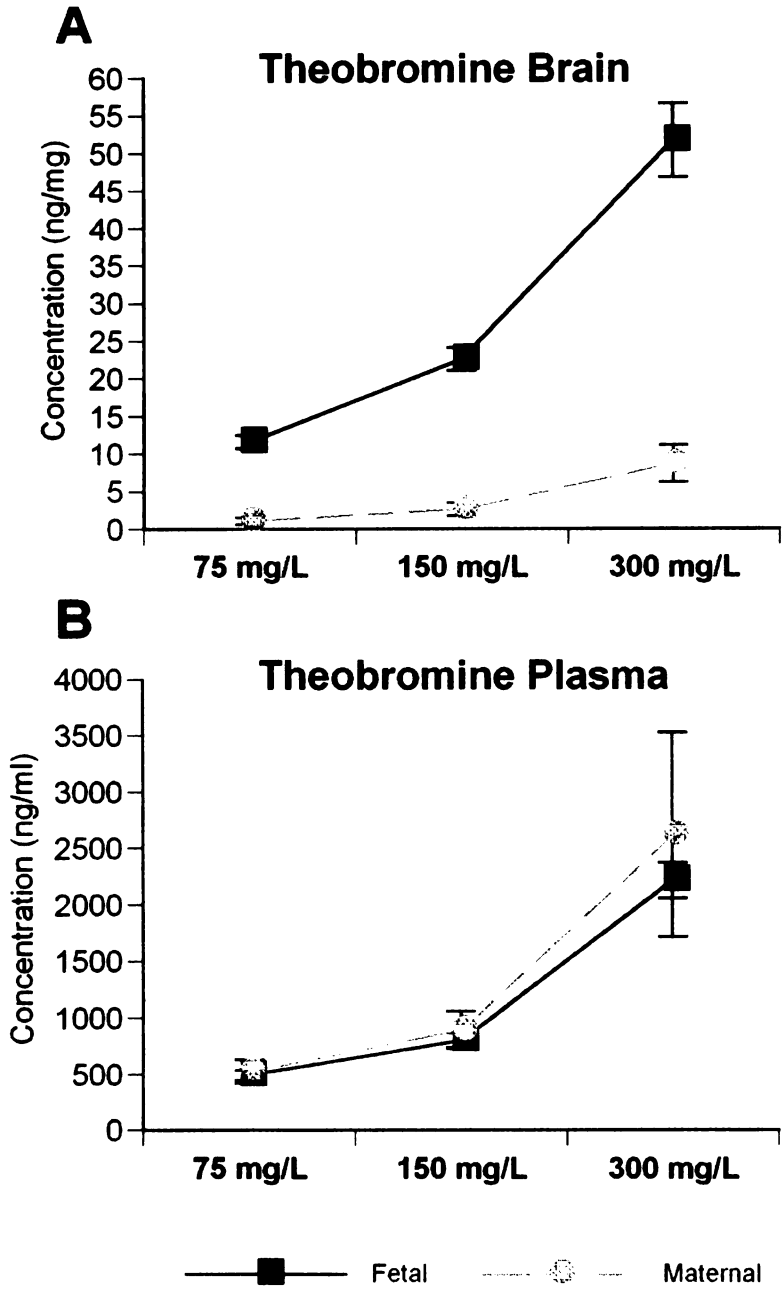


**Figure 2-5.** Concentrations of theobromine in fetal and maternal (A) brain and (B) plasma following prenatal caffeine exposure. Data represent the mean theobromine concentration (± SEM) reached following exposure to each dose. Chronic prenatal exposure to caffeine results in a significant accumulation of theobromine within the fetal brain when compared to the maternal brain that increased by dose. No differences between fetal and maternal plasma concentrations of theobromine were found.

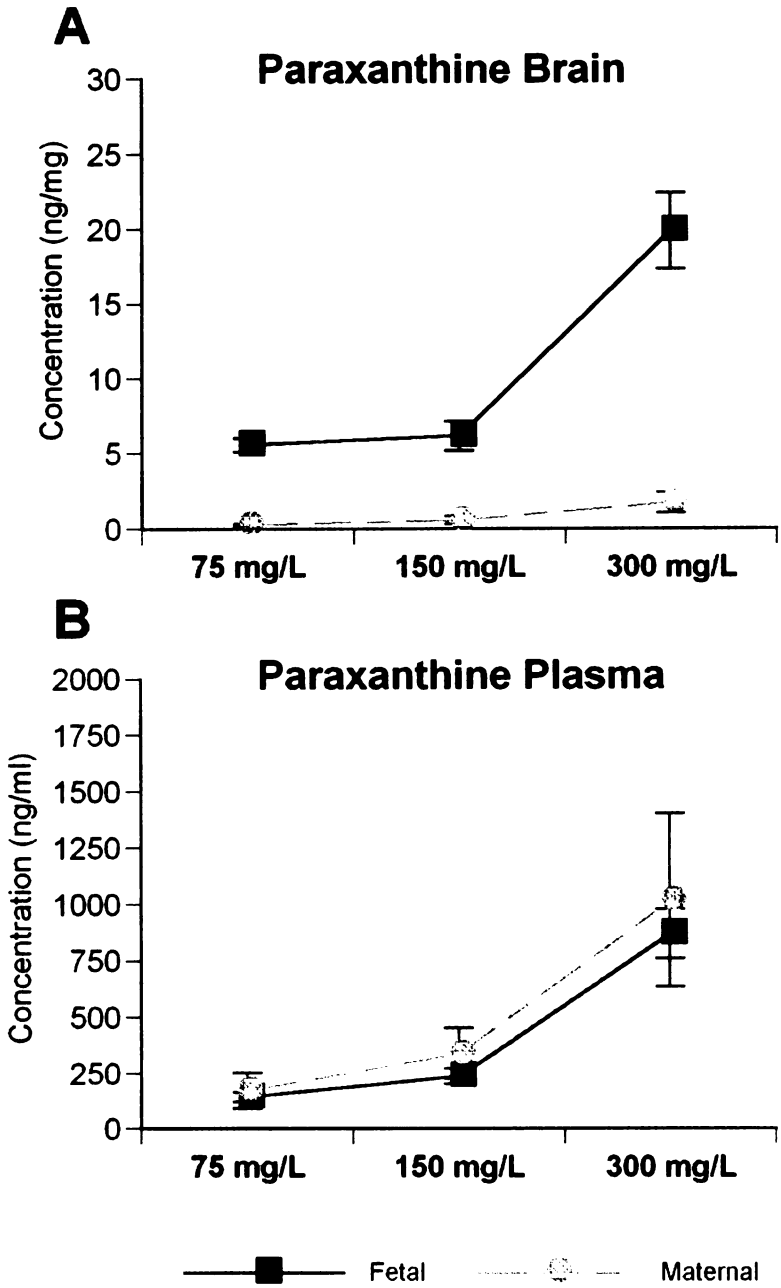


**Figure 2-6.** Concentrations of paraxanthine in fetal and maternal (A) brain and (B) plasma following prenatal caffeine exposure. Data represent the mean paraxanthine concentration (± SEM) reached following exposure to each dose. Chronic prenatal exposure to caffeine results in a significant accumulation of paraxanthine within the fetal brain when compared to the maternal brain that increased by dose. No differences between fetal and maternal plasma concentrations of paraxanthine were found.

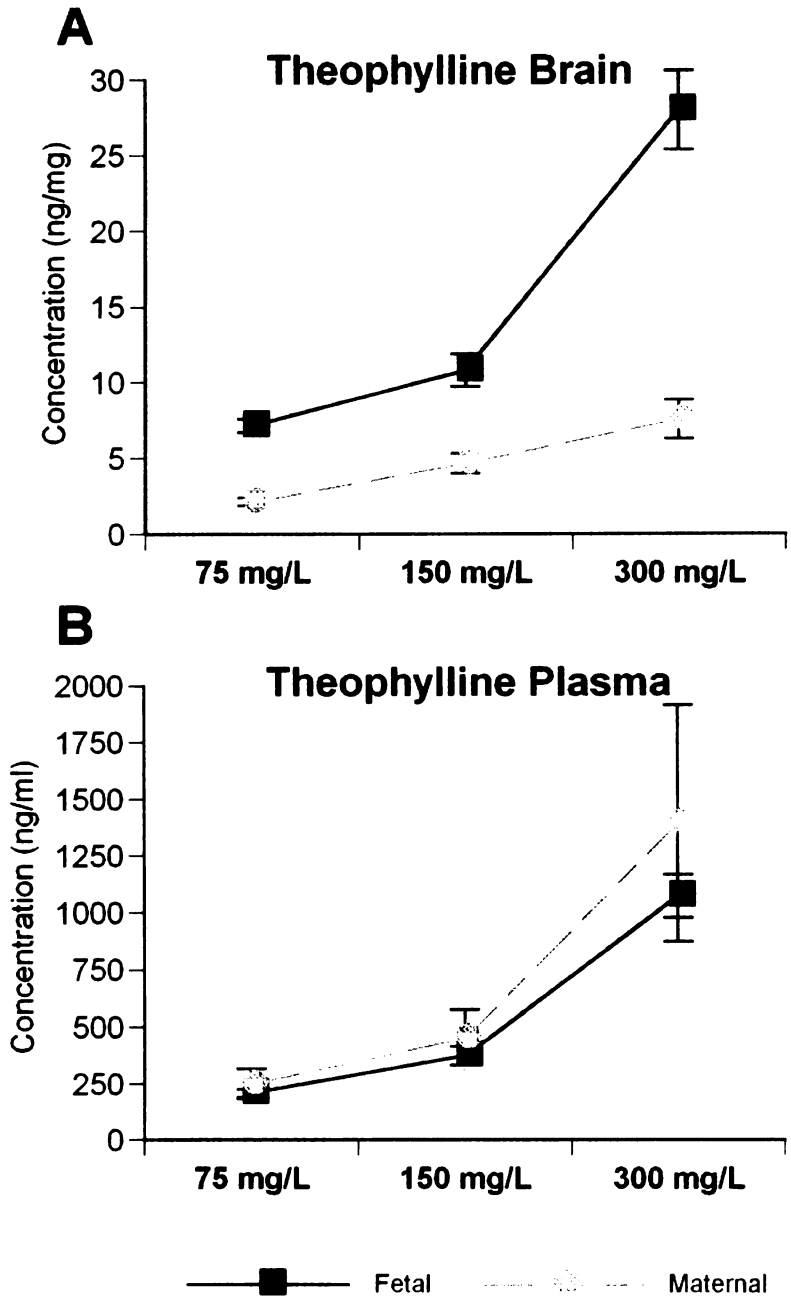


**Figure 2-7.** Concentrations of theophylline in fetal and maternal (A) brain and (B) plasma following prenatal caffeine exposure. Data represent the mean theophylline concentration (± SEM) reached following exposure to each dose. Chronic prenatal exposure to caffeine results in a significant accumulation of theophylline within the fetal brain when compared to the maternal brain that increased by dose. No differences between fetal and maternal plasma concentrations of theophylline were found.

## D. Discussion

This report demonstrates that chronic maternal intake of caffeine results in 3-fold or higher concentrations of caffeine and its metabolites in the fetal brain than in the maternal brain. In the fetal brain, theobromine had the highest concentration followed by lower concentrations of caffeine, paraxanthine, and theophylline, respectively. All of the metabolites showed higher concentrations in the fetal brain as compared to the maternal brain. Thus, the total methylxanthine level in the fetal brain was greatly elevated in comparison to the methylxanthine levels in the maternal brain. In contrast, there were no differences between caffeine and metabolite levels in the fetal and maternal plasma. Importantly, these findings indicate that measurements of plasma levels in newborns underestimate elevated methylxanthine levels in the brain.

Increased doses of caffeine administered to dams resulted in a dose dependent increase in caffeine and metabolite levels in the fetal brain while there were only slight increases in methylxanthine levels in the maternal brain across the three doses. Thus, the increase of caffeine and metabolite levels in the maternal brain appears linear, whereas in the fetal brain, the increase in methylxanthine concentration following exposure to the 300 mg/L dose appears to be non-linear. This suggests that repeated exposure to caffeine, especially to high doses, may lead to an accumulation of caffeine and its metabolites in the fetal brain due to decreased clearance of these drugs during development.

The elevated levels of caffeine in the developing fetal brain are likely due to caffeine's prolonged half-life in the fetus. Once ingested, caffeine is rapidly absorbed

from the gastrointestinal tract and distributed throughout the body within 45 min (Marks & Kelly, 1973; Bonati *et al.*, 1982; Arnaud, 1993). Caffeine is a hydrophobic compound and as such freely crosses all biological membranes including the placental and blood-brain barriers (Ikeda *et al.*, 1982; Kimmel *et al.*, 1984; Tanaka *et al.*, 1984; Abdi *et al.*, 1993). In non-pregnant adults, the half-life of caffeine is estimated to be 2-4 hours (Arnaud, 1987). During the second and third trimesters of pregnancy, the half-life of caffeine is estimated to be 10-15 hours in expectant mothers (Aldridge *et al.*, 1981; Knutti *et al.*, 1981; Brazier *et al.*, 1983). In the developing fetus, the immature cytochrome P-450 1A2 metabolic pathway greatly prolongs the half-life of caffeine and its metabolites (Aranda *et al.*, 1979a). It is estimated that the half-life of caffeine in newborn infants can range from 50-100 hours, with higher estimates for the half-life of caffeine in the fetus (Aranda *et al.*, 1977; Parsons & Neims, 1981; Arnaud, 1993). Thus, once an expectant mother clears caffeine from her system, the drug could maintain substantial levels in the fetus for prolonged periods of time.

The results of the present study demonstrate that methylxanthine levels are higher in the fetal brain than in maternal brain despite the fact that there are no differences between maternal and fetal plasma. The cause of sequestration of methylxanthines in the fetal brain is not well understood. It is known that caffeine freely crosses the placental barrier to reach the fetus. Bidirectional transport of caffeine across the placental barrier would allow equal concentrations of methylxanthines in maternal and fetal plasma (McCall *et al.*, 1982; Kimmel *et al.*, 1984). Elevated levels of caffeine and its metabolites in the fetal brain suggest an inability of caffeine to readily cross from the fetal brain back into circulation. This could be due to various factors including saturation

of carrier-mediated facilitated diffusion systems, protein binding of caffeine in the brain, or caffeine sequestration in relatively lypophillic brain tissue. However, further research is needed to understand the cause of caffeine sequestration in the fetal brain.

Although a direct comparison between the brain and plasma levels in the current data may not be warranted, it does appear that levels of caffeine and its metabolites are higher in plasma than in brain in both the dam and her offspring. Therefore, it is possible that there may be a mechanism to keep caffeine from entering the brain that is better functioning in the adult compared to the fetus, and thus fetal levels of methylxanthines are elevated compared to maternal levels. However, such a mechanism blocking caffeine from entering the brain has not been found.

It is also important to consider the effects of cytochrome P-450 metabolism in the liver and brain on the elevated levels of methylxanthines in the fetus. As mentioned previously, in the developing fetus, the immature cytochrome P-450 1A2 metabolic pathway greatly prolongs the half-life of caffeine and its metabolites (Aranda *et al.*, 1979a). Cytochrome P-450 enzymes are found in brain tissue and therefore may contribute to the elevated levels of methylxanthines seen in the fetal brain. However, the levels of these enzymes in the brain are only 0.5-2% of that found in the liver (Hedlund et al, 2001). Thus, slowed metabolism of P-450 enzymes in the brain is likely making a very minor contribution to the elevated levels of caffeine in the fetal brain.

Chronic maternal intake of caffeine resulted in elevated levels of caffeine as well as elevated levels of theobromine, theophylline, and paraxanthine in the fetal brain. Because these metabolites are biologically active, the levels of each metabolite must be considered to determine the cumulative effects of xanthines on the developing brain. In

rats, theobromine is the major metabolite of caffeine whereas in humans paraxanthine is the major metabolite (Eteng *et al.*, 1997; Fredholm *et al.*, 1999). Thus, it is not surprising that concentrations of theobromine were found to be higher than concentrations of caffeine and the other metabolites in dams and their offspring. Furthermore, caffeine and theobromine are poorly bound by plasma proteins leaving 70-85% of these drugs free to cross the blood-brain barrier (Bonati *et al.*, 1984; Wilkinson & Pollard, 1993). This is unlike theophylline which readily binds plasma proteins and thus does not enter the brain as rapidly (Birkett *et al.*, 1985; Aramayona *et al.*, 1991; Stahle *et al.*, 1991). Within the brain, paraxanthine is equally as potent as caffeine at antagonizing adenosine receptors, whereas theophylline is thought to be 3-5 times more potent than caffeine (Benowitz *et al.*, 1995; Daly, 2000). Theobromine is a weaker adenosine antagonist than caffeine, however has considerable effects as a vasodilator (Shi & Daly, 1999; Daly, 2000). Thus, the availability of these substances to enter the fetal brain and potency of these substances within the brain are important determinants of the total effects of xanthines on brain development.

Caffeine and its major metabolites function as adenosine receptor antagonists (Fredholm, 1980; Daly *et al.*, 1981; Daly & Fredholm, 1998). Elevated levels of these methylxanthines within the fetal brain may cause persistent antagonism of adenosine receptors during brain development. Chronic antagonism of these receptors may alter normal brain development since adenosine is one of the earliest neuromodulators in the developing brain (Rivkees *et al.*, 2001). Current reports in the literature have shown that disruption of adenosine receptor function during development alters neonatal respiratory patterns (Montandon *et al.*, 2008), hypoxic response (Back *et al.*, 2006), seizure activity

(Tchekalarova *et al.*, 2006, 2007), and white matter development (Turner *et al.*, 2002). In humans, maternal consumption of greater than 200 mg caffeine per day has been shown to double the risk of miscarriage (Weng *et al.*, 2008). It has also been found that elevated levels of paraxanthine in fetal plasma are positively correlated with intrauterine growth restriction and small for gestational age newborns (Grosso *et al.*, 2006). Thus, elevated levels of these adenosine receptor antagonists in the fetal brain throughout gestation may have detrimental effects on fetal development.

Caffeine metabolism in rats and humans is very similar, making rats an excellent animal model for studying the effects of prenatal caffeine exposure (Arnaud, 1993). To maintain relevance to humans, doses of 0.075 to 0.3 g/L were utilized in the present study. These doses are well within the range of human caffeine intake and low compared to most caffeine studies, which use a range of 0.2-1 g/L (Aden *et al.*, 2000; Leon *et al.*, 2002; Picard *et al.*, 2008). One drawback to using a prenatal rat model is the short length of gestation (21-24 days). Newborn rats are altricial, or underdeveloped, in comparison to newborn humans, and it is common to consider postnatal days 1-7 in the rat parallel to the third trimester of development in humans (Clancy *et al.*, 2007). In the present study, embryonic pups were used to avoid experimental issues with timing of birth and lack of control over neonatal exposure when suckling. This resulted in a much shorter period of exposure than would be seen in the human fetus if the mother drank caffeine throughout pregnancy. It is likely that the longer duration of exposure in humans could lead to even higher fold accumulation in the fetal brain.

In summary, moderate maternal caffeine intake leads to accumulation of caffeine and its bioactive metabolites in the fetal brain. Elevated levels of these methylxanthines

may disrupt normal adenosine receptor function during development. High maternal caffeine consumption may lead to toxic levels of caffeine and its metabolites in the fetal brain increasing the risk of miscarriage and fetal growth restriction. Assays of newborn plasma may not be indicative of levels of caffeine in the newborn brain. Thus, expectant women should be cautioned to limit or discontinue the use of caffeine during pregnancy due to the prolonged half-life of the drug in the fetus and the potential for accumulation in the fetal brain.

## Chapter 3. Chronic Gestational Caffeine Exposure Alters Glutamate Receptor Expression and Calcium Influx in the Developing Hippocampus

### A. Introduction

Glutamate receptors play a role in numerous developmental processes including activity-dependent migration, synapse formation, and synaptic plasticity. Ionotropic glutamate receptors include the N-methyl-D-aspartate (NMDA), α-amino-3-hydoxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptor subtypes. Of these, NMDA receptors exhibit increased calcium permeability making their activation critical to the induction of numerous calcium-dependent cascades that are integral to processes such at long-term potentiation (Dingledine *et al.*, 1999). NMDA receptors are tetrameric complexes comprised of the NR1, NR2A-D, and NR3A-B subunits. The hippocampus predominantly contains NR1/NR2A and NR1/NR2B oligomers (Dingledine *et al.*, 1999). During development, the expression of these subunits is temporally and spatially regulated. The obligate NR1 subunit is expressed during both embryonic and postnatal periods (Lujan *et al.*, 2005). The NR2B and NR2A subunits undergo a developmental switch in expression such that NR2B subunits are highly expressed during pre- and early postnatal periods after which NR2A subunit expression predominates (Lujan *et al.*, 2005). Each of these subunits confers distinct kinetic properties allowing the function of NMDA receptors to be modulated during development.

