

AN EXAMINATION OF THE EFFECTS OF CHRONIC CAFFEINE AND
WITHDRAWAL FROM CHRONIC CAFFEINE ON FEAR CONDITIONING
IN PRE-ADOLESCENT, ADOLESCENT, AND ADULT C57BL/6J MICE

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ABSTRACT

Caffeine is the most widely used psychoactive substance in the world. While findings suggest that chronic caffeine exerts negligible effects on cognition in adults, the effects of chronic caffeine on cognition in children and adolescents are not well understood. The hippocampus, a brain region important for learning and memory, undergoes extensive structural and functional modifications during pre-adolescence and adolescence. As a result, chronic caffeine may have differential effects on hippocampus-dependent learning and memory in pre-adolescents and adolescents compared to adults. The present study characterized the effects of chronic caffeine and withdrawal from chronic caffeine on hippocampus-dependent (contextual) and hippocampus-independent (cued) fear conditioning in pre-adolescent, adolescent, and adult mice. In addition, we investigated whether exposure to chronic caffeine during pre-adolescence, adolescence, or adulthood had long-lasting effects on conditioning in adulthood. Results indicate that exposure to chronic caffeine during pre-adolescence and adolescence either enhances or impairs contextual conditioning in a concentration-dependent manner. However, withdrawal from chronic caffeine impairs contextual conditioning in pre-adolescent mice only. In addition, exposure to chronic caffeine during pre-adolescence either enhances or impairs retention of contextual memories in adulthood in a concentration-dependent manner. In contrast, exposure to chronic caffeine during adolescence impairs cued conditioning in adulthood. These findings support the hypothesis that exposure to chronic caffeine during pre-adolescence and adolescence compromises hippocampus-dependent learning and memory. Furthermore, exposure to chronic caffeine during adolescence may produce long-lasting deficits in learning and memory in adulthood.

I dedicate this dissertation to my family.

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TABLE OF CONTENTS

ABSTRACT.....	iii
ACKNOWLEDGMENTS	vi
LIST OF TABLES.....	viii
LIST OF FIGURES	ix
CHAPTER	
1. INTRODUCTION	1
2. AGE-DEPENDENT EFFECTS OF CHRONIC CAFFEINE AND WITHDRAWAL FROM CHRONIC CAFFEINE ON FEAR CONDITIONING	23
Introduction.....	23
Methods	28
Results.....	36
Discussion.....	54
3. LONG-LASTING EFFECTS OF EXPOSURE TO CHRONIC CAFFEINE DURING PRE-ADOLESCENCE AND ADOLESCENCE ON FEAR CONDITIONING IN ADULTHOOD.....	68
Introduction.....	68
Methods	70
Results.....	73
Discussion.....	81
4. CONCLUSION.....	89

REFERENCES CITED..... 94

LIST OF TABLES

Table

1. The effects of chronic caffeine on fear conditioning	48
2. The effects of withdrawal from chronic caffeine on fear conditioning	49
3. The effects of chronic caffeine exposure during pre-adolescence, adolescence, and adulthood on fear conditioning in adulthood.....	80

LIST OF FIGURES

Figure

1. Schematic diagram of chronic and withdrawal fear conditioning experiments.....	33
2. Age-dependent effects of chronic caffeine on fear conditioning	38
3. The effect of chronic caffeine on shock-sensitivity.....	40
4. The effect of chronic caffeine on fear conditioning using a reduced conditioning protocol	41
5. Age-dependent effects of withdrawal from chronic caffeine on fear conditioning	43
6. Age-dependent effects of withdrawal from chronic caffeine on recall of a fear memory formed during withdrawal	45
7. Age-dependent effects of withdrawal from chronic caffeine on retention of a fear memory formed during withdrawal	47
8. The effect of chronic caffeine on adenosine 2A receptor (A2AR) levels in the dorsal and ventral hippocampus of pre-adolescent and adult mice	50
9. The age-dependent effect of chronic caffeine on anxiety-related behavior in the elevated zero maze.....	53
10. Schematic diagram of 30 day withdrawal fear conditioning experiment	73
11. The effects of exposure to chronic caffeine during pre-adolescence, adolescence, and adulthood on fear conditioning in adulthood.....	75
12. The effects of exposure to chronic caffeine during pre-adolescence, adolescence, and adulthood on fear conditioning recall in adulthood.....	77
13. The effects of exposure to chronic caffeine during pre-adolescence, adolescence, and adulthood on retention of fear conditioning in adulthood	79

CHAPTER 1

INTRODUCTION

Caffeine, a stimulant found in coffee and tea, is the most widely used psychoactive substance in the world (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999). For thousands of years, caffeine has been safely consumed by humans for its pleasurable mood enhancing effects as well as its ability to fight fatigue and enhance attention (Weinberg & Bealer, 2001). However, caffeine consumption patterns have changed over the past 20-30 years (Reissig, Strain, & Griffiths, 2009; Frary, Johnson, & Wang, 2005). Today, there are many more caffeinated products on the market (Reissig et al., 2009), and these products contain higher concentrations of caffeine than have been reported in the past (Reissig et al., 2009). In addition, some of these caffeinated products are being aggressively marketed to children and adolescents (Reissig et al., 2009). Perhaps as a result of this aggressive marketing, children and adolescents between 12 and 17 are the fastest growing segment of the population for caffeine use (Temple, 2009).

Many scientists and medical professionals have raised concern about the potential consequences of caffeine consumption during childhood and adolescence (Herrera, 2013). This concern stems from several observations. First, children and adolescents are smaller than adults and incidentally ingest a higher mg/kg ratio when they consume the same volume and concentration of caffeine as adults (Luebbe, 2011). Furthermore, children and adolescents who consume caffeine for the first time, possibly in higher doses than typically consumed by adults, have not yet developed tolerance to its effects (Temple, 2009). This size difference and lack of tolerance may partially explain why between 2010 and 2011, 50% of all calls to the National Poison Data System for caffeine

toxicity concerned children under 6 years old (Seifert et al., 2013). Second, several case studies have reported that energy drink consumption is associated with new-onset seizures in adolescents and young adults (Babu, Zuckerman, Cherkes, & Hack, 2011; Calabrò, Italiano, Gervasi, & Bramanti, 2012; Iyadurai & Chung, 2007), which may suggest that young people are either more susceptible to caffeine-induced seizures or are liable to consume doses of caffeine that are detrimental to their health. Third, caffeine use in children is associated with poor performance on cognitive tests (Heatherley, Hancock, & Rogers, 2006), and caffeine use in adolescents is associated with poor academic performance (James, Kristjánsson, & Sigfúsdóttir, 2011). Finally, caffeine consumption during adolescence may be a risk factor for drug abuse later in life (Arria & O'Brien, 2011; Arria et al., 2010). Overall, habitual caffeine consumption during pre-adolescence and adolescence appears to be associated with a number of detrimental effects that deserve further investigation.

The findings that habitual caffeine consumption during childhood is associated with poor performance on cognitive tests (Heatherley et al., 2006), and that caffeine use during adolescence is associated with poor academic performance (James et al., 2011) may suggest that chronic caffeine disrupts normal learning and memory during these developmental periods. Dysfunctional learning not only impacts academic performance, but can interfere with social and emotional functioning (Cortiella & Horowitz, 2014), and may predispose individuals to developing substance use disorders (Beitchman, Wilson, Douglas, Young, & Adlaf, 2001). Therefore, determining whether or not habitual caffeine consumption during pre-adolescence and adolescence has a direct effect on normal learning and memory will be important for informing the public on the potential

consequences of consuming caffeinated products early in life – products that continue to bear no warning labels in the United States and remain unregulated by the Food and Drug Administration (Reissig et al., 2009).

The age-dependent effects of chronic caffeine on learning and memory

The immediate and long-lasting effects of exposure to chronic caffeine during pre-adolescence and adolescence on learning and memory have received little attention. However, a recent study by Ardais and colleagues (2014) demonstrated that exposing adolescent rats (postnatal day 28) to chronic caffeine through their drinking water (at both 0.3 and 1.0 mg/mL) for 22 days improved performance in the novel object recognition task during late adolescence (postnatal day 50). Moreover, findings from two additional rodent studies suggest that exposure to chronic caffeine during pre-adolescence and adolescence can both enhance and impair performance in the novel object recognition task in adulthood. Specifically, Pires and colleagues (2010) found that repeated administration of acute caffeine (3 mg/kg/day i.p.) to pre-adolescent rats for 14 days (postnatal days 25-38) impaired performance in the novel object recognition task when animals were tested in adulthood (between postnatal days 63-70). In contrast, Abreu and colleagues (2011) found that exposing rats to chronic caffeine (0.04 or 0.08% in chow) throughout pre-adolescence, adolescence, and adulthood (postnatal days 21-90) enhanced performance in the novel object recognition task on postnatal day 90 (i.e. in adulthood). Differences in methodology may be responsible for the contradictory results presented by Pires and colleagues (2010) and Abreu and colleagues (2011). For example, chronic caffeine may enhance performance when caffeine is on board during training and testing as was demonstrated by Abreu and colleagues (2011), yet impair performance

when caffeine treatment is terminated prior to training and testing as was demonstrated by Pires and colleagues (2010). Furthermore, Pires and colleagues (2010) administered multiple acute injections of caffeine to female rats, whereas Abreu and colleagues (2011) administered caffeine to male rats in chow. Therefore, the schedule of administration, method of administration, as well as the sex of the animal may determine the effects of repeated exposure to caffeine in different age groups. Despite the conspicuous methodological differences between studies, findings suggest that exposure to chronic caffeine, or repeated exposure to acute caffeine, during pre-adolescence and/or adolescence has immediate and long-lasting effects on learning and memory.

*The age-dependent effects of exposure to chronic caffeine on
hippocampus-dependent learning and memory*

Repeated or chronic exposure to caffeine during pre-adolescence and adolescence might not only alter learning and memory in general, but hippocampus-dependent learning and memory specifically. While the role of the hippocampus in novel object recognition memory is controversial (Barker & Warburton, 2011; Winters, Saksida, & Bussey, 2008), there is evidence that an intact and functional hippocampus is important for performance on this task (Broadbent, Gaskin, Squire, & Clark, 2009). Thus, exposure to chronic caffeine during pre-adolescence and adolescence may compromise forms of learning and memory that engage the hippocampus. This hypothesis is further strengthened by findings that other psychostimulants have a more profound effect on hippocampus-dependent learning and memory during pre-adolescence and adolescence than during adulthood.

While little is known about the effects of chronic caffeine on learning and memory in pre-adolescent and adolescent animals beyond what was previously described, data suggest that chronic exposure to nicotine, another commonly used psychostimulant, exerts immediate and lasting effects on hippocampus-dependent learning and memory when administered during pre-adolescence and adolescence, but not during adulthood (Portugal, Wilkinson, Turner, Blendy, & Gould, 2012). For example, Portugal and colleagues (2012) administered chronic nicotine to pre-adolescent and adolescent mice and analyzed learning and memory using contextual fear conditioning (a hippocampus-dependent form of conditioning (Logue, Paylor, & Wehner, 1997; Phillips & Ledoux, 1992)) after 14 days of chronic nicotine treatment, 48 hours after treatment cessation, or 30 days after treatment cessation. In this study, pre-adolescent mice showed enhanced contextual conditioning during chronic treatment with nicotine. In addition, adolescent mice showed impaired contextual conditioning after 48 hours of withdrawal from a dose of chronic nicotine that did not impair contextual conditioning in adults. Moreover, in the same study, exposure to chronic nicotine during both pre-adolescence and adolescence impaired contextual conditioning in adulthood. Importantly, nicotine had no effect on cued conditioning (a hippocampus-independent form of conditioning (Phillips & Ledoux, 1992)) in any group, which suggests hippocampus-dependent memory systems may be particularly sensitive to the effects of nicotine during pre-adolescence and adolescence. Thus, as a psychostimulant, chronic caffeine may exert a more profound effect on hippocampus-dependent learning and memory in pre-adolescent and adolescent animals compared to adults as well.

Several studies have found that prenatal or early postnatal exposure to chronic caffeine impairs hippocampus-dependent learning and memory in adulthood (Pan & Chen, 2007; Soellner, Grandys, & Nuñez, 2009; Zimmerberg, Carr, Scott, Lee, & Weider, 1991). For example, Soellner and colleagues (2009) demonstrated that gestational exposure to chronic caffeine (~10 mg/kg/day through the dam's drinking water) impaired memory retention in the novel object recognition task, and working/reference memory in the radial arm maze, a task that requires the hippocampus (Meck, Church, & Olton, 1984), of the adult offspring. In addition, Zimmerberg and colleagues (1991) demonstrated that acute daily administration of caffeine (1 or 9 mg/kg by gavage) to rats during their first week of life (postnatal days 1-6) impaired performance on an operant spatial task in adulthood (training and testing occurred between postnatal days 70 and 90). Furthermore, Pan and colleagues (2007) demonstrated that administering chronic caffeine (15-20 mg/kg/day by gavage) to rats between postnatal days 2 and 6 produced deficits in inhibitory avoidance conditioning, a form of conditioning that involves the hippocampus (Martínez, Quirarte, & Diaz-Cintra, 2002), when animals were tested in adolescence (postnatal days 25-42). Therefore, findings suggest that prenatal and early postnatal exposure to chronic caffeine results in long-lasting deficits in hippocampus-dependent conditioning when caffeine is not on board.

There have been several reports that acute caffeine specifically alters hippocampus-dependent learning and memory in normal adult rodents (Angelucci, Cesário, Hiroi, Rosalen, & Cunha, 2002; Angelucci et al., 1999; Corodimas, Stieg, & Pruitt, 2000). For example, Angelucci and colleagues (2002) found that administering

acute caffeine (0.3 mg/kg) to adult rats immediately after training or 30 minutes before testing in the spatial version of the Morris water maze enhanced retention and retrieval, respectively. In addition, Corodimas and colleagues (2000) found that administering acute caffeine (30 mg/kg) to adult rats prior to training in contextual fear conditioning produced deficits in the acquisition of contextual conditioning, yet exerted no effect on cued conditioning. Furthermore, in the same study, administering acute caffeine prior to both training and testing in contextual fear conditioning produced deficits in contextual conditioning, yet exerted no effect on cued conditioning. Interestingly, administering acute caffeine prior to testing alone produced deficits in contextual retrieval. Therefore, acute caffeine has been shown to impair the acquisition of memories that engage the hippocampus, and to both facilitate (at 0.3 mg/kg) and impair (at 30 mg/kg) the retrieval of memories that engage the hippocampus depending on dose.

In contrast to the reported effects of acute caffeine on hippocampus-dependent learning and memory in adults, several studies on the effects of chronic caffeine on hippocampus-dependent learning and memory in adults have found no effect of treatment (Alhaider, Aleisa, Tran, Alzoubi, & Alkadhi, 2010; Alzoubi et al., 2013; Corodimas et al., 2000). For example, Alhaider and colleagues (2010) administered chronic caffeine at 0.3 mg/mL to adult Wistar rats for 4 weeks and found that treatment had no effect on performance in the radial arm water maze. In addition, Corodimas and colleagues (2000) treated adult Sprague Dawley rats with chronic caffeine for 7 days and found that treatment had no effect on contextual fear conditioning. While findings suggest that exposure to chronic caffeine exerts no effect on hippocampus-dependent learning and memory in normal adult rodents, it remains to be discovered if chronic caffeine exerts the

same negligible effects on hippocampus-dependent learning and memory in pre-adolescent and adolescent animals. Again, to date, findings only support the conclusion that repeated (e.g. Pires et al., 2010) or chronic exposure to caffeine during pre-adolescence (e.g. Ardais et al., 2014) and/or adolescence (e.g. Abreu et al., 2011) alters novel object recognition memory. However, no studies have utilized behavioral paradigms with the goal of elucidating the effect of exposure to chronic caffeine during pre-adolescence and adolescence on specific memory systems. As will be discussed in the next section, evidence suggests that the developing hippocampus may be more susceptible to caffeine-induced modifications than the adult hippocampus. Thus, hippocampus-dependent learning and memory may be compromised by chronic caffeine exposure during pre-adolescence and adolescence.

The pre-adolescent and adolescent hippocampus: Vulnerability to psychostimulant-induced modifications

The hippocampus is essential for explicit memory in humans (Eichenbaum, 2001; Squire, 1987) and spatial/contextual learning and memory in rodents (Phillips & LeDoux, 1992; Rudy, 1993; Young, Fox, & Eichenbaum, 1994). In addition, the hippocampus undergoes structural (Pokorný & Yamamoto, 1981; Seress & Ribak, 1995; Zehr, Nichols, Schulz, & Sisk, 2008) and functional (Pyapali, Turner, Wilson, & Swartzwelder, 1999; Swartzwelder, Wilson, & Tayyeb, 1995a, 1995b; White & Swartzwelder, 2004) modifications during adolescence that may make it more vulnerable to psychostimulant-induced modifications (e.g. Slotkin et al., 2008). For example, throughout pre-adolescence and adolescence spine densities in the hippocampus increase (Seress & Ribak 1995a, Seress & Ribak 1995b), granule cells in the infrapyramidal blade of the

dentate gyrus undergo dendritic pruning (Zehr et al., 2008), and synapses become more stable (Insausti, 2010; Zehr et al., 2008).

While the effects of chronic caffeine on hippocampal structure in pre-adolescent and adolescent animals are not known, chronic caffeine has been shown to modify hippocampal structure in a model of age-related cognitive decline. Specifically, Vila-Luna and colleagues (2012) demonstrated that exposing rats to chronic caffeine through the drinking water (0.03-0.08 mg/mL) starting in young adulthood (at ~3 months of age) and ending in middle age (at ~9 months of age) increased the length, branching, and spine density of basal dendrites in CA1 neurons when hippocampal slices were analyzed after a 30 day period of withdrawal (at 10 months of age). Furthermore, in the same study, chronic caffeine prevented age-related deficits in working memory, suggesting these chronic caffeine-associated structural changes may be one mechanism supporting the reversal of age-related working memory deficits. However, there is evidence that the caffeine-induced changes in hippocampal structure reported by Vila-Luna and colleagues (2012) could compromise learning and memory in adolescent mice. For example, while not caffeine, Valladolid-Acebes and colleagues (2013) demonstrated that administering a high-fat diet to adolescent mice increased the spine density of neurons in the CA1 region of the hippocampus; however, this increase in spine density was associated with impaired spatial memory in the object location task – a task that relies on the hippocampus to a greater degree than other forms of object recognition (Barker et al., 2011). These findings are not surprising as inefficient pruning during hippocampal development has been shown to impair learning and memory in humans as well (Molnár & Kéri, 2014). Therefore, while chronic caffeine may prevent memory deficits associated with aging

(Vila-Luna et al., 2012), possibly by modifying hippocampal structure, the effects of exposure to chronic caffeine during adolescence remain unknown.

Importantly, chronic caffeine can modulate synaptic plasticity (a neurophysiological correlate of learning and memory (Martin, Grimwood, & Morris, 2000)) in the hippocampus (for review see Costenla, Cunha, & Mendonça, 2010). However, these effects have primarily been observed in adult animal models of disrupted cognition. For example, chronic caffeine prevents impairment of early-phase long-term potentiation (LTP) in the dentate gyrus of sleep-deprived adult rats, yet has no effect in control rats (Alhaider et al., 2010). In addition, chronic caffeine prevents stress-induced impairment of LTP in adult rats, yet has no effect in control rats (Alzoubi et al., 2012). Interestingly, acute caffeine does not appear to facilitate LTP in normal adult rats either (Stepan et al., 2012), and may actually inhibit post-tetanic potentiation of area CA1 (see Lee et al., 1987). In contrast, Martin & Buño (2003) demonstrated that in hippocampal slices from 13-17 day old (i.e. pre-adolescent) rats a brief pulse of caffeine could evoke a non-decremental LTP of Schaffer collateral excitatory postsynaptic currents that did not require NMDA receptor activation or postsynaptic-free calcium. In addition, Simons and colleagues (2012) demonstrated that treating 14 to 42 day old (i.e. pre-adolescent to adolescent) rats with acute caffeine could induce a robust, long-lasting potentiation of synaptic transmission in area CA2 of the hippocampus. While the effects of chronic caffeine on synaptic plasticity in the pre-adolescent and adolescent hippocampus are not yet known, the findings that acute caffeine can induce LTP in the juvenile hippocampus (Martin & Buño, 2003; Simons et al., 2012), but not the adult hippocampus (Stepan et al.,

2012), supports the notion that chronic caffeine may have age-dependent effects on synaptic plasticity as well.

