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Adolescent caffeine consumption increases adulthood anxiety-related behavior and modifies neuroendocrine signaling

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Abstract

Caffeine is a commonly used psychoactive substance and consumption by children and adolescents continues to rise. Here, we examine the lasting effects of adolescent caffeine consumption on anxiety-related behaviors and several neuroendocrine measures in adulthood. Adolescent male Sprague-Dawley rats consumed caffeine (0.3 g/L) for 28 consecutive days from postnatal day 28 (P28) to P55. Age-matched control rats consumed water. Behavioral testing for anxiety-related behavior began in adulthood (P62) 7 days after removal of caffeine. Adolescent caffeine consumption enhanced anxiety-related behavior in an open field, social interaction test, and elevated plus maze. Similar caffeine consumption in adult rats did not alter anxiety-related behavior after caffeine removal. Characterization of neuroendocrine measures was next assessed to determine whether the changes in anxiety were associated with modifications in the HPA axis. Blood plasma levels of corticosterone (CORT) were assessed throughout the caffeine consumption procedure in adolescent rats. Adolescent caffeine consumption elevated plasma CORT 24 h after initiation of caffeine consumption that normalized over the course of the 28-day consumption procedure. CORT levels were also elevated 24 h after caffeine removal and remained elevated for 7 days. Despite elevated basal CORT in adult rats that consumed caffeine during adolescence, the adrenocorticotrophic hormone (ACTH) and CORT response to placement on an elevated pedestal (a mild stressor) was significantly blunted. Lastly, we assessed changes in basal and stress-induced *c-fos* and corticotropin-releasing factor (*Crf*) mRNA expression in brain tissue collected at 7 days withdrawal from adolescent caffeine. Adolescent caffeine consumption increased basal *c-fos* mRNA in the paraventricular nucleus of the hypothalamus. Adolescent caffeine consumption had no other effects on the basal or stress-induced *c-fos* mRNA changes. Caffeine consumption during adolescence increased basal *Crf* mRNA in the central nucleus of the amygdala, but no additional

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Contributors

CEO, RJN, RLS, SC and RKB contributed to concept development and experimental design of procedures. CEO, RJN, and RKB wrote the paper. CEO, RJN, JS, TS, and SCL conducted behavioral experiments. CEO, RJN, JS, SA, TS performed *in situ* hybridization and quantification. SC and RLS provided materials for pedestal stress experiments, and experimental guidance for the ACTH and CORT assays and *in situ* hybridization. CEO, RJN, RLS, SC and RKB contributed to data analysis and interpretation of findings. All authors approved final version for submission.

Conflicts of Interest

None.

effects of stress or caffeine consumption were observed in other brain regions. Together these findings suggest that adolescent caffeine consumption may increase vulnerability to psychiatric disorders including anxiety-related disorders, and this vulnerability may result from dysregulation of the neuroendocrine stress response system.

Keywords

Adolescence; Corticotropin-releasing hormone; Hypothalamic-Pituitary-Adrenal Axis; Glucocorticoid; Immediate early gene

1. Introduction

Caffeine is the most widely used psychostimulant in the world (Rath, 2012; Warzak et al., 2011). Caffeine consumption has risen in recent years, especially among children and adolescents (Temple, 2009). In fact, 75% of children 5 years or older in the United States consume caffeine on a daily basis (Ahluwalia and Herrick, 2015), and daily caffeine consumption in 9-17 year olds has more than doubled since 1980 (Frery et al., 2005). The rise in caffeine consumption is compounded by the fact that energy drinks that are increasingly marketed to children and young adults contain extremely high concentrations of caffeine.

Although caffeine is thought to be relatively safe, epidemiological studies suggest that caffeine consumption is linked to anxiety disorders. For example, oral caffeine administration precipitates panic attacks in adults diagnosed with panic disorder, generalized social anxiety disorder, and performance social anxiety disorder (Nardi et al., 2009). Genetic studies have linked panic disorder and agoraphobia with single nucleotide polymorphisms in the adenosine A_{2A} receptor, a primary target antagonized by caffeine (Deckert et al., 1998; Lam et al., 2005). Animal studies also report caffeine-induced anxiety in adult rats as measured by the elevated plus maze, light dark box (El Yacoubi et al., 2000), and open field (Noschang et al., 2009) during acute caffeine administration and chronic caffeine consumption. Withdrawal-induced anxiety was also observed 48 hours following chronic caffeine in adult animals (Bhattacharya et al., 1997). While there is substantial evidence to suggest a relationship between caffeine consumption and anxiety in adults, little research has specifically examined the **lasting** effects of caffeine consumption during adolescence and adulthood.

Adolescence is a developmental period characterized by the maturation of the brain during which numerous endogenous and environmental factors impact the maturation process (Arain et al., 2013; Wahlstrom et al., 2010). Here, we explore the notion that adolescent caffeine consumption is an environmental factor that may increase the risk of developing neuropsychiatric conditions such as anxiety. There appears to be a relationship between anxiety and caffeine consumption occurring during early life. A recent study reports that higher levels of caffeine intake in children in the United Kingdom were associated with an increased risk of anxiety (Ruxton, 2014). Similarly, energy drink consumption in Australian young adult males correlates with self-reported anxiety (Trapp et al., 2014). Rats administered acute caffeine also display increased anxiety while consuming caffeine during

adolescence (Ardais et al., 2014). Thus, there is substantial evidence suggesting that caffeine consumption can influence anxiety in both adolescent and adults, although no studies have examined the long-term effects of chronic adolescent caffeine consumption on anxiety-related behaviors.