The dynamic timing of NMDA receptor expression during critical periods of brain development makes these receptors susceptible to the effects of perinatal drug

exposure. Ethanol exposure during prenatal or postnatal development alters many aspects of NMDA receptor expression and function. In the neonatal hippocampus and cortex, NR2A receptor expression is increased in ethanol exposed animals (Nixon *et al.*, 2002, 2004). NMDA receptor-mediated calcium transients are reduced in cerebellar granule neurons after four consecutive days of postnatal ethanol exposure (Gruol *et al.*, 1998). Similarly, there is reduced binding of the NMDA receptor antagonist MK-801 in the hippocampus of ethanol-treated animals (Diaz-Granados *et al.*, 1997). Like ethanol, nicotine and morphine also induce alterations in the expression and function of NMDA receptors in the developing brain. Exposure to nicotine increases NR2A mRNA in the rat forebrain and enhances the duration of NMDA-mediated excitatory postsynaptic potentials (EPSPs) (Hsieh *et al.*, 2002). Similarly, prenatal morphine exposure alters the kinetic properties of NMDA receptors in neonatal hippocampal pyramidal neurons (Yang *et al.*, 2000).

Given the effects of numerous commonly used drugs on NMDA receptor function and expression, it is surprising that prenatal exposure to caffeine and its potential effects on the glutamatergic system have not been previously investigated. Caffeine is the most commonly consumed psychostimulant drug in the world and an estimated 70-95% of women consume this drug during pregnancy (Fredholm *et al.*, 1999). Caffeine readily crosses both the placental and blood brain barriers to reach the fetus (Arnaud, 1993) and accumulates in the developing brain (Chapter 2; Wilkinson & Pollard, 1993). Caffeine acts as an adenosine receptor antagonist, and blockade of adenosine receptors by caffeine results in an increase in neurotransmitter release and an overall increase in neuronal excitability (Fredholm *et al.*, 1999). Specifically, caffeine facilitates glutamate

transmission, particularly in brain regions with the high levels of A1 adenosine receptor expression such as the cortex and hippocampus (Wang, 2007). Therefore, if prenatal exposure is increasing glutamate neurotransmission in the developing brain, caffeine may alter the development and function of critical glutamatergic systems.

The purpose of the present study was to determine the effects of prenatal caffeine on NMDA receptor expression and calcium-dependent signaling in the hippocampus. Total protein expression of the NR1, NR2A, and NR2B NMDA subunits was measured in hippocampal tissue from control and prenatal caffeine-exposed offspring. Phospho-CREB and phospho-ERK proteins were also analyzed following acute caffeine treatment on postnatal days 10 or 20 in control and prenatal caffeine-exposed offspring to determine whether there are long-term changes in calcium-dependent cascades due to prenatal caffeine exposure. Additionally, calcium imaging was performed on hippocampal cultures from control and caffeine-exposed pups. Glutamate was briefly applied during live cell recordings to determine the resulting peak calcium transients. The results reveal that protein expression of the NR1, NR2A, and NR2B subunits is altered by prenatal caffeine, with each subunit exhibiting increased or decreased expression during a specific time period. Further, peak calcium transients were reduced in caffeine-treated neurons, whereas phospho-CREB and phospho-ERK activation were not significantly different between control and treated animals.

## B. Materials and Methods

*Animals*

Pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were provided either caffeinated tap water (75, 150, or 300 mg/L) or tap water alone from gestational day 4 until birth and intake was recorded daily. For Western blot analysis of total receptor expression, offspring were housed with each dam until postnatal day 1, 5, 10, or 20 when hippocampal tissue was collected. Six pups were collected at each time point for each treatment group. For Western blot analysis of phosphorylated receptor expression, offspring were housed with each dam until postnatal days 10 or 20. Pups were administered caffeine (10 mg/kg) orally by pipette and kept on 37°C heating pads for 1.5 hours after which hippocampal tissue was immediately collected. Six pups were collected at each time point for each treatment group. For calcium imaging studies, dams were provided with caffeinated tap water (75mg/L) or tap water alone throughout gestation, and male and female pups were collected on postnatal day 0 for hippocampal cell cultures.

*Primary Hippocampal Cell Cultures*

Within 24 hours of birth, the bilateral hippocampi from male and female rat pups were collected and separately cultured by prenatal treatment. Tissue was dissected into HBSS+ (88ml sterile dH2O, 10ml 10X Hank's Balanced Salt Solution (Gibco, Invitrogen, Carlsbad, CA), 1ml 1.0M pH 7.3 HEPES buffer (Gibco), 1ml penicillin (10,000 units) plus streptomycin (10,000 μg; Gibco)) and incubated in HBSS+ with 2.5%

trypsin for 15 min at 37°C. Supernatant was removed and tissue was rinsed twice with HBSS+ followed by treatment with DNAase and dissociation by trituration. Dissociated cells were seeded on 25mm poly-L-lysine coated coverslips which were stored in 100mm petri dishes with 10ml plating medium (86ml MEM (Gibco), 10ml normal horse serum (Gibco), 3ml 20% filter sterilized glucose, 1ml 100mM sodium pyruvate) and incubated for 4 hours at 37°C, 5% CO2. Coverslips were then transferred to individual 35mm petri dishes, which contained 3ml Neurobasal+ (1ml B-27 supplement (Gibco), 1ml penicillin (10,000 units) plus streptomycin (10,000 μg; Gibco), 125ul L-glutamine (Gibco), and 48ml phenol red free Neurobasal (Gibco)). Cells were allowed to plate until day *in vitro* 3 (DIV3) when they were used for calcium imaging.

*Calcium Imaging*

Cultured hippocampal cells were used for calcium imaging on DIV3 as described (Nunez & McCarthy, 2007). The cell permeable fluorescent indicator fura-2 acetoxymethyl ester (fura-2-AM) was used for all imaging experiments. Cells were loaded with fura-2-AM (3μM) in DMSO (<0.5%) for 30 min at 37°C. Coverslips were then placed on a perfusion chamber mounted to a Nikon Eclipse TE-2000U inverted microscope and rinsed for 30 min with a physiological saline solution (PSS; 137ml NaCl, 20ml KCl, 4ml MgCl2, 12ml CaCl2, 40ml HEPES (Gibco), and 90ml 20% glucose brought to a 4L with ultrapure dH2O) to remove extracellular dye. Illumination was provided by a high-speed Sutter DG-5 wavelength switcher while fluorescent images were obtained using a Roper Coolsnap Cascade 512B cooled CCD camera with on-chip multiplication gain. Universal Imaging Metamorph/Metafluor Imaging software (version

6.0) was used for all image acquisition and analysis. Baseline intracellular calcium measurements were collected over a 5 min period during which cells were superfused with PSS for each new field of view. This was followed by a 1 min exposure to 2, 5, 10, or 50 μM glutamate followed by a 5 min rinse with PSS **(Figure 3-1)**. The maximal intracellular peak calcium response, baseline calcium, and percentage of cells responding to each treatment were analyzed. The intracellular calcium concentrations were not directly measured and thus all references to peak levels are referring to the percent change over baseline resting Fura-2 ratios. The relationship between the calcium ratio and the overall change in the intracellular calcium concentration cannot be assumed to be equal or directly proportional (i.e. a doubling of the ratio is not equivalent to a doubling of the intracellular calcium concentration).

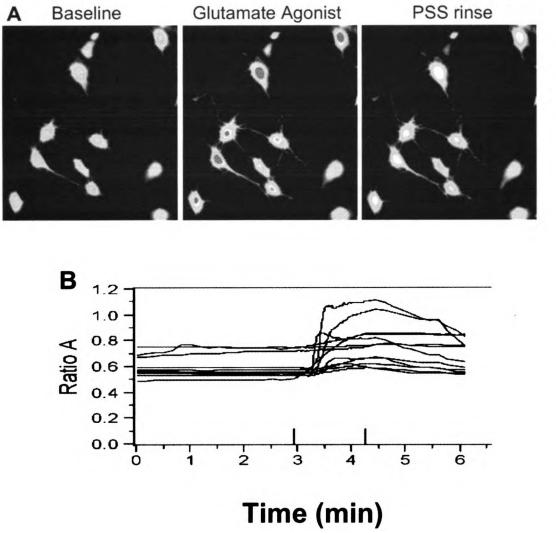


**Figure 3-1.** The change in ratio between resting intracellular calcium and peak responses following glutamate administration are shown in both the hippocampal cell cultures (A) and a corresponding trace recording (B) during live cell imaging. Both the images and trace represent the ratio of bound to unbound intracellular Fura-2.

*Western Blot Analysis*

On postnatal days 1, 5, 10, or 20, hippocampi were microdissected from both hemispheres and immediately flash frozen in 2-methylbutane. Tissue was weighed and sonicated briefly in 10μl/mg tissue buffer (50mM β-glycerophosphate, 1.5mM EGTA, 0.1mM sodium orthovanadate, 1mM DTT, Complete mini protease inhibitor cocktail (Roche)). Protein concentrations were normalized using a BCA protein assay kit (Pierce, Rockford, IL). 100μl homogenate was added to an equal volume of SDS loading buffer (10mM Tris-HCl buffer pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue, 5% β-mercaptoethanol). Samples were boiled for 10min, electrophoresed on 10% Tris-Glycine gels (Invitrogen, Novex, Carlsbad, CA), and transferred to PVDF membranes. For each primary antibody, overnight incubation at 4°C in 5% nonfat milk (Carnation, USA) made with PBS with 0.1% Triton-X was performed. For analysis of total receptor expression, the following primary antibodies were used: NR1 (1:2000, Cell Signaling), NR2A (1:1000, Cell Signaling), NR2B (1:1000, Cell Signaling). For analysis of receptor activity, phospho-CREB (1:2000, Cell Signaling) and phospho-ERK1/2 (1:1000, Cell Signaling) were used. Membranes were washed in PBS with 0.01% Triton-X before being incubated with HRP-conjugated secondary antibodies (1:5000, Pierce) at room temperature for 1 hour. Beta-Actin (1:5000, Sigma) was used as a loading control. Proteins were visualized with enhanced chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL) and exposed to film for several periods to obtain optimal signal. Membranes were probed multiple times by rinsing with stripping buffer (1% SDS, 0.2% glycine, pH 2.0) before incubating with

another primary antibody. Quantification of bands was performed using Scion Image (NIH, Bethesda, Maryland, USA).

*High Performance Liquid Chromatography*

On PN5 and PN10, trunk blood and whole brains from 4 pups in each treatment group were collected for HPLC determination of caffeine and its metabolites. Blood was collected in heparinized glass capillary tubes, immediately transferred to centrifuge tubes, and centrifuged at 1000 g for 10min at 4°C. Plasma was removed and stored at -80°C. Whole brains from pups and dams were removed and immediately flash frozen in 2-methylbutane. Brains were stored at -80°C.

Caffeine, theophylline, 7-(β-hydroxyethyl)theophylline, theobromine, and 1,7-dimethylxanthine (paraxanthine) were obtained from Sigma, St. Louis, MO. Methanol and glacial acetic acid (99% HPLC grade) were obtained from Fisher Scientific. Stock solutions of caffeine (1 mg/ml), theobromine (1 mg/ml), theophylline (1 mg/ml), paraxanthine (1 mg/ml), and 7-(β-hydroxyethyl)theophylline (1 mg/ml) were prepared in 0.1% acetic acid. All stock solutions were stored at -80°C and have been previously shown to be stable for at least 6 months (Hartley *et al.*, 1985). Standards (0-10 μg/ml) were prepared by serial dilution in 0.1% acetic acid from the stock solutions prior to each HPLC run.

Waters C18 solid-phase extraction preparation columns (1.5 ml capacity) were used for rapid sample extraction. The columns were pre-conditioned by drawing 2 x 2 ml methanol followed by 2 x 2 ml water through the column. A single plasma sample (100 μl) and the internal standard 7-(β-hydroxyethyl)theophylline (133 ng/100 μl) were pulled

into the column and allowed to equilibrate for 2 min. The column was washed with 2 x 2 ml water followed by 3 x 1 ml acetone. The eluent was evaporated to dryness using a speed-vac (Savant, SVC100) and reconstituted in 150 ul of 1% glacial acetic acid-methanol (83:17) mobile phase by vortexing. Injections of 20 ul were made for each plasma sample.

Extraction of caffeine and its metabolites in fetal brain was performed by homogenizing each PN5 brain in 1 ml tissue buffer (0.1 M phosphate/citrate containing 15% methanol) and each PN10 brain in 2 ml tissue buffer for 30 sec. Homogenized tissue was sonicated briefly (5 x 1 sec) and centrifuged at 18,000g for 10 min. The supernatant was immediately removed and brought to a final volume of 1.375 ml for PN5 samples and 2.75 ml for PN10 samples using tissue buffer. Injections of 50 µl were made for each brain sample extract.

Two ml of 1.0N NaOH was added to the tissue pellet for determination of protein levels using a standard Lowry assay (Lowry *et al.*, 1951). Each sample was sonicated and vortexed until dissolved. Five µl of each protein sample was added to 95µl NaOH. A single ml of Reagent A (50ml sodium carbonate, 500 µl cupric sulfate, and 500 µl KNA tartrate) was added to each protein sample for 10 min followed by 100µl of Folin-Phenol Reagent for 30 min. Protein standards (12.5, 25, 50 µg/10µl) were run in duplicate. Protein levels were assayed using a spectrophotometer set to a wavelength of 700nm.

A reverse-phase C18 column was used with a Waters 510 HPLC system, Waters 717 autosampler, and Waters 996 Photodiode array for analyses of caffeine and its metabolites. A mobile phase of 1% glacial acetic acid-methanol (83:17) was filtered and

degassed prior to use (Hartley *et al.*, 1985). A flow-rate of 1.0 ml/min was used with UV detection wavelength set at 270nm. Waters Millennium32 Version 4.0 software was used to process the data.

Levels of caffeine and its primary metabolites were determined in plasma using 20 µl injections of plasma extracts for each sample. Assays were performed on individual plasma samples from control and caffeine exposed male and female pups. High (1,250 ng/ml) and low (156.3 ng/ml) standards were injected between every four consecutive samples to ensure retention times were maintained. The peak height for caffeine and its metabolites was used and calculated as [(sample peak height/standard peak height)*(dilution factor)*(1/extraction efficiency)*10] to determine the concentration of each peak of interest (ng/ml).

Levels of caffeine and its primary metabolites were determined in brain using 50 µl injections of brain extracts for each sample. Assays were performed on each individual fetal and maternal brain. High (625 ng/ml) and low (312.5 ng/ml) standards were injected between every four consecutive samples. The peak height was used and calculated as [(sample peak height/standard peak height)*(dilution factor)*1000]/[sample protein content].

*Statistics*

Statistical analyses of the calcium imaging data were performed using one-way analysis of baseline and two-way analysis of variance (treatment, concentration) for the peak calcium transient. The Western blot data were analyzed by one-way analysis of variance by treatment for each individual blot. For all main effects, post-hoc (Tukey)

tests were performed. A level of $p<0.05$ was required to obtain statistical significance. All analyses were performed using the SYSTAT program (version 11.0).

## C. Results

*Peak Calcium Response to Glutamate*

One-way analysis of resting intracellular calcium revealed no differences between baseline calcium ratios in control and caffeine-exposed neurons (**Figure 3-2).** Two-way analysis (treatment, concentration) of the peak calcium transient following brief application of 2, 5, 10, or 50 μM glutamate revealed a significant main effect of treatment [$F(1,1423)=7.129$, $p<0.01$], a main effect of concentration [$F(3,1439)=169.445$, $p<0.01$], and a treatment by concentration interaction [$F(3,1439)=3.506$, $p<0.02$]. To further investigate these findings, the peak calcium response of each cell was classified as a low responder (less than 20% change over baseline), medium responder (20-100% change over baseline), or high responder (over 100% change from baseline). Two-way analysis of variance (treatment, concentration) revealed no significant effects of treatment or concentration in low responding neurons **(Figure 3-3A).** In medium responding neurons, there was a significant main effect of glutamate concentration [$F(3, 662)=25.605$, $p<0.001$], but no effect of treatment and no treatment by concentration interaction **(Figure 3-3B).** In high responding neurons, there was a significant main effect of treatment [$F(1,472)=4.902$, $p<0.003$] as well as a significant main effect of glutamate concentration [$F(3, 472)=4.954$, $p<0.003$] **(Figure 3-3C).** These findings indicate that caffeine-exposed neurons exhibit a significantly reduced peak calcium transient when compared to control neurons. Further, in both treatment groups, application of higher concentrations of glutamate induces a larger calcium transient.

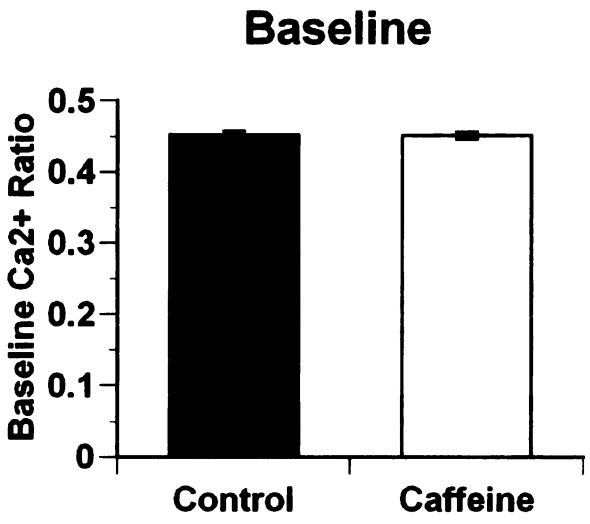


**Figure 3-2**. Baseline resting intracellular Ca2+ ratio in cultured hippocampal neurons from control and prenatal caffeine-treated neurons. There were no significant differences between resting intracellular Ca2+ ratios between treatments. Data represent the average resting Fura-2 ratio ± SEM.

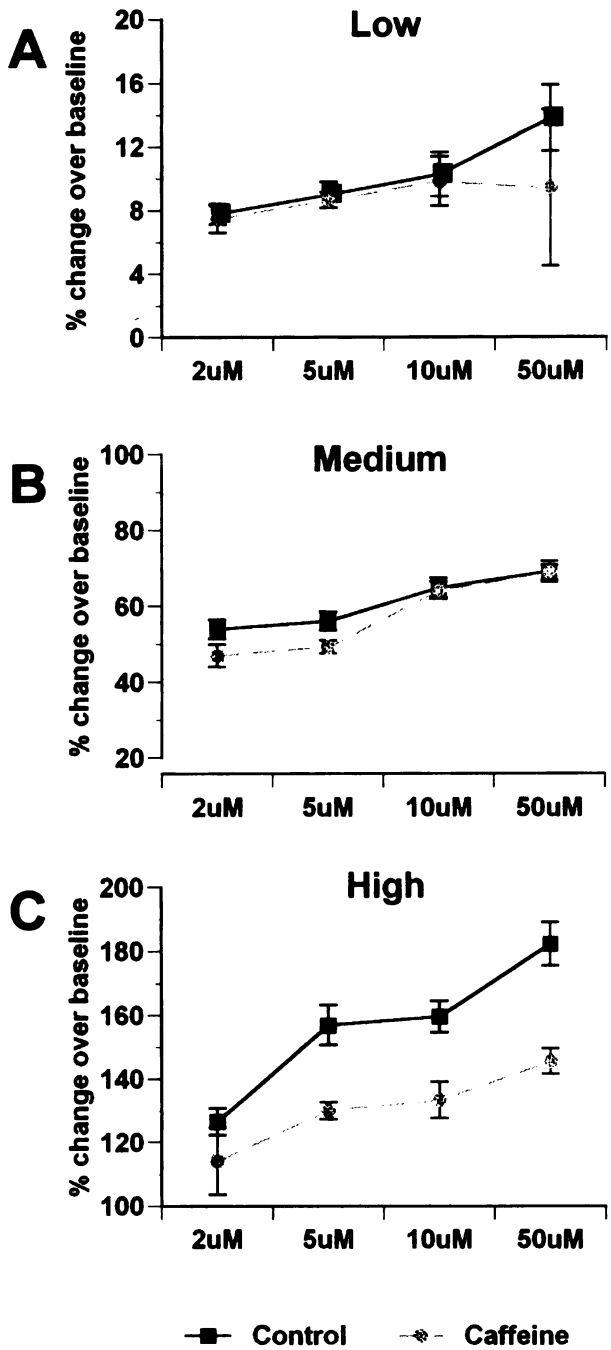


**Figure 3-3.** Peak calcium transients following brief application of 2, 5, 10, or 50 μM glutamate in hippocampal neurons cultured from control or caffeine-exposed pups. A calcium transient less than 20% over baseline was classified as a low response (A), 20-100% change over baseline as a medium response (B), and higher than 100% change from baseline as a high response (C). In high responding neurons, caffeine treatment resulted in a reduced calcium transient. Data represent the mean calcium peak ± SEM.