To our knowledge, only one study has examined the effect of chronic caffeine-induced neurochemical changes in the adolescent hippocampus. In this study, Ardais and colleagues (2014) found that chronic caffeine increased adenosine A1 receptor levels (caffeine is an adenosine receptor antagonist, which will be discussed in the next section), yet decreased BDNF, proBDNF, GFAP, and SNAP-25 in the hippocampus (the authors probed for these proteins due to their known roles in “cognition and synaptic integrity”). Interestingly, while chronic caffeine is known to increase the expression of adenosine A1 receptor in the adult hippocampus (Fredholm, 1982), chronic caffeine has no effect on BDNF levels in the normal adult hippocampus (Alzoubi et al., 2012). Therefore, evidence suggests that chronic caffeine may have age-dependent effects on BDNF levels in the hippocampus. While the consequences of these effects are not known, BDNF is essential for LTP (Pang et al., 2004), which may suggest that chronic caffeine exposure during adolescence can compromise LTP. However, the significance of these neurochemical changes, as well as their persistence, deserve further investigation.

Despite the limited information available on the effects of chronic caffeine on the adolescent hippocampus, several studies have found that chronic exposure to other psychostimulants during adolescence can induce functional changes in the hippocampus that last into adulthood. For example, Adriani and Laviola (2004) found that adolescent mice repeatedly administered acute injections of nicotine for 10 days during mid-adolescence showed a down-regulation of AMPA receptor subunits (GluR2/3) in the hippocampus in adulthood, whereas animals exposed to the same schedule of nicotine

treatment in adulthood showed no changes in GluR2/3 levels in the hippocampus. In another study, mice chronically treated with nicotine during adolescence showed an increase in choline transporter levels, as measured by hemicholinium-3 (HC3) binding, in the hippocampus in adulthood (Slotkin, Bodwell, Ryde, & Seidler, 2008). Furthermore, Calabrese and colleagues (2013) found that repeated exposure to amphetamine during adolescence increased *bdnf* transcripts and *cfos* mRNA in the hippocampus in adulthood. While no adult control groups were used in any of the aforementioned studies except the one conducted by Adriani and Laviola, these studies seem to support the notion that pre-adolescence and adolescence may be critical periods for psychostimulant effects on hippocampal function.

Overall, findings suggest that modifying the structure and function of the developing hippocampus can permanently affect this region (e.g. White & Swartzwelder, 2004). In contrast, psychostimulant-induced modifications in the adult hippocampus are less severe than in the adolescent hippocampus (e.g. Valzachi et al., 2013; Adriani & Laviola, 2004). Furthermore, the adult hippocampus may show a greater return to a pre-drug state after a period of protracted withdrawal compared to the adolescent hippocampus (e.g. Trauth, Seidler, McCook, & Slotkin, 1999). Therefore, exposure to chronic caffeine during pre-adolescence and adolescence could permanently alter hippocampal structure and function, yet these changes may not be as severe or persistent in the normal adult hippocampus.

Caffeine mechanism of action

Caffeine is an adenosine receptor antagonist, and therefore many of the behavioral effects of caffeine are mediated by adenosine receptors (Nehlig, Daval, & Debry, 1992;

Svenningsson, Nomikos, & Fredholm, 1999). Furthermore, adenosine, the endogenous ligand for adenosine receptors, plays an important role in regulating cellular and molecular processes underlying learning and memory (Dias, Rombo, Ribeiro, Henley, & Sebastião, 2013). For example, adenosine regulates neurotransmitter release (Sebastião & Ribeiro, 2009a, 2009b; Van Dort, Baghdoyan, & Lydic, 2009), neuronal excitability (Li et al., 2011), and synaptic plasticity (Mendonça & Ribeiro, 2001) in many brain regions (Fredholm, Chen, Cunha, Svenningsson, & Vaugeois, 2005). To date, four adenosine receptor subtypes have been cloned (i.e. A1, A2A, A2B, and A3) (Fredholm, 2010); however, A1R and A2AR are the main targets of caffeine in the central nervous system (Fredholm et al., 1999). Therefore, the effects of caffeine on cognition are likely the result of A1R and/or A2AR blockade (Fredholm et al., 1999).

A1R and A2AR are G-protein coupled receptors (GPCRs) (Fredholm, 2010), meaning their activation results in the release of receptor-associated subunits ($\alpha_{i/o}$ and α_s , respectively), which can modulate intracellular signal transduction pathways (Grishina & Berlot, 1997). For example, when A1Rs are activated, the α subunits of G_i can inhibit adenylyate cyclase and decrease the production of cAMP (Dias et al., 2013), a second messenger that activates PKA (a kinase that is important for learning and memory (Kandel, 2012)). Furthermore, A1R activation can modulate inwardly rectifying potassium channels at postsynaptic sites, resulting in hyperpolarization, which can decrease neuronal responsiveness to excitatory inputs (Fredholm et al., 2005). In contrast, when A2ARs are activated, the α_s subunit can increase levels of cAMP by activating adenylyate cyclase (Dias et al., 2013). Overall, the activation of pre-synaptic A1Rs typically decreases the probability of neurotransmitter release by inhibiting adenylyate

cyclase, whereas the activation of pre-synaptic A2ARs typically enhances the probability of neurotransmitter release by activating adenylate cyclase (Dias et al., 2013). It should be noted that both A1R and A2AR can be found pre-synaptically, post-synaptically, and non-synaptically (Vizi, Fekete, Karoly, & Mike, 2010). In addition, adenosine receptors are expressed by both neurons and glial cells (Boison, Sandau, Ruskin, Kawamura, & Masino, 2013). The ubiquitous presence of adenosine receptors in the central nervous system highlights the important neuromodulatory role of the adenosinergic system. In the hippocampus, adenosine balances inhibition and excitation towards homeostasis (Dias et al., 2013). Therefore, caffeine (and other adenosine receptor antagonists) have the ability to interfere with hippocampal homeostasis. However, the degree to which caffeine can disrupt homeostasis (i.e. increase excitation) in the hippocampus depends on the relative balance of adenosine receptors in this brain region, which will be discussed below.

Adenosinergic modulation of hippocampal function

Again, the main targets of caffeine in the central nervous system are A1Rs and A2ARs and both of these receptors are expressed in the hippocampus (Fastbom, Pazos, Probst, & Palacios, 1987; Johansson, Georgiev, Lindström, & Fredholm, 1997; Swanson, Drazba, & Rivkees, 1995). Furthermore, both adenosine receptor agonists and antagonists have been shown to modulate synaptic plasticity in the hippocampus (Arai, Kessler, & Lynch, 1990; Mendonça & Ribeiro, 1994; Sekino, Ito, Miyakawa, Kato, & Kuroda, 1991). Specifically, adenosine (Arai et al., 1990) and other adenosine receptor agonists (Mendonça & Ribeiro, 2001) prevent LTP in the hippocampus. In contrast, A1R antagonism has been shown to facilitate LTP (Mendonça & Ribeiro, 1994), whereas A2AR antagonism has been shown to attenuate LTP (Costenla et al., 2011).

Similar to the effects of caffeine, the effects of adenosine receptor agonists and antagonists on hippocampal plasticity depends on age. For example, Costenla and colleagues (2011) found that in young adult rats (2-3 months old) selective A2AR antagonism with SCH58261 attenuated LTP in the hippocampus by approximately 36%, yet in aged animals (18-20 months) SCH58261 attenuated LTP by approximately 63%. Thus, A2AR antagonism has a greater effect on LTP in aged animals. In the same study the selective A1R antagonist DPCPX increased LTP magnitude in young adult rats by approximately $42 \pm 6\%$, yet had no effect in middle-aged adults (6-8 months) or aged (18-20 months) rats. Thus, A1R antagonism has a greater effect on LTP in younger adult animals. Given that chronic caffeine affects learning and memory in aged animals (e.g. Sallaberry et al., 2013), but not normal adult animals (e.g. Corodimas et al., 2000), it may be the case that the effects of chronic caffeine on cognition are primarily mediated by A2AR blockade as opposed to A1R blockade.

Interestingly, while acute A2AR antagonism attenuates LTP in normal adults (Costenla et al., 2011), chronic A2AR antagonism has no effect on LTP in normal adults (Batalha et al., 2013). In contrast, chronic A2AR antagonism has been shown to ameliorate stress-induced deficits in LTP in adult rats (Batalha et al., 2013). Therefore, acute and chronic adenosine receptor antagonism may have different, or even opposite effects on LTP. Furthermore, in agreement with these findings, acute and chronic adenosine receptor antagonism have been shown to elicit effects on learning and memory that are diametrically opposed (for review see Jacobson, von Lubitz, Daly, & Fredholm, 1996). This is an important point because, as was previously described, acute caffeine

induces LTP in the pre-adolescent hippocampus (Martin & Buño, 2003), which may suggest that chronic caffeine has the ability to compromise LTP in this age group.

Adenosinergic modulation of learning and memory

Many studies have demonstrated that adenosine receptor agonists disrupt learning and memory in adult rodents (Normile, Gaston, Johnson, & Barraco, 1994; Normile & Barraco, 1991; Ohno & Watanabe, 1996; Zarrindast & Shafaghi, 1994), yet adenosine receptor antagonists facilitate learning and memory in adult rodents (Angelucci et al., 2002; Angelucci et al., 1999; Hauber & Bareiss, 2001; Pereira et al., 2002). For example, Ohno and Watanabe (1996) found that the selective A1R agonist N6-cyclohexyladenosine (CHA) significantly increased the number of errors in a working memory task when infused directly into the dorsal hippocampus of adult rats. However, pretreatment with the A1R antagonist 1, 3-dipropyl-8-cyclopentylxanthine (DPCPX) prevented the increase in working memory errors induced by direct infusion of CHA. A1R agonists have been shown to specifically impair the acquisition of contextual fear memory in adult rats as well (Corodimas & Tomita, 2001). It should be noted that while acute A1R antagonism can ameliorate deficits induced by A1R agonism (Ohno & Watanabe, 1996), A1R antagonism has no effect on memory in normal adults (Kopf et al., 1999; Normile & Barraco, 1991). In contrast, chronic A1R antagonism has been shown to produce deficits in long-term memory in the Morris water maze (Vollert, Forkuo, Bond, & Erikson, 2013).

While the effects of acute A1R antagonism on learning and memory depend on whether or not A1R is pharmacologically activated, acute A2AR antagonism has been shown to enhance memory consolidation in normal adults. For example, blockade of

adenosine A2ARs has been shown to enhance memory consolidation and retention in the passive avoidance task (Kopf, Melani, Pedata, & Pepeu, 1999). Moreover, mice lacking adenosine A2ARs show improved spatial recognition memory (Wang et al., 2006). Overall, chronic A2AR antagonism appears to enhance memory (Kopf, Melani, Pedata, & Pepeu, 1999), whereas chronic A1R antagonism appears to produce deficits in memory (Vollert, Forkuo, Bond, & Erikson, 2013).

Age-dependent changes in adenosinergic signaling

As animals age, adenosine receptors undergo adaptive changes. Several studies have found that there is a consistent decrease in the density of A1Rs (e.g. Castillo et al., 2009 found a decrease between postnatal days 21 and 180, whereas Meerlo et al., 2004 found a gradual decrease in 3, 24, and 30 month old animals) and an increase in the density of A2ARs in the hippocampus (e.g. Costenla et al., 2011 found a gradual increase in rats that were 2-3, 6-8, and 18-20 months old; Cunha, Constantino, Sebastião, & Ribeiro, 1995 found an increase when hippocampi were dissected at 24 months compared to 6 weeks), cerebral cortex (Cunha et al., 1995), and whole brain (Castillo et al., 2009). Furthermore, age-related changes in adenosine receptor levels in the hippocampus correspond to altered modulation of hippocampal synaptic transmission by A1R and A2AR (Rebola et al., 2003; Sebastiao, Cunha, Mendonça, & Ribeiro, 2000). Specifically, A2AR-mediated facilitation of synaptic transmission in the hippocampus is larger in aged rats (24 months old) compared to young rats (6 weeks old) due to the increase in A2AR density on hippocampal nerve terminals in older animals (Sebastiao et al., 2000). Interestingly, Costenla and colleagues (2011) found that there are a greater number of glutamatergic terminals that express A2AR in the hippocampus of aged rats (18-20

months old) compared to young rats (2-3 months old), which may explain why chronic caffeine improves cognition in aged animals (Cunha & Agostinho, 2010), but not normal adults (Alhaider et al., 2010; Corodimas et al., 2000). Overall, A1Rs appear decrease as animals' age and A2ARs increase, which has consequences for adenosine receptor mediated plasticity and the effects of chronic caffeine on learning and memory.

This change in receptor density and function, whereby adenosine potentially activates A2ARs more and A1Rs less in the aged animal relative to the younger adult animal, may help us predict the age-dependent effects of caffeine. For example, while studies suggest that chronic caffeine exerts no effect on learning and memory in normal adult animals (Alhaider et al., 2010; Corodimas et al., 2000), chronic caffeine has been shown to prevent or reverse learning and memory deficits caused by aging (Vila-Luna et al., 2012), neurodegenerative diseases (Cunha & Agostinho, 2010), and sleep deprivation (Alhaider et al., 2010). Interestingly, some of these effects of chronic caffeine on cognition in adults can be mimicked by A2AR antagonists, but not by A1R antagonists (Dall'Igna et al., 2007; Prediger, Batista, & Takahashi, 2005). Therefore, differences in central expression of A1Rs or A2ARs could determine the effect of chronic caffeine on learning and memory in pre-adolescent, adolescent, and adult animals. Specifically, chronic caffeine may have beneficial effects in a system that expresses high levels of A2AR and low levels of A1R, but detrimental effects in a system that expresses low levels of A2AR and high levels of A1R.

*The adolescent amygdala and prefrontal cortex: Vulnerability to
caffeine-induced modifications*

While the main focus up to this point has been on the potential effects of chronic caffeine on the pre-adolescent and adolescent hippocampus/hippocampus-dependent learning and memory, there are other brain regions that display structural and functional differences during adolescence compared to adulthood (Casey, Jones, & Levita, 2010) that may be affected by exposure to chronic caffeine. For example, the amygdala is undergoing dendritic pruning (Cunningham, Bhattacharyya, & Benes, 2002; Zehr et al., 2006) and shows an exaggerated response to affective cues during adolescence relative to childhood and adulthood (Ernst et al., 2005; Guyer et al., 2008). Importantly, presynaptic A1Rs mediate excitatory transmission in the amygdala (Rau, Ariwodola, & Weiner, 2014), which could suggest that caffeine has the ability to disinhibit the adolescent amygdala to a greater degree than the adult amygdala. The prefrontal cortex also continues to develop throughout adolescence and into adulthood (Casey, Jones, & Levita, 2010). For example, the prefrontal cortex displays higher levels of dendritic pruning during childhood and adolescence (Cunningham et al., 2002) compared to adulthood, and is not fully myelinated until the mid to late 20's in humans (Arain et al., 2013). Furthermore, adenosine receptors in this region are important for arousal (Van Dort et al., 2009), and changes in levels of arousal can regulate the consolidation of long-term memories (McGaugh, 2013). Moreover, there is evidence that exposure to nicotine during adolescence may lead to prefrontal neuroadaptations that are more pronounced than if exposure occurs in adulthood (Schochet, Kelley, & Landry, 2004). Overall, caffeine may modulate learning and memory differently in adolescent animals by increasing excitation

in the amygdala and/or prefrontal cortex, which might change affective processing or levels of arousal.

Interestingly, the functional and structural changes occurring in the prefrontal cortex, the amygdala, and the circuitry connecting these structures during adolescence appears to underlie a behavioral phenotype associated with increased consumption of caffeine. Specifically, adolescent animals engage in risk-taking and sensation seeking behaviors (Johnson, Blum, & Giedd, 2010; Steinberg et al., 2008), and findings suggest that sensation seeking is associated with higher intake of caffeine (Herz, 1999). The relative delay in prefrontal cortex development (i.e. the prefrontal cortex is not fully myelinated until adulthood, whereas subcortical regions are myelinated earlier in life (Arain et al., 2013; Giedd, 2004)) and thus decreased ability to exert neuromodulatory control over the limbic cortex (Kalsbeek, Voorn, Buijs, Pool, & Uylings, 1988; Lewis, Sesack, Levey, & Rosenberg, 1998) contribute to the emotional disturbance and poor decision making displayed during adolescence (Arain et al., 2013). Specifically, the amygdala is not yet fully connected to the prefrontal cortex during adolescence (Casey, Jones, & Hare, 2008), which may explain the enhanced amygdala activity during this developmental period (Ernst et al., 2005; Guyer et al., 2008). Therefore, altering the development of the prefrontal cortex can potentially have long-term consequences for the proper inhibitory control to be exerted over the amygdala, which may lead to emotional disorders (Arnsten & Rubia, 2012). Likewise, connections between the prefrontal cortex and the hippocampus are important for attention (Wall & Messier, 2001) and memory as well (Frankland & Bontempi, 2005), and pre-natal exposure to chronic caffeine has been

shown to impair both working and reference memory in the radial arm maze (Soellner et al., 2009).

*Fear conditioning: A model for studying hippocampus-dependent and
hippocampus-independent learning and memory*

Many of the brain regions that display structural and functional changes during adolescence compared to adulthood are important for different aspects of contextual and cued fear conditioning (e.g. the hippocampus is important for contextual conditioning (Fanselow & Poulos, 2005), the amygdala is important for the acquisition and expression of fear memories (Ciocchi et al., 2010), and the prefrontal cortex is important for processing contextual information, fear memory retrieval, and extinction learning (Maren, 2011)). Thus, in the following studies, we employed contextual fear conditioning in order to examine the immediate (see Chapter 2) and long-lasting (see Chapter 3) effects of chronic caffeine exposure during pre-adolescence, adolescence, and adulthood on hippocampus-dependent (contextual) and hippocampus-independent (cued) learning and memory.

In fear conditioning, an innocuous conditioned stimulus (CS) (e.g. a tone) is presented, which co-terminates with an aversive unconditioned stimulus (US) (e.g. a footshock) that activates an unconditioned fear responses (URs) (e.g. startle or freezing) (Kim & Jung, 2006). The CS-US associations that are formed then come to elicit conditioned responses (CRs), like freezing (previously described as “crouching”), which are similar to innate fear responses (Blanchard & Blanchard, 1969). Contextual fear conditioning training results in the formation of two associations: an association between the training context and the US (contextual fear conditioning), which depends upon the

hippocampus, whereas the association between the CS and the US (cued fear conditioning) does not depend critically upon the hippocampus (Phillips & LeDoux, 1992). Thus, by employing contextual and cued fear conditioning, we were able to determine if chronic caffeine specifically acts on hippocampus-dependent learning and memory in pre-adolescent and adolescent animals, or if chronic caffeine has more general effects on learning and memory in these age groups. The findings presented in this study will be important for determining the age-dependent effects of exposure to chronic caffeine on learning and memory.

CHAPTER 2

AGE-DEPENDENT EFFECTS OF CHRONIC CAFFEINE AND WITHDRAWAL FROM CHRONIC CAFFEINE ON FEAR CONDITIONING

Introduction

Caffeine is the only licit psychoactive drug available to minors in all 50 states (Luebbe & Bell, 2009). Possibly as a result of caffeine's availability, approximately 75-95% of children and adolescents consume caffeine on a regular basis (Frary et al., 2005; James et al., 2011). In addition, the number of youth who consume caffeine and the quantities consumed appear to be growing (Temple, 2009), yet there is currently limited information available on how caffeine affects the pre-adolescent and adolescent brain (Temple, 2009).