The anxiogenic nature of acute caffeine administration has prompted several studies to examine the effect of acute caffeine administration on hypothalamic-pituitary-adrenal (HPA) axis activation. Although there is inconsistent evidence to support a causal role of HPA functioning in the manifestation of anxiety behavior (Armario et al., 2012), the activity of the HPA axis is thought to significantly impact the pathogenesis of stress-related disorders such as depression and anxiety (Jezova and Hlavacova, 2008; McEwen, 2005). The fact that acute caffeine administration increases HPA activity supports a potential link between caffeine-induced anxiety and HPA axis function. In humans, acute caffeine injected into sleeping subjects significantly increases plasma adrenocorticotropin hormone (ACTH) and cortisol (Lin et al., 1997). Studies in rats corroborate this link where higher doses of caffeine elevate plasma ACTH (Patz et al., 2006) and corticosterone (CORT) levels (Nicholson, 1989; Patz et al., 2006). Caffeine also increases levels of the stress-related neuropeptide, corticotropin-releasing factor (CRF), in cultured hypothalamic neurons (Nicholson, 1989). Pharmacological blockade of endogenous CRF release attenuates the increases in plasma CORT seen in response to acute caffeine (Nicholson, 1989). Together, these studies suggest that caffeine enhances both anxiety and the activity of the HPA axis.

The effect of chronic caffeine consumption, especially in adolescents, on anxiety and HPA axis function remains unclear. The present studies were designed to assess the effects of adolescent caffeine consumption on anxiety and HPA axis function in adulthood and in the absence of caffeine. We first conducted several tests of anxiety-related behaviors following 28 days of caffeine consumption occurring during adolescence (postnatal days 28-55; P28-55) or adulthood (P67-94). Based on the observation that adolescent, but not adult, caffeine consumption produced significant lasting enhancements in anxiety-related behaviors, we further characterized the effect of chronic caffeine consumption during adolescence on concurrent and subsequent HPA axis function to determine whether dysregulation of HPA axis activity was associated with enhanced anxiety behavior.

2. Materials and Methods

2.1 Rats and Housing

Male Sprague-Dawley rats (Charles River) were received on either P21 (adolescent studies) or P60 (adult studies) and pair housed with *ad libitum* food and water. Separate cohorts of rats were used for each experimental procedure, except where noted. All experimental procedures were conducted during the light period of a 12 h light/dark cycle and were completed in accordance with the guidelines established by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder.

2.2 Caffeine Consumption Procedure

Seven days after arrival, caffeine-consuming rats were given access to a single bottle containing caffeine in water (0.3 g/L) for 28 days based on previously published procedures (Ardais et al., 2014; O'Neill et al., 2015). Caffeine consumption procedures occurred continuously between P28-P55 in adolescent studies and P67-94 in adult studies. Age-matched control groups continued to receive water throughout the procedure. Caffeine and water consumption were recorded by measuring the amount of fluid consumed throughout the procedure. To avoid the potential stress effects of housing rats singly during adolescence, rats were pair-housed throughout the procedure. Total consumption of water and caffeine for each cage were divided by in half to estimate individual fluid consumption. Caffeine intake (mg/kg/day) was further estimated by adjusting the intake by each animal's individual body weight. Following 28 days of caffeine exposure, the caffeine solution was replaced with water for the remainder of the experiment. Behavioral testing was initiated at least 7 days (unless specified otherwise) after the last caffeine consumption (adolescent studies: P62-69; adult studies: P102-109). Likewise, brain tissue and blood were collected in the absence of caffeine between P62-P69 (except where noted), periods corresponding to adulthood. Behavioral testing and blood sampling were completed in separate cohorts.

2.3 Behavioral Tests

2.3.1 Elevated Plus Maze—Rats were subjected to the elevated plus maze between P62-67 (Adolescent studies) or P101-109 (Adult studies). An additional cohort of adolescent and adult rats was tested on the elevated plus maze during the last week of caffeine consumption (Adolescent: P50-55; Adult: P90-94) to evaluate the direct effects of caffeine consumption on anxiety measures. The elevated plus maze consisted of four arms (50 × 10 cm each) joined by a central platform (10 × 10 cm). Two arms are enclosed with 40 cm high walls, while the other two are “open”. The entire apparatus was elevated 75 cm from the floor. The elevated plus maze procedures were conducted in a fully lit room. Rats were put in the center of the maze and allowed to explore the arms for 5 min. Time spent in the open arms was recorded manually by a technician who was blind to the experimental groups. Open arm time was defined as more than half of the rat's body being in the open arm.

2.3.2 Social Interaction—Rats were tested in a social interaction test between P62-67 (adolescent studies) or P101-109 (adult studies). Each rat was allocated a standard plastic tub cage with a wire lid and bedding located in a designated testing room. Rats were placed in the test cage with a novel age-matched conspecific. An observer, blind to the experimental treatment, timed the exploratory behaviors initiated by the rat being tested to the conspecific over a 3 min test period. Exploratory social behaviors included only sniffing and grooming interactions. Other types of interaction like wrestling, boxing, or chasing were not observed during the test.