*Total NMDA Receptor Expression in the Developing Hippocampus*

Analysis of the total protein expression of the NR1 subunit in the developing hippocampus revealed no statistical difference between control and caffeine-exposed animals on postnatal day 1 **(Figure 3-4A)**. However, a statistically significant main effect of NR1 receptor expression was found on postnatal day 5 [$F(3,6)=7.046$, $p<0.03$] **(Figure 3-4B)**, postnatal day 10 [$F(3,7)=5.543$, $p<0.03$] **(Figure 3-4C)**, and postnatal day 20 [$F(3,7)=8.229$, $p<0.02$] **(Figures 3-4D)**, with caffeine exposure increasing NR1 expression. **Figure 3-4E** shows representative immunoblots of total NR1 protein expression at each postnatal time point.

Analysis of the total protein expression of the NR2A subunit in the developing hippocampus revealed no statistical differences between control and caffeine-exposed animals on postnatal days 1 or 5 **(Figure 3-5A, B)**. However, a main effect of treatment was found on both postnatal day 10 [$F(3,8)=32.068$, $p<0.01$] and postnatal day 20 [$F(3,8)=10.159$, $p<0.02$] **(Figure 3-5C, D)**. Representative immunoblots of total NR2A protein expression at each postnatal time point are shown in **Figure 3-5E**.

Analysis of the total protein expression of the NR2B subunit in the developing hippocampus revealed a main effect of treatment on postnatal day 1 [$F(3,7)=6.554$, $p<0.02$] with caffeine-exposed animals having reduced NR2B protein expression as compared to controls **(Figure 3-6A)**. However, there was no significant main effect between groups on postnatal days 5, 10, or 20. Representative immunoblots of total NR2B protein expression at each postnatal time point are shown in **Figure 3-6E**.

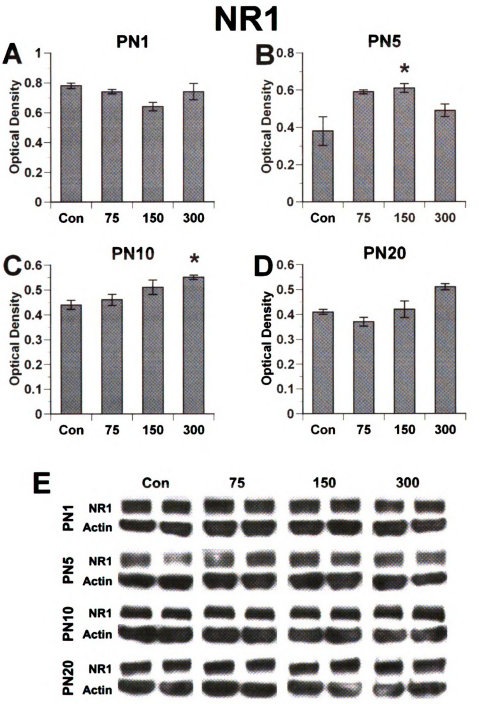


**Figure 3-4.** Total NR1 protein expression in the developing hippocampus on postnatal day 1 (A), postnatal day 5 (B), postnatal day 10 (C), and postnatal day 20 (D) in control and caffeine-exposed (75, 150, or 300 mg/L) offspring. Data represent the mean optical density ± SEM. (E) Representative immunoblots from each treatment group (n=3) at each postnatal time period. A main effect of treatment was found on postnatal days 5, 10, and 20. * indicates significant difference from control (Tukey; $p<0.05$).

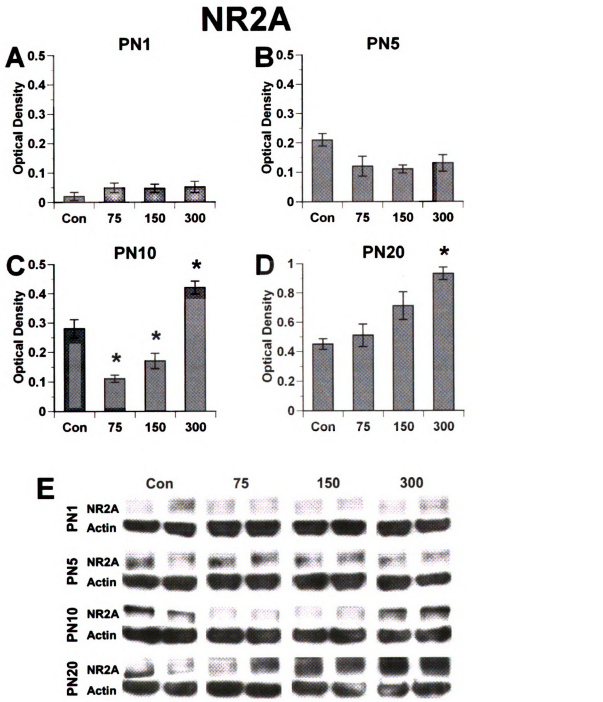


**Figure 3-5.** Total NR2A protein expression in the developing hippocampus on postnatal day 1 (A), postnatal day 5 (B), postnatal day 10 (C), and postnatal day 20 (D) in control and caffeine-exposed (75, 150, or 300 mg/L) offspring. Data represent the mean optical density ± SEM. (E) Representative immunoblots from each treatment group (n=3) at each postnatal time period. A main effect of treatment was found on postnatal days 10 and 20. * indicates significant difference from control (Tukey; $p<0.05$).

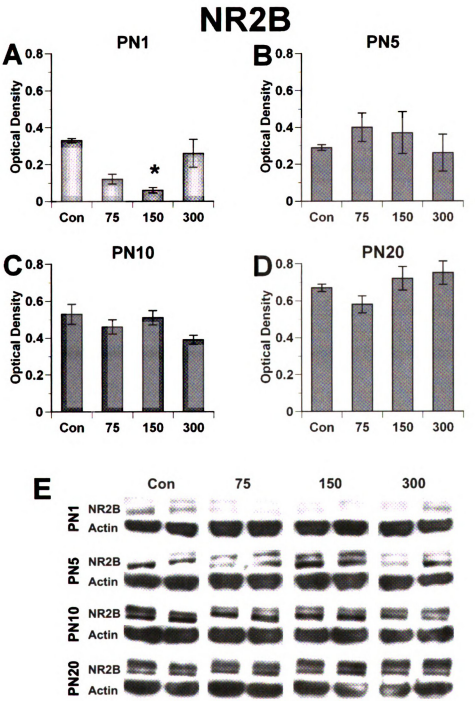


**Figure 3-6.** Total NR2B protein expression in the developing hippocampus on postnatal day 1 (A), postnatal day 5 (B), postnatal day 10 (C), and postnatal day 20 (D) in control and caffeine-exposed (75, 150, or 300 mg/L) offspring. Data represent the mean optical density ± SEM. (E) Representative immunoblots from each treatment group (n=3) at each postnatal time period. A main effect of treatment was found on postnatal day 1. * indicates significant difference from control (Tukey; $p<0.05$).

*Activation of Calcium Dependent Cascades*

Analysis of the protein expression of phospho-CREB following acute exposure to caffeine revealed no significant differences between prenatal control and prenatal caffeine-exposed groups on postnatal day 10 or postnatal day 20 **(Figure 3-7A, B)**. Representative immunoblots of phosho-CREB protein expression for both time periods are shown in **Figure 3-7C**.

Similarly, analysis of the protein expression of phospho-ERK1/2 following acute caffeine exposure revealed no significant differences between control and caffeine-exposed animals on postnatal day 10 or postnatal day 20 **(Figure 3-8A, B)**. Representative immunoblots of phosph-ERK1/2 protein expression are shown in **Figure 3-8C**.

*High Performance Liquid Chromatography*

Analysis of brain and plasma samples in PN5 and PN10 offspring exposed to prenatal caffeine revealed no remaining caffeine, theobromine, theophylline, or paraxanthine at either postnatal period. Thus, caffeine and its primary metabolites are metabolized from the brain and circulation within 5 days of birth in rats.

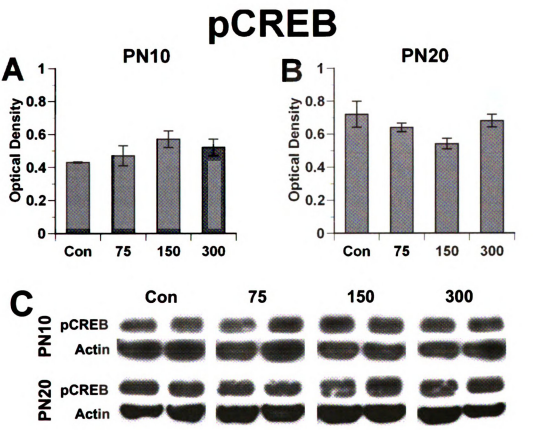


**Figure 3-7.** Phosphorylated-CREB (cAMP response element binding protein) expression in the developing hippocampus on postnatal day 10 (A) and postnatal day 20 (B) following acute caffeine treatment in control and caffeine-exposed (75, 150, or 300 mg/L) offspring. Acute caffeine treatment consisted of a single dose caffeine (10 mg/kg) administered orally by pipette followed by a 1.5 hour delay prior to tissue collection. Data represent the mean optical density ± SEM. (C) Representative immunoblots from each treatment group (n=3) at both postnatal time periods. No significant differences in pCREB activation were found between control and caffeine-exposed offspring.

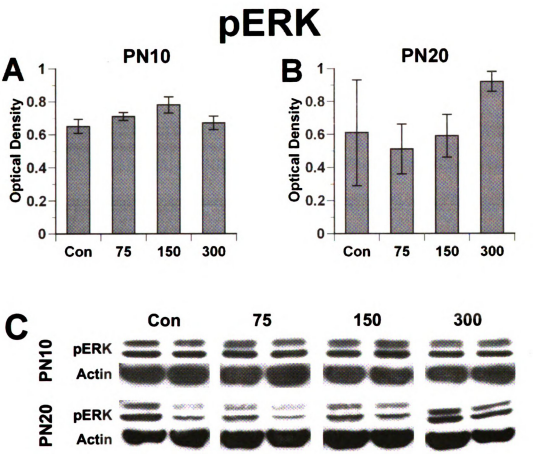


**Figure 3-8.** Phosphorylated-ERK1/2 (extracellular signal related kinase 1/2) expression in the developing hippocampus on postnatal day 10 (A) and postnatal day 20 (B) following acute caffeine treatment in control and caffeine-exposed (75, 150, or 300 mg/L) offspring. Acute caffeine treatment consisted of a single dose caffeine (10 mg/kg) administered orally by pipette followed by a 1.5 hour delay prior to tissue collection. Data represent the mean optical density ± SEM. (C) Representative immunoblots from each treatment group (n=3) at both postnatal time periods. No significant differences in pERK activation were found between control and caffeine-exposed offspring.

**D. Discussion**

Results from the present study demonstrate that chronic prenatal exposure to caffeine alters the expression of NMDA receptors in the neonatal hippocampus and reduces glutamate-induced calcium transients in caffeine-exposed primary hippocampal neurons. The NR1, NR2A, and NR2B subunits all exhibited slight differences in protein expression at various postnatal periods. The NR1 subunit showed increased expression on postnatal days 5, 10, and 20 in caffeine-exposed offspring. In contrast, the NR2B subunit showed decreased expression in caffeine-exposed animals on postnatal day 1, but this effect was no longer observed on postnatal day 5. On postnatal day 10, the NR2A subunit had increased expression only in offspring prenatally exposed to the highest dose of caffeine. Offspring exposed to the two lower doses of caffeine showed reduced NR2A expression on postnatal day 10, but by postnatal day 20 the NR2A subunit showed elevated expression in the caffeine-exposed groups. The changes in subunit expression at PN5 and later are not likely due to a direct action of caffeine and its metabolites in the brain since HPLC analysis showed no detectable levels of these metabolites at these time points. Interestingly, prenatal caffeine exposure also reduced glutamate-induced calcium transients in hippocampal cultures from treated offspring. Specifically, caffeine-treated hippocampal cells showed diminished maximal calcium transients after glutamate administration when compared to the maximal calcium transients seen in control cultures. Despite this reduction in total calcium influx in caffeine-treated cells, there were no treatment differences detected between the levels of phosphorylated ERK and CREB

proteins, suggesting that sufficient levels of intracellular calcium are still being attained to induce these calcium-dependent cascades.

The altered expression of NMDA receptors in the neonatal hippocampus following prenatal caffeine treatment corresponds to the period of peak expression for each subunit. It is well known that the NMDA receptors are spatially and temporally regulated during brain development (Lujan *et al.*, 2005). In general, NMDA receptor expression is elevated during development and expression levels decrease as the brain matures. Periods of peak receptor expression have been found to correlate with neonatal periods of synaptogenesis during the brain growth spurt, which occurs during the first three postnatal weeks in rats (Ritter *et al.*, 2002). The NR1 subunits are expressed during both prenatal and postnatal development, with peak levels of expression occurring around postnatal days 3-14 in the rat (Ritter *et al.*, 2002). The NR2A and NR2B subunits undergo a developmental switch in expression, allowing the diverse pharmacological properties of each subunit to predominate during distinct periods of brain development. The NR2B subunit is characterized by slower channels kinetics and decreased channel open probability, whereas the NR2A subunit is characterized by fast channel kinetics and increased channel open probability (Lau & Zukin, 2007). The NR2B subunit is expressed prenatally and maintains high levels of expression until postnatal day 7 after which levels decline (Ritter *et al.*, 2002). In contrast, NR2A expression levels are very low during early prenatal and postnatal development. NR2A expression increases around postnatal day 10 and peaks around postnatal day 21 (Ritter *et al.*, 2002). The current findings suggest that prenatal caffeine treatment may alter this delicate timing of peak receptor expression. For instance, caffeine-exposed offspring showed increases in NR1

subunit expression only at the time points of postnatal days 5, 10, and 20 which correspond to the highest levels of expression for this subunit. Similarly, altered NR2B subunit expression was found only on postnatal day 1 when expression of these subunits is highest. NR2A subunit expression was altered on postnatal days 10 and 20 when elevated expression of this subunit becomes apparent. Thus, prenatal caffeine exposure may selectively increase or decrease subunit expression during neonatal periods normally associated with high levels of subunit expression. This may disrupt the delicate timing of subunit expression, which mediates many important developmental processes.

NMDA receptor expression is dynamically regulated during development and is influenced by neuronal activity. It has been found that chronic activation of NMDA receptors results in receptor internalization and degradation via the ubiquitin-proteasome system (Lau & Zukin, 2007). Further, it is believed that immature synapses are especially susceptible to activity-dependent remodeling with NR1/NR2B receptor complexes undergoing rapid internalization during development. Therefore, if prenatal caffeine exposure is increasing glutamate neurotransmission and thereby increasing glutamate receptor activity, it is possible that the increase in activity results in receptor internalization and degradation resulting in less overall NMDA receptor expression. This may be a factor mediating the decreased maximal calcium response seen following prenatal caffeine exposure.

It is also possible that prenatal caffeine exposure is altering the expression of AMPA receptors in the developing hippocampus, which may affect glutamate-mediated signaling. AMPA receptors are expressed during prenatal and postnatal development although many of these receptors are thought to be quiescent during early development.

GluR1 subunit expression is detectable in the rat cortex as early as embryonic day 15 (Martin *et al.*, 1998). Unlike the developmental profiles of other subunits, GluR1 expression remains fairly constant in the hippocampus during prenatal and postnatal development (Ritter *et al.*, 2002). Expression of GluR2/3 subunits is also detectable during embryonic development. In the rat hippocampus, GluR2 subunits show a rapid increase in expression immediately following birth, which peaks at postnatal day 10 and then rapidly declines to prenatal levels of expression by postnatal day 18 (Ritter *et al.*, 2002). The effects of prenatal caffeine exposure on AMPA receptor expression is currently unknown yet it is possible that increased glutamate neurotransmission is increasing activation of AMPA receptors during development and altering their expression in the hippocampus. It is also possible that changes in AMPA receptor expression may alter NMDA receptor function due to the AMPA receptor-mediated depolarization needed to fully activate NMDA receptors. Further research is needed to fully elucidate the effects of prenatal caffeine exposure on AMPA receptor expression.

Another factor that requires consideration is subunit-dependent NMDA receptor desensitization. NMDA receptor desensitization occurs after prolonged exposure to glutamate and is likely an important neuroprotective mechanism (Dingledine *et al.*, 1999). Changes in subunit expression between control and caffeine-exposed offspring could alter subunit-mediated receptor kinetics and thereby alter Ca2+ permeability. For instance, receptors containing NR1/NR2A assemblies exhibit faster rise and decay currents when compared to receptors comprised of NR1/NR2B assemblies (Chen *et al.*, 1999). As discussed previously, the NR2B subunit is characterized by slower channels kinetics and decreased channel open probability, whereas the NR2A subunit has fast

channel kinetics and increased channel open probability. Thus, in response to glutamate NR2A-containing receptors open more quickly and desensitize more rapidly than do NR2B-containing receptors (Yashiro & Philpot, 2008). Since NR2B subunit-containing receptors have slow deactivation they likely carry more overall charge and allow more Ca2+ entry during a single synaptic event (Sobczyk *et al.*, 2005). Therefore, it is possible that altered expression of the NR2A and NR2B subunits following prenatal caffeine exposure may account for the decreased maximal calcium transient seen in caffeine-exposed neurons. Unfortunately, the expression profile of these subunits in day *in vitro* 3 hippocampal cultures was not assessed making the kinetic profiles of these neurons unable to be determined. Electrophysiological studies of caffeine-exposed neurons would provide invaluable information of how receptor kinetics may be altered.

Several other factors could be reducing the maximal calcium influx in caffeine-exposed neurons. Importantly, baseline resting calcium levels did not differ between treatments as this could have many consequences on overall cell function and calcium signaling. However, because calcium entry through NMDA receptors can induce intracellular calcium release as well as membrane depolarization sufficient to open voltage-gated calcium channels, it cannot be ruled out that any alteration in calcium influx via these mechanisms may contribute to the reduced peak calcium amplitude seen in caffeine-exposed cultures. Future studies utilizing specific antagonists to block calcium influx via these mechanisms are needed.

Interestingly, the images obtained during the live calcium imaging recordings suggest that peak calcium levels increase most in the nuclear portion of the cell rather than being contained in the somatic cytosol. It has recently been shown that there is no

nuclear barrier to calcium in hippocampal neurons, such that influx of calcium at the cell membrane readily propagates calcium towards and into the nuclear compartment (Eder & Bading, 2007). Thus, the findings obtained in the current study may suggest that signaling of nuclear calcium is altered. However, it is unknown whether the appearance of increased calcium in the nuclear compartment is merely an artifact of the calcium imaging protocol used for these studies. For instance, the shape of the cells in culture may influence the appearance of the recording such that a spherical shape may result in the highest light exposure in the center, or nuclear portion, of the cell. To fully elucidate the cellular compartments involved in the calcium signal, much higher resolution would be needed. Thus, the location of the calcium signal within the soma cannot be described in precise detail.

Calcium influx through NMDA receptors is known to activate numerous intracellular cascades including the cAMP and MAPK/ERK (mitogen-activated protein kinase/extracellular signal-related kinase) pathways (Poser & Storm, 2001). Activation of the MAPK/ERK pathway modulates phosphorylation of CREB (cAMP response element binding protein) (Lynch, 2004; Malenka & Bear, 2004). CREB activation is an important transcription factor for long-term protein synthesis and plays an essential role in hippocampal synaptic plasticity (Lynch, 2004; Malenka & Bear, 2004). Because ERK and CREB activation occur as downstream events following NMDA-receptor mediated calcium influx, the present study sought to determine whether altered calcium influx following prenatal caffeine exposure could alter activation of these signaling factors. Previously, caffeine has been shown to activate ERK in the hippocampus and cortex (Okano *et al.*, 2008). Similarly, glutamate administration *in vitro* has been shown to

induce phosphorylation of CREB in primary hippocampal cultures (Mabuchi *et al.*, 2001). Interestingly, removal of calcium from the culture medium abolished glutamate-induced CREB phosphorylation, suggesting that CREB activation is dependent upon calcium influx (Mabuchi *et al.*, 2001). Moreover, neither the AMPA/kainate receptor antagonist CNQX nor the voltage-gated calcium channel antagonist nifedipine blocked glutamate-induced CREB phosphorylation. However, CREB phosphorylation was blocked by application of the NMDA receptor antagonist MK-801, suggesting that CREB activation is dependent on calcium influx via NMDA receptors (Mabuchi *et al.*, 2001). The findings presented in this study suggest that CREB and ERK activation in the hippocampus are not altered in offspring exposed to caffeine prenatally. Thus, it is likely that there is still sufficient calcium influx to activate these cascades or that calcium influx is no longer altered by postnatal days 10 and 20. Nevertheless, these findings suggest that prenatal caffeine exposure does not alter these critical signaling cascades.