Chronic caffeine has been shown to reverse age-related deficits in learning and memory, deficits induced by sleep deprivation, and deficits in models of impaired cognition (e.g. Alzheimer's disease models and attention deficit disorder models) (for review see Cunha & Agostinho, 2010). However, chronic caffeine does not appear to affect learning and memory in normal adult humans (Herz, 1999; Koppelstaetter et al., 2008; Warburton, 1995) or rodents (Alhaider et al., 2010; Alzoubi et al., 2013; Corodimas et al., 2000). Likewise, human studies suggest that 24-48 hours of withdrawal from chronic caffeine has no effect on memory in adults (Addicott & Laurienti, 2009; Comer, Haney, Foltin, & Fischman, 1997; Lane & Phillips-Bute, 1998).

While chronic caffeine and withdrawal from chronic caffeine may have no effect on learning and memory in adulthood, habitual caffeine consumption in early and middle adolescence is associated with ADHD-like symptoms (Dosh et al., 2010) and negative academic outcomes (James et al., 2011). In addition, habitual caffeine consumption

during childhood is associated with poor performance on cognitive tests (Heatherley et al., 2006). Furthermore, Bernstein and colleagues (1998) found that children who habitually consumed caffeine performed worse on a test of attention 24 hours after caffeine cessation. Thus, because attention is essential for normal learning (Chun & Turk-Browne, 2007), children may experience withdrawal-induced learning deficits. Overall, there appears to be a relationship between caffeine consumption during pre-adolescence and adolescence and impaired cognition.

Few studies have been conducted to address the causal relationship between exposure to chronic caffeine during pre-adolescence and adolescence and changes in cognition in non-human animals. The information available suggests that repeated exposure to chronic caffeine during pre-adolescence (between postnatal days 25 and 38) impairs discriminatory ability in the novel object recognition task in adulthood (i.e. when animals are 9-10 weeks old) (Pires, Pamplona, Pandolfo, Prediger, & Takahashi, 2010), yet continuing caffeine treatment throughout pre-adolescence, adolescence, and adulthood (between postnatal days 21 and 90) enhances object recognition memory when animals are tested with caffeine on board (Abreu, Silva-Oliveira, Moraes, Pereira, & Moraes-Santos, 2011). These findings may suggest that exposure to chronic caffeine during pre-adolescence and adolescence specifically alters hippocampus-dependent learning and memory as object recognition memory engages the hippocampus (Broadbent et al., 2009; Winters et al., 2008). However, to our knowledge, no studies have provided conclusive evidence that exposure to chronic caffeine during pre-adolescence or adolescence specifically affects hippocampus-dependent learning and memory as opposed to learning and memory in general. Moreover, it has not yet been determined if

exposure to chronic caffeine during pre-adolescence and adolescence enhances or disrupts learning and memory.

Caffeine is a non-specific adenosine receptor antagonist (Fredholm et al., 1999), and the cognitive and behavioral effects of caffeine are known to be due to the blockade of adenosine A1 and A2A receptors (A1Rs and A2ARs) (Fisone, Borgkvist, & Usiello, 2004). Furthermore, the hippocampus expresses high levels of A1Rs, yet lower levels of A2ARs throughout life (Costenla et al., 2010; Cunha et al., 1995; Fastbom et al., 1987; Fredholm et al., 2005; Rosin & Robeva, 1998). However, between late adolescence (postnatal day 42) and adulthood (postnatal day 180) the density of A1Rs decrease and the density of A2ARs increase in the hippocampus (Cunha et al., 1995), which could define the effects of chronic caffeine on hippocampal function. For example, Costenla and colleagues (2011) found that selective A1R blockade increases LTP magnitude in young adult rats, but has no effect on middle-aged or aged rats. Furthermore, in the same study, selective A2AR blockade attenuated LTP to a greater degree in aged rats compared to middle-aged and adult rats. Therefore, age-related differences in adenosine receptor levels could define the effect of caffeine on hippocampal LTP, a neurophysiological correlate of learning and memory (Martin, Grimwood, & Morris, 2000).

In addition to age-related changes in adenosinergic signaling, the hippocampus is undergoing structural (Pokorný & Yamamoto, 1981; Seress & Ribak, 1995; Zehr, Nichols, Schulz, & Sisk, 2008) and functional (Pyapali, Turner, Wilson, & Swartzwelder, 1999; Swartzwelder, Wilson, & Tayyeb, 1995a, 1995b; White & Swartzwelder, 2004) modifications during adolescence that may make it more vulnerable to psychostimulant-induced modifications. In support, exposure to the psychostimulant nicotine induces more

pronounced and longer-lasting modifications in the adolescent hippocampus compared to the adult hippocampus (Slotkin et al., 2008).

Overall, the goal of the present study was to investigate the age-dependent effects of chronic caffeine and withdrawal from chronic caffeine on contextual and cued fear conditioning. In fear conditioning, learning to associate the context with a shock unconditioned stimulus (US) is hippocampus and amygdala dependent (Phillips & LeDoux, 1992), whereas forming an association between an auditory cued conditioned stimulus (CS) and the shock, is independent of the hippocampus but dependent on the amygdala (Phillips & LeDoux, 1992). Therefore, we were able to dissociate the effects of chronic caffeine on hippocampus-dependent conditioning from the effects of chronic caffeine on learning and memory in general.

We hypothesized that chronic caffeine would enhance contextual conditioning in pre-adolescent and adolescent mice, but not adult mice due to previous findings that chronic exposure to caffeine starting in pre-adolescence and continuing through testing enhances object recognition (Abreu et al., 2011; Ardais et al., 2014), and that chronic exposure to the psychostimulant nicotine enhances contextual conditioning in pre-adolescent mice, but not adult mice (Portugal et al., 2012). In addition, we hypothesized that withdrawal from chronic caffeine would impair both contextual and cued learning and memory in pre-adolescent and adolescent mice because previous research suggests that adolescent rats show a decreased response to an acute challenge of caffeine during withdrawal compared to adults (Rhoads et al., 2011), which may suggest adolescent animals show tolerance to the effects of caffeine. Furthermore, signs of withdrawal may be associated with an increased sensitivity to endogenous adenosine (Nehlig et al., 1992),

and adenosine receptor agonism has been shown to impair both contextual and cued learning and memory (Corodimas & Tomita, 2001).

We also examined the effect of exposure to chronic caffeine on adenosine A2AR levels in the pre-adolescent and adult hippocampus, as findings from a radioligand binding study suggest that A2AR levels increase in the hippocampus between postnatal day 42 and postnatal day 180 (Cunha et al., 1995), yet it is not known if A2AR levels increase between pre-adolescence and adulthood. Furthermore, there is evidence that chronic antagonism of A2ARs mimics the effects of chronic caffeine on cognition in prenatal animals (Silva et al., 2013) and animals with age-related cognitive deficits (for review see Cunha & Agostinho, 2010), suggesting that differences in A2AR levels may underlie the effects of chronic caffeine on cognition. Finally, we investigated the age-dependent effects of chronic caffeine on anxiety-related behavior as a control for changes in anxiety, because chronic caffeine was recently shown to increase anxiety-related behavior in adolescent rats (Ardais et al., 2014) and anxiety can influence the expression of fear (Davis, Walker, & Lee, 1997; Bannerman et al., 2004; Helmstetter, 1993).

Identifying the age-dependent cognitive and behavioral effects of chronic caffeine, as well as the neural substrates of these effects, will be important for determining how chronic caffeine might impact the developing brain differently than the adult brain. Importantly, our fear conditioning results will help determine if chronic caffeine has generalized effects on learning and memory in pre-adolescent and adolescent animals, or if specific learning and memory systems are affected by chronic caffeine during these developmental periods.

Methods

Subjects

Male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). C57BL/6J mice were selected because robust fear conditioning has been previously demonstrated in this strain (Balogh & Wehner, 2003; Bolivar, Pooler, & Flaherty, 2001; Owen, Logue, Rasmussen, & Wehner, 1997). Housing rooms were illuminated on a 12 hour light/dark cycle with lights on at 7:00 AM and mice received *ad libitum* access to food and water. All behavioral procedures were performed between 11:00 AM and 5:00 PM and were approved by the Temple University Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Mice arrived one week prior to the start of experiments so that they could acclimate to the colony room. Pre-adolescent mice were shipped with dams and were postnatal day (PND) 16 on the day of arrival, adolescent mice were PND 31 on the day of arrival, and adult mice were PND 64 on the day of arrival. The neurodevelopmental and behavioral changes that occur in humans are analogous to those that happen in rodents (Schneider, 2013; Spear, 2000). For example, at approximately PND 30 male mice exhibit signs of puberty, increased risk-seeking, impulsivity, and reward sensitivity, and begin exhibiting social behavior that is more characteristic of adult mice (Schneider, 2013; Terranova, Laviola, de Acetis, & Alleva, 1998). In mice, early adolescence occurs between PND 21 - 28, middle-adolescence occurs between PND 34 - 46, late adolescence occurs between PND 46 - 59, and adulthood has been described as PND 60 and beyond (Hefner & Holmes, 2007; Laviola, Macrì, Morley-Fletcher, & Adriani, 2003; Spear,

2000). Therefore, at the start of all experiments, pre-adolescent mice were PND 21, adolescent mice were PND 38, and adult mice were PND 70. Mice were housed 2 per cage.

Apparatus

Fear conditioning Training and testing of contextual fear conditioning occurred in four identical chambers (17.78 cm X 19.05 cm X 38.10 cm) that were housed in sound attenuating boxes (Med-Associates, St. Albans, VT). The front and back walls of the training chambers were constructed from Plexiglas and the side walls were aluminum. The bottoms of the training chambers were composed of stainless steel grid floors (rods were 2 mm in diameter and spaced 1 cm apart) connected to a scrambled shock generator. Background noise (69 dB) during training and testing was provided by ventilation fans. Stimulus administration during training and testing was controlled by a computer running Med-PC software. Cued fear conditioning was tested in an altered context, which consisted of four identical chambers with different dimensions than the training context (20.32 cm X 22.86 cm X 17.78 cm). The front and back walls of the altered context were constructed from Plexiglas, the side walls were constructed from aluminum, and the floors were constructed from smooth plastic. Additionally, the altered context chambers were located in a different room from the training chambers. A vanilla scent was used to further distinguish the altered context from the training context. Thus, the altered context chambers differed from the training chambers in size, construction, tactile cues, visual cues, and olfactory cues. The training and testing chambers used in the present study were previously described by Davis and colleagues (2005).

Zero maze The zero maze was constructed of white Plexiglas and consisted of a 5.5 cm wide circular track with an inside diameter of 34 cm, a mid-track circumference of 121 cm, and an elevation of 40 cm. The maze has two open quadrants with a raised 2 mm edge and two closed quadrants with walls 11 cm high. Lighting, measured using a lux meter, was held at 40 lux. White noise (69dB) was generated by a fan in the room.

Behavioral Procedures

Fear Conditioning The procedure for fear conditioning was similar to that used in previous studies (Davis, James, Siegel, & Gould, 2005; Gould & Higgins, 2003).

Training was initiated by placing mice in the training context and activating the house lights. Baseline freezing was then scored for 120 s at which point a 30 s white noise conditioned stimulus (CS, 85 dB) was activated that co-terminated with a 2 s 0.62 mA footshock unconditioned stimulus (US). Immediate freezing was then scored during a 120 s inter-trial interval and was followed by a second CS-US pairing. The end of the training session consisted of a 30 s interval that terminated when the house lights turned off. The training session lasted a total of 5.5 min. For the reduced training protocol, mice were trained with one 15 s white noise CS that co-terminated with a 2 s 0.62 mA footshock US as was described previously (Gould et al., 2004). Freezing was defined as the absence of all movement except respiration (Blanchard & Blanchard, 1969) and was scored using a time-sampling procedure for 1 s during 10 s intervals. Freezing was the dependent variable and the measure of learning and memory for all fear conditioning experiments.

Contextual fear conditioning was tested 24 h after training by returning mice to the training chambers, activating the house lights, and scoring freezing for 5 min. Cued fear conditioning was tested 1 h after contextual fear testing by placing mice in the

altered context, activating the house lights, and scoring freezing for 6 min. During the first 3 min, freezing was scored in the absence of the CS. During the last 3 min, freezing was scored in the presence of the CS. The sessions terminated when the house lights were turned off. A solution of 70% ethanol was used to clean the conditioning chambers after training and testing.

Shock sensitivity Mice were tested for shock sensitivity in the same chambers used for fear conditioning as described in previous studies (Gulick & Gould, 2009; Kenney, Wilkinson, & Gould, 2010) with minor modifications. In brief, after 3 min of acclimation to the chambers, mice were exposed to a range of 2 s foot shocks (0.10-0.80 mA), which escalated by 0.10 mA over a testing period that lasted approximately 20 min. There were three presentations at each shock intensity, with a 20 s inter-stimulus interval and a 90 s inter-trial interval. Motor movements were scored during each shock presentation (0 = no response; 1 = hop; 2 = jump; 3 = run; 4 = horizontal jump; 5 = vertical jump) as described previously (Gulick & Gould, 2009). A solution of 70% ethanol was used to clean the chambers after the end of each session.

Zero Maze The effects of chronic caffeine on anxiety-related behavior were tested using a zero maze. Mice were placed in a closed quadrant and allowed to explore the zero maze for a period of 5 min. During this time mice were video recorded and distance traveled was tracked using PanLab Smart software. Videos were manually scored for anxiety-related variables (i.e. time spent in both open and closed quadrants, number of transitions between quadrants, and rearing) as described in previous studies (Shepherd, Grewal, Fletcher, Bill, & Dourish, 1994; Tarantino, Gould, Druhan, & Bucan, 2000). An entry into an arm was defined when the mouse's back legs had crossed into that quadrant.

Rearing was defined when the mouse raised itself upright on its hind legs. A solution of 70% ethanol was used to clean the zero maze after the end of each session.

Drug Administration and Experimental Design

For all experiments, caffeine (C0750; Sigma-Aldrich) was dissolved in filtered water and administered through drinking bottles at 0 mg/mL, 1.0 mg/mL, or 3.0 mg/mL. The selection of chronic caffeine concentrations was based on previous work examining the effects of chronic caffeine (Dall'Igna et al., 2003; Jaszyna, Gasior, Shoaib, Yasar, & Goldberg, 1998; Rossi et al., 2009). Drinking bottles and mice were weighed daily between 3:00 PM and 5:00 PM on the first 12 days of chronic treatment. Bottles were changed every 2-3 days as described previously (Boeck et al., 2009; da Silva et al., 2003).

The effects of chronic caffeine on fear conditioning

To investigate the effects of chronic caffeine on fear conditioning, PND 21, 38, and 70 mice were administered water, 1.0 mg/mL, or 3.0 mg/mL caffeine through drinking bottles for 14 days. Mice were trained in fear conditioning on day 13 of chronic treatment and tested for contextual and cued conditioning on day 14 of chronic treatment ($n = 8$). A separate cohort of PND 21 mice were administered chronic caffeine at 1.0 mg/mL for 14 days, trained using a reduced 1 CS-US fear conditioning protocol on day 13 of chronic treatment, and tested for contextual and cued conditioning on day 14 of chronic treatment ($n = 9-11$) (see Figure 1 for a schematic diagram of the chronic fear conditioning experiments).

The effects of withdrawal from chronic caffeine on fear conditioning

To examine the effects of withdrawal from chronic caffeine on fear conditioning, a separate cohort of PND 21, 38, and 70 mice were administered water, 1.0 mg/mL, or

3.0 mg/mL caffeine through drinking bottles for 12 days ($n = 8$ per group). All bottles were changed and filled with water on day 12. On day 13 mice were trained in fear conditioning and on day 14 mice were tested for contextual and cued conditioning. Finally, on day 21 mice were re-tested for contextual and cued recall (see Figure 1 for a schematic diagram of the withdrawal fear conditioning experiment).

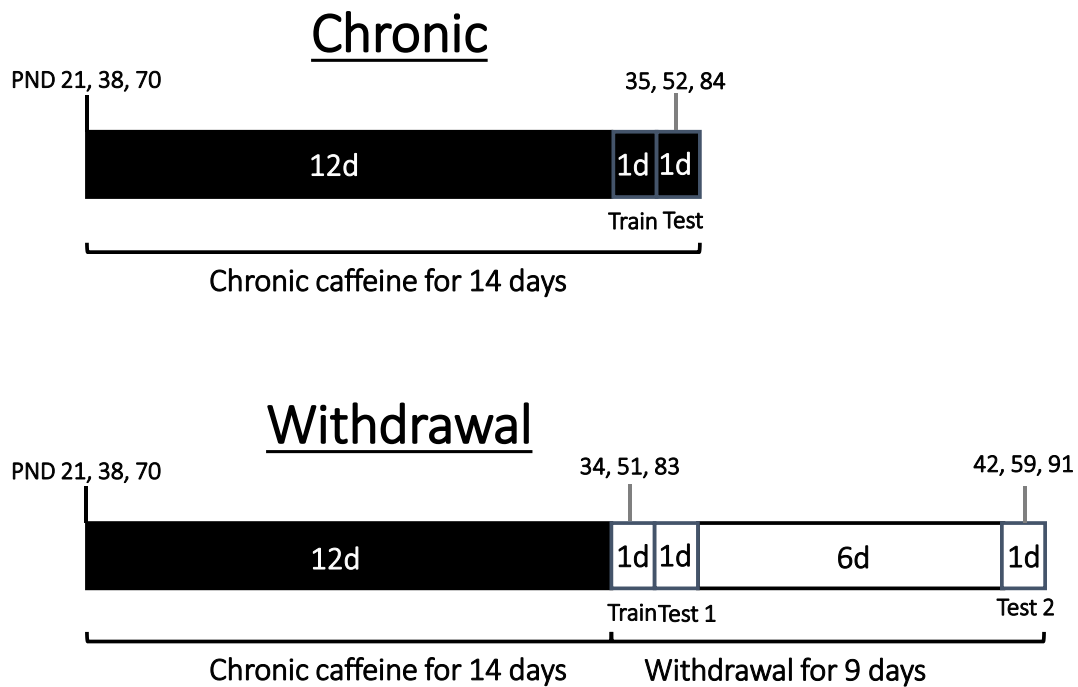


Figure 1: Schematic diagram of chronic and withdrawal fear conditioning experiments.

The effects of chronic caffeine on anxiety-related behavior and shock-sensitivity

To investigate the effects of chronic caffeine on anxiety-related behavior and shock-sensitivity, PND 21, 38, and 70 mice were administered water, 1.0 mg/mL, or 3.0 mg/mL caffeine through drinking bottles for 14 days. On day 13 of treatment mice were

tested in the zero maze and on day 14 of treatment mice were tested for shock sensitivity ($n = 8-12$ per group).

Western blotting: Adenosine A2A receptor levels

PND 21 and PND 70 mice were administered water or chronic caffeine at 3.0 mg/mL for 13 days. Between 10:00 AM and 2:00 PM on day 13 of chronic caffeine treatment, mice were euthanized via cervical dislocation. The dorsal and ventral hippocampus was dissected (1:1 ratio), flash frozen on dry ice, and stored at -80°C for later processing. Tissue was sonicated in RIPA buffer (9806; Cell Signaling, Danvers, MA) containing protease and phosphatase inhibitors (PI-78445; Thermo Scientific, Rockford, IL). Homogenates were then spun at 12,000 rpm for 30 min at 4°C and the total protein concentration of the resulting supernatants was determined by DCTM Protein Assay (500-0112; Bio-Rad, Hercules, CA).