2.3.3 Open Field—Rats were tested for locomotor activity in an open field. Tests were conducted between P62-67 (adolescent studies) or P101-109 (adult studies). Locomotor activity was recorded in plexiglass chambers (San Diego Instruments) measuring 41 × 41 × 38 cm with 16 pairs of photobeams spaced 2.5 cm apart on both x-and y-axes. All locomotor

tests were performed during the light phase of the light:dark cycle. Central and peripheral activity was recorded for 10 min after introduction into the chamber.

2.4 Pedestal Stress Exposure

Rats were exposed to an elevated pedestal 7 days after the cessation of the caffeine consumption procedures during adolescence (P62). These studies were conducted only following adolescent caffeine consumption due to the lack of effects on all behavioral measures in adults that consumed caffeine for an equivalent duration. Exposure to an elevated pedestal represents a psychological stress sufficient to induce physiological indications of stress (Pace et al., 2005). Exposure to the pedestal occurred within 1 h of the onset of the light cycle, a period considered the trough of the circadian CORT cycling. Rats were placed on a pedestal (27 cm²) elevated 60 cm off the floor for 5 min and returned to their home cage until euthanasia 30 min after the onset of pedestal exposure. Control rats remained in their home cages. Brain samples were immediately collected and flash frozen for 30 s in a beaker of isopentane chilled to approximately –30 °C by a surrounding slurry of ethanol and dry ice. Brains were then stored at –80°C until sectioning and subsequent exposure to *in situ* hybridization procedures. Trunk blood was collected in EDTA-coated tubes and centrifuged at 2000 rpm for 10 min at 4°C. Blood plasma was pipetted into 0.5 mL microcentrifuge tubes and stored at –80°C until analysis for CORT and ACTH.

2.5 Blood Sampling

2.5.1 Time course of caffeine-related HPA axis activity throughout the caffeine consumption procedure—Acute caffeine exposure has been shown to activate the HPA axis (Nicholson, 1989; Patz et al., 2006). Therefore, we assessed how chronic caffeine consumption impacted HPA axis function. Blood samples were collected via tail nick within 1 h of light cycle onset (i.e. circadian trough of HPA axis activity) on days 1, 14, and 28 of the caffeine consumption procedure. We also collected blood samples on day 29 of the procedure, which corresponds to 24 h after caffeine consumption ended. Plasma was frozen at –80°C for subsequent CORT analysis.

2.5.2 Circadian peak and trough of basal plasma CORT level—In order to examine whether adolescent caffeine consumption altered circadian peak and trough levels of plasma CORT at the time of behavioral assessments, blood samples were taken from a separate cohort of caffeine-treated rats and control rats 7 days after cessation of caffeine treatment (P62). Blood samples were taken via tail nick within 1 h following the onset of the light cycle for determination of basal CORT level at the circadian trough of HPA axis activity, and within 1 h of the onset of the dark cycle for determination of plasma CORT levels at the circadian peak of HPA axis activity. Plasma was frozen at –80°C for subsequent CORT analysis.

2.5.3 Acute ACTH challenge—Another cohort of caffeine consuming rats and controls were administered ACTH (1 mg/kg, i.p.) 7 d following cessation of caffeine consumption (P62) to determine adrenal cortex sensitivity (Cole et al., 2000). Blood samples were taken via tail nick 30 minutes after ACTH administration, and plasma was frozen at –80°C for subsequent CORT analysis.

2.6 CORT Enzyme Linked ImmunoSorbent Assays (ELISA)

Total CORT levels were assayed according to the manufacturer's instructions (Arbor Assays, #K014-H5, Ann Arbor, MI) with the following modification. Plasma samples were diluted 1:50 with assay buffer and heat-inactivated at 80 °C for 1 hr in order to denature corticosteroid binding globulin. To avoid inter-assay variability, all samples from each experiment were run on the same assay plate with intra-assay coefficient of variances ranging from 8.07-9.65% for each assay plate.

2.7 ACTH assay

Plasma concentrations of ACTH were determined in duplicate (200 µl plasma) by a competitive radioimmunoassay procedure (Diasorin, kit #27130, Stillwater, MN, USA). The procedure was completed using the manufacturer's instructions. To avoid inter-assay variability, all samples were run on the same assay plate with intra-assay coefficient of variances of 5.15%.

2.8 In situ hybridization and autoradiography

Previously published methods were used for in situ hybridization histochemistry (Day and Akil, 1996). Briefly, brains were section into 12 µm sections on a cryostat, thaw-mounted on polylysine-coated slides and stored at -80°C. A [³⁵S]-UTP-labeled riboprobe against *c-fos* mRNA (provided by Dr. T. Curran, St Jude Children's Hospital, Memphis TN) or *Crf* mRNA (provided by Dr. R. Thompson at the University of Michigan) was generated using standard transcription methods. Sections were fixed in 4% paraformaldehyde for 1 h, acetylated in 0.1 M triethanolamine with 0.25% acetic anhydride for 10 min, and dehydrated through graded alcohols. Sections were hybridized overnight at 55°C with a [³⁵S]-UTP-labeled riboprobe diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 2× saline sodium citrate (SSC), 50 mM PBS, pH 7.4, 1× Denhardt's solution, and 0.1 mg/ml yeast tRNA. The following day, sections were treated with RNase A, 200 µg/ml at 37 °C for 1 h, and washed to a final stringency of 0.1× SSC at 65°C (1 hour). Dehydrated sections were exposed to X-ray film (BioMax MR; Eastman Kodak, Rochester, NY) for region- and probe-appropriate times (1–3 weeks) prior to film development.