Interestingly, the alterations seen in NMDA receptor expression and function after prenatal caffeine exposure are similar to the findings from prenatal ethanol exposure studies. Prenatal ethanol exposure results in diminished glutamate-induced calcium transients in primary cultures from treated rats (Lee *et al.*, 1994; Spuhler-Phillips *et al.*, 1997). Furthermore, NR2A receptor expression in the neonatal hippocampus and cortex is increased in ethanol exposed animals (Nixon *et al.*, 2002, 2004). During prenatal development, ethanol acts as a neuronal stimulant rather than a depressant due to the reverse in the chloride gradient in GABAergic neurons. Specifically, the intracellular chloride concentration is elevated in immature neurons leading to depolarization following activation of GABA receptors (Ben-Ari, 2002). Thus, the actions of ethanol

and caffeine may be functionally similar in the developing brain, with both drugs leading to an increase in overall neuronal excitation and a consequent disruption of normal NMDA-receptor expression and function.

In summary, prenatal caffeine exposure alters the peak developmental expression of NMDA subunits and diminishes the maximal glutamate-induced calcium transients in treated neurons. Caffeine is known to antagonize adenosine receptors, which normally function to reduce neuronal activity in the brain (Fredholm, 2007). When adenosine receptors are inhibited, there is an overall increase in glutamate neurotransmission particularly in brain regions with an abundance of adenosine receptors such as the cortex and hippocampus (Wang, 2007). The ability of caffeine to increase neuronal stimulation via blockade of adenosine receptors may lead to these alterations in NMDA receptor function due to chronic increased stimulation of these glutamatergic systems during development. Alterations in NMDA receptor expression or function may have long-term consequences due to the importance of these receptors in synapse formation, synaptic plasticity, and numerous other critical developmental processes.

## Chapter 4. Chronic prenatal caffeine exposure impairs novel object recognition and radial arm maze behaviors in adult rats.

### A. Introduction

Caffeine, a methylxanthine, is one of the most widely consumed psychoactive drugs in the world due to its presence in coffee, tea, chocolate, soft drinks and numerous medications (Fredholm *et al.*, 1999). Caffeine is rapidly absorbed throughout the body and brain. At moderate doses, caffeine exerts its stimulatory effects on behavior directly via blockade of adenosine receptors. Of the four characterized adenosine receptors (A1, A2A, A2B, and A3), caffeine has its primary actions at the A1 and A2A receptors (Fredholm, 1980). A1 receptors are the most abundant of the adenosine receptors and are highly expressed throughout the cerebral cortex, hippocampus, and cerebellum (Fastbom *et al.*, 1987; Jarvis *et al.*, 1987; Svenningsson *et al.*, 1997; Dunwiddie & Masino, 2001). In the developing rat brain, the A1 receptor is one of the earliest receptors expressed (Rivkees *et al.*, 2001) with mRNA detectable by gestational day 14 and adult patterns of distribution established by gestational day 20 (Weaver, 1996). A2A receptors are restricted in expression to dopaminergic regions of the brain (Svenningsson *et al.*, 1999), with mRNA expression detectable in the striatum by gestational day 14 in rats (Weaver, 1993). Developmentally, adenosine plays a key role in the inhibition of neuronal activity (Fisone *et al.*, 2004), formation of axons (Turner *et al.*, 2002), and neuroprotection (Rivkees *et al.*, 2001). Gestational exposure to caffeine may inhibit the actions of adenosine within the fetal brain, thereby altering normal brain development.

Maternal consumption of caffeine during pregnancy is of concern due to the extended half-life of the drug in the fetus. Caffeine rapidly crosses both the placental and blood-brain barriers to reach the fetal brain (Ikeda *et al.*, 1982; Tanaka *et al.*, 1984; Arnaud, 1993). In the fetus, the metabolism and elimination of caffeine are slowed due to a lack of cytochrome P-450 activity, resulting in a prolonged half-life (Aranda *et al.*, 1979a). The half-life of caffeine is estimated to be 50-100 hours at the end of gestation, which may lead to accumulation of caffeine in the fetal brain (Aranda *et al.*, 1977; Parsons & Neims, 1981; Arnaud, 1993). In the fetus, the half-life of caffeine is further protracted due to slowed maternal metabolism of caffeine during pregnancy. During the last trimester of pregnancy, the half-life of caffeine is estimated to be 10-15 hours in the expectant mother (Aldridge *et al.*, 1981; Knutti *et al.*, 1981; Brazier *et al.*, 1983). Thus, the altered half-life of caffeine in the fetus and expectant mother may greatly prolong the actions of caffeine within the fetal brain.

Perinatal exposure to caffeine causes persistent alterations in brain development. In the neonatal rat brain, concentrations of protein and cholesterol are decreased following low dose (1 or 2 mg/100 g body weight) caffeine exposure (Nakamoto *et al.*, 1988). Caffeine injections (50 mg/kg/day) on postnatal days 1-12 cause an enduring increase in the dendritic length of pyramidal neurons within the rat prefrontal cortex (Juarez-Mendez *et al.*, 2006). In juvenile rats, caffeine exposure throughout gestation and lactation diminishes the stimulatory locomotor response to MK-801, an NMDA receptor antagonist, suggesting a persistent change in NMDA receptor function (da Silva *et al.*, 2005). Thus, developmental exposure to caffeine may cause long-term alterations in the brain.

Perinatal exposure to caffeine also alters juvenile and adult locomotor behavior and emotional reactivity. Numerous studies have shown decreased levels of activity in both juvenile and adult rat offspring following developmental caffeine exposure (Hughes & Beveridge, 1991; Zimmerberg *et al.*, 1991). Caffeine exposed offspring exhibit heightened emotional reactivity such that male offspring have increased defecation and decreased exploration in the open field and longer latencies to emerge from a light-dark box (Hughes & Beveridge, 1986, 1991). However, increased activity and reduced anxiety following postnatal caffeine exposure have also been shown. Juvenile rats show increased pain sensitivity and reduced anxiety following caffeine exposure (15-20 mg/kg/day) across postnatal days 2-6 (Pan & Chen, 2007). Further, juvenile rats exhibit hyperactivity, which persists into adulthood in an open field task after exposure to caffeine (20 mg/kg/day) across postnatal days 7-11 (Tchekalarova *et al.*, 2005).

The long-term behavioral effects of chronic prenatal exposure to caffeine on measures of learning and memory have not extensively investigated. Rat pups exposed to 1 or 9 mg/kg caffeine during the first week of life exhibit hypoactivity at two weeks of age and impairment in a spatial operant learning task during adulthood (Zimmerberg *et al.*, 1991). Following neonatal caffeine exposure, passive avoidance learning is significantly impaired in male and female juvenile rats (Pan & Chen, 2007). In adult animals, neonatal caffeine (15-20 mg/kg/day on PN2-6) treated female rats have significantly improved retention of passive avoidance learning at 24 and 72 hours after training, while male rats have significantly reduced retention (Fisher & Guillet, 1997). Thus, prenatal caffeine exposure may alter adult behavior in a sexually dimorphic fashion, although these sex differences may be undetectable in pre- or peri-pubertal

animals. Importantly, sex differences in response to perinatal caffeine exposure may help determine whether males or females are more susceptible to caffeine induced developmental effects.

The aim of the present study was to document the effect of chronic prenatal exposure to caffeine on cognitive behavior in adult male and female rats. The study investigated whether daily oral administration of caffeine (approximately 10mg/kg) to pregnant rat dams could alter long-term learning and memory in offspring. Specifically, prenatal caffeine-treated and control offspring underwent testing in adulthood in three well-established learning and memory paradigms, including the novel object recognition task, radial arm maze, and Morris water maze.

## B. Materials and Methods

*Prenatal Caffeine Treatment*

Female Sprague-Dawley rats 60-70 days of age were mated within our colony at Michigan State University. Males were housed with females for 4 days to ensure that one estrous cycle was completed. Males were removed after 4 days and this day was counted as gestational day 4 to approximate the day of birth. From gestational day 4 until the day of birth, pregnant dams were administered *ad libitum* tap water (n=4) or 75 mg/L caffeinated tap water (n-4) with intake measured daily. Caffeine administration was started early in gestation to determine the effects of chronic exposure since it has been shown previously that caffeine can accumulate in the fetal brain (Wilkinson & Pollard, 1993). The dose was chosen after calculating metabolic body weight (=body weight^3/4) (Nehlig & Derby, 1994). Oral administration was used to maintain the human relevance of the study.

On postnatal day 2 (day of birth is postnatal day 0) the sex of each offspring was determined and litters were culled to 4 males and 4 females. No observational differences in litter size, sex ratio, or maternal nest building, retrieval, and nursing behavior were noted following birth. Each litter contained approximately 12-14 pups and there was no difference in mean body weights between control (6.57 ± 0.104 g) and caffeine-treated (6.75 ± 0.243 g) offspring. The pups were weaned on postnatal day 25 (PN25) to 2-3 animals per cage such that animals of the same litter and sex were housed together. Offspring were housed in a temperature and humidity controlled environment with a 12 h light/dark cycle (lights off at 0500 hours) through adulthood. *Ad libitum*

standard chow and tap water were available except during radial arm maze training. The experiments were approved by the Michigan State University Institutional Animal Use and Care Committee and followed NIH guidelines for animal use.

*Behavioral Tests*

To minimize the stress associated with behavioral testing, animals were handled for two minutes daily for two weeks prior to the start of testing. In the first set of behavior testing, twenty-nine male and female offspring from four litters (control male, n=5; control female, n=8; prenatal caffeine-treated male, n=8; and prenatal caffeine-treated female, n=8) underwent three neurobehavioral tests chosen to measure hippocampal-dependent and -independent learning and memory. Object recognition testing, radial arm maze, and Morris water maze were assessed between PN 110-150, sequentially. The testing order was chosen so that less stressful behavior tasks were performed prior to more stressful tasks. The stress induced by each task was previously determined by the defecation and grooming behavior of animals during each task, with almost no defecation and grooming behaviors in the object recognition test and very high levels of defecation and grooming in the Morris water maze. In the second set of behavior tests, thirty-two male and female offspring from four litters (control male, n=8; control female, n=8; prenatal caffeine-treated male, n=8; and prenatal caffeine-treated female, n=8) underwent 10 consecutive days of testing in the advanced Morris water maze. Prior to each behavior test, the animals were placed in the testing room 30 min before testing to allow habituation to the environment. All testing was performed between noon and 4 p.m. During each test, the experimenter remained outside the testing

room except between trials. The behavior room has numerous extra-maze cues such as shelving, counters, and signs. Testing was always performed under dim illumination. Each test was recorded and tracked using Smart tracking system (San Diego Instruments). Behavior was scored by at least two observers blind to the treatment groups.

*Novel Object Recognition Test*

The novel object recognition task was used to assess non-spatial memory. The testing arena was a 40 cm x 60 cm open area to which rats were habituated for 10 min over two days prior to pre-training and training. During pre-training, the animals were allowed to explore two identical objects (A, A) for 3 min. Memory retention testing was performed at both 3 and 24 hours after pre-training. To determine memory retention at 3 hours, animals were allowed to investigate a familiar object (A) and a novel object (B) for 3 min. Memory retention was again tested at 24 hours following pre-training with the familiar object (A) and a novel object (C). The objects used were small plastic toys of varying color and shape. Objects were placed 12 cm from each wall and 18 cm from each other in the maze. Objects were counterbalanced within the arena during each trial and rinsed with 70% alcohol between trials. Time exploring each object was assessed, which included approaching within 2 cm of the object with the face, sniffing, biting, and pawing the object. Sitting or standing on the object was not counted as exploration. The training exploration time was calculated as exploration of objects A1 + A2. The exploration time during the 3 and 24 hour retention tasks were calculated as A + B and A + C respectively. The discrimination index was calculated as the (exploration of novel

object – exploration of familiar object) / (total exploration time). A discrimination index above zero indicates the animal explored the novel object more than the familiar object and retained a memory of the familiar object.

*Radial Arm Maze*

The radial arm maze was used to assess hippocampal-dependent working and reference memory. The radial arm maze is a spatial navigation memory task involving primarily the hippocampus and prefrontal cortex (Olton, 1987). Two weeks prior to behavior testing, animals were individually housed and began food deprivation until 90% of their starting body weight was reached. During this time, animals were handled, weighed, and habituated to a cereal reward daily. The eight-arm maze consisted of a 25 cm diameter round center platform from which 65 cm walled arms emerge. To reduce the stress of the novel environment, rats were pre-trained to the radial arm maze for one session per day over 7 consecutive days. During each pre-training session, a cereal reward was moved closer to the end of each arm to ensure the rats learned to search the entire length of the arm for the reward. Each animal was allowed 10 min to consume the rewards in all 8 arms.

Radial arm maze training occurred over 10 consecutive days, one trial per day. During training, food cups were placed at the end of each arm to ensure that the rats could not see the cereal reward from the center platform. Four of the eight food cups were baited with a reward and the sequence of baiting (arms 2, 3, 5, and 7) remained constant. Animals were placed onto the center platform at the beginning of each trial and were allowed 10 min to retrieve all cereal rewards. After eating all 4 cereal rewards or

once 10 min had elapsed, animals were placed in their home cage. Each day animals were given food pellets (gram amount given equaled 12-16% of their body weight) after the completion of the behavior session. The maze was cleaned with ethanol between each trial. Upon completion of radial arm maze testing, standard chow was again freely available to the rats. Memory acquisition was assessed as the decrease in the number of errors between each trial. Reference memory errors were scored as entries into an arm that was not baited such that animals would need to recall from previous days which arms were not baited to avoid error. Working memory errors were scored as reentries into an arm from which food had already been eaten such that animals would have to recall within a given trial which arms they had already visited.

*Standard Morris Water Maze*

The standard Morris water maze (Morris, 1984) was used as a measure of hippocampal-dependent spatial learning and memory. The maze was a 2 m diameter, 1 m tall circular pool in which the water was kept at 26 ± 1 °C. During pre-training, rats underwent three consecutive trials from separate starting points. Rats were allowed 60 sec per trial to find the non-submerged platform. Between each of the three trials, rats were allowed to sit upon the platform for 20 sec. If an animal did not find the platform within the allowed time, an experimenter would guide the animal to the platform.

Morris water maze training began two days after pre-training. Rats underwent 12 non-consecutive 60 sec trials starting at random from one of four cardinal directions. At the start of each trial, rats were placed into the water facing the wall of the maze. The platform (8 inch diameter) was submerged beneath water made opaque with non-toxic

white paint and always remained in the same location within the maze. Rats were removed from the maze after finding the platform or once the allowed time had elapsed and were dried and returned to a holding cage. The inter-trial interval was approximately 30 min. Latency to find the platform and path length were recorded using the Smart tracking system.

A probe test was done on the same day after the completion of the 12 trials. During the 30 second probe test, the platform was completely removed from the water. The number of times the animals crossed the location where the platform was previously located was assessed.

*Advanced Morris water maze*

Three paradigms were tested in the Morris water maze across nine consecutive days; Days 1-3 examined place learning, days 4-6 examined reversal, and days 7-9 examined working memory. The water maze and recording software used was the same as described previously.

For the place learning task, rats underwent 4 non-consecutive trials per day starting once from each cardinal direction. At the start of each trial, rats were placed into the water facing the wall of the maze. The platform was submerged beneath the opaque water and remained in the same location all three days. Rats were given 60 sec to find the hidden platform and if unsuccessful were guided to the platform location by an experimenter. All animals were allowed to rest for 20 sec on the platform before being returned to their home cage. No pre-training occurred prior to day 1 of testing. A 30 sec

probe test in which the platform was completely removed from the water was performed on day 3 after the completion of the place learning trials.

For the reversal task, rats underwent 4 non-consecutive trials per day starting once from each cardinal direction and were again allowed 60 sec per trial. However, the platform was located opposite to its position during the place learning task. The platform remained in the new location all three days.

During the working memory task, rats underwent 4 consecutive trials per day starting from the same location. The platform was moved to a new location each day and the starting location was also varied each day. The rats were allowed 60 sec per trial to find the submerged platform and then were allowed a 20 sec rest on the platform before beginning the next trial.

*Statistics*

Statistical assessment of weekly water intake was performed using a two-way repeated measures analysis of variance (week, treatment). For assessment of the object recognition test, two-way analysis of variance (sex, treatment) was performed on the pre-training, 3 hr memory, and 24 hr memory trials. Radial arm maze and Morris water maze were assessed by two-way repeated measures analysis of variance (sex, treatment). All analyses were performed using the SYSTAT program (version 11.0).

## C. Results

*Water Intake*

During the time of caffeine exposure (gestational day 4 through birth), the average water intake in control and caffeine-treated dams significantly increased each week (**Figure 4-1**; within-group analyses – $F(2,12)=23.054$, $p<0.001$) and showed a significant week by treatment interaction [$F(2,12)=6.885$, $p<0.01$] with caffeine-treated animals having greater intake than controls during week 3. However, there was no significant main effect of treatment on water intake.

*Novel Object Recognition Test*

*Total exploration time*

During pre-training, two-way analysis of variance revealed a significant effect of treatment [$F(1,25)=18.94$, $p<0.001$] on the total amount of time spent exploring the novel objects (**Figure 4-2A**). Caffeine-treated animals spent less total time exploring the novel objects during pre-training. However, the total time spent exploring the novel and familiar objects was not significantly different between treatments during the 3 hour and 24 hour recognition memory tests. At 3 hours, there was a significant effect of sex [$F(1,25)=5.455$, $p<0.05$] on total exploration time, with females spending more time ($54.1 \pm 5.3$ sec) exploring than males ($37.8 \pm 3.8$ sec) yet this effect was not seen at 24 hours.

*Memory Retention Testing*

At 3 hours following pre-training, there was no significant effect of treatment, sex, or treatment by sex interaction on the novelty discrimination index (**Figure 4-2B**). At 24 hours following pre-training, two-way analysis of variance showed a significant effect of treatment [$F(1,25)=7.652$, $p<0.02$] but no significant effect of sex or treatment by sex interaction on the discrimination index of novel object exploration (**Figure 4-2B**). These findings indicate that at 24 hours following pre-training, the caffeine-treated animals do not distinguish between the novel and familiar objects as well as control animals.

*Radial Arm Maze*

*Pre-training*

Across the 7 consecutive days of pre-training in the radial arm maze, a two-way repeated measures ANOVA revealed no significant between-group effect of treatment, sex, or treatment by sex interaction on the total time required to retrieve all food rewards. However, there was a significant within-group effect of time [$F(6,150)=49.879$, $P<0.001$] indicating that animals learned the task (**Table 4-1**).

*Training*

There was a significant between-group effect of treatment on the number of working memory errors [$F(1, 25)=4.357$, $P<0.05$] across the five trial blocks (trials 1-2, 3-4, 5-6, 7-8, 9-10) of the radial arm maze. Working memory errors were assessed as the number of reentries into arms already entered during the same trial. Caffeine-treated

animals made more errors than controls (**Figure 4-3A**). A significant within-group difference by trial block was observed [$F(4,100)=2.988$, $p<0.03$] indicating that animals learned the task across the five blocks.

Between-group analyses also revealed a significant main effect of treatment on the number of reference memory errors [$F(1, 25)=6.503$; $P<0.02$] with caffeine-treated animals making more errors than controls (**Figure 4-3B**). Reference memory errors were assessed as entries into an arm that never contained a reward. A significant within-group difference was also found across the 5 trial blocks [$F(4,100)=3.201$, $P<0.02$] representing a decrease in reference memory errors in both treatment groups as the task was acquired.

There was no significant between-group effect of treatment, sex, or treatment by sex interaction on path length. Path length was measured as the total distance in cm covered across each of the 5 trial blocks. There was a significant within-group effect of path length [$F(4,100)=8.601$, $P<0.001$] indicating that as the rats learned the task they decreased their traveling distance to obtain the rewards (**Table 4-1**).

Across the 10 consecutive days of testing, there was no significant between-group effect of treatment, sex, or treatment by sex in the total time required for animals to retrieve all 4 food rewards. As in pre-training, there was a significant within-group effect of time [$F(9,225)=3.672$, $P<0.003$] across the trial blocks indicating acquisition of the task (**Table 4-1**).

Two-way ANOVA revealed no significant effect of treatment or treatment by sex interaction on body weights at the start of food deprivation and at the conclusion of food deprivation (**Table 4-1**). However, a significant effect of sex was observed for body weights prior to food deprivation [$F(1,25)=335.97$, $p<0.001$] and at the conclusion of

food deprivation [$F(1,25)=374.42$, $p<0.001$]. Thus, both male and female rats began food deprivation at a healthy weight and lost weight to the same extent during radial arm maze testing.