Samples (10 μg) were loaded with an equal volume of Laemmli sample buffer (161-0737; Bio-Rad) containing 5% β -mercaptoethanol into 4-20% TGX gradient gels (456-1093; Bio-Rad). Electrophoresis was conducted at 100 V for 90 min in 1XTris-Glycine-SDS running buffer (161-0732; Bio-Rad). Proteins were then transferred onto methanol pre-soaked LF-PVDF membranes (162-0262; Bio-Rad) at 400 mA constant current in ice-cold Tris-Glycine buffer (161-0734; Bio-Rad). Membranes were washed 3 times for 5 min with filtered phosphate buffered saline (PBS) (28372; Thermo Scientific) then blocked for 1 h at room temperature in Odyssey blocking buffer (927-40000; LI-COR, Lincoln, NB) diluted 1:1 with PBS (dOB-PBS). Membranes were incubated overnight at 4°C in dOB-PBS with .1% Tween (P9416; Sigma) containing anti-A2AR (365235; Santa Cruz, CA, 1:500). After incubation with anti-A2AR, membranes were

washed 3 times for 20 min in PBS with .1% Tween (PBST). Membranes were incubated for 1 h at room temperature in dOB-PBST containing anti- α/β tubulin (2148S; Cell Signaling, 1:1000). Membranes were then washed 3 times for 20 min in PBST and incubated for 1 h at room temperature in dOB-PBST with .01% SDS (PRV6553; Promega) containing IRDye anti-mouse (926-68180; LI-COR, 1:20,000) and IRdye anti-rabbit (926-32211; LI-COR, 1:10,000) secondary antibodies. After washing 3 times for 20 minutes in PBST, membranes were rinsed once for 5 min in PBS and imaged using LI-COR imaging system. Bands were quantified using iS LITE software.

Data Analysis

Analysis of variance (ANOVA) was used to evaluate all data. One-way ANOVAs were followed by Dunnett's post-hoc tests using the water treated group as the reference group when a significant treatment effect was detected. Two-way ANOVAs were followed by Bonferonni post-hoc tests when a significant interaction was detected, or planned t-tests when a significant main effect was detected. One-way ANOVAs were used to analyze treatment effects on each fear conditioning measure (i.e. freezing during immediate, baseline, context, pre-CS, and CS) and each zero maze measure (i.e. time spent in both open and closed quadrants, number of transitions between quadrants, and rearing) within each age group. Two-way (3 treatment groups X 8 shock levels) repeated measures ANOVAs were used to analyze the effect of treatment on shock sensitivity within each age group, and two-way (2 treatment x 2 age group) ANOVAs were used to analyze A2AR levels in the dorsal and ventral hippocampus. Graph Pad Prism 5.0F (GraphPad, San Diego, CA, USA) was used for all of the statistical analyses.

Results

The age-dependent effects of chronic caffeine on fear conditioning

The effects of exposure to 14 days of chronic caffeine (at 0, 1.0, or 3.0 mg/mL) on hippocampus-dependent (contextual) and hippocampus-independent (cued) fear conditioning (Phillips & LeDoux 1992; Anagnostaras, Maren, & Fanselow, 1999; Kim and Fanselow, 1992; Logue, Paylor, & Wehner, 1997) were investigated in 3 age groups of mice (Figure 2). A significant effect of treatment was found in adolescent and pre-adolescent mice for contextual conditioning (adolescent: [$F(2, 21) = 19.35, p < 0.001$]; pre-adolescent: [$F(2, 21) = 38.49, p < 0.001$]). Subsequent post-hoc tests revealed that adolescent and pre-adolescent mice exposed to chronic caffeine at 1.0 mg/mL exhibited enhanced contextual conditioning when compared to water treated controls within each respective age group ($p < 0.05$). In addition, post-hoc tests revealed that pre-adolescent and adolescent mice exposed to chronic caffeine at 3.0 mg/mL exhibited decreased contextual conditioning when compared to water treated controls within each respective age group ($p < 0.05$). No significant effect of treatment on contextual conditioning was found in adult mice [$F(2, 21) = 1.94, p > 0.05$]. Thus, chronic caffeine has concentration-dependent effects on contextual conditioning in adolescent and pre-adolescent mice, but not adult mice. These results suggest that chronic caffeine alters hippocampus-dependent conditioning in adolescent and pre-adolescent mice, yet chronic caffeine has no effect on hippocampus-dependent conditioning in adult mice at the concentrations tested.

Age-dependent effects were observed for pre-CS freezing as well. One-way ANOVAs revealed a significant effect of treatment on pre-CS freezing in adolescent [$F(2, 21) = 29.72, p < 0.001$] and pre-adolescent [$F(2, 21) = 7.16, p < 0.01$] mice, but not

adult mice [$F(2, 21) = 1.57, p > 0.05$]. Post-hoc tests revealed that adolescent mice treated with chronic caffeine at 1.0 mg/mL exhibited increased pre-CS freezing in the altered context compared to water treated control mice, and pre-adolescent mice treated with chronic caffeine at 3.0 mg/mL exhibited decreased pre-CS freezing in the altered context compared to water treated control mice (all p 's < 0.05).

No significant effects of treatment on baseline freezing or cued fear conditioning were observed in any age group (adult baseline: [$F(2, 21) = 0.53, p > 0.05$]; adolescent baseline: [$F(2, 21) = 2.38, p > 0.05$]; adult cued: [$F(2,21) = 2.10, p > 0.05$]; adolescent cued: [$F(2, 21) = 0.62, p > 0.05$]; pre-adolescent cued: [$F(2, 21) = 0.61, p > 0.05$]), which suggests that treatment with chronic caffeine had no effect on freezing behavior or hippocampus-independent conditioning. However, there was a significant effect of treatment on immediate post-shock freezing in all age groups (adult: [$F(2, 21) = 6.45, p < 0.05$]; adolescent: [$F(2, 21) = 13.06, p < 0.05$]; pre-adolescent: [$F(2, 21) = 9.28, p < 0.05$]). Post-hoc tests revealed that chronic caffeine at 1.0 mg/mL increased immediate freezing in all age groups compared to water-treated controls within each respective age group (all p 's < 0.05) (see Table 1 for a summary of the effects of chronic caffeine on fear conditioning). To rule out potential non-specific effects of chronic caffeine treatment on response to shock, shock sensitivity testing was run next.

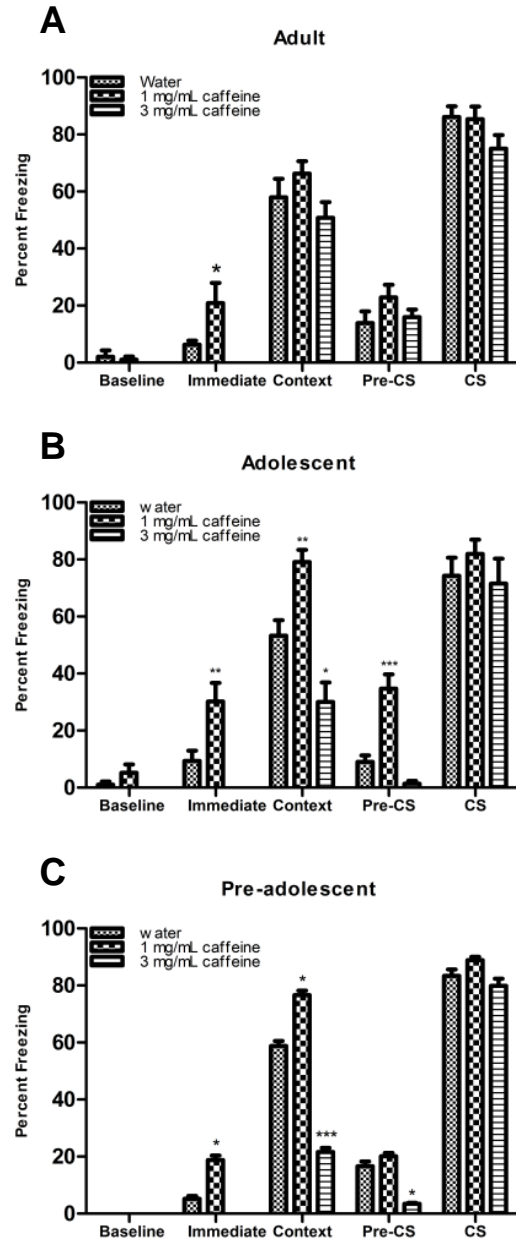


Figure 2: Age-dependent effects of chronic caffeine on fear conditioning ($n = 8$). No concentration of chronic caffeine tested affected baseline or CS freezing in any age group, yet chronic caffeine at 1.0 mg/mL increased immediate freezing in all age groups (A-C). In adolescent and pre-adolescent mice only, chronic caffeine at 1.0 mg/mL enhanced contextual fear conditioning, yet chronic caffeine at 3.0 mg/mL produced deficits in contextual fear conditioning (B and C). Chronic caffeine at 1.0 mg/mL increased pre-CS freezing in adolescent mice only (B) and chronic caffeine at 3.0 mg/mL decreased pre-CS freezing in pre-adolescent mice only (C). Error bars indicate SEM, (*) indicates $p < 0.05$, (**) indicates $p < 0.01$, and (***) indicates $p < 0.001$ compared to water treated mice from each respective age group.

Shock sensitivity during chronic caffeine treatment was tested in all age groups (Figure 3). A significant main effect of shock level was found within each age group (adult: [$F(7, 154) = 155.8, p < 0.0001$]; adolescent: [$F(7, 147) = 171.8, p < 0.0001$]; pre-adolescent: [$F(7, 147) = 136.4, p < 0.0001$]). In addition, a significant main effect of treatment was observed in adult [$F(2, 154) = 4.60, p < 0.05$], but not adolescent [$F(2, 147) = 3.38, p > 0.05$] or pre-adolescent [$F(2, 147) = 2.60, p > 0.05$] mice. Planned comparisons revealed that adult mice treated with chronic caffeine at 1.0 mg/mL showed an increased response to shock compared to adult mice treated with chronic caffeine at 3.0 mg/mL [$t(134) = 2.55, p < 0.05$]; however, neither caffeine treated group was significantly different from water (water vs. 1.0 mg/mL: [$t(126) = 1.05, p > 0.05$]; water vs. 3.0 mg/mL: [$t(134) = 1.47, p > 0.05$]). Given the lack of shock x treatment interaction for all age groups, and the lack of a significant difference observed between caffeine and water treated adult mice, it was concluded that the observed chronic caffeine-induced changes in fear conditioning could not be attributed to differences in response to shock.

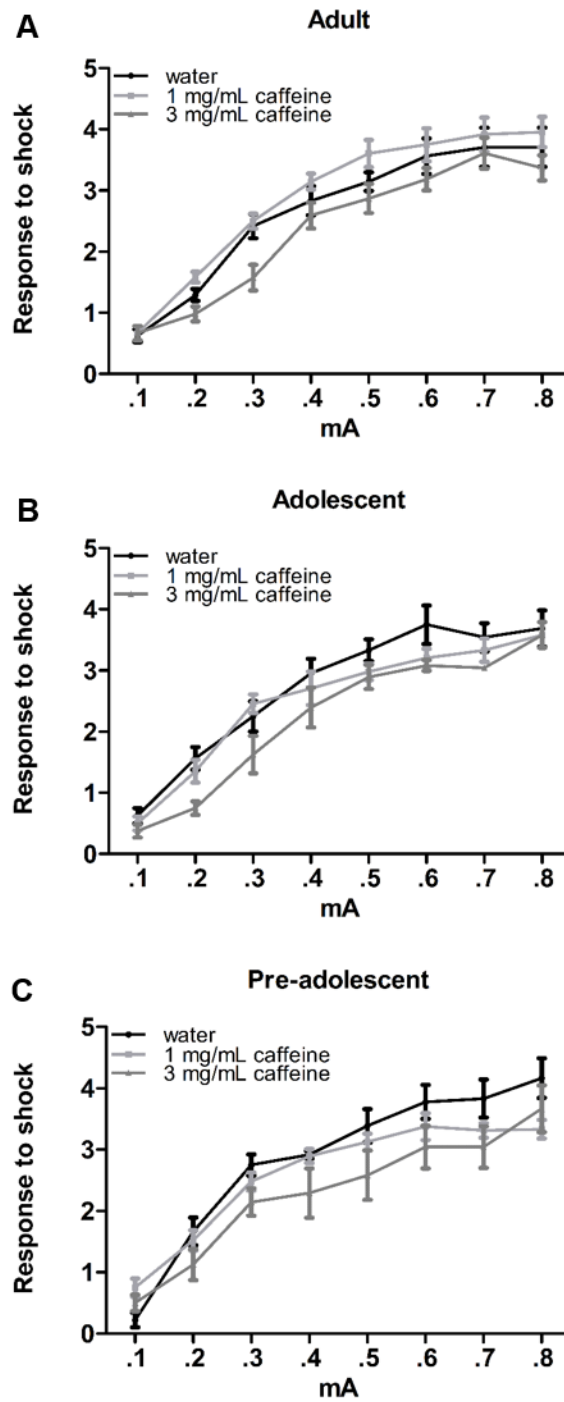


Figure 3: The effect of chronic caffeine on shock sensitivity ($n = 8-12$). Chronic caffeine has no effect on response to shock in adult (A), adolescent (B), or pre-adolescent (C) mice compared to water treated, age-matched controls. Error bars indicate SEM.

The effect of decreasing the level of conditioning on chronic caffeine-induced enhancement of contextual conditioning vs. cued conditioning

We next sought to determine if the lack of effect of chronic caffeine at 1.0 mg/mL on cued conditioning was due to a ceiling effect (Figure 4). Thus, we conditioned pre-adolescent mice with a single CS-US pairing and a shorter duration CS. Pre-adolescent mice treated with chronic caffeine at 1.0 mg/mL showed enhanced contextual conditioning [$t(18) = 2.70, p < 0.05$], yet decreasing the number of CS-US pairings and the duration of the CS did not result in caffeine-enhanced cued conditioning [$t(18) = 0.64, p > 0.05$]. In addition, chronic caffeine had no effect on pre-CS freezing [$t(18) = 1.66, p > 0.05$]. Therefore, we concluded that the lack of treatment effect on cued conditioning was not due to a ceiling effect.

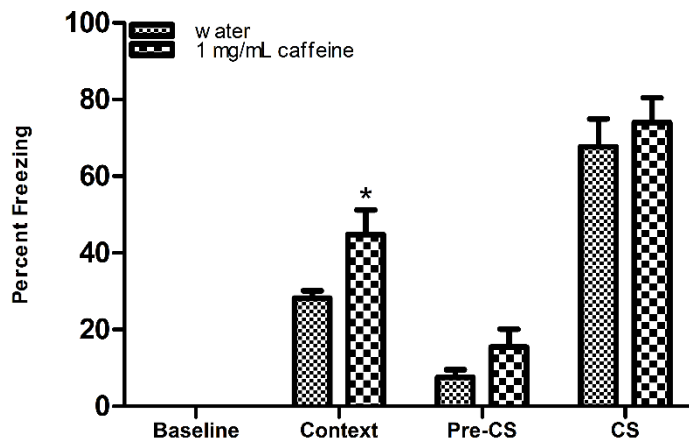


Figure 4: The effect of chronic caffeine on hippocampus-dependent (contextual) and -independent (cued) conditioning using a reduced fear conditioning protocol ($n = 9-11$). Chronic caffeine at 1.0 mg/mL had no effect on pre-CS or CS freezing in pre-adolescent mice using a reduced conditioning protocol. In contrast, caffeine at 1.0 mg/mL enhanced hippocampus-dependent contextual conditioning using the same protocol. Error bars indicate SEM, (*) indicates $p < 0.05$ compared to water treated mice.

The age-dependent effects of withdrawal from chronic caffeine on fear conditioning

To explore the age-dependent effects of withdrawal from chronic caffeine on contextual and cued conditioning, adult, adolescent, and pre-adolescent mice were treated with chronic caffeine for 12 days and withdrawn from treatment 24 h prior to fear conditioning training (Figure 5). A significant effect of treatment on contextual conditioning in pre-adolescent mice was found [$F(2, 21) = 20.07, p < 0.001$]. Post-hoc tests revealed that withdrawal from chronic caffeine at both 1.0 mg/mL and 3.0 mg/mL produced deficits in contextual conditioning in pre-adolescent mice (all p 's < 0.05). In addition, there was an effect of treatment on adult immediate freezing [$F(2, 21) = 4.16, p < 0.05$]; however, post-hoc tests were n.s. ($p > 0.05$). No other significant effects of withdrawal from chronic caffeine were observed for any other fear conditioning measure in any age group (adult context: [$F(2, 21) = 2.42, p > 0.05$]; adolescent context: [$F(2, 21) = 1.79, p > 0.05$], adult pre-CS: [$F(2, 21) = 2.96, p > 0.05$]; adolescent pre-CS: [$F(2, 21) = 0.77, p > 0.05$]; pre-adolescent pre-CS: [$F(2, 21) = 0.28, p > 0.05$]; adult CS: [$F(2, 21) = 0.04, p > 0.05$]; adolescent CS: [$F(2, 21) = 2.27, p > 0.05$]; pre-adolescent CS: [$F(2, 21) = 2.48, p > 0.05$]). Thus, withdrawal from chronic caffeine disrupts contextual conditioning in pre-adolescent mice, yet has no effect on contextual conditioning in adolescent or adult mice (see Table 2 for a summary on the effects of withdrawal from chronic caffeine on fear conditioning).

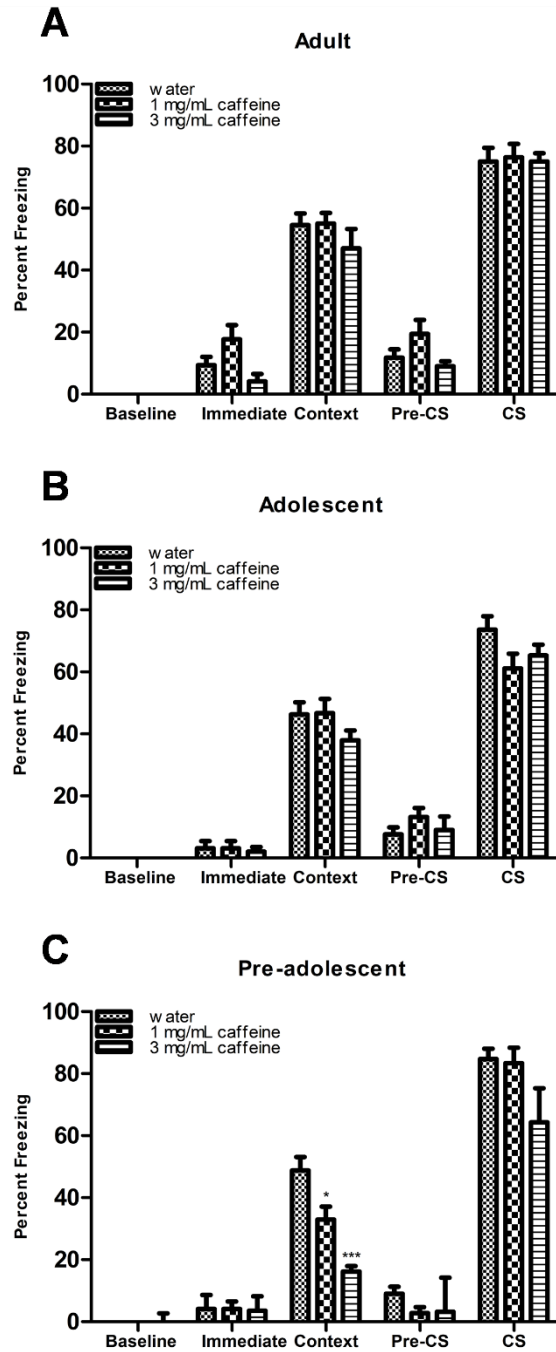


Figure 5: Age-dependent effects of withdrawal from chronic caffeine on fear conditioning ($n = 8$). Withdrawal from chronic caffeine had no effect on baseline, immediate, context, pre-CS, or CS freezing in adolescent or adult mice (A and B). Pre-adolescent mice withdrawn from chronic caffeine at both 1.0 mg/mL and 3.0 mg/mL showed deficits in contextual fear conditioning, but not cued fear conditioning (C). Error bars indicate SEM, (*) indicates $p < 0.05$ and (***) indicates $p < 0.001$ compared to water treated mice from each respective age group.

To investigate the effects of withdrawal from chronic caffeine on recall of a fear memory acquired during withdrawal, mice were retested for contextual and cued memory 7 days after Test 1 (i.e. Test 2) (Figure 6). One-way ANOVAs did not reveal any significant differences in recall of contextual or cued conditioning between treatment groups within each age group (adult context: [$F(2, 21) = 2.42, p > 0.05$]; adolescent context: [$F(2, 21) = 1.79, p > 0.05$]; pre-adolescent context: [$F(2, 21) = 0.54, p > 0.05$]; adult cued: [$F(2, 21) = 0.04, p > 0.05$]; adolescent cued: [$F(2, 21) = 0.69, p > 0.05$]; pre-adolescent cued: [$F(2, 21) = 2.78, p > 0.05$]). A significant effect of treatment on pre-CS freezing was observed in adolescent mice [$F(2, 21) = 5.52, p < 0.05$]; however, post-hoc tests were n.s. ($p > 0.05$).