2.9 Image Quantification

Autoradiographic images were digitized using a light box with even illumination, camera (CCD camera, Sony XC-77, Tokyo, Japan) and analyzed using Scion Image (version 4 for PC). Regions of interest were determined by anatomical landmarks including white matter distribution of unstained tissue sections and compared to a standard rat brain atlas (Paxinos and Watson, 1998). A region-specific template was placed over the region of interest, a background sample was taken over an area of white matter, and a signal threshold was calculated as mean gray value of background + 3.5 standard deviations. Only pixels with gray values above these criteria were included in the analysis. Mean integrated gray values were computed from the product of the number of pixels comprising the positive signal and their average gray level within the region of interest. The signal and area were multiplied, giving the integrated density value. This is the value that is used for statistical analysis. Regions of interest are defined in figure captions.

2.10 Data Analysis

Body weight and consumption data (mg/kg/day and ml/day, respectively) were analyzed using a two-way mixed design ANOVA with consumption group (between) and days (within) as factors. Effects of caffeine consumption on anxiety-related behaviors in adult and adolescent studies were analyzed using an unpaired t-test. Basal and stress-induced plasma CORT and ACTH values were analyzed using a two-way between subjects design ANOVA with consumption group and time of day or stress as factors. The time course of plasma CORT across the period of caffeine exposure was analyzed using a two-way mixed design ANOVA with consumption group (between) and day (within) as factors. Stress-induced *c-fos* and *Crf* mRNA expression were analyzed using a two-way between subjects design ANOVA with consumption group and stress as factors. In all cases, significant interactions and main effects were followed by either post-hoc or planned comparisons using one-way ANOVAs or Bonferroni's correction.

3. Results

3.1 Caffeine consumption

The volume of fluid consumed per day (ml/day) and body weights (g) were recorded throughout the caffeine consumption procedure in adolescent rats (Figure 1) and adult rats (Supplemental Figure 1). Total caffeine consumption (mg/kg/day) was calculated for the caffeine group based on fluid consumed and the concentration of the caffeine-containing solution. Figure 1 displays data from the cohort of adolescent rats ($N = 15$) used for the pedestal stress test, blood sampling and *in situ* hybridization. No differences between the water and caffeine groups were observed in either body weight (Adolescent: Figure 1B) or volume of fluid consumed (Adolescent: Figure 1C). There was a significant increase in body weight (Days: $F_{7,210} = 1307.0$, $p < 0.0001$) and total fluid consumed (Days: $F_{7,210} = 102.9$, $p < 0.0001$) in adolescent rats over the course of the procedure. Adolescent caffeine consumption averaged approximately 30.0 ± 0.7 mg/kg/day (Figure 1D). Similarly, there were no group differences between adult water- and caffeine-consuming rats and intake, although caffeine consumption was generally lower than that observed in adolescent animals (Supplemental Figure 1). Analogous consumption data (not shown) were collected for each cohort of adolescent and adult rats that were used to examine anxiety behaviors. No significant group differences were detected in any of the cohorts and all data is comparable to previously published results using this procedure (O'Neill et al., 2015).

3.2 Adolescent, but not adult, caffeine consumption increases adulthood anxiety behaviors following withdrawal from caffeine

To determine if caffeine consumption confined to the adolescent period affected anxiety behaviors in adulthood we examined three different anxiety-related behavioral tests in different cohorts of rats. Seven days withdrawal from caffeine consumption during adolescence decreased the total percent time spent on the open arms and open arm entries of the elevated plus maze (Figure 2A; $t_{17} = 3.081$, $p < 0.01$; $t_{17} = 2.37$, $p < 0.05$) compared to the control group. Rats that consumed caffeine during adulthood showed no significant differences on percent time spent on the open arms (Figure 2B; $t_{18} = .813$, $p = 0.43$) or open arm entries ($t_{17} = .0596$; $p = 0.56$) on the elevated plus maze at 7-day withdrawal. We also