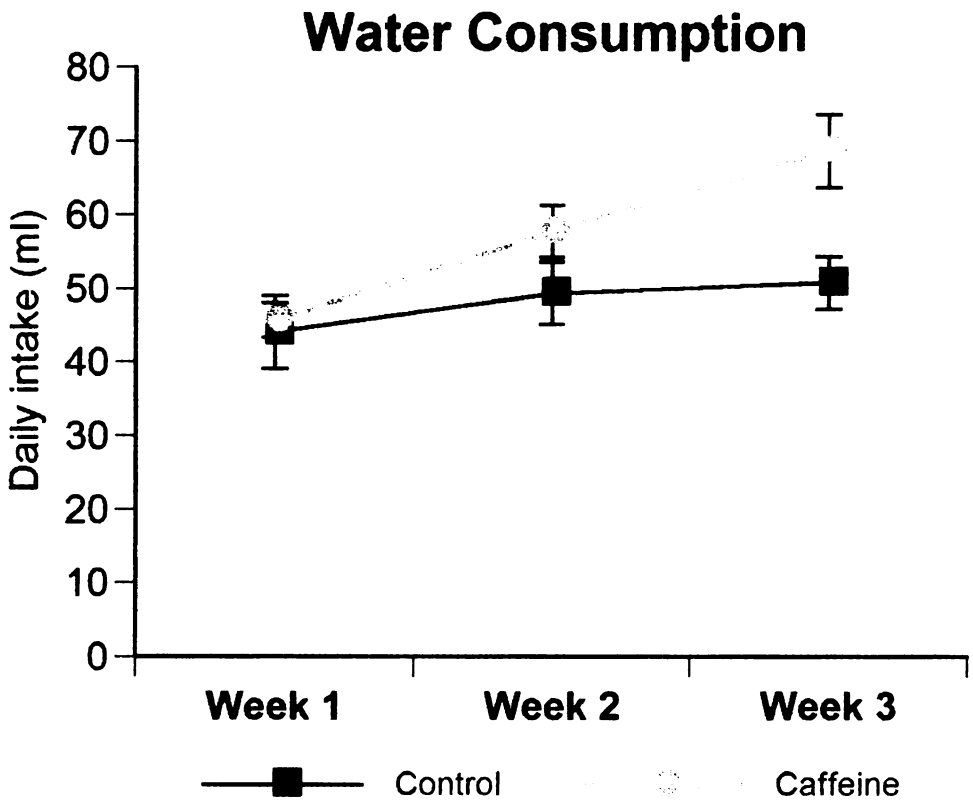


**Figure 4-1**. Maternal intake of either water or caffeinated tap water across each gestational week. Data represent the mean daily intake ± SEM for each week. Water intake increased throughout gestation but did not significantly differ by treatment.

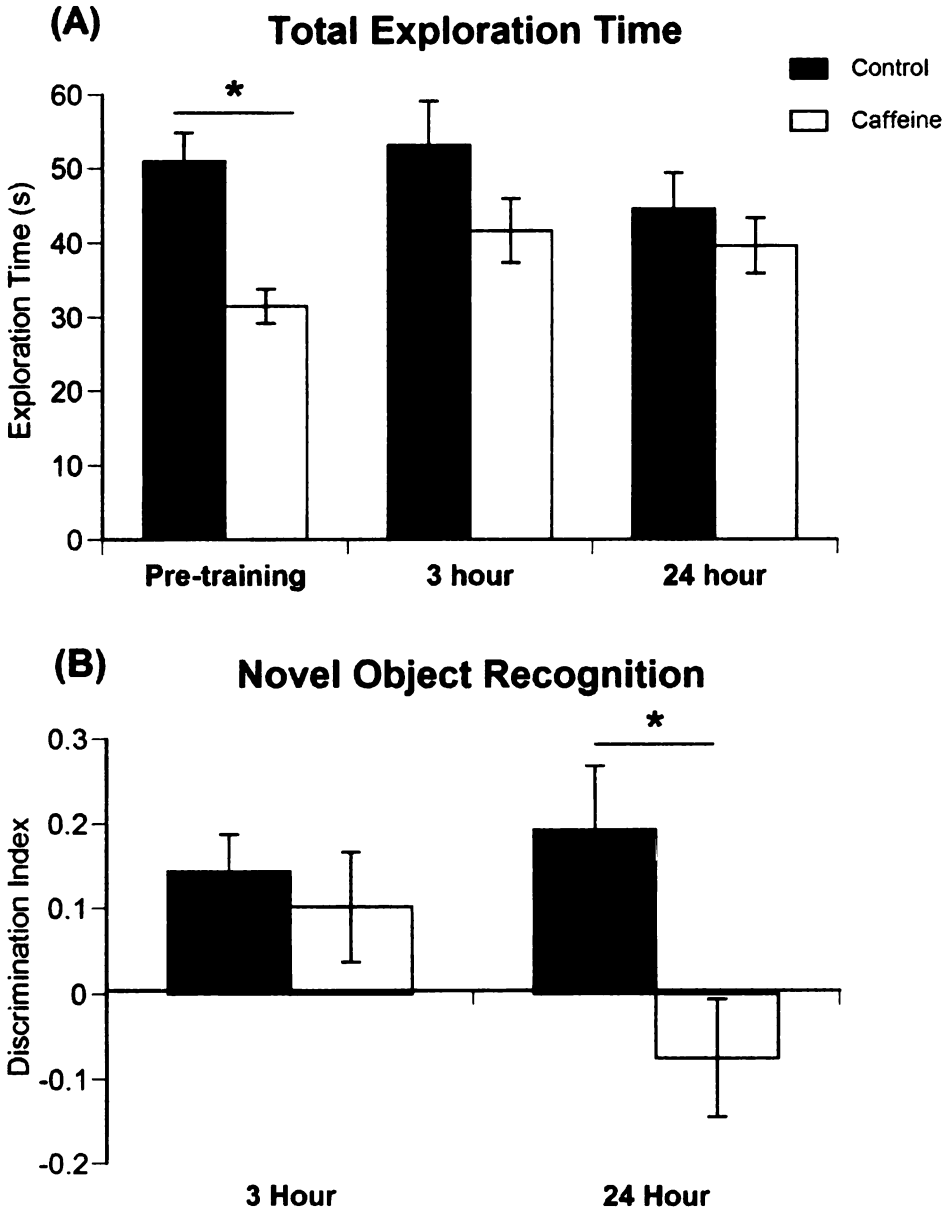


**Figure 4-2.** (A) Effects of prenatal caffeine on the total time spent exploring both objects in the novel object recognition test during each 3 min trial. Data represent the mean exploration time in seconds ± SEM for pre-training, 3 hour recognition memory, and 24 hour recognition memory. There was a significant effect of treatment on total exploration time during pre-training, with caffeine-treated animals exploring the objects less. (B) Effects of prenatal caffeine exposure on memory retention of the novel object at 3 and 24 hours after pre-training. Data represent the percent of time ± SEM spent exploring the novel object. Prenatal exposure to caffeine significantly reduced memory retention of the novel object at 24 hours after pre-training. Controls n=13; Caffeine n=16.

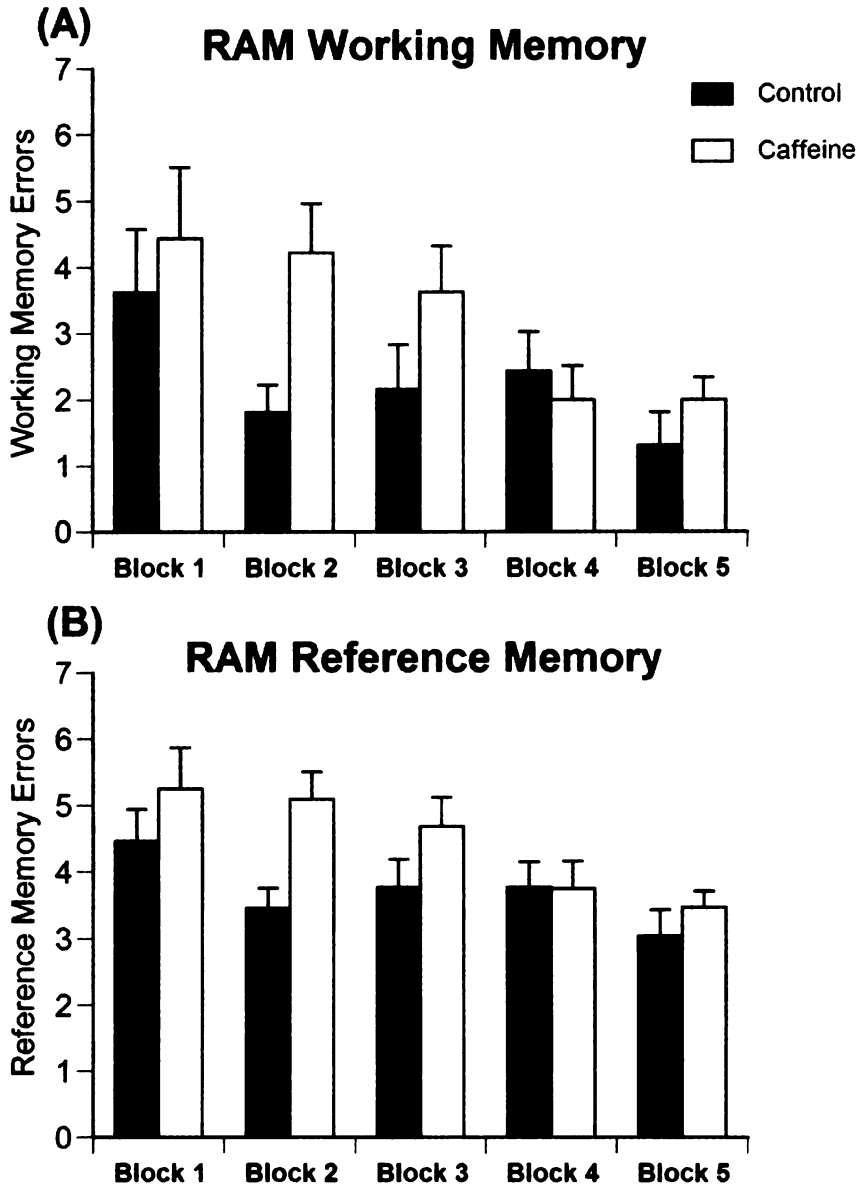


**Figure 4-3**. Effects of prenatal caffeine exposure on the number of (A) working memory errors and (B) reference memory errors performed during Radial arm maze testing, with caffeine-treated animals performing significantly more memory errors. Data represent the average number of errors ± SEM performed during each block (Trials 1-2, 3-4, 5-6, 7-8, 9-10). Control n=13; Caffeine=n=16.

| | Control Male (n=5) | Control Female (n=8) | Caffeine Male (n=8) | Caffeine Female (n=8) |
|---|---|---|---|---|
| **Pre-Training Time (s)** | | | | |
| **Day 1** | 482 ± 68 | 569 ± 31 | 361 ± 36 | 551 ± 38 |
| **Day 2** | 285 ± 56 | 385 ± 53 | 255 ± 54 | 315 ± 32 |
| **Day 3** | 215 ± 19 | 266 ± 64 | 212 ± 39 | 180 ± 29 |
| **Day 4** | 230 ± 41 | 253 ± 64 | 185 ± 31 | 194 ± 41 |
| **Day 5** | 151 ± 15 | 127 ± 24 | 124 ± 12 | 243 ± 62 |
| **Day 6** | 171 ± 33 | 189 ± 31 | 136 ± 22 | 139 ± 16 |
| **Day 7** | 228 ± 42 | 208 ± 19 | 137 ± 8 | 181 ± 28 |
| **RAM Training – Path Length (cm)** | | | | |
| **Block 1** | 3862 ± 658 | 5042 ± 795 | 4129 ± 325 | 5857 ± 785 |
| **Block 2** | 3546 ± 309 | 4272 ± 310 | 5354 ± 589 | 4705 ± 389 |
| **Block 3** | 4244 ± 720 | 3724 ± 369 | 4434 ± 567 | 4555 ± 440 |
| **Block 4** | 3598 ± 264 | 3626 ± 569 | 3523 ± 450 | 3065 ± 222 |
| **Block 5** | 3278 ± 167 | 2793 ± 445 | 2908 ± 239 | 3270 ± 253 |
| **RAM Training – Time to Retrieve Rewards (s)** | | | | |
| **Block 1** | 116 ± 11 | 164 ± 40 | 115 ± 12 | 171 ± 12 |
| **Block 2** | 99 ± 3 | 119 ± 10 | 147 ± 22 | 115 ± 15 |
| **Block 3** | 133 ± 31 | 112.8 ± 10 | 104 ± 6 | 112 ± 9 |
| **Block 4** | 115 ± 5 | 116.1 ± 16 | 93 ± 12 | 84 ± 6 |
| **Block 5** | 113 ± 8 | 106.5 ± 11 | 93 ± 16 | 96 ± 9 |
| **Body Weights (g)** | | | | |
| **Start Weight** | 435 ± 8 | 418 ± 12 | 247 ± 6 | 238 ± 10 |
| **End Weight** | 393 ± 8 | 378 ± 10 | 227 ± 4 | 226 ± 7 |

**Table 4-1.** Mean (± SEM) values of radial arm maze pre-training time (s), training path lengths (cm), time to retrieve all cereal rewards (s), and body weights before and after food deprivation.

*Standard Morris Water Maze*

*Pre-training*

Two-way analysis of variance revealed no significant effect of treatment, sex, or treatment by sex interaction on the pre-training component of the Morris water maze (**Figure 4-4A**). This indicates that the animals did not exhibit motor or sensory deficits that would interfere with the procedural learning of the task.

*Training*

Two-way repeated measures ANOVA revealed no significant effect of treatment, sex, or treatment by sex interaction across the 3 trial blocks (trials 1-4, trials 5-8, trial 9-12; **Figure 4-4A**) on water maze latency. There was a significant within-group difference across the three trial blocks [$F(2,50)=32.65$, $P<0.001$] indicating that all animals were able to decrease their latency to find the platform across the trial blocks. Additionally, no significant effect of treatment, sex, or treatment by sex interaction was observed in the probe trial, indicating that all animals were successfully able to spatially locate the region of the platform by the final trial.

Between-group analyses revealed a significant main effect of treatment on path length [$F(1,25)=6.992$; $P<0.02$] across the 3 trial blocks, with caffeine-treated animals swimming less distance than controls (**Figure 4-4B**). Path length was measured as the total distance (cm) swam in each trial averaged across the 3 trial blocks. A significant within- group difference was also found across the 3 trial blocks [$F(2,50)=37.372$, $P<0.001$] along with a significant within-group block by treatment effect [$F(2,50)= 3.466$,

P<0.04] due to the increased path length of both groups in the first trial block when compared to path length in the second and third trial blocks.

*Advanced Morris Water Maze*

*Place Learning Task*

Two-way repeated measures ANOVA revealed no significant effect of treatment, sex, or treatment by sex interaction across the 3 days of place learning (Day 1: trials 1-4, Day 2: trials 5-8, Day 3: trial 9-12; **Figure 4-5A**) on latency to find the platform. There was a significant within-group difference across the three days [$F(2,54)=88.05$, $P<0.001$] indicating that all animals were able to decrease their latency to find the platform as they learned the task. Additionally, no significant effect of treatment, sex, or treatment by sex interaction was observed in the probe trial conducted on Day 3, indicating that all animals were successfully able to spatially locate the region of the platform by the final trial.

Between-group analyses revealed no significant difference of treatment, sex, or treatment by sex interaction on place learning path length. Path length was measured as the total distance (cm) swam averaged across the 4 trials swam each day. A significant within-group difference was also found across the 3 days [$F(2,54)=62.54$, $P<0.001$] indicating that the path length decreased as animals learned to find the platform (**Table 4-2**).

*Reversal Task*

Two-way repeated measures ANOVA revealed no significant effect of treatment, sex, or treatment by sex interaction across the 3 days of reversal testing (Day4: trials 1-4,

Day 5: trials 5-8, Day6: trial 9-12; **Figure 4-5B**) on latency to find the hidden platform. There was a significant within-group difference across the three days [$F(2,54)=16.14$, $P<0.001$] indicating that all animals were able to decrease their latency to find the platform as they learned the task. Additionally, there was a significant within-group day by treatment by sex.interaction [$F(2,54)=3.578$, $P<0.035$].

Between-group analyses revealed no significant difference of treatment, sex, or treatment by sex interaction on reversal path length. A significant within-group difference was also found across the 3 days of reversal testing [$F(2,54)=15.297$, $P<0.001$] along with a significant within-group day by treatment by sex effect [$F(2,54)= 3.432$, $P<0.04$] (**Table 4-2**).

*Working Memory Task*

Two-way repeated measures ANOVA revealed no significant effect of treatment, sex, or treatment by sex interaction on latency to find the platform across the 4 trials of each of the three days of repeated acquisition working memory testing. There was a significant within-group difference across the four trials each day (Day 7: [$F(3,81)=8.65$, $P<0.001$], Day 8: [$F(3,81)=21.41$, $P<0.001$], Day 9: [$F(3,81)=13.694$, $P<0.001$] indicating that animals were able to find the platform more rapidly each day during the consecutive trials.

Two-way repeated measures ANOVA revealed no significant effect of treatment, sex, or treatment by sex interaction on path length across the 4 trials of each of the three days of repeated acquisition working memory testing. There was a significant within-group difference across the four trials each day (Day 7: [$F(3,81)=6.82$, $P<0.001$], Day 8:

[F(3,81)=17.15, P<0.001], Day 9: [F(3,81)=13.16, P<0.001] indicating that the path length shortened during each consecutive trial as the animals learned the location of the hidden platform (**Table 4-2**).

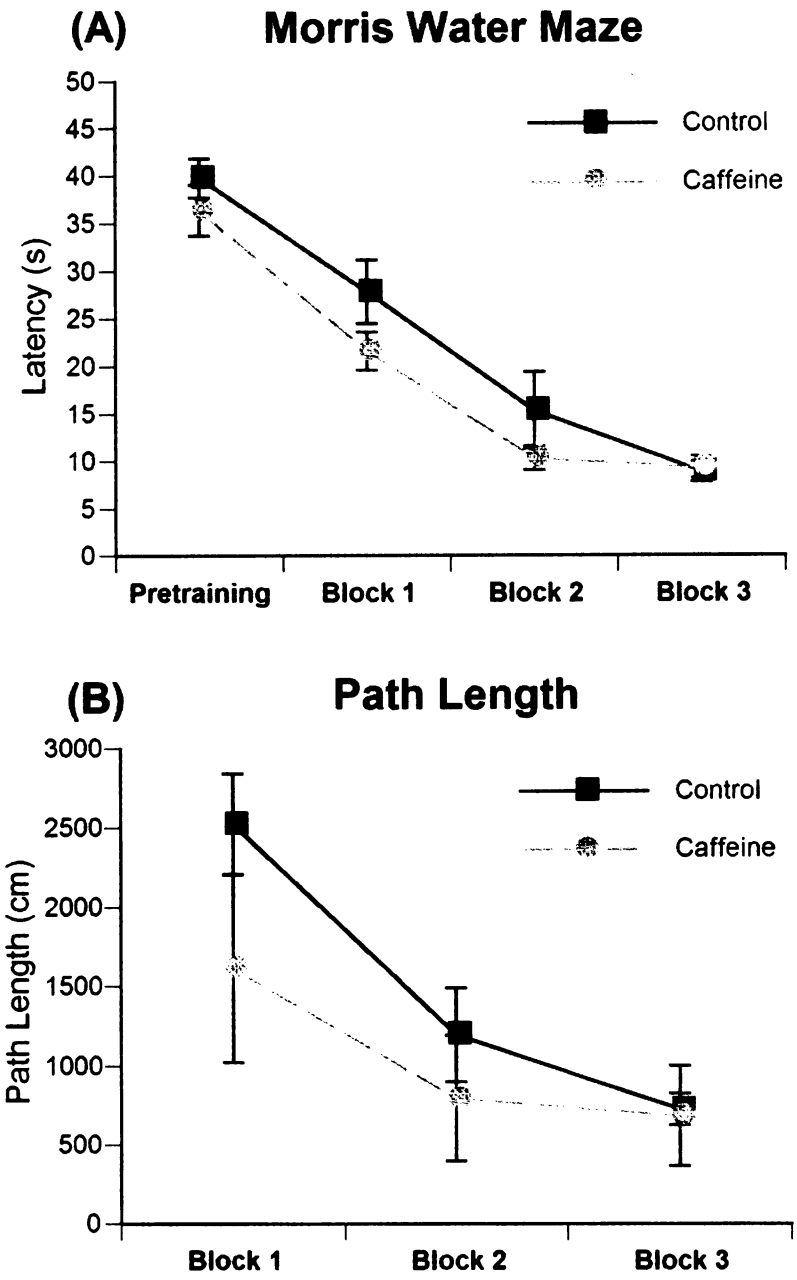


**Figure 4-4.** (A) Effects of prenatal caffeine on latency to find the hidden platform during pre-training and across the three trial blocks (block 1=trials 1-4, block 2 = trials 5-8, block 3 = trials 9-12) in the Morris water maze. Data represent the average latency ± SEM needed to find the platform during each 60 s trial. (B) Effects of prenatal caffeine on path lengths in the Morris water maze. Path length was measured as the total distance in centimeters ± SEM swam during each trial averaged across the 3 trial blocks. Control n=15; Caffeine n=16.