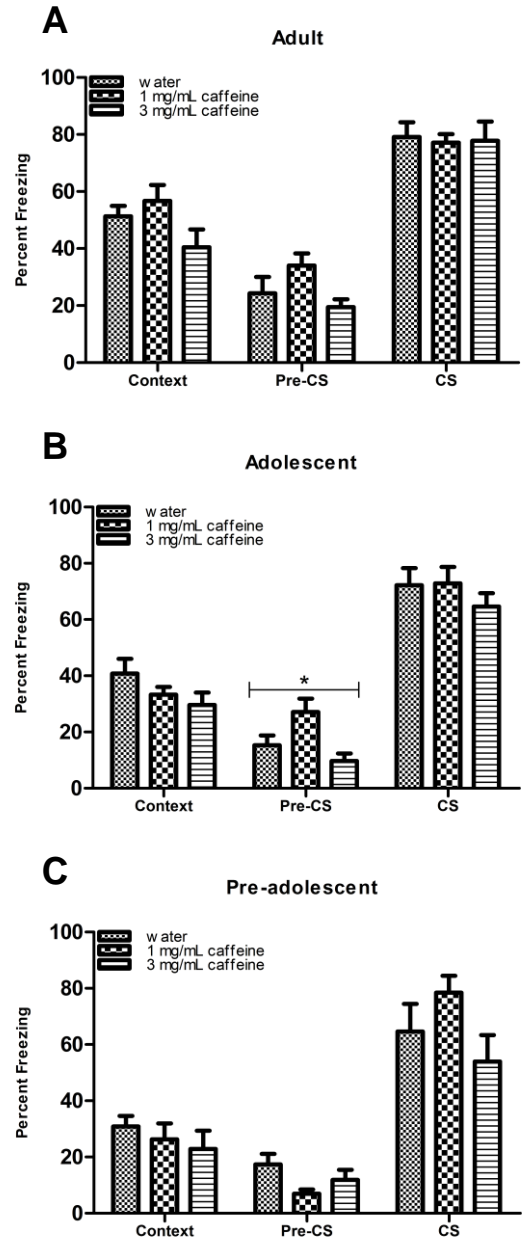


Figure 6: Age-dependent effects of withdrawal from chronic caffeine on recall of a fear memory formed during withdrawal ($n = 8$). Withdrawal from chronic caffeine had no effect on recall of fear conditioning in adult (A), adolescent (B), or pre-adolescent (C) mice. Error bars indicate SEM.

We next computed difference scores between Test 1 and Test 2 to determine if there were treatment-related changes in memory retention (Figure 7), because pre-adolescent mice showed chronic caffeine withdrawal-induced deficits in hippocampus-

dependent contextual conditioning on Test 1, yet normal recall on Test 2. Withdrawal from chronic caffeine had no effect on retention of contextual or cued memories in any age group (adult context: [$F(2, 21) = 0.48, p > 0.05$]; adolescent context: $F(2, 21) = 1.18, p > 0.05$]; pre-adolescent context: $F(2, 21) = 2.94, p > 0.05$]; adult cued: [$F(2, 21) = 0.13, p > 0.05$]; adolescent cued: [$F(2, 21) = 2.40, p > 0.05$]; pre-adolescent cued: [$F(2, 21) = 0.62, p > 0.05$]. Furthermore, withdrawal from chronic caffeine had no effect on pre-CS freezing (adult: [$F(2, 21) = 0.19, p > 0.05$]; adolescent: [$F(2, 21) = 1.84, p > 0.05$]; pre-adolescent: [$F(2, 21) = 0.67, p > 0.05$]). Therefore, the findings that withdrawal from chronic caffeine induced contextual conditioning deficits in pre-adolescent mice on Test 1 that were not present at Test 2 (see Figures 5 and 6), may reflect a transient recall deficit.

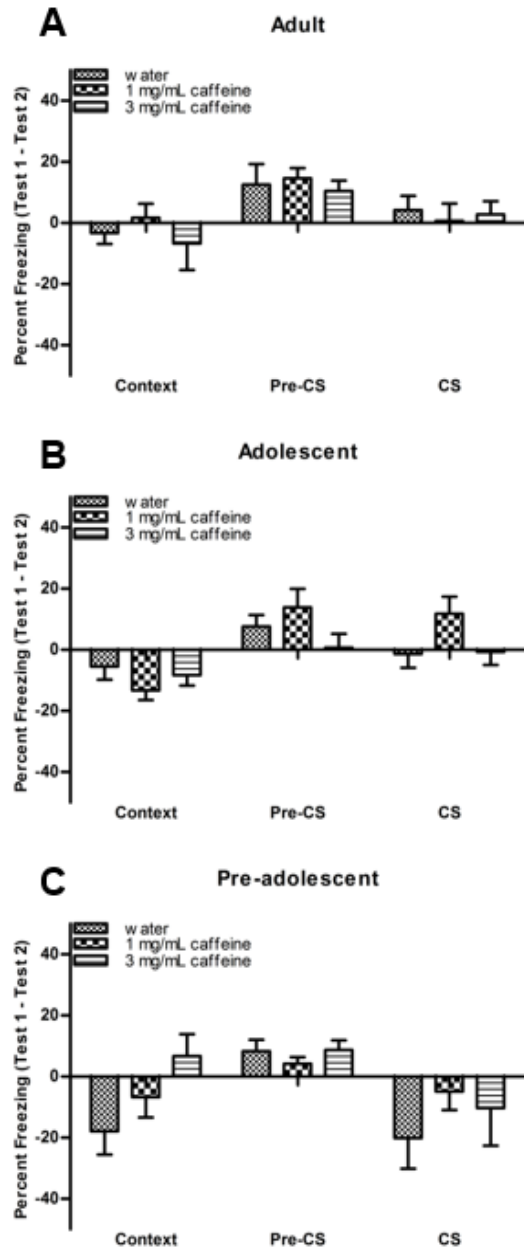


Figure 7: Age-dependent effects of withdrawal from chronic caffeine on retention of a fear memory formed during withdrawal ($n = 8$). Withdrawal from chronic caffeine had no effect on retention of fear conditioning in adult (A), adolescent (B), or pre-adolescent (C) mice. Error bars indicate SEM.

Table 1: Chronic Caffeine			
		1 mg/mL	3 mg/mL
Adult	<i>Baseline</i>	–	–
	<i>Immediate</i>	↑ *	–
	<i>Contextual</i>	–	–
	<i>Pre-CS</i>	–	–
	<i>CS</i>	–	–
Adolescent	<i>Baseline</i>	–	–
	<i>Immediate</i>	↑ **	–
	<i>Contextual</i>	↑ **	↓ *
	<i>Pre-CS</i>	↑ ***	–
	<i>CS</i>	–	–
Pre-adolescent	<i>Baseline</i>	–	–
	<i>Immediate</i>	↑ *	–
	<i>Contextual</i>	↑ *	↓ ***
	<i>Pre-CS</i>	–	↓ *
	<i>CS</i>	–	–

Table 1: The effects of chronic caffeine on fear conditioning.

		1 mg/mL	3 mg/mL
Adult	<i>Baseline</i>	–	–
	<i>Immediate</i>	–	–
	<i>Contextual</i>	–	–
	<i>Pre-CS</i>	–	–
	<i>CS</i>	–	–
Adolescent	<i>Baseline</i>	–	–
	<i>Immediate</i>	–	–
	<i>Contextual</i>	–	–
	<i>Pre-CS</i>	–	–
	<i>CS</i>	–	–
Pre-adolescent	<i>Baseline</i>	–	–
	<i>Immediate</i>	–	–
	<i>Contextual</i>	↓ *	↓ ***
	<i>Pre-CS</i>	–	–
	<i>CS</i>	–	–

Table 2: The effects of withdrawal from chronic caffeine on fear conditioning.

Age differences and treatment-dependent changes in adenosine 2A receptor levels in the dorsal and ventral hippocampus

Western blotting was used to examine treatment-induced changes in adenosine 2A receptor (A2AR) levels in the dorsal and ventral hippocampus of pre-adolescent and adult mice treated with chronic caffeine for 13 days (at 0 or 3.0 mg/mL) (Figure 8). Two-way ANOVAs did not reveal any significant interactions or main effects of age or treatment on A2AR levels in the dorsal hippocampus (age: [$F(1, 20) = 1.20, p > 0.05$]; treatment: [$F(1, 20) = 0.00, p > 0.05$]; interaction: [$F(1, 20) = 1.73, p > 0.05$]) or the ventral hippocampus (age: [$F(1, 20) = 2.96, p > 0.05$]; treatment: [$F(1, 20) = 0.01, p > 0.05$];

interaction: [$F(1, 20) = .31, p > 0.05$]). These findings are in agreement with previous studies, which showed no effect of chronic caffeine treatment on A2AR levels in the adult hippocampus (Fredholm, 1982; Hawkins, Dugich, Porter, Urbancic, & Radulovacki, 1988).

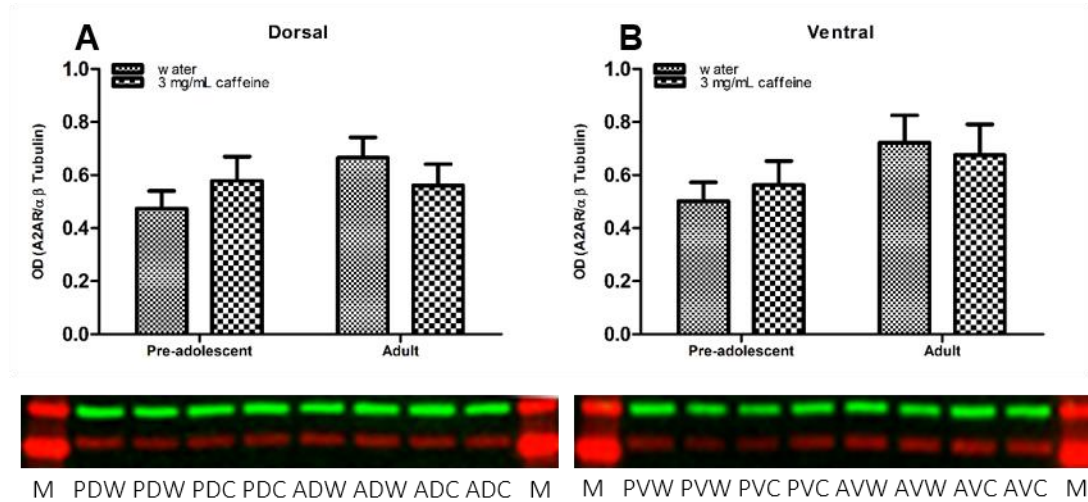


Figure 8: The effect of chronic caffeine on A2AR levels in the dorsal and ventral hippocampus of pre-adolescent and adult mice ($n = 6$). Green bands = α/β tubulin (50 kDa), Red bands = A2AR (39 kDa). Chronic caffeine at 3.0 mg/mL had no effect on A2AR levels in the dorsal hippocampus (A) or the ventral hippocampus (B). Error bars indicate SEM. Representative blots are shown below each graph (from left to right: M=molecular weight marker; PDW=pre-adolescent, dorsal, water; PDC=pre-adolescent, dorsal, chronic caffeine at 3.0 mg/mL; ADW=adult, dorsal, water; ADC=adult, dorsal, chronic caffeine at 3.0 mg/mL; PVW=pre-adolescent, ventral, water; PVC=pre-adolescent, ventral, chronic caffeine at 3.0 mg/mL; AVW=adult, ventral, water; AVC=adult, ventral, chronic caffeine at 3.0 mg/mL).

The age-dependent effects of chronic caffeine on anxiety-related

behavior in the zero maze

To examine the age-dependent effects of caffeine on anxiety-related behavior, the time spent in the open quadrants of the zero maze was analyzed in pre-adolescent, adolescent, and adult mice treated with chronic caffeine for 13 days (at 0, 1.0, or 3.0

mg/mL) (Figure 9A). A significant effect of treatment on time spent in the open quadrants was found in pre-adolescent mice [$F(2, 32) = 4.14, p < 0.05$]. Post-hoc tests revealed that pre-adolescent mice treated with chronic caffeine at 3.0 mg/mL spent more time in the open quadrants of the zero maze (all p 's < 0.05). A significant effect of treatment on time spent in open quadrants was found in adolescent mice [$F(2, 32) = 4.63, p < 0.05$]; however, post-hoc tests were n.s. ($p > 0.05$). There was no effect of treatment on time spent in open quadrants in adult mice [$F(2, 32) = 0.75, p > 0.05$]. Thus, chronic caffeine at 3.0 mg/mL decreases anxiety-related behavior in pre-adolescent mice, but has no effect on anxiety-related behavior in adult mice.

The effect of treatment on rearing and transitions between quadrants was examined in the zero maze as well (see Tarantino et al., 2000) (Figure 9B and C). A significant effect of treatment on rearing in pre-adolescent mice [$F(2, 32) = 9.72, p < 0.001$], but not adolescent [$F(2, 32) = 0.75, p > 0.05$] or adult mice [$F(2, 32) = 1.14, p > 0.05$] was found. Post-hoc tests revealed that pre-adolescent mice treated with chronic caffeine at 3.0 mg/mL reared significantly more than the water-treated control mice ($p < 0.05$). In addition, a significant effect of treatment on transitions was found in both pre-adolescent and adolescent mice (pre-adolescent: [$F(2, 32) = 5.60, p < 0.05$]; adolescent: [$F(2, 32) = 7.57, p < 0.001$]), but not adult mice [$F(2, 32) = 1.43, p > 0.05$]. Post-hoc tests revealed that pre-adolescent and adolescent mice treated with chronic caffeine at 3.0 mg/mL were more active in the zero maze than water treated control mice (all p 's < 0.05). Together, the zero maze results indicate that chronic caffeine at 3.0 mg/mL decreases anxiety-related behavior and/or increases activity in pre-adolescent mice, and that chronic caffeine increases activity in adolescent mice. Interestingly, pre-adolescent

mice treated with chronic caffeine at 3.0 mg/mL display increased rearing whereas adolescent mice do not, suggesting that caffeine-induced changes in behavior differ between these age groups.

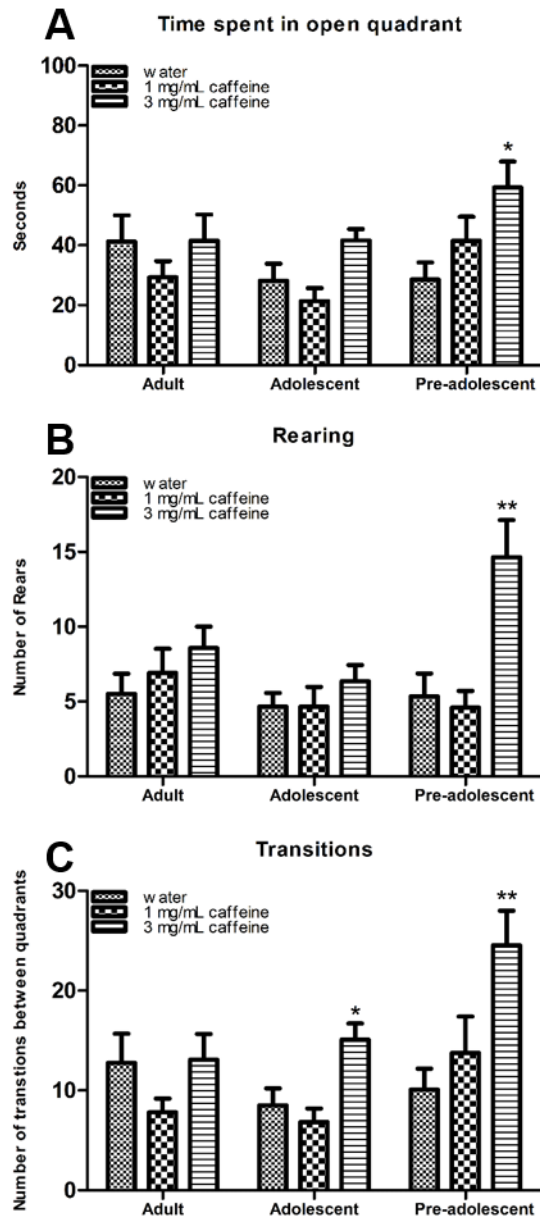


Figure 9: The age-dependent effect of chronic caffeine on anxiety-related behavior and/or activity levels in the zero maze ($n = 8-12$). Caffeine treatment altered the time spent in the open quadrant of the zero maze in adolescent and pre-adolescent mice, but not adult mice (A). Chronic caffeine at 3.0 mg/mL increased rearing in pre-adolescent mice, and increased transitions in both adolescent and pre-adolescent mice, but not adult mice (B and C). Error bars indicate SEM, (*) indicates $p < 0.05$, (**) indicated $p < 0.01$ compared to water treated mice from each respective age group.

Discussion

In the present study, the effects of chronic caffeine and withdrawal from chronic caffeine on hippocampus-dependent (contextual) and hippocampus-independent (cued) conditioning were investigated in pre-adolescent, adolescent, and adult mice. Chronic caffeine had dose-dependent effects on contextual conditioning in pre-adolescent and adolescent mice, but not adult mice. Specifically, chronic caffeine at 1.0 mg/mL enhanced contextual conditioning in pre-adolescent and adolescent mice, whereas chronic caffeine at 3.0 mg/mL produced deficits in contextual conditioning in pre-adolescent and adolescent mice. In contrast, withdrawal from both concentrations of chronic caffeine impaired contextual conditioning in pre-adolescent, but not adolescent or adult mice. Cued conditioning was not affected by chronic caffeine or withdrawal from chronic caffeine in any age group. Together, our results demonstrate that chronic caffeine and withdrawal from chronic caffeine have age-dependent effects on hippocampus-dependent learning and memory as opposed to learning and memory in general.

Again, the findings that chronic caffeine and withdrawal from chronic caffeine alter contextual but not cued conditioning in pre-adolescent and adolescent mice, suggest that caffeine exerts selective effects on hippocampus-dependent learning and memory during these developmental periods. In support, learning to associate the context with a shock unconditioned stimulus (US) is hippocampus and amygdala dependent (Phillips & LeDoux, 1992), whereas forming an association between an auditory cued conditioned stimulus (CS) and a shock, is independent of the hippocampus but dependent on the amygdala (Phillips & LeDoux, 1992; Kim et al., 1993; Logue et al., 1997). Therefore, the difference in the effects of caffeine on cued versus contextual fear conditioning suggest

that caffeine may differentially alter the neural circuitry underlying contextual fear conditioning compared to the neural circuitry underlying cued fear conditioning. Thus, our data suggest that chronic caffeine may be acting on the hippocampus, or structures that modulate hippocampal function, to alter contextual conditioning in pre-adolescent and adolescent mice. It is unlikely that the lack effect of chronic caffeine on cued conditioning is due to a ceiling effect, because we demonstrated that freezing to the CS is not altered by caffeine following training that produces lower levels of conditioning (i.e. a one shock presentation).

The effects of chronic caffeine on contextual conditioning in pre-adolescent and adolescent mice were concentration-dependent as well. Specifically, chronic caffeine at 1.0 mg/mL enhanced contextual conditioning in pre-adolescent and adolescent mice, but not adult mice. However, chronic caffeine at 3.0 mg/mL produced deficits in contextual conditioning in pre-adolescent and adolescent mice, but not adult mice. The concentration-dependent effects of chronic caffeine on contextual conditioning in pre-adolescent and adolescent mice may be the result of age-related changes in the hippocampus. In support, the hippocampus undergoes structural (Pokorný & Yamamoto, 1981; Seress & Ribak, 1995; Zehr, Nichols, Schulz, & Sisk, 2008) and functional (Pyapali, Turner, Wilson, & Swartzwelder, 1999; Swartzwelder, Wilson, & Tayyeb, 1995a, 1995b; White & Swartzwelder, 2004) modifications during adolescence, and the developing hippocampus may respond differently to caffeine than the adult hippocampus. Specifically, caffeine may increase excitation in the pre-adolescent and adolescent hippocampus to a greater degree than the adult hippocampus. This increased excitation could enhance hippocampus-dependent learning and memory; however, higher

concentrations of caffeine may result in conditioning deficits due to excitotoxic effects. Indeed, it has been previously demonstrated that acute caffeine can induce LTP (a neurophysiological correlate of learning and memory (Martin, Grimwood, & Morris, 2000)) in the juvenile hippocampus (Martin & Buño, 2003; Simons et al., 2012), but not the adult hippocampus (Stepan et al., 2012). Therefore, future work should examine the hypothesis that chronic caffeine induces age-dependent changes in hippocampal excitability and LTP.