examined open field exploration in both adolescent and adult caffeine consuming rats. Caffeine during adolescence (Figure 2C), but not adulthood (Figure 2D), decreased percent time spent in the center of the open field (Adolescent: $t_8 = 4.450$; $p < 0.01$; Adulthood: $t_{14} = 0.936$, $p = 0.37$). Importantly, we observed no group differences in total locomotion in either adolescent ($t_6 = .814$, $p = 0.45$) or adult rats ($t_{14} = 1.76$, $p = 0.10$) at 7-day withdrawal from caffeine, suggesting that these effects were not confounded by locomotor suppression that is observed at earlier stages of caffeine withdrawal (Holtzman, 1983). Finally, we examined social interaction in rats that consumed caffeine during adolescence and adulthood. Adolescent caffeine consumption decreased the total amount of social interaction (Figure 2E; $t_{16} = 2.135$, $p < 0.05$) compared with age-matched conspecifics following 7-day withdrawal from caffeine. No significant differences were observed in social interaction at 7-day withdrawal following caffeine consumption during adulthood (Figure 2F, $t_{18} = 0.046$, $p = 0.96$). Together, these data indicate that chronic adolescent, but not adult, caffeine consumption increases anxiety-related behavior 7 days after cessation of caffeine consumption when compared to the behavior of respective water consuming control rats. However, these results may be confounded by differences in the amount of caffeine consumed by adolescent and adult rats. Adolescents rats consumed higher caffeine amounts (~30 mg/kg/day) compared to adults (~23 mg/kg/day). To identify whether these differing doses produced differential effects on anxiety, we tested both adolescent and adult rats on the elevated plus maze during the last week of caffeine consumption (Figure 3). Similar to previously published work that tested the anxiogenic effects during caffeine consumption (El Yacoubi et al., 2000), we observed decreased percent time spent on the open arms (Adolescent, $t_{38} = 3.02$, $p < 0.005$; Adult, $t_{16} = 3.21$, $p < 0.01$) and open arm entries (Adolescent, $t_{38} = 2.11$, $p = 0.051$; Adult, $t_{16} = 2.64$, $p < 0.05$) in both adolescent and adult rats (Figure 3).

3.3 Chronic caffeine consumption during adolescence produces persistent dysregulation of HPA axis

We next examined the effects of chronic caffeine consumption during adolescence on various markers of HPA axis activity. Figure 4A illustrates CORT levels throughout the adolescent caffeine consumption procedure beginning 24 h after the introduction of caffeine in the drinking water, at a midpoint of the procedure (day 14), and on the last day of caffeine consumption (day 28). CORT levels were also assessed at 24 h after the removal of caffeine (day 29). Blood samples for each of these time points were taken within 1 h of the onset of the light cycle and are thought to represent basal CORT levels. A two-way ANOVA revealed a significant day X treatment interaction ($F_{3,48} = 4.058$, $p < 0.05$), and significant main effects of treatment ($F_{1,48} = 22.73$, $p < 0.001$) and day ($F_{1,48} = 4.701$, $p < 0.01$). Subsequent post hoc analysis indicates that water-consuming controls showed no significant changes in basal CORT levels across the course of the study. Caffeine-consuming rats, on the other hand, had significantly elevated basal CORT levels on Day 1 ($t_{16} = 3.52$, $p < 0.005$), Day 14 ($t_{16} = 3.66$, $p < 0.005$) and Day 29 ($t_{16} = 3.66$, $p < 0.005$) compared to water-consuming controls. CORT levels on Day 28 were not significantly different from water controls ($t_{16} = 0.90$, $p = 0.3782$). Within group comparisons indicate a significant difference between Day 1 and Day 14 ($t_{48} = 2.627$, $p < 0.05$) and Day 1 and Day 28 ($t_{48} = 3.021$, $p < 0.01$) of rats that consumed caffeine during adolescence. Similarly, CORT levels on Day 29

were also significantly different from Day 14 ($t_{48} = 3.410$, $p < 0.01$) and Day 28 ($t_{48} = 3.803$, $p < 0.001$). These data suggest that 24 h after the initial introduction of caffeine CORT is elevated compared to controls, and by Day 14 and 28 of caffeine exposure rats have become tolerant to the HPA axis activating effects of caffeine. Interestingly, 24 h after the removal of caffeine (Day 29) basal CORT levels were increased in the rats previously treated with caffeine.

In a separate cohort of rats, we examined basal CORT levels 7 days after the removal of caffeine (Figure 4B) at both the circadian trough (light cycle onset) and peak (dark cycle onset) of HPA activity. A two-way ANOVA revealed a significant treatment X time interactions ($F_{1,38} = 4.128$, $p < 0.05$) and a significant main effect of time ($F_{1,38} = 151.2$, $p < 0.0001$). There was no significant main effect of treatment. Subsequent post hoc analysis found that adolescent caffeine consumption elevated basal CORT at the circadian trough compared to controls ($t_{38} = 2.725$, $p < 0.05$), but not at the circadian peak.

Due to the lasting increase in basal CORT levels following adolescent caffeine consumption, we also examined whether prior caffeine consumption altered stress-induced HPA activity. We observed a significant treatment X stress interaction ($F_{1,26} = 3.974$, $p < 0.05$) where a pedestal stress challenge increased ACTH levels in the control rats, but not in the rats that consumed caffeine during adolescence caffeine rats (Figure 4C). Similarly, pedestal stress increased CORT levels in both groups ($F_{1,39} = 74.53$, $p < 0.0001$) but that increase was also significantly blunted following adolescent caffeine consumption as revealed by a significant treatment X stress interaction (Figure 4D; $F_{1,39} = 15.90$, $p < 0.001$).