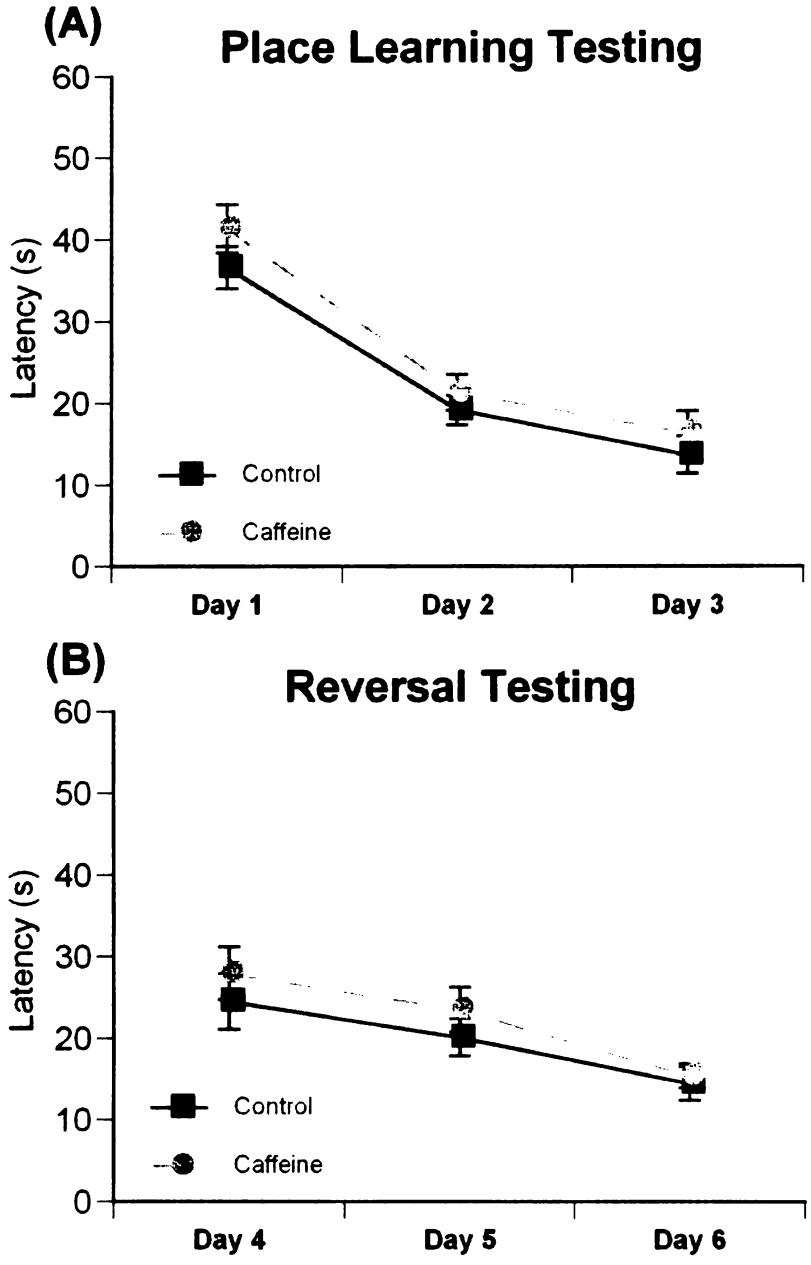


**Figure 4-5.** Effects of prenatal caffeine on latency to find the hidden platform during (A) place learning and (B) reversal in the Morris water maze across the four trials conducted each day over the first 6 days of testing. Data represent the average latency ± SEM needed to find the platform during each 60 s trial. Control n=15; Caffeine n=16.

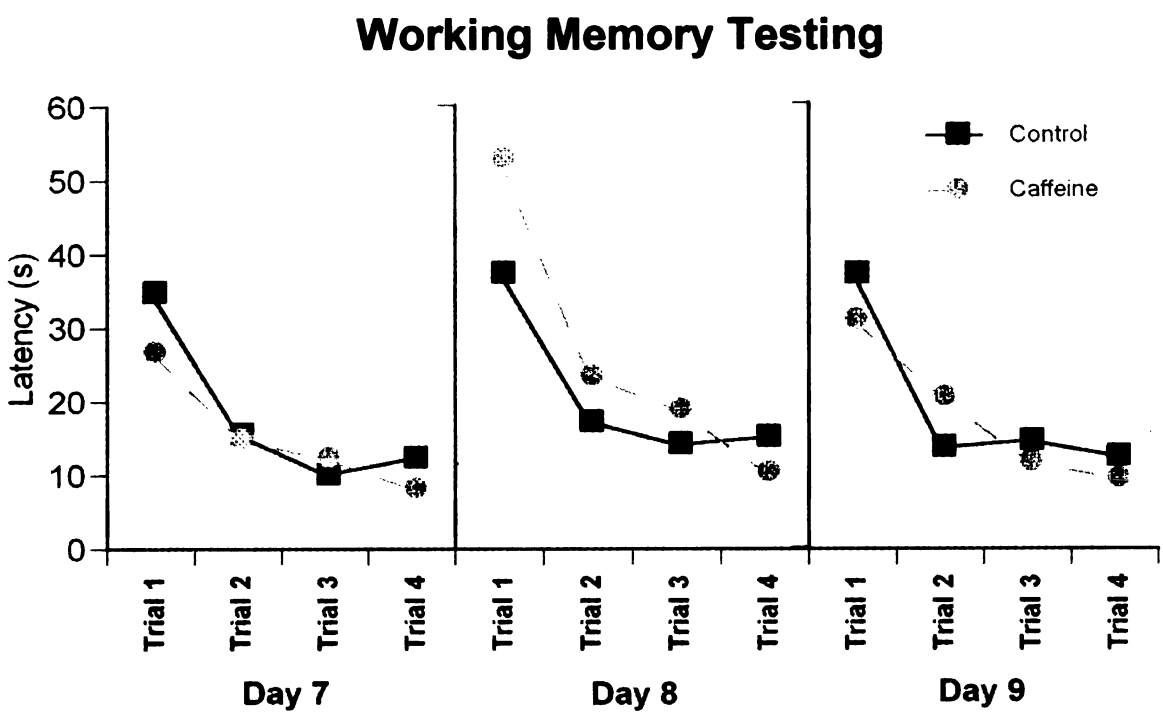


**Figure 4-6**. Effects of prenatal caffeine on latency to find the hidden platform during working memory tasks in the Morris water maze across the four trials conducted each day over the last 3 days of the total 9 days of testing. Data represent the average latency ± SEM needed to find the platform during each 60 s trial. Control n=15; Caffeine n=16.

| Place Learning Path Length (cm) | Control Male (n=7) | Control Female (n=8) | Caffeine Male (n=8) | Caffeine Female (n=8) |
|---|---|---|---|---|
| **Day 1** | 2182 ± 182 | 2571 ± 257 | 2732 ± 161 | 2125 ± 186 |
| **Day 2** | 1524 ± 181 | 1252 ± 189 | 1360 ± 247 | 1320 ± 167 |
| **Day 3** | 770 ± 156 | 1232 ± 268 | 1171 ± 205 | 890 ± 224 |
| **Reversal Path Length (cm)** | | | | |
| **Day 4** | 1449 ± 249 | 2249 ± 408 | 1911 ± 255 | 1613 ± 325 |
| **Day 5** | 1264 ± 227 | 1884 ± 245 | 1927 ± 279 | 1327 ± 326 |
| **Day 6** | 968 ± 237 | 1188 ± 198 | 737 ± 91 | 1148 ± 175 |
| **Working Memory Path Length (cm)** | | | | |
| **Day 7 - Trial 1** | 2753 ± 973 | 2318 ± 636 | 2026 ± 744 | 1658 ± 403 |
| **Day 7 - Trial 2** | 930 ± 372 | 1722 ± 603 | 1269 ± 296 | 1023 ± 395 |
| **Day 7 - Trial 3** | 854 ± 219 | 788 ± 300 | 548 ± 129 | 1233 ± 475 |
| **Day 7 - Trial 4** | 591 ± 290 | 583 ± 193 | 473 ± 130 | 713 ± 303 |
| **Day 8 - Trial 1** | 3192 ± 597 | 2905 ± 975 | 3802 ± 762 | 3639 ± 812 |
| **Day 8 - Trial 2** | 1247 ± 202 | 1549 ± 443 | 2886 ± 809 | 729 ± 125 |
| **Day 8 - Trial 3** | 1310 ± 318 | 1102 ± 266 | 1839 ± 196 | 1114 ± 308 |
| **Day 8 - Trial 4** | 1647 ± 401 | 944 ± 221 | 841 ± 75 | 814 ± 191 |
| **Day 9 - Trial 1** | 1684 ± 273 | 3650 ± 734 | 2301 ± 604 | 2115 ± 537 |
| **Day 9 - Trial 2** | 814 ± 180 | 1069 ± 349 | 1553 ± 399 | 1338 ± 525 |
| **Day 9 - Trial 3** | 1083 ± 457 | 1352 ± 369 | 777 ± 297 | 1029 ± 199 |
| **Day 9 - Trial 4** | 1117 ± 391 | 798 ± 137 | 869 ± 358 | 544 ± 100 |

**Table 4-2.** Mean (± SEM) values for advanced Morris water maze path length (cm) in the place learning, reversal, and working memory tasks.

**D. Discussion**

In this report, it was demonstrated that chronic prenatal exposure to a moderate dose of caffeine disrupts learning and memory behaviors in adult male and female rats. Specifically, it was shown that prenatal caffeine exposure impairs 24 hour memory retention in the novel object recognition task and impairs spatial learning in the radial arm maze. Previous studies in rats have shown that prenatal caffeine exposure alters the concentration of DNA, protein, and cholesterol in the newborn brain (Nakamoto *et al.*, 1988), decreases fetal brain weight (Tanaka *et al.*, 1987), delays developmental milestones (Tchekalarova *et al.*, 2005), and alters juvenile behavior (Pan & Chen, 2007). However, the long-term effects of prenatal caffeine exposure have not been extensively studied. Unlike many previous studies that used a high dose of caffeine and an injection or gavage administration paradigm, a low dose of approximately 10 mg/kg/day was used along with self-administration in the drinking water. This dose and method of administration was chosen to maintain human relevance and to avoid the stress associated with injections or gavage. No main effects of treatment on water intake were found between control and caffeine-treated dams indicating that caffeine-treated dams were not averse to the taste of the caffeinated water. These findings demonstrate that chronic oral intake of caffeine throughout gestation can lead to subtle alterations in adult cognitive behavior in rats.

The novel object recognition test, which measures the natural propensity of a rat to explore a novel versus familiar object (Ennaceur & Delacour, 1988), revealed that after a 24 hour inter-trial interval, exploration of the novel object is reduced in animals

exposed to prenatal caffeine. This task has both an exploratory behavior component as well as a memory retention component such that an animal must have sufficiently explored the familiar object during the pretest phase in order to distinguish between it and a novel object later during the test phase (Ennaceur & Delacour, 1988; Ennaceur & Meliani, 1992). Caffeine-treated animals exhibited less total exploration time during pre-training than controls. This is similar to previous findings in other behavioral paradigms showing prenatal caffeine-treated adult animals exhibit decreased exploratory behavior, which may be due to altered emotional reactivity in caffeine treated animals (Hughes & Beveridge, 1986). However, the total exploration time of control and caffeine-treated animals during the 3 and 24 hour tests were not significantly different. At 3 hours, both groups had a discrimination index greater than zero indicating preference for the novel over the familiar object. After a 24 hour delay, caffeine-treated animals had a discrimination index less than zero indicating that they could no longer distinguish between the familiar and novel objects indicating a deficit in memory, exploratory behavior, or attentional processes.

A deficit in the performance of caffeine-treated animals in the radial arm maze was also found, with caffeine-treated animals performing significantly more reference and working memory errors than control animals. Increased errors performed by caffeine-treated animals indicate that prenatal caffeine exposure may alter the developing hippocampus and prefrontal cortex affecting both working and reference memory in adults.

Unlike the findings in the radial arm maze, no deficits were found in the Morris water maze. In the standard version of the Morris water maze employed, there was

approximately a 30-minute delay between trials, with all 12 trials and the probe trial run on the same day. Thus, the memory retention demands of this version of the water maze task were greatly diminished. However, in the advanced Morris water maze task, the memory demands were increased in both the reversal and working memory paradigms and still there were no differences between control and caffeine-treated animals. This may be due to many differences between the Morris water maze and radial arm maze tasks, including level of difficulty, delay between trials, appetitive vs. aversive motivation used, and the type of memory tested.

In the simple version of the water maze, the caffeine-treated animals had a significantly shorter path length when compared to controls, indicating that caffeine-treated animals performed as well as, or better, than controls in directly finding the platform. The decrease in path length in caffeine-treated animals may be due to the aversive motivation used in the water maze. If prenatal caffeine exposure increases anxiety in juveniles and adults, as has been shown previously (Hughes & Beveridge, 1986, 1991), these animals may utilize a more direct escape route to avoid the stressful conditions inherent to the water maze. Previous studies have shown that animals with prior exposure to stressful experiences have enhanced learning and memory in the water maze when compared to unstressed animals (Yang *et al.*, 2003).

It is also possible that prenatal caffeine exposure alters motivational or exploratory aspects of behavior. It has been shown previously that perinatal caffeine exposure alters locomotor activity, increases emotional reactivity, and increases adrenal weight, particularly in males (Hughes & Beveridge, 1986, 1991). Each of the tasks presented in this study has a motivational aspect such that caffeine-treated animals may

have differences in motivation to explore a novel object or a novel arena. As previously discussed, in the simple version of the Morris water maze, there is a facilitating effect of caffeine treatment on path length. The caffeine-treated animals appear to be more motivated to find the hidden platform than control animals, with the largest difference in path length occurring during the first trial. Perhaps this difference may be attributed to decreased exploratory behavior such that caffeine-treated animals are less motivated to explore the maze in their initial exposure to the novel area. However, no differences were found in path length or latency to find the platform on any of the advanced Morris water maze tasks. Nevertheless, it is possible that prenatal caffeine exposure may be altering motivational and exploratory aspects of behavior making it difficult to know whether and how these changes may alter learning and memory testing.

Another concern is that gestational caffeine intake may alter maternal behavior and the intrauterine environment of dams, either of which may alter behavior of the offspring. Recently, it has been shown that wildtype mice born to dams heterozygous for the A1 adenosine receptor exhibit hyperactivity as adults, similar to wildtype mice exposed to caffeine (0.3g/L) from gestational day 1 to postnatal day 21 (Bjorklund *et al.*, 2008). Dams heterozygous for the A1 receptor express approximately half the number of A1 receptors in the cortex and hippocampus. Similarly, chronic caffeine exposure leads to a blockade of approximately half the A1 adenosine receptors in the brain. These studies suggest that alterations in maternal A1 receptor function may have long-lasting changes on offspring behavior. Bjorklund et al. (2008) hypothesize that the alterations in offspring behavior are likely due to changes in the prenatal intrauterine environment rather than maternal behavior, since they observed no differences in maternal behavior.

Others have shown no changes in maternal behavior, as assessed by nesting activity as well as licking and grooming behaviors, in dams exposed to caffeine (Picard *et al.*, 2008) and no effects of cross-fostering on reduced effects of caffeine on the offspring (Ajarem & Brain, 1993). Therefore, it seems unlikely that the behavioral changes reported are due to alterations in maternal behavior, however, it is not possible to completely rule out the effects of the intrauterine environment and maternal behavior as important factors.

The mechanisms by which prenatal caffeine exposure may alter the brain are not well understood. It is known that caffeine targets the adenosine receptor in both the developing and adult brain (Fredholm *et al.*, 1999), however it is unknown whether caffeine exerts the same action at these receptors in the fetus and adult. Prenatal exposure to caffeine decreases A1 adenosine receptor expression in the fetal rat brain (Aden *et al.*, 2000; Leon *et al.*, 2002). In the brain of young adult rats, A1 receptor levels are up-regulated in the cortex, hippocampus, and cerebellum following postnatal caffeine treatment (Guillet & Kellogg, 1991). Thus, exposure to caffeine during development can have long-term effects on adenosine receptor expression. Further, the levels of mGluRs are decreased in the fetal brain following caffeine and theophylline treatment (Leon *et al.*, 2005a). Enduring changes in adenosine or glutamate receptor expression following perinatal caffeine exposure may contribute to changes in learning and memory.

Recent studies in transgenic animals have shown that altered adenosine receptor expression impairs learning and memory behavior. Similar to the current findings, transgenic rats over-expressing the adenosine A2A receptor were found to have impaired novel object recognition and increased errors in a 6-arm radial tunnel maze while showing no impairments in place learning and reversal tasks in the Morris water maze

(Gimenez-Llort *et al.*, 2007). A1 receptor knockout animals also show no impairments in the Morris water maze, while showing slowed habitutation to the 6-arm tunnel maze and impaired paired pulse facilitation of long-term potentiation (Gimenez-Llort *et al.*, 2005). Thus, if chronic prenatal caffeine is altering adenosine receptor expression in adults, it seems likely that animals may have impaired learning and memory which is not detected using the Morris water maze alone.

In the current study, no sex differences were found in learning and memory behaviors as a result of prenatal caffeine exposure. This finding was surprising given the previous literature on sex dependent effects of developmental caffeine exposure. In adult rats, it has been shown that neonatal caffeine exposure (15-20 mg/kg/day on PN2-6) has differential sex effects on passive avoidance learning with females exhibiting enhanced memory retention at 24 and 72 hours after training, while males exhibit significantly reduced retention at both time periods (Fisher & Guillet, 1997). Interestingly, the same neonatal caffeine treatment does not cause gender specific effects in juvenile passive avoidance memory tests (Fisher & Guillet, 1997). Prenatal caffeine exposure has also been shown to alter passive avoidance learning in a sex dependent fashion. Adult female offspring of caffeine treated dams (60 mg/kg/day on GD13-19) showed significantly enhanced retention at 25 days after training when compared to placebo treated controls (Swenson *et al.*, 1990). Sexually dimorphic effects of prenatal caffeine exposure have also been shown on measures of locomotion and anxiety in the open field, with male animals exhibiting decreased activity and increased defecation (Hughes & Beveridge, 1991). In humans, mean intake of approximately 300 mg/day caffeine during the third trimester of pregnancy was associated with an increased risk of lowered birth weight in

males, but not in females (Vik *et al.*, 2003). Thus, the current literature suggests that males may be more susceptible to the detrimental effects of developmental caffeine exposure. However, the current study and others (Zimmerberg *et al.*, 1991) suggest that both males and females may be affected in specific learning and memory paradigms. Future research is needed to determine why developmental caffeine exposure renders males more susceptible to some detrimental effects of caffeine, while affecting other behaviors equally in both sexes.

In summary, prenatal caffeine exposure alters some learning and memory behaviors in adult male and female rats. Caffeine's ability to cross both the placental and blood-brain barrier allows it to enter the fetal brain where it has actions at adenosine receptors. Developmental changes in the expression or function of adenosine receptors within the hippocampus and cortex following prenatal exposure to caffeine may contribute to the long-term alterations in learning and memory reported here. Thus, pregnant women should consume caffeine with caution as it may have long-term developmental consequences on multiple facets of behavior including cognition.

## Chapter 5: Chronic prenatal caffeine exposure increases hippocampal volume in juvenile rats

### A. Introduction

Caffeine is the most widely consumed psychoactive stimulant in the world (Fredholm *et al.*, 1999). Caffeine is commonly consumed during pregnancy despite uncertainty about the neurodevelopmental effects of prenatal caffeine exposure. Consumption during pregnancy is of concern because caffeine and its metabolites cross both the placental and blood brain barriers where they accumulate in the fetal brain (Ikeda *et al.*, 1982; Tanaka *et al.*, 1984; Arnaud, 1993). As shown previously in Chapter 2, caffeine and its metabolites are each higher in the fetal brain when compared to the maternal brain regardless of dose.

Caffeine and its metabolites are methylxanthines that function as adenosine receptor antagonists. Of the four known adenosine receptors expressed within the brain, the A1 and A2A receptors mediate the stimulatory effects of caffeine (Fredholm, 1980). The A1 receptors are located throughout the cortex, hippocampus, and cerebellum (Fastbom *et al.*, 1987) whereas the A2A receptors are located in regions of the brain containing axon terminals of dopaminergic neurons, primarily the striatum (Svenningsson *et al.*, 1999). The A1 and A2A receptors are expressed in the fetal brain with mRNA detectable for both receptors by gestational day 14 and thus may be antagonized by prenatal caffeine (Weaver, 1993; Weaver, 1996).