Caffeine is an adenosine receptor antagonist (Fredholm et al., 1999) and thus the observed effects of chronic caffeine on contextual conditioning are likely mediated at least in part by adenosine receptor antagonism. Interestingly, antagonism of the inhibitory adenosine A1 receptor (A1R) has been shown to facilitate hippocampal LTP (Mendonça & Ribeiro, 1994), whereas antagonism of the facilitatory adenosine A2A receptor (A2AR) has been shown to attenuate hippocampal LTP (Costenla et al., 2011). Therefore, it could be the case that chronic caffeine at 1.0 mg/mL enhances contextual conditioning by blocking A1Rs in the hippocampus, and that chronic caffeine at 3.0 mg/mL impairs contextual conditioning by blocking A2ARs in the hippocampus. However, reducing the threshold for LTP may not necessarily result in enhanced learning and memory.

Although A2AR antagonism attenuates LTP (Costenla et al., 2011), A2AR antagonism has also been shown to enhance memory consolidation and retention in the passive avoidance task (Kopf et al., 1999). Moreover, mice lacking adenosine A2ARs show improved spatial recognition memory (Wang et al., 2006). Therefore, the ability of chronic caffeine at 1.0 mg/mL to enhance contextual conditioning in pre-adolescent and adolescent mice may be the result of a caffeine-induced decrease in A2AR activity in the

hippocampus as opposed to decreased A1R activity. In support, despite the findings that A1R antagonism facilitates LTP (Mendonça & Ribeiro, 1994), chronic antagonism of A1Rs has been shown to impair long-term memory in the Morris water maze (Vollert, Forkuo, Bond, & Eriksen, 2013). Consequently, the ability of chronic caffeine at 3.0 mg/mL to impair contextual conditioning in pre-adolescent and adolescent mice may be the result of a caffeine-induced decrease in A1R activity in the hippocampus. Therefore, while somewhat counterintuitive based on the synaptic plasticity literature, future studies should examine the hypothesis that chronic caffeine at 1.0 mg/mL depresses A2AR activity in the pre-adolescent and adolescent hippocampus to a greater degree than in the adult hippocampus. Moreover, future studies should examine the hypothesis that chronic caffeine at 3.0 mg/mL decreases A1R activity in the pre-adolescent and adolescent hippocampus to a greater degree than in the adult hippocampus.

The ability of chronic caffeine to exert age-dependent effects on contextual conditioning may be due to developmental changes in the adenosinergic system. In other words, caffeine may have an increased ability to depress excitation in a system that expresses higher levels of facilitatory A2ARs and a greater ability to enhance excitation in a system that expresses higher levels of inhibitory A1Rs. In fact, between late adolescence (postnatal day 42) and adulthood (postnatal day 180) the density of inhibitory A1Rs decrease and the density of facilitatory A2ARs increase in the hippocampus (Cunha et al., 1995). Furthermore, Costenla and colleagues (2011) found that selective A1R blockade increases LTP magnitude in young adult rats, but has no effect on middle-aged or aged rats. Moreover, in the same study, selective A2AR blockade attenuated LTP to a greater degree in aged rats compared to middle-aged and

adult rats. Therefore, chronic caffeine likely has a greater effect on suppressing A1R activity in developing animals (leading to increased excitation), while chronic caffeine likely has a greater effect on suppressing A2AR activity in adult animals (leading to decreased excitation). However, it will be important to elucidate the relative expression of adenosine receptors across development to generate a better model of how chronic caffeine might exert age-dependent effects on hippocampal LTP and hippocampus-dependent learning and memory.

In addition to age-related changes in adenosine receptor levels in the hippocampus, there may be age-related differences in caffeine-induced neuroadaptations. For example, a recent paper demonstrated that chronic caffeine at 1.0 mg/mL increases levels of A1R in the hippocampus of adolescent rats (Ardais et al., 2014). Thus, in light of our findings that a higher concentration of caffeine (i.e. 3.0 mg/mL) has no effect on A2AR levels in the pre-adolescent hippocampus, chronic caffeine may shift the balance of A1R/A2AR in the developing hippocampus (i.e. there may be a higher ratio of A1Rs to A2ARs). Therefore, chronic caffeine could modulate contextual conditioning by changing the balance of A1R/A2AR in the hippocampus. It should be noted that inhibitory A1Rs are the predominant adenosine receptor in the hippocampus throughout life (Costenla et al., 2010; Cunha et al., 1995; Fastbom et al., 1987; Fredholm et al., 2005; Rosin & Robeva, 1998). Therefore, the net effect of non-specifically blocking adenosine receptors in the hippocampus is increased excitation. However, caffeine-induced A1R upregulation may suppress excitation by increasing the ability of endogenous adenosine to inhibit neurotransmitter release, thereby counteracting the effects of caffeine. Future

studies should determine age-related changes in caffeine-induced A1R upregulation and if these changes are associated with changes in contextual conditioning.

Alternatively, chronic caffeine may exert effects beyond the adenosinergic system to modulate contextual conditioning in pre-adolescent and adolescent mice. In support, doses of caffeine that produce higher than average plasma levels of caffeine act on different molecular substrates (e.g. phosphodiesterases and GABA_A receptors) than lower doses of caffeine (Fredholm et al., 1999). Furthermore, higher doses of caffeine have been shown to act on different brain regions and exert different behavioral effects than lower doses. For example, higher doses of caffeine can produce deficits in cognition and increase glucose utilization in the shell of the nucleus accumbens (Fredholm et al., 1999; Hyman, Malenka, & Nestler, 2006; Kaminer, 2010; Luebbe & Bell, 2009; Nehlig, 1999; Nehlig, Armspach, & Namer, 2010). Therefore, chronic caffeine at 3.0 mg/mL could disrupt contextual conditioning by acting on the nucleus accumbens and/or the hippocampus. To support this claim, lesions to the nucleus accumbens have been shown to disrupt contextual fear conditioning, but not cued fear conditioning (Levita, Dalley, & Robbins, 2002). Likewise, NMDA and AMPA receptor blockade within the nucleus accumbens impairs object recognition (Sargolini et al., 2003), a task that also engages the hippocampus and is altered by pre-adolescent exposure to caffeine (Pires et al., 2010; Abreu et al., 2011). Therefore, while contextual conditioning is hippocampus-dependent, chronic caffeine-induced changes in the nucleus accumbens could also specifically disrupt contextual conditioning while leaving cued conditioning intact. Future studies will need to determine if chronic caffeine has age-dependent effects on the hippocampus, nucleus accumbens, and/or other brain regions involved in contextual conditioning.

The effects of acute caffeine on hippocampus-dependent learning and memory in adults are known to be dose-dependent. For example, low doses of caffeine (e.g. 0.3 mg/kg) enhance retention and retrieval in the Morris water maze task (Angelucci et al., 2002), and high doses of caffeine (e.g. 30 mg/kg) disrupt acquisition and retrieval of contextual fear conditioning (Corodimas et al., 2000). However, in normal adults chronic caffeine has no effect on performance in the radial arm water maze (Alzoubi et al., 2013; Alhaider et al., 2010), object recognition (Botton et al., 2010), inhibitory avoidance (Sallaberry et al., 2013), or contextual fear conditioning (Corodimas et al., 2000) when administered in a wide range of doses and for different periods of time (0.3 mg/mL for 3 months, 0.3 mg/mL for 4 weeks, 10 mg/kg/day for 4 days, 1 mg/mL for 30 days, or through 5-25 mg s.c. pellets for 7 days, respectively). Therefore, it is possible that pre-adolescent and adolescent mice do not develop the same degree of tolerance to the effects of caffeine on hippocampus-dependent learning and memory; however, before this conclusion can be made, the effects of acute caffeine on hippocampus-dependent learning and memory in pre-adolescent and adolescent mice will need to be determined. Furthermore, if tolerance to the effects of caffeine on contextual conditioning are to be assessed, acute and chronic doses will need to be adjusted to produce comparable plasma caffeine levels.

We also found that chronic caffeine at 1.0 mg/mL increased immediate freezing in all age groups. Increased immediate freezing could be due to an effect of chronic caffeine on shock sensitivity and/or an increase in an unconditioned fear response. Importantly, if the US footshock is perceived as more intense, more conditioning may occur (i.e. chronic caffeine at 1.0 mg/mL may enhance contextual conditioning by

increasing response to shock). In fact, it has been proposed that increased reactivity to a foot shock may make fear conditioning ‘easier to learn and remember’ (Kazdoba, Del Vecchio, & Hyde, 2007). Likewise, if the US footshock is perceived as less intense, less conditioning may occur (i.e. chronic caffeine at 3.0 mg/mL may produce deficits in contextual conditioning by decreasing response to shock). We did not find chronic caffeine-induced changes in response to shock or cued conditioning, indicating that not only was there no difference in shock sensitivity, but the effect of chronic caffeine on contextual conditioning is likely specific (i.e. enhanced conditioning would have enhanced both contextual and cued conditioning). Likewise, adult mice showed increased immediate freezing, yet did not show enhanced contextual conditioning, again suggesting a lack of relationship between the conditioned response and the training experience.

To determine if chronic caffeine affected anxiety, which could have an effect on unconditioned fear responding (Davis, Walker, & Lee, 1997; Bannerman et al., 2004; Helmstetter, 1993), we analyzed anxiety-related behavior in the zero maze. Chronic caffeine at 1.0 mg/mL did not decrease time spent in the open quadrant of the zero maze, a measure of anxiety, in any age group. In light of these findings, while somewhat speculative, it may be the case that the auditory CS preceding the shock during fear conditioning training led to enhanced unconditioned freezing. Interestingly, it has previously been shown that the bed nucleus of the stria terminalis is involved in light-enhanced unconditioned fear responses (Walker et al., 1997). Thus, it could be the case that caffeine acts on a brain region that is involved in auditory-enhanced unconditioned fear responses. In support, previous findings suggest that caffeine improves transmission in the peripheral and central brain auditory pathways (Dixit, Vaney, & Tandon, 2006).

Although our data suggest that changes in baseline anxiety as a result of exposure to chronic caffeine at 1.0 mg/mL are not likely responsible for enhanced immediate freezing in any age group, chronic caffeine at 1.0 mg/mL did significantly increase pre-CS freezing in adolescent mice, which may indicate enhanced fear generalization – a phenomenon that has been observed in a variety of anxiety disorders (Jovanovic, Kazama, Bachevalier, & Davis, 2012; Reinecke, Becker, Hoyer, & Rinck, 2010). Therefore, due to recent findings that exposure to chronic caffeine at 1.0 mg/mL during adolescence enhances anxiety-related behavior in rats (Ardais et al., 2014), it may be the case that under some conditions chronic caffeine at 1.0 mg/mL can modulate anxiety (e.g. after a fear conditioning experience). The increase in pre-CS freezing in adolescent mice treated with chronic caffeine at 1.0 mg/mL could be due to decreased locomotor activity, and/or decreased specificity of the context-shock association as well. However, Ardais and colleagues (2014) demonstrated that exposure to chronic caffeine at 1.0 mg/mL during adolescence has no effect on distance traveled in the open-field. Therefore, it may be the case that increased pre-CS freezing is due to a change in the specificity of the context-shock association, which would agree with the findings that chronic caffeine at 1.0 mg/mL enhances contextual conditioning.

Although chronic caffeine at 3.0 mg/mL did not produce deficits in cued fear conditioning in pre-adolescent or adolescent mice, it did alter behavior in the zero maze. Specifically, exposure to chronic caffeine at 3.0 mg/mL during adolescence increased transitions in the zero maze, but had no effect on time spent in the open arm, suggesting an increase in activity. In contrast, exposure to chronic caffeine at 3.0 mg/mL during pre-adolescence increased both transitions and rearing, as well as time spent in the open

quadrant of the zero maze, suggested an increase in activity and/or a decrease in anxiety. It should be noted that an increase in exploratory behavior, including increased activity (i.e. increased transitions), in anxiety paradigms has been interpreted as a release of exploratory inhibition (Bailey & Crawley, 2009), and has been observed after animals are treated with anxiolytic drugs (Crawley & Goodwin, 1980). Therefore, exposure to chronic caffeine at 3.0 mg/mL during pre-adolescence and adolescence increases activity and/or decreases anxiety-related behavior. While it would be reasonable to conclude that changes increased activity levels may be responsible for the observed contextual conditioning deficits in pre-adolescent and adolescent mice treated with chronic caffeine at 3.0 mg/mL, treatment did not affect cued conditioning, which suggest that chronic caffeine at 3.0 mg/mL specifically impaired contextual conditioning rather than reducing freezing due to increases in activity levels.

While we do not know the brain regions responsible for our behavioral effects, it could be the case that chronic caffeine at 3.0 mg/mL disrupts ventral hippocampus function in pre-adolescent mice. In support, rats with neonatal ventral hippocampal lesions show enhanced locomotion and show impairments in place learning and memory during adolescence (Lipska, 2004; Tseng, Chambers, & Lipska, 2009). Again, while we do not yet know if chronic caffeine is acting directly on the hippocampus to modulate contextual conditioning in pre-adolescent and adolescent mice, or the contribution of the dorsal vs. ventral hippocampus to these effects, spatial memory depends on the dorsal hippocampus (Moser & Moser, 1995), whereas the ventral hippocampus is more involved in stress responses and emotional behavior (Henke, 1990) (for review see Fanselow & Dong, 2010). Therefore, our findings that chronic caffeine at 3.0 mg/mL disrupts

contextual conditioning in addition to decreasing anxiety-related behavior in the zero maze of both pre-adolescent and adolescent mice may suggest that chronic caffeine acts on the dorsal and ventral hippocampus.

The adenosine A2A receptor plays an important role in the effects of chronic caffeine on hippocampal development early in life (Silva et al., 2013) as well chronic caffeine reversal of age-related cognitive impairments (for review see Cunha & Agostinho, 2010). Therefore, we hypothesized that the age-dependent effects of chronic caffeine at 3.0 mg/mL would be associated with differences in adenosine A2AR levels between pre-adolescent and adult mice as adolescent rats have lower levels of A2ARs in the hippocampus than adult rats (Cunha et al., 1995). However, we did not observe any differences in A2AR levels in the dorsal or ventral hippocampus between pre-adolescent or adult mice using western blotting. Furthermore, there was no effect of chronic caffeine on A2AR levels in the dorsal or ventral hippocampus of either age group. Therefore, the age-associated increase in A2AR levels that has been previously been described between adult and aged animals (Cunha et al., 1995), may not be present when comparing pre-adolescent and adult mice. In other words, aging may not result in a gradual increase in A2ARs in the hippocampus. Instead, A2AR levels in the hippocampus may be similar between pre-adolescence and adulthood, yet decrease in adolescence and increase in sentinel animals. As stated previously, the age-dependent effects of chronic caffeine on contextual conditioning may be driven by changes in A1R levels within the hippocampus, which should be further investigated in future studies.

The effects of withdrawal from chronic caffeine on cognition have received little attention in the rodent literature. Therefore, we also examined the effects of withdrawal

from chronic caffeine on contextual and cued conditioning in pre-adolescent, adolescent, and adult mice. Withdrawal from chronic caffeine specifically disrupted contextual conditioning in pre-adolescent mice regardless of concentration, yet had no effect on contextual or cued conditioning in adolescent or adult mice. Thus, while pre-adolescent mice show enhanced contextual conditioning during treatment with chronic caffeine at 1.0 mg/mL, they show deficits during withdrawal from the same concentration.

Withdrawal from chronic caffeine is associated with a variety of symptoms in humans including fatigue, decreased alertness, difficulty concentrating, irritability, and depressed mood (Juliano & Griffiths, 2004). In addition, negative symptoms associated with withdrawal from chronic caffeine have been reported in children (Bernstein et al., 1998), adolescents (Bernstein, Carroll, Thuras, Cosgrove, & Roth, 2002), and adults (Juliano & Griffiths, 2004) alike. Therefore, while withdrawal from chronic caffeine is associated with negative symptoms in all age groups, our data suggests that only pre-adolescent mice show withdrawal-induced cognitive deficits. Interestingly, Luebke and colleagues (2011) found that children who experience more intense withdrawal symptoms after caffeine cessation tend to drink more caffeine rather than less. Thus, the findings that chronic caffeine may have positive effects on cognition in pre-adolescent animals, whereas withdrawal from chronic caffeine may have negative effects on cognition in pre-adolescent animals could indicate that children may be more subject to developing caffeine dependence than adolescents or adults. Furthermore, the findings that both chronic and withdrawal from chronic caffeine at 3.0 mg/mL impaired contextual conditioning in pre-adolescent mice may suggest that exposure to chronic caffeine at 3.0 mg/mL produces lasting effects on hippocampus-dependent learning and memory even

after caffeine cessation (Chapter 3 will explore this hypothesis). It should also be mentioned that the conditioning deficits that were present 48 hours after withdrawal (i.e. at Test 1) were no longer present in pre-adolescent mice one week after withdrawal (i.e. at Test 2). Furthermore, withdrawal had no effect on retention of contextual memories. These findings may suggest that withdrawal impairs attention in pre-adolescent mice as normal recall at Test 2 may be indicative of normal contextual learning and normal retention may be indicative of normal contextual memory. The conclusion that withdrawal from chronic caffeine during pre-adolescence impairs attention would be in agreement with findings in humans that withdrawal from habitual caffeine consumption impairs attention in children (Bernstein et al., 1998). Thus, future studies should explore the hypothesis that pre-adolescent animals show impaired attention during withdrawal from chronic caffeine.

In conclusion, the results of the present study are the first to identify age-dependent differences in the effects of chronic caffeine on fear conditioning. Chronic caffeine at 1.0 mg/mL enhanced hippocampus-dependent contextual conditioning in pre-adolescent and adolescent mice. In contrast, chronic caffeine at 3.0 mg/mL impaired hippocampus-dependent contextual conditioning in pre-adolescent and adolescent mice. Furthermore, withdrawal from chronic caffeine at both concentrations tested impaired contextual conditioning in pre-adolescent mice, but not adolescent mice. No effects of chronic treatment or withdrawal from chronic treatment were observed in adult mice. Overall, our results indicate that chronic caffeine and withdrawal from chronic caffeine exert age-dependent effects on hippocampus-dependent learning and memory, but not hippocampus-independent learning and memory. These effects may be due to differences

in the pre-adolescent, adolescent, or adult adenosinergic system, differences in the developing vs. mature hippocampus, and/or age-related differences in other brain regions that modulate hippocampus-dependent learning and memory.

CHAPTER 3

EFFECTS OF EXPOSURE TO CHRONIC CAFFEINE DURING PRE-ADOLESCENCE, ADOLESCENCE, AND ADULTHOOD ON FEAR CONDITIONING IN ADULTHOOD

Introduction

Exposure to psychostimulants during adolescence has been shown to induce neural adaptations that persist into adulthood (Adriani & Laviola, 2004; Boeck et al., 2009; Counotte et al., 2011; Slotkin et al., 2008; Wheeler et al., 2013). In addition, psychostimulant exposure during pre-adolescence and adolescence is associated with learning and memory deficits in adulthood (Portugal, Wilkinson, Turner, Blendy, & Gould, 2012; Sherrill, Stanis, & Gulley, 2013). Caffeine is the most widely used psychostimulant in the world (Fredholm et al., 1999), and one of the only licit psychostimulants available to children and adolescents (Luebbe & Bell, 2009). Therefore, there is a need to determine if chronic caffeine consumption during pre-adolescence and adolescence has long-lasting effects on learning and memory.