In order to assess if the decrease in stress-induced CORT release observed in the adolescent caffeine consumption group could be related to decreased sensitivity of the adrenal gland to stress-induced ACTH secretion, we administered ACTH (1 mg/kg, ip) to a different cohort of rats and measured plasma CORT levels 30 min after the injection (Figure 4E). Adolescent caffeine consumption resulted in lower CORT levels in response to the ACTH challenge ($t_{16} = 2.159$; $p < 0.05$) indicating there is likely decreased adrenal function in these rats.

3.4 Basal and stress-induced alterations in *c-fos* mRNA expression following adolescent caffeine consumption

We next evaluated how adolescent caffeine consumption influences neural signaling both basally and in response to a mild stressor using immediate early gene *c-fos* expression as a marker of relative neuronal activity. Consequently, we collected brain tissue from rats exposed to the elevated pedestal and analyzed *c-fos* mRNA expression in several brain regions that have been implicated in stress and/or anxiety (**Supplemental Figure 2**). The paraventricular nucleus (PVN) of the hypothalamus (Figure 5A) shows a stress-induced increase in *c-fos* mRNA expression in both the adolescent caffeine-consuming and water-consuming control rats ($F_{1,26} = 36.36$, $p < 0.0001$). A significant treatment X stress interaction ($F_{2,26} = 4.389$, $p < 0.05$) was also observed, but no significant main effect of treatment. Subsequent post-hoc tests revealed that non-stressed rats that consumed caffeine during adolescence had higher basal levels of *c-fos* mRNA expression compared with water controls ($t_{26} = 2.245$, $p < 0.05$). This increased basal activity in the PVN may relate to the

persistent increases in basal CORT that we observed following adolescent caffeine consumption (Figure 3B).

We also observed several interesting alterations in two subregions of the amygdala. In the central nucleus of the amygdala (CeA), a significant stress-induced increase in *c-fos* mRNA expression (Figure 5B) was observed ($F_{1,25} = 6.313$, $p < 0.05$). A significant treatment X stress interaction ($F_{1,25} = 5.834$, $p < 0.05$) was also observed where post hoc analysis revealed that stress-induced *c-fos* expression was significantly increased in the water control rats ($t_{25} = 5.588$, $p < 0.01$), but not in the adolescent caffeine consuming rats. The basolateral amygdala (BLA) shows increased *c-fos* mRNA expression following pedestal stress ($F_{1,26} = 12.87$, $p < 0.01$), but no significant main effect of treatment or significant treatment X stress interaction were detected. (Figure 5C). The anteroventral division of the bed nucleus of the stria terminalis (BSTav) has previously been shown to be stress reactive (Newsom et al., 2012), however a two-way ANOVA found no significant treatment X stress interaction or main effects of stress or treatment (Figure 5D). We also examined stress-induced *c-fos* mRNA expression in the prefrontal cortex (PFC, Figure 5E). Our analysis included both the prelimbic (PL) and infralimbic (IL) subregions of the PFC since no differential effects between the subregions was detected. A two-way ANOVA revealed a significant main effect of stress ($F_{1,26} = 87.86$, $p < 0.0001$), but no main effect of treatment or treatment X stress interaction. Although the nucleus accumbens (NAc) shell is not typically examined in stress-related studies, we have previously shown alterations in the expression of several proteins within the NAc shell following exposure to caffeine during adolescence (O'Neill et al., 2015). A two-way ANOVA revealed a main effect of stress ($F_{1,26} = 80.26$, $p < 0.0001$), but no significant treatment X stress interaction or main effect of treatment (Figure 5F).

3.5 Expression of *Crf* mRNA is elevated in the central amygdala following adolescent caffeine consumption

To determine whether adolescent caffeine consumption alters additional aspects of central processing of stressful stimuli, we evaluated the mRNA expression of the stress-related neuropeptide, *Crf*, in stress- and/or anxiety-related brain regions (**Supplemental Figure 2**). Utilizing the same tissue from the caffeine- and water-consuming rats that underwent pedestal stress, we evaluated *Crf* mRNA expression levels. No significant effects of caffeine consumption or stress-induced changes in *Crf* mRNA expression were observed in the PVN, PFC, or BSTav (Figure 6). Interestingly, a two-way ANOVA showed that *Crf* mRNA expression in the CeA (Figure 6B) was significantly increased in rats that consumed caffeine during adolescence ($F_{1,26} = 5.328$, $p < 0.05$). No interactive effects or main effect of stress were detected for the CeA.

4. Discussion

We examined the effects of chronic caffeine consumption during adolescence on anxiety-related behaviors and neuroendocrine activity in adulthood following cessation of caffeine consumption. Our findings reveal that adolescent caffeine consumption increases anxiety in several behavioral measures including elevated plus maze, social interaction, and open field activity. These behavioral effects were not observed in adult rats that consumed caffeine for

the same length of time. Rats that consume caffeine during adolescence also exhibit subsequent dysregulation of HPA axis function. Specifically, prior adolescent caffeine consumption increased young adulthood basal CORT at the circadian trough, decreased stress-induced release of ACTH and CORT, and decreased sensitivity of adrenal glands to ACTH. Dysregulation of stress reactivity as a result of prior adolescent caffeine exposure was not only evident at the level of the HPA axis response, but was also evident in some central neural responses. Specifically, we observed alterations in *c-fos* mRNA expression following stress in the PVN, CeA, and NAc, as well as basal increases in *Crf* mRNA in the CeA. Previous studies demonstrate that acute caffeine administration activates the HPA axis (Nicholson, 1989; Patz et al., 2006), and has anxiogenic effects (Ardais et al., 2014; Nardi et al., 2009). Our findings extend this work by demonstrating that chronic caffeine consumption during adolescence produces alterations in HPA activity, stress reactivity and anxiety that persist into adulthood even after removal of caffeine.