The effects of antagonism of A1 and A2A adenosine receptors during development are not well understood, however, caffeine could act at these receptors to produce neurotoxic effects in the developing brain. In neonatal rats, apoptotic cell death is significantly increased in the cortex, hippocampus, thalamus, and hypothalamus 12 hours after a single injection of caffeine (100 mg/kg on postnatal day 3; Black *et al.*, 2008) and 24 hours following three caffeine injections (50 mg/kg on postnatal day 7; (Kang *et al.*, 2002). At lower doses (2.5, 5, or 10 mg/kg/day on postnatal days 4-10), caffeine does not significantly increase cell death (Desfrere *et al.*, 2007). However, exposure to low dose caffeine during development results in decreased cell proliferation in the dentate gyrus and subventricular zone and also causes a transient and dose-dependent decrease in astrocyte expression in the hippocampus and striatum of neonatal rats (Desfrere *et al.*, 2007). Caffeine (1g/L) administered throughout gestation and lactation increases acetylcholinesterase activity in the hippocampus of neonatal rats, supporting the hypothesis that caffeine acts at the A1 and A2A receptors during development to increase neuronal activity (da Silva *et al.*, 2008). Therefore, caffeine could act at the A1 and A2A receptors to dose-dependently alter cell proliferation and cell death in the developing brain.

Caffeine induced alterations in cell proliferation and cell death during development could result in gross anatomical changes within the brain. Prenatal caffeine exposure significantly decreases brain weight in newborn rats (Tanaka *et al.*, 1987). Neonatal caffeine administration (50 mg/kg/day on postnatal days 1-12) increases the dendritic length of pyramidal neurons in the prefrontal cortex in both juvenile and adult rats (Juarez-Mendez *et al.*, 2006). The increase in dendritic length of the pyramidal

neurons of the prefrontal cortex may be a direct result of adenosine receptor antagonism in the cortex. It may also be an indirect effect of increased activity in other regions of the brain. For instance, the prefrontal cortex receives glutamatergic inputs from the hippocampus suggesting that A1 receptor antagonism in the hippocampus may increase activity in the prefrontal cortex. However, the effects of developmental caffeine exposure on the hippocampal anatomy are unknown. The aim of the present study was to document the effect of moderate chronic prenatal exposure to caffeine (10 mg/kg/day) on hippocampal volume in juvenile and adult male and female rats. The findings suggest that daily oral administration of caffeine to pregnant rat dams increases the volume of the hippocampus and dentate gyrus in juvenile offspring but that this volume difference does not persist into adulthood.

## B. Materials and Methods

*Animals*

Female Sprague-Dawley rats 60-70 days of age were mated within our colony at Michigan State University. From gestational day 4 until the day of birth, pregnant dams were administered *ad libitum* tap water or 75 mg/L caffeinated tap water. Daily water intake was recorded. Oral administration was used to avoid the stress associated with injections or gavage administration.

On postnatal day 2 (PN2), litters were culled to 4 males and 4 females. On PN25, pups were weaned to 2-3 animals per cage according to litter and sex. All animals were housed in a temperature and humidity controlled environment with a 12 h light/dark cycle (lights on at 0500 hours) with standard chow available *ad libitum*. The experiments were approved by the Michigan State University Institutional Animal Care and Use Committee and followed NIH guidelines for animal use.

Juvenile (n=16) and adult (n=27) animals were perfusion fixed on either PN30 or PN150, respectively, with 4% paraformaldehyde in 0.1M phosphate buffer. Brains were kept in fixative overnight and then transferred to a para-sucrose (4:30%) solution and stored until sectioned. Coronal 50μm sections were collected through the entire brain with every third section mounted on gelatin-coated slides and stained with methylene-blue azure II.

*Cavalieri Estimation of Volume*

The volume of the anterior to posterior bilateral extent of the hippocampus and dentate gyrus was measured using the Cavalieri method (Gundersen *et al.*, 1999). For analysis, the volume of the hippocampal formation was subdivided into two regions of interest: 1) Ammon's horn which is comprised of the CA1-CA3 fields and 2) the dentate gyrus (**Figure 5-1**). The delineation of each region of interest was made using a rat brain atlas for each section (Paxinos and Watson, 2005). The anterior-most tissue section used for volume analysis was a randomly chosen section within 150 μm of the start of the hippocampus and dentate gyrus. Each subsequent tissue section used for volume analysis was approximately 300 μm (or six tissue sections) from the previous section. The entire extent of the hippocampus and dentate gyrus were analyzed with the posterior-most tissue section being the last section in the series to contain Ammon's horn or dentate gyrus in either hemisphere.

A total of 12-14 sections were analyzed for each juvenile animal and a total of 18-20 sections were analyzed for each adult animal. Analysis was performed using a 4X objective and 75μm grid spacing with the StereoInvestigator software (MicroBrightField version 8.03, Williston, Vermont), which was coupled to a Nikon Eclipse 80i microscope with a motor-driven LEP stage and Optronics Microfire cooled CCD camera. The Cavalieri method was used to determine the surface area of each region, which was multiplied by the tissue thickness to determine the volume of each plane, using the following equation:

$$\text{Volume} = \Sigma p \times (a/p) \times t \times s$$

where Σp is the total number of grid points overlying a region of interest, (a/p) is the area represented by each point on the grid, t is the tissue thickness per section, and s is the section sampling interval. The total volume of a brain region was determined by summing the area across all the tissue planes (12-20 total) through the entire depth of the tissue samples (between 3600-6000μm).

Measurements of a 2400μm length cross-section of the brain were taken to ensure that the volume of the brain did not alter hippocampal volume measurements. Because tissue can shrink during the staining process it is important to determine that volumetric differences are not merely the result of a total brain volume difference. Differences in brain volume were determined by drawing a contour around each brain section to find the area of that section. The anterior most section sampled was the first section to contain hippocampus and the last section was that located 2400μm posterior of the first section sampled. The volume of this section of brain was determined using the equation previously described.

The extent of the hippocampus was determined by counting the number of sections that contained hippocampus. This number was then multiplied by the thickness of each section to determine the approximate anterior to posterior extent of the hippocampus.

*Statistics*

Statistical assessment of weekly water intake was performed using two-way repeated measures analysis of variance (week, treatment). For all other analyses, two-

way analysis of variance (sex, treatment) was performed. All statistics were performed using the Systat program (version 11.0).

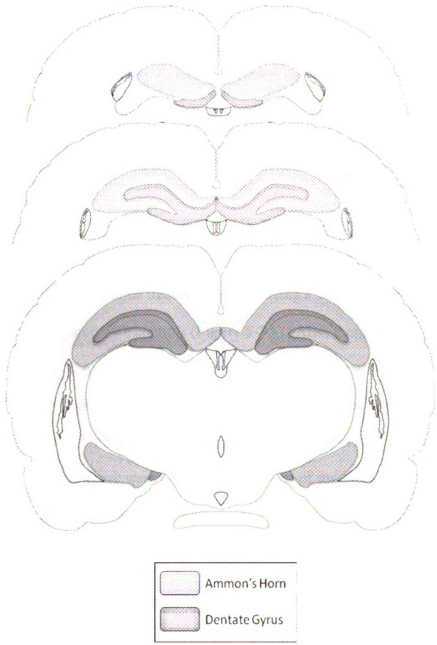


**Figure 5-1.** Representative coronal sections highlighting the rat hippocampus to show the delineation of the Ammon's horn and dentate gyrus used for the bilateral volume measurements (Swanson, 1998). The entire area within the medium gray area was used to determine the area of Ammon's horn, while the entire dark gray area area was used to determine area of the dentate gyrus. Both areas combined were used to determine the area of the hippocampal formation.

## C. Results

During the time of caffeine exposure (gestational day 4 through birth), the average water intake in control and caffeine-treated dams significantly increased each week (**Figure 5-2**; within-group analyses, $p=0.00$). However, there was no significant main effect of treatment on water intake.

In juvenile male and female rats, two-way analysis of variance revealed a significant effect of treatment on total hippocampal formation volume [$F(1,12)=7.324$, $p<0.020$] with caffeine treated juveniles having a 20.6% greater hippocampal volume when compared with controls (**Figure 5-3A**). Also, there was a significant effect of treatment on Ammon's horn volume [$F(1,12)=5.124$, $p<0.05$] and dentate gyrus volume [$F(1,12)=12.603$, $p<0.01$], with caffeine treated juveniles having a 19.6% larger volume of Ammon's horn and a 20.0% larger dentate gyrus volume when compared to controls (**Table 5-1**). There were no significant effects of sex or treatment by sex interactions across any of the volume measurements.

In adult male and female rats, there were no significant effects of treatment or treatment by sex interactions across any of the volume measurements. However, two-way analysis of variance showed a significant effect of sex on hippocampal volume [$F(1,23)=21.853$, $p<0.000$] with females having a 9.6% decrease in volume when compared to males (**Figure 5-3B**). There was also a significant effect of sex on Ammon's horn volume [$F(1,23)=15.344$, $p<0.01$] and dentate gyrus volume [$F(1,23)=21.646$, $p<0.01$] with an 8.1% and 12.2% increase in male volumes, respectively, when compared to females (**Table 5-1**).

There was no significant effect of treatment on the volume of the 2400μm extent of whole brain analyzed for juveniles or adults. However, there was a significant effect of treatment [$F(1,12)= 16.124$, $p<0.01$] on the anterior to posterior length of the hippocampus in juveniles such that caffeine treated animals had a mean extent of 4406 ± 49μm whereas control animals had an extent of 3675 ± 165μm.

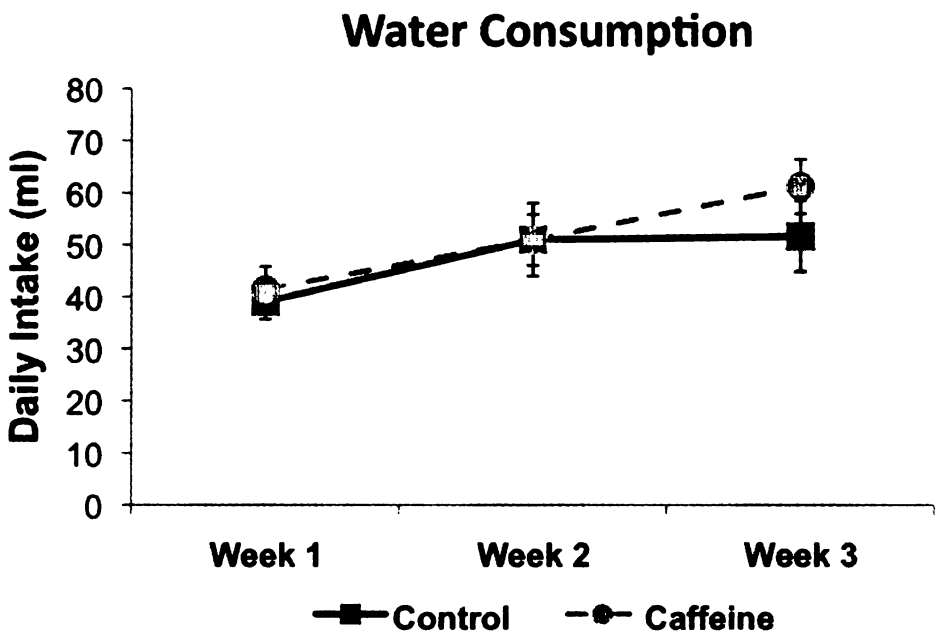


**Figure 5-2.** Maternal intake of water (n=4) or caffeinated tap water (n=4) from gestational day 4 until the day of birth (gestational day 23 ± 1 day). Data represent the mean daily intake ± SEM for each gestational week. Water intake increased throughout gestation, but there was no main effect of treatment on intake.

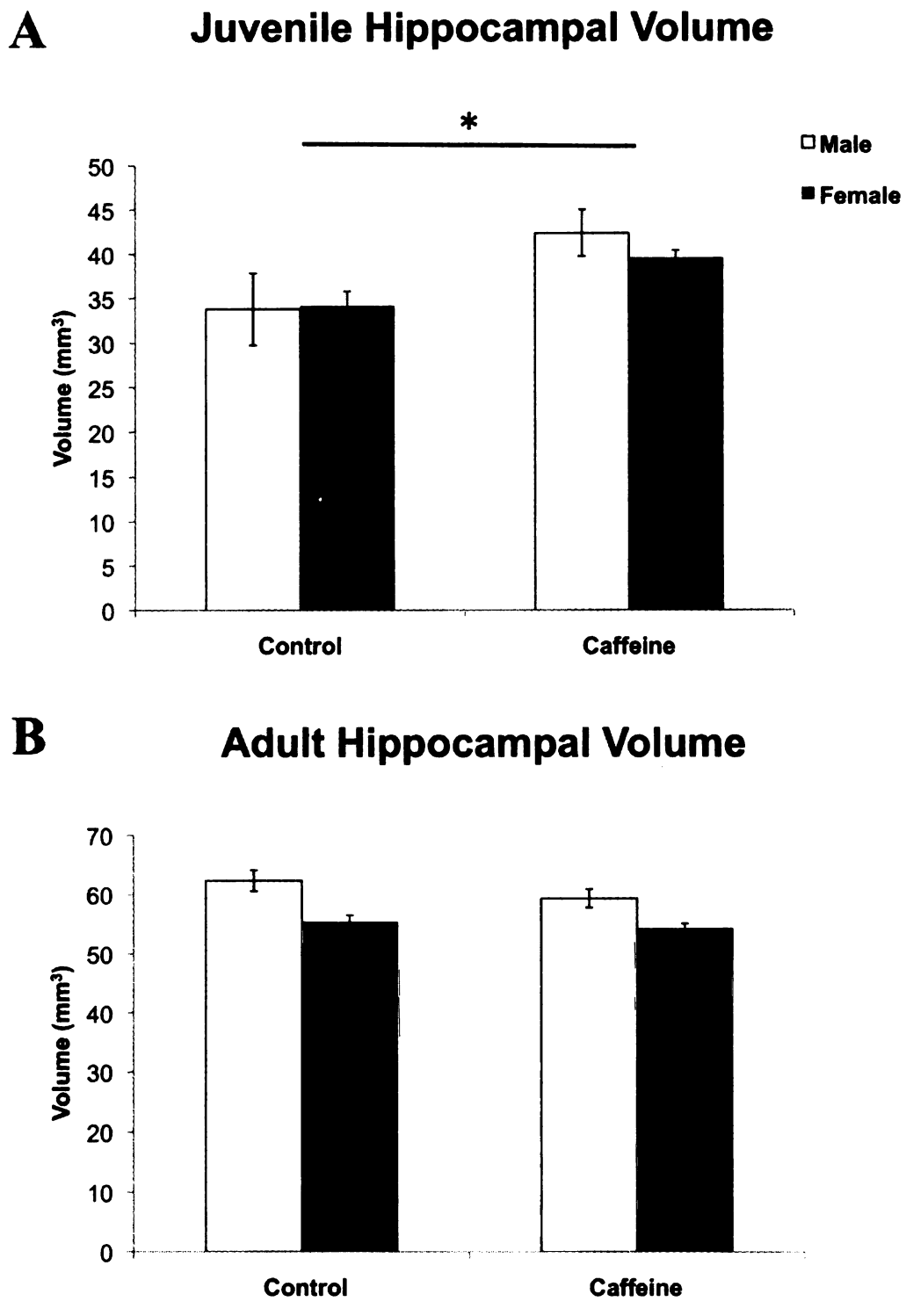


**Figure 5-3.** Effect of prenatal caffeine on total hippocampal volume in (A) juveniles (n-16) and (B) adults (n=27). Data represent the average volume ± SEM. Juvenile rats show a significant main effect of treatment ($p<0.05$) whereas adult rats show no main effect of treatment but a main effect of sex ($p<0.00$).

| | Hippocampal Formation | Ammon's Horn | Dentate Gyrus |
|---|---|---|---|
| **Juvenile** | | | |
| Control Male (n=4) | 33.8 (4.0) | 23.7 (3.2) | 10.0 (0.9) |
| Control Female (n=4) | 34.1 (1.7) | 24.1 (1.6) | 10.1 (0.5) |
| Caffeine Male (n=4) | 42.4 (2.6) | 29.7 (2.1) | 12.8 (0.6) |
| Caffeine Female (n=4) | 39.5 (0.8) | 27.6 (0.6) | 11.9 (0.3) |
| **Adult** | | | |
| Control Male (n=5) | 62.3 (1.8) | 39.4 (1.2) | 22.9 (0.7) |
| Control Female (n=7) | 55.4 (1.1) | 35.8 (0.7) | 19.6 (0.6) |
| Caffeine Male (n=7) | 59.3 (1.6) | 37.9 (1.0) | 21.4 (0.7) |
| Caffeine Female (n=8) | 54.2 (0.8) | 35.1 (0.5) | 19.1 (0.4) |

**Table 5-1.** Volumes ($mm^3$) for the whole hippocampal formation, Ammon's horn, and dentate gyrus for control and caffeine-treated juveniles and adults. Data represent the average volume (± SEM).

**D. Discussion**

Results for the present study demonstrate that chronic prenatal caffeine exposure to a moderate dose of caffeine increases total hippocampal volume in juvenile rats but that this volume increase does not persist into adulthood. Specifically, chronic prenatal caffeine exposure increases the volume of the hippocampus in juveniles by approximately 20% when compared with controls. This volume increase occurs in both caffeine-treated males and females, however males exhibit the greatest change. Interestingly, this treatment effect does not persist into adulthood and it is unknown what changes may occur during puberty to eliminate the caffeine-induced alterations in hippocampal volume.

These data suggest that changes in volume that result from prenatal caffeine exposure are dependent on the overall anterior to posterior extent of the hippocampus. Analysis of the total number of sections containing hippocampus or dentate gyrus revealed that caffeine-treated animals have an approximate 700 μm greater length of the hippocampus when compared to controls. Because most studies do not report the anterior to posterior extent of the hippocampus, it is not known whether this difference accounts for the volume differences seen after other prenatal drug exposures.

The sex difference found in adult animals was not surprising given that this difference has been widely reported. Adult male rats have increased volume (Diamond *et al.*, 1983), increased cell number (Juraska *et al.*, 1989), and increased dendritic arborization of the hippocampus when compared adult females. Although the caffeine-treated juvenile animals appear to have a sex difference, there was no main effect of sex

between caffeine-treated males and females. Therefore, the sex-dependent effects on hippocampal volume are not observed prior to puberty.

After a thorough review of the literature, the present study appears to be the first to document the effects of prenatal caffeine exposure on hippocampal volume, making it difficult to compare these findings to the previous reports. However, numerous developmental drug exposure studies using other drugs of abuse have shown alterations in hippocampal volume and neuronal number in pre-pubertal and adult rats. Postnatal exposure to alcohol during PN days 4-9 has been shown to decrease the volume of CA1 and CA3 in PN10 rats, with an associated decrease in CA1 pyramidal cell number (Livy *et al.*, 2003). In PN30 rats exposed to ethanol perinatally, CA1 volumes were larger while the number of pyramidal neurons in the CA1 was lower than controls (Gonzalez-Burgos *et al.*, 2006). Similar to the present findings, perinatal cocaine exposure resulted in a greater volume of the dentate granule cell layer in juveniles but this effect was reversed in adults (Ismail & Bedi, 2007). In the same animals, juveniles showed a decrease in the total number of pyramidal cells whereas adults showed a decrease in both pyramidal and granule cells when compared to controls. Therefore, it is not uncommon for drug exposure during perinatal development to alter hippocampal volume and neuron number. However, it is unknown why alterations in volume and neuron number are often transient and do not persist into adulthood.

The relationship between hippocampal volume and behavior has just recently begun to be elucidated. For instance, hippocampal volume measurements taken using MRI technology are important predictors of disease states. Diseases such as depression, schizophrenia, bipolar disorder, and post-traumatic brain disorder have been associated

with shrinkage of the hippocampus (Czeh & Lucassen, 2007). Each of these diseases is associated with specific behaviors, which may or may not be related to the associated changes in hippocampal volume, but further research will likely determine whether there is a direct structure-function relationship. Interestingly, the use of MRI techniques to study these diseases in humans has provided some evidence that the hippocampal volume can change throughout the lifespan. In some depressed patients who receive anti-depressant treatment, the volume of the hippocampus is normalized (Czeh & Lucassen, 2007).

Many MRI studies in humans have also correlated decreased hippocampal volume to decreased memory, although this sentiment is widely debated (Van Petten, 2004). Interestingly, meta-analysis of the relationship between memory abilities and hippocampal volume has shown a positive relationship between smaller hippocampal volumes and increased memory abilities in healthy adolescents. However, hippocampal volume varies widely in normal healthy adults (Van Petten, 2004). In rats, approximately 26% of dentate gyrus granule cells labeled during peak granule cell proliferation in neonates die, whereas granule cells developed in adulthood are much more stable (Dayer *et al.*, 2003). Therefore, it is possible that the hippocampus is reorganizing and changing volumes throughout the lifespan and that alterations in volume in juveniles are lost during hippocampal reoganization in adulthood. Further, it is likely that hippocampal volume may be directly related to learning and memory behaviors (Van Petten, 2004), however more research into this structure-function relationship is needed.