Findings suggest that caffeine exposure during adolescence may increase susceptibility to mental illness, drug use, and other problem behavior (James et al., 2011; Kristjansson, Sigfusdottir, Frost, & James, 2013; Temple, 2009; Whalen et al., 2008). For example, Arria and colleagues (2010) found that consumption of caffeinated energy drinks in the second year of college predicted the frequency of tobacco use and new illegal use of prescription stimulants in the third year of college (Arria et al., 2010). These findings may suggest that habitual caffeine consumption during adolescence produces neural adaptations that result in a greater preference for stimulants. Moreover, there has been speculation that caffeine-induced neural adaptations may persist into adulthood

(Temple, 2009). However, the long-lasting effects of exposure to chronic caffeine during pre-adolescence and adolescence are unclear.

Most research on the developmental effects of chronic caffeine on learning and memory has been conducted in prenatal (Silva et al., 2013; Soellner et al., 2009; Swenson, Beckwith, Lamberty, Krebs, & Tinius, 1990) and early postnatal animals (Pan & Chen, 2007; Zimmerberg et al., 1991). For example, Soellner and colleagues (2009) found that exposing rats to chronic caffeine from gestational day 4 until birth impaired non-spatial memory, reference memory, and working memory in adulthood. Furthermore, early postnatal exposure to chronic caffeine (postnatal days 1 through 6 only) has been shown to produce deficits in spatial learning and memory in adulthood (Zimmerberg et al., 1991). Overall, studies on the effects of chronic caffeine exposure during prenatal and early postnatal life suggest that chronic caffeine impairs hippocampus-dependent learning and memory in adulthood when caffeine is no longer on board. However, it is not yet clear if exposure to chronic caffeine during pre-adolescence and adolescence exerts comparable effects.

Two studies have been conducted on the effects of caffeine exposure during pre-adolescence and/or adolescence on learning and memory during adulthood with disparate findings. Pires and colleagues (2010) found that administering caffeine to rats throughout pre-adolescence (i.e. post-natal day 25-28) via daily injections impaired short-term object-recognition in adulthood. In contrast, Abreu and colleagues (2011) found that administering chronic caffeine to rats starting in pre-adolescence (postnatal day 21) through testing in adulthood (postnatal day 90) enhanced long-term object recognition memory. While the schedule and time-course of chronic caffeine administration was

different in these two studies, the results suggest that pre-adolescent and/or adolescent exposure to chronic caffeine can both impair and enhance learning and memory in adulthood depending on whether or not caffeine treatment continues through testing. Furthermore, although the hippocampus is involved in object recognition memory, it is not yet known whether chronic caffeine affects hippocampus-dependent learning and memory specifically. However, it has previously been shown that psychostimulants alter the development of the hippocampus (Slotkin et al., 2008), and have effects on hippocampus-dependent learning and memory that persist into adulthood (Portugal et al., 2012). Therefore, the present study examined the effects of exposure to chronic caffeine during pre-adolescence and adolescence on hippocampus-dependent (contextual) (Phillips & LeDoux, 1992) and hippocampus-independent (cued) (Phillips & LeDoux, 1992) learning and memory in adulthood.

Methods

Subjects

Male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice received *ad libitum* access to food and water and were maintained on a 12 hour light/dark cycle (lights on at 7:00 AM). All behavioral procedures were conducted between 11:00 AM and 5:00 PM and approved by the Temple University Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Mice arrived to the animal facility one week prior to the start of experiments. Mice were postnatal day (PND) 16, 31, and 64 on the day of arrival. At the start of all

experiments, pre-adolescent mice were PND 21, adolescent mice were PND 38, and adult mice were PND 70. Mice were housed 2-3 per cage.

Apparatus

Training and testing of contextual fear conditioning occurred in four identical chambers (17.78 cm X 19.05 cm X 38.10 cm) housed in sound attenuating boxes (Med-Associates, St. Albans, VT). Background noise (69 dB) during training and testing was provided by ventilation fans. Stimulus presentation during training and testing was controlled by a computer running Med-PC software. Cued fear conditioning was tested in an altered context, which consisted of four identical chambers with different dimensions and composition than the training context (20.32 cm X 22.86 cm X 17.78 cm). Additionally, a vanilla scent was used to distinguish the altered context from the training context. The training and testing chambers used in the present study were previously described by Davis and colleagues (2005).

Behavioral Procedures: Contextual and Cued Fear Conditioning

Mice were trained and tested in fear conditioning according to Gould and Higgins (2003). Briefly, mice were placed in the training context and observed for freezing behavior for 1 s every 10 s. Training began with the activation of the house lights. Baseline freezing was then scored for 120 s at which point a 30 s white noise conditioned stimulus (CS, 85 dB) was activated and co-terminated with a 2 s 0.62 mA footshock unconditioned stimulus (US). Immediate freezing was scored during a 120 s inter-trial interval and was followed by a second CS-US pairing. The end of the training session consisted of a 30 s interval that terminated when the house lights turned off. Mice were tested for contextual conditioning 24 h later by returning mice to the training chambers,

activating the house lights, and scoring freezing for 5 min. One hour after contextual testing, cued conditioning was tested by placing mice in the altered context, activating the house lights, and scoring freezing for 6 min. For the first 3 min of cued testing, mice were scored for generalized freezing in the absence of the CS. For the last 3 min of cued testing, mice were scored for freezing in the presence of the CS. Cued testing terminated when the house lights were turned off. A solution of 70% ethanol was used to clean the conditioning chambers at the end of training and testing.

Drug Administration and Experimental Design

Caffeine (Sigma C0750) was dissolved in filtered water and administered through drinking bottles at 0, 1.0, or 3.0 mg/mL. The selection of caffeine concentrations was based on previous work examining the effects of chronic caffeine (Dall'Igna et al., 2003; Jaszyna et al., 1998; Rossi et al., 2009). Drinking bottles were changed every 2-3 days as described previously (Boeck et al., 2009; da Silva et al., 2003). All bottles were replaced with filtered water on day 14. Mice were then left undisturbed for 30 days prior to training in fear conditioning except for weekly water and cage changes.

To investigate the effects of chronic caffeine on fear conditioning after a 30 day period of withdrawal, mice were trained in fear conditioning on day 44 ($n = 8-12$) and tested for contextual and cued fear conditioning on day 45. One week after the initial testing, mice were re-tested for contextual and cued recall (see Figure 10 for a schematic diagram of the experiment).

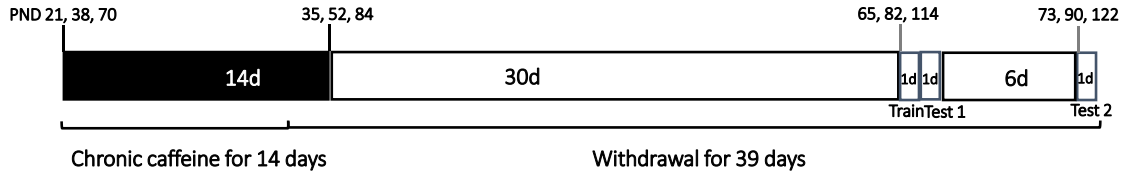


Figure 10: Schematic diagram of 30 day withdrawal experiment.

Data Analysis

One-way ANOVAs were used to analyze fear conditioning measures (i.e. freezing during immediate, baseline, context, pre-CS, and CS) within each age group. One-way ANOVAs for fear conditioning effects were followed by Dunnett’s post-hoc tests using the water treated group within each age group as the reference group when significant treatment effects were detected. Graph Pad Prism 5.0F (GraphPad, San Diego, CA, USA) was used for all of the statistical analyses.

Results

The effects of chronic caffeine exposure during pre-adolescence, adolescence, and adulthood on fear conditioning in adulthood

The effects of chronic caffeine exposure (at 0, 1.0, or 3.0 mg/mL) during adolescence and pre-adolescence on fear conditioning in adulthood were investigated in 3 age groups of mice (Figure 11). A significant effect of treatment was found in adolescent mice for cued fear conditioning [$F(2, 28) = 5.72, p < 0.05$]. Post-hoc tests revealed that exposure to chronic caffeine at 1.0 mg/mL and 3.0 mg/mL during adolescence produced deficits in cued conditioning during adulthood (all p 's < 0.05). No other significant effects were observed for any other fear conditioning measure in any other age group (adult immediate: [$F(2, 24) = 0.23, p > 0.05$]; adolescent immediate: [$F(2, 28) = 2.24, p >$

0.05]; pre-adolescent immediate: [$F(2, 26) = 1.08, p > 0.05$]; adult context: [$F(2, 24) = 0.42, p > 0.05$]; adolescent context: [$F(2, 28) = 0.98, p > 0.05$]; pre-adolescent context: [$F(2, 26) = 2.14, p > 0.05$]; adult pre-CS: [$F(2, 24) = 2.66, p > 0.05$]; adolescent pre-CS: [$F(2, 28) = 1.70, p > 0.05$]; pre-adolescent pre-CS: [$F(2, 26) = 0.56, p > 0.05$]; adult CS: [$F(2, 24) = 0.29, p > 0.05$]; pre-adolescent CS: [$F(2, 26) = 1.05, p > 0.05$]).

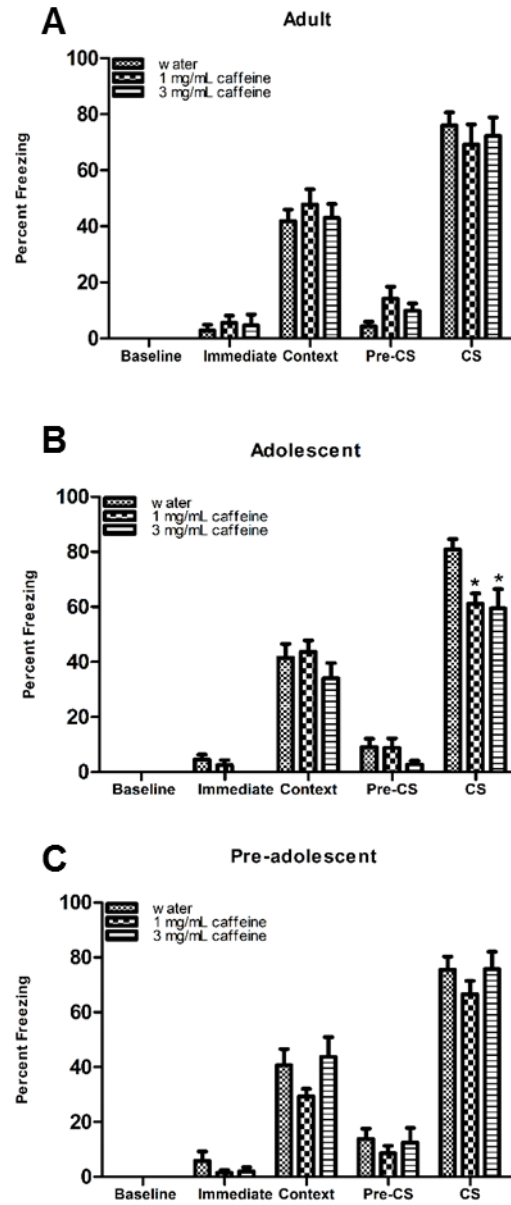


Figure 11: The effects of exposure to chronic caffeine during pre-adolescence, adolescence, and adulthood on contextual fear conditioning in adulthood ($n = 8-12$). Exposure to chronic caffeine at both 1.0 mg/mL and 3.0 mg/mL during adolescence produced deficits in hippocampus-independent cued conditioning in adulthood (B). No significant long-term effects of exposure to chronic caffeine during pre-adolescence or adulthood on fear conditioning were observed 30 days after withdrawal (A and C). Error bars indicate SEM, (*) indicates $p < 0.05$, (**) indicated $p < 0.01$, and (***) indicates $p < 0.001$ compared to water treated mice from each respective age group.

Mice were then retested 7 days later for memory recall (Figure 11). A one-way ANOVA detected a significant effect of treatment on contextual memory in mice that were exposed to chronic caffeine during pre-adolescence [$F(2, 26) = 15.34, p < 0.001$]. Post-hoc tests revealed that mice treated with chronic caffeine at 3.0 mg/mL during pre-adolescence showed enhanced recall of a contextual memory, and mice treated with chronic caffeine at 1.0 mg/mL during pre-adolescence showed deficits in recall of a contextual memory (all p 's < 0.05). No other significant effects were observed for any other fear conditioning measure in any other age group (adult context: [$F(2, 24) = 0.54, p > 0.05$]; adolescent context: [$F(2, 28) = 1.19, p > 0.05$]; adult pre-CS: [$F(2, 24) = 0.07, p > 0.05$]; adolescent pre-CS: [$F(2, 28) = 0.38, p > 0.05$]; pre-adolescent pre-CS: [$F(2, 26) = 0.85, p > 0.05$]; adult CS: [$F(2, 24) = 0.23, p > 0.05$]; adolescent CS: [$F(2, 28) = 1.48, p > 0.05$]; pre-adolescent CS: [$F(2, 26) = 1.63, p > 0.05$]).

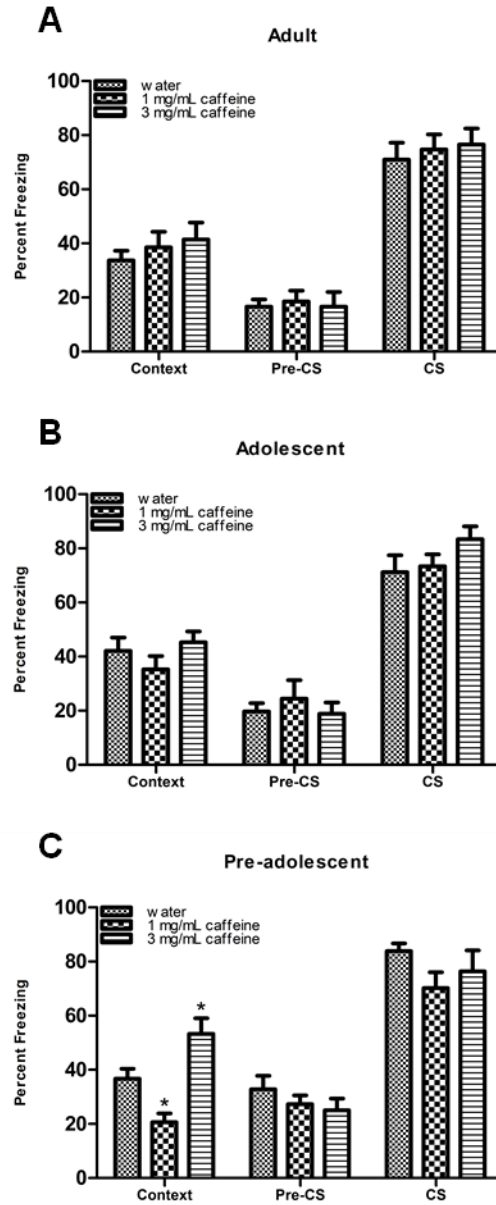


Figure 12: The effects of chronic caffeine exposure during pre-adolescence and adolescence on fear conditioning recall 7 days after Test 1 ($n = 8-12$). Exposure to chronic caffeine at 3.0 mg/mL during pre-adolescence enhances contextual fear recall in adulthood, yet exposure to chronic caffeine at 1.0 mg/mL during pre-adolescence produces deficits in contextual recall in adulthood (C). No significant long-term effects of chronic caffeine exposure during pre-adolescence or adulthood on fear conditioning were observed (A and C). Error bars indicate SEM, (*) indicates $p < 0.05$ compared to water treated mice from each respective age group.

To determine if the difference in recall at Test 2 was due to a change in memory retention, difference scores were analyzed between Test 1 and Test 2 (Figure 13). Results suggest that exposure to chronic caffeine during adolescence alters retention of cued memories in adulthood [$F(2, 28) = 5.72, p < 0.01$]. Post-hoc tests revealed that prior exposure to chronic caffeine at both 1.0 mg/mL and 3.0 mg/mL enhances cued memory retention in adulthood (p 's < 0.05). Furthermore, results suggest that exposure to chronic caffeine during pre-adolescence alters contextual memory retention in adulthood [$F(2, 26) = 5.52, p < 0.05$], and post-hoc tests revealed that exposure to chronic caffeine at 3.0 mg/mL during pre-adolescence enhances retention of contextual memories in adulthood ($p < 0.05$). There was also an effect of chronic caffeine exposure during adolescence on contextual memory retention in adulthood [$F(2, 28) = 3.66, p < 0.05$]; however, post-hoc tests were n.s. ($p > 0.05$). Together, the fear conditioning results suggest that exposure to chronic caffeine during adolescence has long-lasting effects on hippocampus-independent cued conditioning and retention. In contrast, exposure to chronic caffeine during pre-adolescence has long-lasting effects on retention of hippocampus-dependent contextual memories.

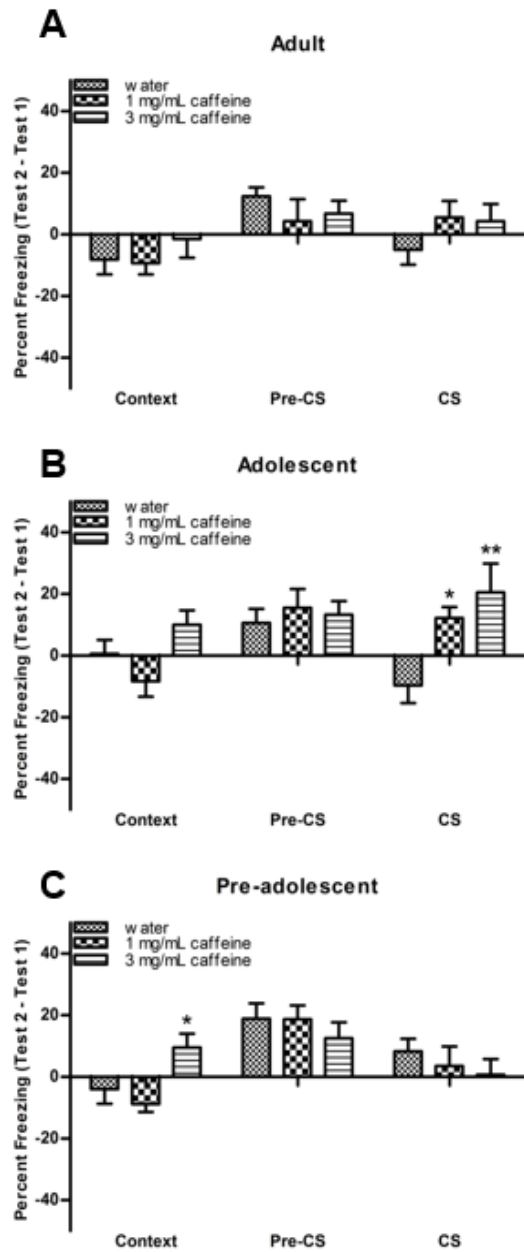


Figure 13: The effects of chronic caffeine exposure during pre-adolescence and adolescence on retention of fear memories ($n = 8-12$). Exposure to chronic caffeine during adolescence enhanced retention of cued memories in adulthood (B). Exposure to chronic caffeine at 3.0 mg/mL during pre-adolescence enhanced retention of contextual memories in adulthood, yet exposure to chronic caffeine at 1.0 mg/mL during pre-adolescence impaired retention of contextual memories in adulthood (C). No significant long-term effects of exposure to chronic caffeine during adulthood were observed (A). Error bars indicate SEM, (*) indicates $p < 0.05$, and (**) indicates $p < 0.01$ compared to water treated mice from each respective age group.