Adolescence is a developmental period characterized by the maturation of several brain systems that aid in the brain's ability to perform functions such as self-control, decision-making, emotional processing and risk-taking (Wahlstrom et al., 2010). Adolescent brain maturation is controlled by several known factors including sex hormones, eating and sleeping behaviors, exposure to stress, and exposure to drugs including therapeutic, licit and illicit drugs (Arain et al., 2013). All of these factors have the potential to positively and negatively impact brain development during this time. Here, we demonstrate that consumption of the widely used psychostimulant drug, caffeine, during adolescence increases anxiety that persists into adulthood even after the removal of caffeine. Caffeine use in children and young adults supports these findings in that higher levels of caffeine intake were associated with an increased risk of anxiety (Ruxton, 2014; Trapp et al., 2014). Thus, our findings are congruent with existing data in humans suggesting that caffeine consumption during development may render individuals more vulnerable to the development of anxiety-related disorders such as panic disorder, post-traumatic stress disorder and generalized anxiety disorder. We did not observe analogous vulnerabilities to anxiety in adult rats that consumed caffeine suggesting that these effects are specific to caffeine consumption during adolescence.

Studies examining caffeine consumption in children and adolescents have reported average caffeine intake between 100-400 mg of caffeine per day (Frary et al., 2005; Rudolph et al., 2014; Temple, 2009). Although these amounts are typically not adjusted for body weight, estimated caffeine doses would range between 1-10 mg/kg depending on the consumption and body weight measures throughout adolescence. The rats in our studies consumed significantly more caffeine (~30 mg/kg/day) comparatively. However, due to pharmacokinetic differences between the two species, it is difficult to compare drug dosing in this manner. The half-life of caffeine in adult rats is far shorter ($t_{1/2} \approx 1$ hr) than that of adult humans ($t_{1/2} \approx 5$ hrs), suggesting that a lower mg/kg intake in humans could potentially show similar effects (Arnaud, 1987; Bonati et al., 1982). In addition, studies demonstrate that caffeine clearance rates are extremely slow in neonates with a half-life greater than 40 hrs (Aranda et al., 1979), and become progressively faster through early

development until late adolescence when the clearance rates are similar to adults (Bienvenu et al., 1990; Ginsberg et al., 2002; Latini et al., 1980).

It remains unclear how adolescent caffeine consumption may produce these persistent effects on anxiety behavior. Caffeine is a non-selective adenosine receptor antagonist that has a multitude of effects on the brain. Chronic caffeine produces withdrawal symptoms that emerge 24 hrs after caffeine removal and dissipate within 48 hrs (Finn and Holtzman, 1986; Holtzman, 1983; Rhoads et al., 2011). Since we conducted behavioral and neuroendocrine testing 7 days following caffeine consumption, the effects observed are likely not due to acute withdrawal symptoms previously measured. However, the results may reflect a caffeine-induced protracted withdrawal state existing within specific neurobiological and neuroendocrine systems. Previous studies demonstrated that caffeine administration activates the HPA axis (Nicholson, 1989; Patz et al., 2006). We therefore explored whether adolescent caffeine consumption altered the functioning of the HPA axis.

We explored whether adolescent consumption of caffeine altered subsequent basal and stress-induced HPA axis functioning that may relate to the observed changes in anxiety behavior. We observed enhanced CORT levels after 24 h of caffeine consumption. Rats developed tolerance following chronic caffeine consumption with no CORT elevations detected on the last day (Day 28) of caffeine consumption. We also observed a robust increase in plasma CORT 24 h after the removal of caffeine that persisted for at least 7 days consistent with the emergence of a withdrawal state. Alterations in plasma cortisol have been associated with several psychiatric disorders including depression and panic disorder (Belmaker and Agam, 2008; Strohle and Holsboer, 2003; Veen et al., 2011). We found that adolescent caffeine consumption also impaired plasma ACTH and CORT release in response to an elevated pedestal challenge. Surprisingly, we did not observe a stress-induced increase in plasma ACTH in the rats that consumed caffeine, but did observe a stress-induced increase in plasma CORT. This is likely due to the fact that samples were collected 30 min after the beginning of the pedestal stress, and plasma ACTH peaks between 5 and 15 min while plasma CORT peaks at 30 min following the onset of stress (Kovacs and Sawchenko, 1996). CORT elevations produced by mimicking HPA axis activation with peripheral administration of ACTH was also blunted in the caffeine-consuming rats suggesting that adolescent caffeine consumption decreases adrenal gland sensitivity to ACTH. A blunted HPA response to psychological stress has been seen in humans with panic disorder compared to healthy controls following administration of a psychosocial test (Jezova et al., 2004; Petrowski et al., 2013; Takai et al., 2007).