The development of the hippocampus and dentate gyrus occurs during gestation and early neonatal periods. In the rodent hippocampus, all of the pyramidal cells and

neurons in Ammon's horn are formed prior to birth (Bayer, 1980a, b). Unlike neurons that form Ammon's horn, neurogenesis of dentate granule cells occurs postnatally and continues at a reduced rate throughout life. In the rat, dentate gyrus development takes place between gestational day 14 and the second postnatal week of life, with a peak of activity around postnatal day 5-7 (Schlessinger *et al.*, 1975). Granule cells increase their dendritic trees until PN14 after which the branches are sculpted to form adult-like morphological features (Rahimi & Claiborne, 2007). In humans, hippocampal development occurs over a prolonged period of gestation. By gestational week 15, neurogenesis of pyramidal neurons is visible in Ammon's horn with adult-like morphological features of the CA1-CA3 fields established by the 23-25$^{th}$ gestational week (Arnold & Trojanowski, 1996a, b). Neurogenesis in the granule cell layer of the dentate gyrus begins around gestational week 14 (Humphrey, 1967) and continues postnatally (Seress *et al.*, 2001). Therefore, it is not surprising that prenatal exposure to drugs during these critical stages of hippocampus and dentate gyrus formation can alter the overall morphology of both structures in treated offspring.

Caffeine's actions within the developing brain and how these actions may affect hippocampal development are not well understood. The A1 and A2A receptors, which mediate caffeine's stimulatory actions, are expressed in the fetal hippocampus of both rodents and humans (Weaver, 1993; Weaver, 1996). It is known that adenosine, acting at the A1 receptors, depresses glutamatergic activity and regulates giant depolarizing potentials within the hippocampus during the first week of postnatal life in rodents (Safiulina *et al.*, 2005). Giant depolarizing potentials are a trait of the developing hippocampus and are characterized by recurrent spontaneous membrane depolarizations

(Ben-Ari *et al.*, 1989). Giant depolarizing potentials are instrumental in shaping the synaptic connections of the hippocampal circuitry and are mediated by the actions of GABA and glutamate in the developing brain (Safiulina *et al.*, 2006). Alterations in the frequency of giant depolarizing potentials may alter the development of hippocampal neural networks (Dzhala *et al.*, 1999). If caffeine antagonizes the adenosine receptors in the hippocampus, primarily the A1 receptors, such that adenosine can no longer regulate or depress the frequency of the giant depolarizing potentials, the development of the hippocampus may be negatively affected. Further, glutamatergic NMDA receptors play a fundamental role in activity-dependent migration, synapse formation, and functional connectivity of the developing hippocampus (Ben-Ari & Holmes, 2006). Caffeine may increase glutamate release in the developing hippocampus thereby disrupting these processes, which may account for the increase in volume observed in the present study.

Caffeine also has numerous indirect effects on hippocampal development. Acute administration of caffeine has been shown to increase hippocampal cell death in the neonatal brain (Kang *et al.*, 2002; Black *et al.*, 2008), decrease cell proliferation in the dentate gyrus and astrocyte expression in the hippocampus (Desfrere *et al.*, 2007), and increase acetylcholinesterase activity in the hippocampus (da Silva *et al.*, 2008). It is possible that caffeine-induced alterations in cell death, neurogenesis, glial cell number, or activity dependent synaptogenesis may account for changes in hippocampal volume.

In summary, exposure to a moderate dose of caffeine throughout gestation alters the volume of the hippocampus in juveniles, but these differences are no longer present in adult offspring. The use of caffeine during pregnancy is of concern because it crosses both the placental and blood-brain barriers to reach the developing fetal brain. The

actions of caffeine within the fetal brain are not well understood but caffeine may act as an antagonist of adenosine receptors within the developing fetal brain, which may alter pyramidal and granule cell neurogenesis, migration, and synaptic connectivity in the hippocampus and dentate gyrus. As the hippocampus plays a critical role in learning and memory, care should be taken to reduce caffeine consumption during pregnancy until a more thorough understanding of its effects on neurodevelopment becomes available.

## Chapter 6: Discussion

Caffeine is the most widely used psychoactive drug in the world. Although it is estimated that 70-95% of women continue to consume caffeine during pregnancy (Fredholm *et al.*, 1999), the effects of prenatal caffeine exposure have not been well investigated. The use of caffeine during pregnancy is of concern because it crosses both the placental and blood brain barriers to reach the developing fetus (Arnaud, 1993) where the half-life of caffeine and its primary metabolites are greatly prolonged due to an immaturity of cytochrome P-450 metabolism (Aranda *et al.*, 1979a). The slowed metabolism of caffeine and its metabolites in the fetus results in methylxanthine accumulation in the developing brain (Chapter 2; Wilkinson & Pollard, 1993) and poses a potential risk to neural development if unsafe levels are reached. Caffeine is an adenosine receptor antagonist and adenosine is a well-known critical neuromodulator during brain development (Fisone *et al.*, 2004). Adenosine functions to reduce neuronal activity thus providing protection against excitotoxicity (Fredholm, 2007). When adenosine receptors are inhibited, there is an overall increase in glutamate neurotransmission particularly in brain regions with an abundance of adenosine receptors such as the cortex and hippocampus (Wang, 2007). Given the widespread effects of caffeine and the frequency of prenatal exposure, it is imperative to better understand the effects of the drug on the developing brain. The objectives of this dissertation were to determine whether caffeine and its primary metabolites accumulate in the fetal brain and what effects these drugs have on glutamate receptor expression, glutamate-mediated calcium transients, hippocampal-dependent learning and memory behaviors, and hippocampal anatomy.

In order to elucidate the effects of caffeine exposure, pregnant rats were exposed to chronic moderate doses of caffeine throughout gestation. The model of chronic exposure to moderate doses of caffeine in the drinking water was chosen to ensure that findings from this dissertation are as relevant to the human situation as possible. Self-reports of pregnant women who consume caffeine estimate their daily intake to range between 100-300 mg (Christian & Brent, 2001). This range is likely due to current medical recommendations, which suggest that pregnant women reduce intake to less than 300 mg/day. Metabolism of caffeine and its primary metabolites is known to occur at a faster rate in rats than in humans. For instance, the half-life of caffeine in adult humans is approximately 2-4 hours whereas the half-life in adult rats is approximately 1-2 hours (Bonati *et al.*, 1984-1985). Therefore, much of the previous literature suggests use of a metabolic body weight conversion to determine approximately equivalent doses between rats and humans. Metabolic body weight (=body weight^3/4) calculations used were based on findings that 20 mg/kg in a rat is equivalent to 4-6 cups of coffee in a 70 kg human (Nehlig & Derby, 1994). The dose most commonly used in this dissertation was 75 mg/L of which pregnant rats consumed approximately 40-60 ml/day, or an intake of approximately 10 mg/kg/day in the rat, which is approximately equivalent to consumption of 2-3 cups of coffee per day in humans. This dosing regimen aligns well to self-reports of human consumption. The additional higher doses of 150 and 300 mg/L described in Chapters 2 and 3 were used to allow comparisons to the previous literature, in which doses of 300-1000 mg/L are most common. Thus, the studies in this dissertation not only align well to the human condition they also present novel findings of neurodevelopmental effects following low to moderate exposure to prenatal caffeine.

Rats are an excellent animal model of caffeine exposure due to the similar pharmacokinetics of the drug between rats and humans. In both rats and humans, caffeine is metabolized into the three primary metabolites paraxanthine, theobromine, and theophylline with nearly superimposable plasma curves (Fredholm *et al.*, 1999). However, one drawback to using a prenatal rat model is the short length of gestation (21-24 days) in rats. Newborn rats are underdeveloped in comparison to newborn humans and it is common to consider postnatal days 1-7 in the rat parallel to the third trimester of development in humans (Clancy *et al.*, 2007). This shortened period of gestation in rats results in a much shorter period of prenatal exposure than would be seen in the human fetus if a mother drank caffeine throughout pregnancy. Furthermore, in humans extensive brain development occurs during gestation whereas in rats the brain growth spurt occurs primarily during postnatal development (Bayer, 1980a, b). For the studies presented in this dissertation, rats were exposed to caffeine only during prenatal development. This period of exposure was chosen to avoid differences between placental and lactational transfer, dosing, and metabolism of caffeine. However, the differences between gestational length and prenatal brain development between rats and humans should not be overlooked. It is possible that the findings following prenatal exposure in rats under reflect the neurodevelopmental consequences of prenatal caffeine exposure in humans.

The studies in this dissertation focused primarily on the effects of prenatal caffeine exposure on the developing hippocampus. The hippocampus was chosen because it has an abundance of A1 adenosine receptors, which are likely to be antagonized during caffeine exposure. The hippocampus is also well known to be a brain

region particularly susceptible to prenatal insult. For instance, hippocampal development is impaired following prenatal exposure to alcohol, hypoxia-ischemia, seizures, anesthetic medications, and stress. The hippocampus is also well characterized both anatomically and behaviorally, which lends to enhanced experimentation of this region. Prior to this dissertation work, the effects of prenatal caffeine on the developing hippocampus had not been well investigated, making this a valuable region for analysis.

The first objective of this dissertation (Chapter 2) was to determine whether chronic moderate exposure to prenatal caffeine resulted in an accumulation of caffeine and its primary metabolites in the fetal brain. A previous study showed that caffeine and its metabolites accumulate in the fetal brain, however this study used a single maternal dose of 5 or 25 mg/kg administered by gavage (Wilkinson & Pollard, 1993). Because the pharmacokinetics of caffeine may differ following bolus and intermittent intake, it was important to reevaluate these findings with an *ad libitum* administration paradigm. Furthermore, it was important to assess whether repeated exposures to caffeine result in higher-fold accumulation due to the prolonged half-life and lack of metabolism of caffeine in the fetus. Results from the first objective show that caffeine and its metabolites do accumulate in the fetal brain when compared with the maternal brain. Specifically, it was found that maternal intake of caffeine results in at least 3-fold higher concentrations of caffeine and its metabolites in the fetal brain than in the maternal brain. If the levels of all four bioactive methylxanthines are considered in combination, the levels in the fetal brain are greatly elevated in comparison to the levels in the maternal brain. In contrast, no differences were found between caffeine and metabolite levels in the fetal and maternal plasma. Importantly, these findings indicate that measurements of

plasma levels in newborns may underestimate actual bioactive methylxanthine levels in the brain. Therefore, studies of methylxanthine levels in humans, which are performed in plasma, may greatly underestimate the actual risk posed to the developing brain of offspring whose mothers consume caffeine.

The results from this study have numerous implications for caffeine use during pregnancy. As discussed previously, caffeine and its metabolites are greatly elevated in the fetal brain when compared to the maternal brain, even following exposure to the lowest dose. It is known that at high concentrations caffeine can elicit actions beyond antagonism of adenosine receptors. These actions include inhibition of phosphodiesterase activity, blockade of GABA(A) receptors, and direct effects at numerous ion channels, including sodium and potassium channels (Daly, 2000). In animal studies, caffeine administration of doses greater than 20 mg/kg produces depressant rather than stimulant effects on motor behavior (Fredholm *et al.*, 1999). Although the exact mechanisms behind this biphasic response are unknown, it is believed that the behavioral changes likely result from inhibition of phosphodiesterase activity or changes in GABA(A) receptor activity. With regards to elevated levels of methylxanthines in the developing brain, it is currently unknown whether the levels reached can elicit any of the actions described above, however it is worth considering that there may be effects beyond antagonism of adenosine receptors. The current findings also show that methylxanthine levels reached in the fetal brain increase in a dose-dependent manner with increasing maternal intake. Therefore, mothers who consume large amounts of caffeinated beverages each day during pregnancy will likely be exposing the brains of their developing offspring to extremely high methylxanthine concentrations.

Numerous follow-up studies would provide additional invaluable information regarding the elevated levels of methylxanthines within the developing brain. In the current study, analysis was performed on whole brains. It would be highly informative to determine whether caffeine is accumulating at different rates in various regions of the developing brain. This analysis was not performed in the current study because it proved very difficult to dissect the hippocampus, cortex, and other brain regions of interest from the same fetal brain. However, the use of brain slicing and Palkovits punch technique could allow for this type of analysis. Further, it would be interesting to determine whether prenatal caffeine exposure is altering neurotransmitter levels within the developing brain. High performance liquid chromatography with electrochemical detection could be used to determine the levels of GABA and glutamate following prenatal caffeine exposure. Similar techniques could also be used to determine the levels of adenosine within the brain, and it is unlikely that sensitivity would be an issue as demonstrated in Chapter 2. Such studies would provide a better understanding of how prenatal caffeine exposure is altering neurotransmission and which receptors may consequently be most affected. Although it is hypothesized that glutamate neurotransmission is increased in the developing hippocampus following caffeine exposure, such a study would provide more direct evidence for this hypothesis.

The second objective of this dissertation (Chapter 3) was to determine the effects of prenatal caffeine exposure on NMDA receptor expression and glutamate-mediated calcium signaling in the neonatal hippocampus. The results showed that the NR1, NR2A, and NR2B subunits all exhibit slight differences in protein expression at various postnatal periods, which correspond to peak periods of expression for each subunit. Interestingly,

prenatal caffeine exposure also reduced glutamate-induced calcium transients in hippocampal cultures from treated offspring. Caffeine-treated hippocampal cells showed diminished maximal calcium transients after glutamate administration when compared to the maximal calcium transients seen in control cultures. However, no treatment differences were detected between the levels of phosphorylated ERK and CREB proteins, suggesting that these calcium-dependent cascades are not affected by prenatal caffeine exposure. The results from these studies indicate that prenatal caffeine exposure is likely altering glutamate-mediated neurotransmission and consequently altering expression of the NR1, NR2A, and NR2B subunits. However, the changes found in subunit expression appear to be subtle and transient. Therefore, it is unknown whether prenatal caffeine exposure is causing long-term alterations in glutamate-receptor expression and whether these changes are of enough magnitude to alter glutamate-receptor dependent processes such as learning and memory or LTP. It does appear from these studies that early alterations in receptor expression may affect glutamate-mediated calcium transients. However, it is unknown whether the reduced maximal peak response seen in caffeine-exposed hippocampal neurons results from a reduction in overall receptor number or from a shift in subunit composition that could lead to altered receptor kinetics. Further, it is possible that changes in AMPA receptor expression may be altered following prenatal caffeine exposure. Increased glutamate neurotransmission is likely also acting on AMPA receptors, which are expressed during prenatal and early postnatal development. Therefore, there may be changes in AMPA receptor expression, which may reduce the AMPA-mediated depolarization needed to fully activate NMDA receptors. It is also possible that the reduced calcium transients in caffeine-exposed cultures are mediated by

changes in intracellular calcium-induced calcium release or voltage-gated calcium channels. Thus, it cannot be ruled out that any alteration in calcium influx via these mechanisms may contribute to the reduced peak calcium amplitude until specific antagonists are used to block these sources of calcium.

Although subtle changes in glutamate receptor expression could have numerous developmental consequences, further research is needed to fully understand the extent and magnitude of these changes following prenatal caffeine exposure. One obvious follow-up study would be to repeat the calcium imaging with the use of specific voltage-gated calcium channel antagonists as well as blockers of intracellular calcium channels, such as the ryanodine receptors. A preliminary study of this type was conducted during the course of this dissertation, yet it was very difficult to determine whether the antagonists were working correctly as the responses following glutamate-administration did not appear to be different regardless of whether antagonists were used in the medium. For this reason, the use of antagonists was not further pursued for these dissertation studies. However, if this study could be repeated while performing both electrophysiology as well as calcium imaging, one could test whether the antagonists were working correctly prior to conducting the live cell imaging. This setup would also provide invaluable information of changes in receptor kinetics that may occur following prenatal caffeine exposure. Another intriguing study would be to perform fluorescent triple-label immunohistochemistry for the NR1, NR2A, and NR2B subunits in tissue sections. Like Western blots analyses, such a study would allow for determination of changes in subunit expression, although it might be difficult to detect subtle changes. More importantly, it would allow for region specific determinations of subunit expression

within the CA1-3 fields and the dentate gyrus as well as localization of specific subunits to specific cells within the subdivisions of the hippocampus. Such a study would further our understanding of both receptor trafficking and region specific expression following caffeine exposure.

The third objective of this dissertation (Chapter 4) was to determine the long-term effects of prenatal caffeine exposure on learning and memory behaviors. For this study, behavioral assessments in the novel object recognition task, radial arm maze, and Morris water maze were conducted. The results reveal impaired behavior in caffeine-exposed offspring in both the novel object recognition task and radial arm maze, but not in the Morris water maze. Importantly, these results indicate that prenatal caffeine exposure has long-term neurodevelopmental effects on behavior, however these effects are subtle. Although the caffeine-exposed animals had more performance errors than control animals, they learned each task after repeated trials. In the Morris water maze, the caffeine-exposed animals actually performed as well as or slightly better than control animals. Thus, the behavioral effects appear to be subtle yet persistent and do not affect all hippocampal-dependent behaviors. Importantly, these findings have potential implications for children exposed to caffeine prenatally and support previous findings showing deficits in learning following caffeine-exposure in animals. Many learning deficits are not detected in children until they are in elementary or middle school. Because learning deficits are often subtle and not easily detectable early in development, there is a lack of longitudinal research investigating whether prenatal caffeine exposure results in long-term alterations of learning in children. It does not seem likely that

prenatal caffeine exposure results in large learning and memory deficits, however even subtle changes could hinder educational success.

An intriguing follow-up to the behavioral studies performed in this dissertation would be to determine whether there are long-term alterations in LTP following prenatal caffeine exposure. If changes in LTP were found, this would be the first study reported in the literature with regards to prenatal caffeine. Further, such a study would provide a more direct link between the hypothesis that glutamate receptor expression or function is altered following prenatal caffeine and that these changes affect learning and memory behaviors. Another intriguing follow-up study would be to utilize adenosine receptor knockout mice to repeat many of the experiments presented in this dissertation. The use of adenosine receptor knockout animals would further elucidate whether the effects of prenatal caffeine exposure on glutamate receptor expression, calcium transients, and learning and behavior are mediated through antagonism of the A1 receptor.

The final objective of this dissertation (Chapter 5) was to determine whether there are anatomical alterations in the hippocampus following prenatal caffeine exposure. Specifically, stereological analysis with the Cavalieri estimator method was used to determine whether there are hippocampal volume differences between control and caffeine-exposed offspring. The results reveal a significant increase in total hippocampal volume in caffeine-treated juvenile rats, which does not persist into adulthood. These results are similar to previous findings showing that neonatal caffeine administration (50 mg/kg/day on postnatal days 1-12) increases dendritic length of pyramidal neurons in the prefrontal cortex in both juvenile and adult rats (Juarez-Mendez *et al.*, 2006). If the hypotheses presented in this dissertation are correct, the increase in hippocampal volume

in juveniles may result from altered glutamatergic activity in the developing brain since glutamate receptor activity is known to play a key role in cell proliferation and activity-dependent cell survival. However, because glutamate receptor activity was not directly tested in the current studies, this remains speculative. Since measures of hippocampal volume provide limited information, follow-up studies utilizing more in-depth anatomical measurements are necessary to fully understand the effects of prenatal caffeine exposure on hippocampal anatomy. One intriguing follow-up would be a study of Golgi-impregnated neurons to determine if (similar to neurons in the prefrontal cortex) hippocampal neurons exhibit increased dendritic length and arborization. If this were the case, this may help explain why overall volume of the hippocampus is increased at specific ages. Further, it would be interesting to increase the number of time periods sampled to better understand how hippocampal size is being regulated between puberty and adulthood.

Overall, this dissertation has added substantial novel findings to the area of prenatal caffeine research, an area that has been vastly understudied in the past few decades. Like most research, the findings presented here have raised far more questions about the consequences of prenatal caffeine exposure than they have answered. Future research is needed to further elucidate the subtle molecular and cellular consequences of prenatal caffeine exposure. It would be extremely interesting and clinically important to utilize a perinatal model for such research to more effectively recreate gestational exposure in humans. The findings using such a model are likely to be more robust and substantially different from the results of prenatal exposure alone. Although this dissertation highlighted many changes in the hippocampus, numerous brain regions may

be affected by prenatal caffeine exposure. Therefore, molecular and cellular analysis is needed in other brain regions. A few recent studies have highlighted changes in the prefrontal cortex indicating that perhaps more research into this area is currently being pursued. Lastly, a better understanding of the actions of caffeine within the developing brain is needed. In this dissertation, it was hypothesized that caffeine is increasing glutamate neurotransmission via adenosine receptor blockade. Although this hypothesis is reasonable based on the known actions of adenosine, the developing brain does not always respond to drugs in the same fashion as the adult brain. Therefore, additional experiments must be done to fully understanding the relationship between caffeine, adenosine, and glutamate neurotransmission in the developing brain.

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