Table 3: The Effects of Prior Exposure to Chronic Caffeine 30 Days After Withdrawal

		Test 1			Test 2		
		1 mg/mL	3 mg/mL		1 mg/mL	3 mg/mL	
Adult	<i>Baseline</i>	-	-		<i>Baseline</i>	-	-
	<i>Immediate</i>	-	-		<i>Immediate</i>	-	-
	<i>Contextual</i>	-	-	Adult	<i>Contextual</i>	-	-
	<i>Pre-CS</i>	-	-		<i>Pre-CS</i>	-	-
	<i>CS</i>	-	-		<i>CS</i>	-	-
Adolescent	<i>Baseline</i>	-	-		<i>Baseline</i>	-	-
	<i>Immediate</i>	-	-	Adolescent	<i>Immediate</i>	-	-
	<i>Contextual</i>	-	-		<i>Contextual</i>	-	-
	<i>Pre-CS</i>	-	-		<i>Pre-CS</i>	-	-
	<i>CS</i>	↓ *	↓ *		<i>CS</i>	-	-
Pre-adolescent	<i>Baseline</i>	-	-		<i>Baseline</i>	-	-
	<i>Immediate</i>	-	-		<i>Immediate</i>	-	-
	<i>Contextual</i>	-	-	Pre-adolescent	<i>Contextual</i>	↓ *	↑ *
	<i>Pre-CS</i>	-	-		<i>Pre-CS</i>	-	-
	<i>CS</i>	-	-		<i>CS</i>	-	-

Table 3: The effects of exposure to chronic caffeine during pre-adolescence, adolescence, and adulthood on fear conditioning in adulthood.

Discussion

In the present study, chronic caffeine was administered to pre-adolescent, adolescent, and adult mice for 14 days. Following a 30 day washout period where caffeine was not given, mice were trained in contextual fear conditioning and tested for contextual and cued conditioning 24 hours later (i.e. Test 1). Mice were then retested for recall of contextual and cued conditioning 7 days after the initial test (i.e. Test 2). Difference scores comparing the change in freezing between Test 1 and Test 2 were then computed as a measure of memory retention. Prior exposure to chronic caffeine during pre-adolescence had no effect on contextual or cued conditioning at Test 1; however, prior exposure to chronic caffeine at 1.0 mg/mL during pre-adolescence impaired recall of contextual memories at Test 2 and prior exposure to chronic caffeine at 3.0 mg/mL during pre-adolescence enhanced recall of contextual memories at Test 2. Furthermore, prior exposure to chronic caffeine at 3.0 mg/mL during pre-adolescence enhanced retention of contextual memories. In contrast, prior exposure to chronic caffeine during adolescence impaired cued conditioning, yet enhanced cued retention regardless of concentration. Exposure to chronic caffeine during adulthood had no effect on contextual or cued conditioning later in adulthood. Overall, prior exposure to chronic caffeine during pre-adolescence altered recall and retention of hippocampus-dependent contextual memories in adulthood depending on concentration, and prior exposure to chronic caffeine during adolescence impaired hippocampus-independent cued conditioning in adulthood independent of concentration.

The finding that exposure to chronic caffeine during adolescence impaired cued conditioning in adulthood may suggest that chronic caffeine disrupts the development of

brain regions required for the formation of associative memories in cued fear conditioning. As the amygdala contains the neural circuitry that supports the acquisition of the CS-US association of auditory cued fear conditioning (Phillips & LeDoux, 1992), it might seem plausible that exposure to chronic caffeine during adolescence exerts long-lasting effects on amygdala function which interfere with auditory cued memories in adulthood. However, the amygdala is also essential for contextual conditioning. Therefore, other brain regions that modulate cued conditioning could be affected by chronic caffeine exposure during adolescence.

An alternative explanation for a deficit in cued conditioning in the absence of a deficit in contextual conditioning could be that exposure to chronic caffeine during adolescence had lasting effects on ventral hippocampal function. In support, ventral hippocampus lesions have been found to disrupt both contextual and cued conditioning (Esclassan & Coutureau, 2009). Thus, if chronic caffeine exposure during adolescence enhanced dorsal hippocampus function in adulthood, this could have masked ventral hippocampal dysfunction. Therefore, even in the absence of an effect of treatment on contextual conditioning, chronic caffeine may have long-term effects on hippocampal function. The idea that exposure to chronic caffeine during adolescence could enhance function in the dorsal hippocampus during adulthood seems plausible given that mice exposed to chronic caffeine at 3.0 mg/mL during pre-adolescence showed enhanced recall and retention of contextual memories.

Exposure to chronic caffeine during pre-adolescence and adolescence may disrupt the function of the hippocampus in adulthood by interfering with the development of the adenosinergic system in this region. Caffeine is a non-specific adenosine receptor

antagonist (Fredholm et al., 1999), and the hippocampus expresses high levels of adenosine A1 receptors, yet lower levels of adenosine A2A receptors throughout life (Costenla et al., 2010; Cunha et al., 1995; Fastbom et al., 1987; Fredholm et al., 2005; Rosin & Robeva, 1998). However, between late adolescence and adulthood the density of A1Rs decrease and the density of A2ARs increase in the hippocampus (Cunha et al., 1995). Interestingly, it was recently demonstrated that chronic caffeine at 1.0 mg/mL increases levels of A1Rs in the adolescent hippocampus (Ardais, 2014). However, it is not yet known if chronic caffeine-induced A1R upregulation in the hippocampus is sustained into adulthood, or if caffeine exposure induces a uniform upregulation of A1Rs across the dorso-ventral axis in the hippocampus. With that said, endogenous adenosine acting at A1Rs has been shown to blunt excitatory neurotransmission in the hippocampus (Yoon & Rothman, 1991). Therefore, increased A1R levels in the ventral hippocampus could produce deficits in contextual and cued conditioning. Likewise, increased A1R levels in the dorsal hippocampus could produce deficits in contextual conditioning. Future studies should therefore examine the effect of exposure to chronic caffeine during pre-adolescence and adolescence on A1R levels in the hippocampus during adulthood. The decrease in A1R levels between late adolescence and adulthood observed by Cunha and colleagues (1995) may be important for maintaining normal learning and memory as animals age, and prior caffeine exposure may interfere with A1R downregulation.

Another possible explanation for the observed cued deficit in absence of a contextual conditioning deficit could be that prior caffeine treatment disrupted hearing or development of the auditory system. Interestingly, Mujica-Mota and colleagues (2014) recently demonstrated that exposure to 120 mg/kg/day of caffeine for 14 days exerted a

detrimental effect on hearing recovery after acoustic trauma. Furthermore, in utero exposure to chronic caffeine delays the development of auditory startle in male mice (West et al., 1986). However, the fact that mice exposed to chronic caffeine during pre-adolescence do not show deficits in cued conditioning suggest that the auditory system is likely not affected by caffeine.

Exposure to chronic caffeine during pre-adolescence had no effect on contextual or cued conditioning at Test 1; however, at Test 2 recall of the hippocampus-dependent contextual memory was affected in a concentration-dependent manner. Specifically, exposure to chronic caffeine at 1.0 mg/mL during pre-adolescence impaired contextual recall at Test 2. However, difference scores suggest that chronic caffeine at 1.0 mg/mL had no effect on retention. In other words, the difference between recall at Test 1 and recall at Test 2 was comparable between the group treated with chronic caffeine at 1.0 mg/mL during pre-adolescence and the group treated with water during pre-adolescence. Therefore, these results may indicate a subtle conditioning deficit that only reached significance at Test 2. In other words, these findings may indicate a learning deficit as opposed to a recall deficit. However, future work will be necessary to determine whether exposure to chronic caffeine at 1.0 mg/mL during pre-adolescence affects contextual learning and/or memory. Interestingly, exposure to chronic caffeine at 3.0 mg/mL enhanced contextual recall at Test 2. In addition, the difference between recall at Test 1 and recall at Test 2 was significantly greater in mice treated with chronic caffeine at 3.0 mg/mL during pre-adolescence. Therefore, exposure to the higher concentration of chronic caffeine during pre-adolescence enhanced retention of contextual conditioning in adulthood. These findings may suggest that prior exposure to chronic caffeine at 3.0

mg/mL during pre-adolescence enhances contextual memory as opposed to contextual learning in adulthood. Alternatively, prior exposure to chronic caffeine at 3.0 mg/mL during pre-adolescence may produce a deficit in extinction of contextual memories, or change the way that memories decay during adulthood. In order to determine if chronic caffeine at 3.0 mg/mL during pre-adolescence impairs extinction in adulthood, extinction training could be employed in future studies. To determine if chronic caffeine at 3.0 mg/mL during pre-adolescence has an effect on memory decay in adulthood, retention tests could be administered at different time points post-training as well.

Our findings that exposure to chronic caffeine at 3.0 mg/mL during pre-adolescence enhances recall and retention of contextual conditioning in adulthood are in agreement with the findings presented by Abreu and colleagues (2011) that exposure to chronic caffeine during pre-adolescence enhances object recognition memory in adulthood in rats. However, Abreu and colleagues (2011) continued treatment through adulthood, which may suggest that rodents that consume chronic caffeine during pre-adolescence do not become tolerant to the effects of caffeine on cognition in adulthood. Interestingly, Abreu and colleagues (2011) treated adolescent rats with chronic caffeine at 1.0 mg/mL, and this concentration of caffeine was found to produce deficits in recall of contextual conditioning in the present study. Therefore, continuing treatment with chronic caffeine at 1.0 mg/mL from pre-adolescence into adulthood may enhance learning and memory, while discontinuing treatment with chronic caffeine at 1.0 mg/mL in adolescence produces recall deficits in adulthood. Future studies will be necessary to determine the significance of continuing caffeine treatment from pre-adolescence into adulthood on contextual conditioning.

It may be argued that the observed effects were not due to early exposure, but rather are the result of withdrawal. However, in the present study, 30 days were allowed to elapse following cessation of chronic caffeine treatment. It is expected that this extended period of withdrawal precluded potential confounding effects of acute caffeine withdrawal on contextual fear conditioning. In humans, caffeine withdrawal symptoms (e.g. headache, fatigue, and decreased ability to concentrate) begin 12-24 hours after cessation of habitual caffeine consumption, peak between 20-51 hours, and last between 2 and 9 days (Juliano & Griffiths 2004). Interestingly, rodents show a similar time-course of withdrawal-associated behavior. For example, Sukhotina and colleagues (2004) treated adult Swiss mice with increasing doses of caffeine (40-100 mg/kg i.p., twice daily) for 8 days. Twenty-four hours after the last injection of caffeine, significant increases in defensive behavior and decreases in locomotor activity were observed. In addition, an acute injection of caffeine (1 mg/kg) 24 hours after cessation of chronic caffeine reversed these withdrawal-associated behaviors (i.e. the acute dose of caffeine reduced the withdrawal-associated increase in defensive behavior and decrease in locomotor activity). However, these changes were no longer apparent at 72 hours after caffeine cessation. Furthermore, Kaplan and colleagues (1993) treated adult CD-1 mice with chronic caffeine via s.c. pumps (97 or 194 mg/kg/day) for 6 days and observed locomotor depression 24 and 48 hours after caffeine cessation. However, locomotor activity returned to control levels by days 4 and 6 after withdrawal. Additionally, Khaliq and colleagues (2012) treated 6-7 week old (180-200 g) albino Wistar rats with 30 mg/kg caffeine for 5 days and administered saline on the 6th day to initiate withdrawal. 30 minutes after withdrawal, rats were tested for memory in the Morris water maze. The

authors found that caffeine withdrawal significantly impaired memory function. However, in Chapter 2 it was shown that 24-48 hours of withdrawal from chronic caffeine has no effect on contextual or cued conditioning in adolescent or adult mice, and that 7 days after withdrawal from chronic caffeine pre-adolescent mice show recall of contextual and cued conditioning that is comparable to water treated mice. Therefore, while the time-course and schedule of chronic caffeine administration, as well as the species being treated likely has an effect on the precise length of acute withdrawal (i.e. repeated administration of 30 mg/kg of caffeine impairs memory in the Morris water maze 24 hours after the last treatment in adult rats, yet continuous exposure to chronic caffeine at 1.0 mg/mL or 3.0 mg/mL has no effect on contextual conditioning 24-48 hours after withdrawal), data suggest that the effects of acute withdrawal on contextual and cued conditioning in C57BL/6J mice are likely not present 30 days after caffeine cessation.

This is the first study to examine the effects of chronic caffeine during pre-adolescence and adolescence on hippocampus-dependent and hippocampus-independent learning and memory in adulthood. Together, our results indicate that exposure to chronic caffeine during adolescence impairs cued conditioning, yet enhances recall and retention of cued memories in adulthood. In addition, exposure to chronic caffeine during pre-adolescence alters retention of contextual memories in adulthood depending on concentration. Future work will be necessary to determine what brain regions are involved in the observed effects and to determine if chronic caffeine has age-dependent effects on extinction of fear memories. Furthermore, future work will be necessary to

determine the long-lasting neuroadaptations underlying the observed behavioral effects which may include changes in adenosine receptor levels or adenosinergic signaling.

CHAPTER 4

Conclusion

The experiments described above revealed several findings regarding the age-dependent effects of chronic caffeine. In Chapter 2, we demonstrated that exposure to chronic caffeine at 1.0 mg/mL during pre-adolescence and adolescence enhanced contextual conditioning, yet exposure to chronic caffeine at 3.0 mg/mL during pre-adolescence and adolescence produced deficits in contextual conditioning. In contrast, exposure to chronic caffeine during adulthood exerted no effect on contextual conditioning. Furthermore, withdrawal from chronic caffeine during adolescence or adulthood exerted no effect on contextual conditioning. However, withdrawal from both concentrations of chronic caffeine during pre-adolescence produced deficits in contextual conditioning. Interestingly, neither chronic caffeine nor withdrawal from chronic caffeine altered cued conditioning in any age group. Therefore, chronic caffeine and withdrawal from chronic caffeine have age-dependent effects on hippocampus-dependent contextual conditioning, but not hippocampus-independent cued conditioning. These results suggest that habitual caffeine consumption during childhood and adolescence may have specific effects on explicit memory, a form of memory that depends critically on the hippocampus (Eichenbaum, 2001; Squire, 1987).

The experiments described in Chapter 3 examined the long-lasting effects of exposure to chronic caffeine during pre-adolescence, adolescence, and adulthood on contextual and cued conditioning 30 days after withdrawal (i.e. when all age groups reached adulthood). Exposure to chronic caffeine at 1.0 mg/mL during pre-adolescence impaired recall of contextual memories in adulthood, yet exposure to chronic caffeine at

3.0 mg/mL during pre-adolescence enhanced recall and retention of contextual memories in adulthood. Furthermore, exposure to chronic caffeine during adolescence impaired cued conditioning in adulthood. In contrast, exposure to chronic caffeine during adulthood had no effect on contextual or cued conditioning later in adulthood.

If contextual conditioning is enhanced by chronic caffeine at 1.0 mg/mL during adolescence (see Chapter 2), yet produces deficits in cued conditioning in adulthood (see Chapter 3) (i.e. after a 30 day washout period where caffeine is not administered), this may suggest that chronic caffeine has immediate effects on the dorsal hippocampus, yet interferes with the development of the ventral hippocampus. In other words, the deficit in cued conditioning observed in mice that were treated with chronic caffeine during adolescence and tested in adulthood may be the result of disrupted function in the ventral hippocampus. In support, inactivation of the ventral hippocampus produces deficits in both contextual and cued conditioning, yet inactivation of the dorsal hippocampus produces deficits in contextual conditioning only (Esclassan et al., 2009). Interestingly, the ventral hippocampus plays an important role in emotional behavior (Henke, 1990) (for review see Fanselow & Dong, 2010). Thus, if exposure to chronic caffeine during adolescence disrupts ventral hippocampus function in adulthood, this would be in agreement with previous findings that exposing adolescent rats to chronic caffeine leads to increased emotionality in adulthood (Anderson & Hughes, 2008). While additional experiments will be necessary to determine which brain regions are affected by exposure to chronic caffeine, the data presented in Chapters 2 and 3 clearly demonstrate that exposure to chronic caffeine during pre-adolescence and adolescence exerts immediate and long-lasting effects on learning and memory, while exposure to chronic caffeine during adulthood exerts no such effects.

Overall, the findings presented in the current study suggest that habitual caffeine consumption during childhood and adolescence could compromise normal learning and memory in addition to the function of brain regions underlying contextual conditioning such as the hippocampus (Logue et al., 1997; Phillips & LeDoux, 1992). Furthermore, by altering normal cognition, habitual caffeine consumption during childhood and adolescence could potentially impact academic performance, interfere with social and emotional functioning (Cortiella & Horowitz, 2014), or perhaps predispose individuals to developing substance use disorders (Beitchman, Wilson, Douglas, Young, & Adlaf, 2001). Indeed, previous studies have found an association between caffeine consumption during adolescence and poor academic performance, emotional instability (James et al., 2011), and illicit drug use (Arria & O'Brien, 2011; Arria et al., 2010). Furthermore, the findings that pre-adolescent mice display cognitive deficits during withdrawal from chronic caffeine may suggest that children are at a greater risk for caffeine withdrawal-induced deficits in cognition than adolescents or adults. Given that children show irregular patterns of caffeine consumption (e.g. children consume less caffeine during the weekdays than on the weekends (Pollak & Bright, 2003)), this age group should be monitored more closely for caffeine withdrawal-related problems in school.

Again, previous studies in humans have demonstrated that caffeine consumption during adolescence is associated with mental illness, drug use, and other problem behavior (James et al., 2011; Kristjansson, Sigfusdottir, Frost, & James, 2013; Temple, 2009; Whalen et al., 2008). However, it is not yet clear whether caffeine consumption is more common in young people who are predisposed to developing cognitive, emotional, and behavioral problems, or if caffeine consumption during adolescence precipitates these

problems. In light of our findings that exposure to chronic caffeine during pre-adolescence and adolescence disrupts normal learning and memory, and alters anxiety-related behavior, we propose that caffeine consumption during childhood and adolescence has the ability to produce maladaptive cognitive, emotional, and behavioral states. Given that it would be unethical to examine the causal relationship between chronic caffeine consumption and cognitive and emotional function in children and adolescent humans, these data provide compelling evidence to limit caffeine intake in younger people. However, future work will be necessary to determine what doses of caffeine (if any) are appropriate for children and adolescents.

It should be noted that caffeine may have beneficial effects on cognition in aged animals (Prediger et al., 2005; Vila-Luna et al., 2012), and may be neuroprotective/reverse cognitive deficits in models of disrupted cognition (Cunha & Aghostino, 2010; Alhaider et al., 2010). Furthermore, it may not be advisable to recommend that children and adolescents who are currently caffeine consumers discontinue caffeine use. In support, Pires and colleagues (2010) found that treating pre-adolescent rats with caffeine for 14 days impaired performance in the novel object recognition task when animals were tested in adulthood without caffeine on board. In contrast, Abreu and colleagues (2011) found that exposing rats to chronic caffeine throughout pre-adolescence, adolescence, and adulthood enhanced performance in the novel object recognition task when caffeine was on board in adulthood. Therefore, in future human studies it will be important to report the age at which subjects first become habitual caffeine consumers (i.e. during childhood, adolescence, or adulthood) as we do

not yet have a good understanding of how the age when caffeine consumption is initiated contributes to the learning and memory effect of caffeine later in life.

In conclusion, future studies will be necessary to determine exactly how chronic caffeine affects cognition in pre-adolescent and adolescent animals (i.e. what brain regions are involved), what doses of caffeine can be tolerated without altering learning and memory, and whether discontinuing caffeine consumption after initiating caffeine consumption at an early age will be beneficial or detrimental for cognitive function later in life. Overall, hippocampus-dependent memory systems appear to be more sensitive to the effects of chronic caffeine exposure during pre-adolescence and adolescence. As stated previously, this could be due to developmental changes in hippocampal structure (Pokorny & Yamamoto, 1981; Seress & Ribak, 1995; Zehr et al., 2008) and function (Pyapali et al., 1999; Swartzwelder et al., 1995a, 1995b; White & Swartzwelder, 2004). Alternatively, as caffeine is an adenosine receptor antagonist (Fredholm et al., 1999) these effects could be mediated by age-related changes in the adenosinergic system. While previous studies have demonstrated that the hippocampus is vulnerable to psychostimulant-induced modifications during pre-adolescence and adolescence, this is the first study to demonstrate that exposure to chronic caffeine during these developmental periods can specifically affect hippocampus-dependent conditioning, while leaving hippocampus-independent cued conditioning intact. Furthermore, this is the first study to demonstrate that exposure to chronic caffeine during adolescence may have enduring effects on cued conditioning in adulthood.

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