The effects of adolescent caffeine consumption partially recapitulate effects observed after chronic stress and CORT administration. This is not unexpected since we, and others find that caffeine administration induces HPA activation (Nicholson, 1989; Patz et al., 2006). Thus, similar to our findings, chronic stress in mice decreases adrenal sensitivity to ACTH (Uschold-Schmidt et al., 2012). Contrary to our findings, the altered adrenal sensitivity following chronic stress was associated with lower basal CORT levels whereas we observed higher basal CORT levels. Additionally, chronic CORT administration decreased Crf mRNA expression in the PVN while increasing Crf mRNA expression in the CeA (Makino et al., 1994; Shepard et al., 2000). We observed similar enhancements in Crf expression

following adolescent caffeine consumption, although we did not see changes in PVN *Crf* expression. These findings suggest caffeine consumption may only partially mimic the effects of chronic CORT/stress paradigms.

Caffeine consumption during adolescence also produced differential expression of *c-fos* and *Crf* mRNA in brain regions that have been implicated in stress and anxiety (Bhatnagar and Dallman, 1998; Kearns and Spencer, 2013; Newsom et al., 2012; Weinberg et al., 2007). Most brain regions we examined (PFC, BLA, NAcSh, PVN, and CeA) showed a stress-induced increase in *c-fos* mRNA expression in response to pedestal stress. It is important to note that PVN *c-fos* mRNA expression was elevated in non-stressed rats that consumed caffeine throughout the adolescent period compared to non-stressed water controls. This effect may underlie the basal increases in CORT observed at the circadian trough in rats that consumed caffeine during adolescence. In the CeA, a stress-induced increase in *c-fos* mRNA expression was observed in water control rats, but not caffeine-consuming rats. We also observed increases in *Crf* mRNA expression in the CeA following adolescent caffeine consumption. Chronic elevations in CORT have been shown to increase *Crf* mRNA expression in the CeA that corresponds with increased anxiety behaviors (Makino et al., 1994; Shepard et al., 2000). It is unclear whether these changes correspond with an increase or decrease in CRF protein expression. Administration of CRF directly into the CeA induces anxiety while infusion of CRF antagonists into the CeA block these effects (Davis et al., 1994; Heinrichs et al., 1996; Heinrichs et al., 1992; Rassnick et al., 1993; Swiergiel et al., 1993). In fact, basal increases in *Crf* mRNA expression within the CeA have been suggested as the reason for increased anxiety exhibited by Fawn-hooded rats (Altemus et al., 1994), a strain known to exhibit decreased social interaction as well as increased freezing behavior in response to stress (Kantor et al., 2000; Overstreet et al., 1992). The observation that *Crf* mRNA, and possibly *c-fos* mRNA, expression is increased following adolescent caffeine consumption suggests increased output from the CeA that may enhance fear- and anxiety-related responses.

Rats exposed to caffeine as adults show no persistent alterations in anxiety-related behaviors following the removal of caffeine and there were no differences in overall anxiety behaviors relative to the age of testing. This is somewhat surprising since both acute and chronic caffeine increase anxiety behavior when testing is conducted in the presence of caffeine (El Yacoubi et al., 2000; Nardi et al., 2009; Noschang et al., 2009), and short withdrawal (48 hrs) also increases anxiety on the elevated plus maze in adult animals (Bhattacharya et al., 1997). This suggests that caffeine consumption during adolescence, when the brain is undergoing rapid developmental changes (Arain et al., 2013; Wahlstrom et al., 2010), produces more lasting effects on behavioral reactivity to psychologically stressful events. A significant caveat to the adolescent-specific effects is that caffeine consumption by adolescent animals was higher than that consumed by adult animals (~30 mg/kg/day vs. ~23 mg/kg/day, respectively). These amounts of caffeine consumption were sufficient to induce anxiety in both age groups when tested during the caffeine consumption phase as was shown previously (El Yacoubi et al., 2000), but it is unclear whether this dosing difference could produce differential outcomes during withdrawal. Previous work demonstrates that high levels of caffeine consumption (40-100 mg/kg/day) in adult rats was ineffective in altering

plasma CORT levels and the development of withdrawal-induced conditioned taste aversion at withdrawal time points similar to our studies (Dingle et al., 2008; Pettenuzzo et al., 2008). In addition, the adolescent-specific effects may be attributable to differential clearance of caffeine, potentially compounding the relative dosing differences. This is unlikely since pharmacokinetic studies demonstrate that caffeine clearance rates of adolescents are faster in early adolescence and become quite similar to the clearance rates of adults by late adolescence (Bienvenu et al., 1990; Ginsberg et al., 2002; Latini et al., 1980).

5. Conclusions

The data presented here indicate that caffeine consumption during adolescence has effects on the expression of anxiety-related behavior and neuroendocrine functioning that persist into adulthood in the absence of continued caffeine consumption. These data suggest that caffeine consumption during adolescent development may increase vulnerability to the development of psychiatric disorders. Given the increasing prevalence of caffeine consumption among children and adolescents (Ahluwalia and Herrick, 2015; Frary et al., 2005; Temple, 2009), it is important to enhance awareness of the potentially deleterious long-term effects of caffeine consumption during adolescent development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Adolescent caffeine consumption increases anxiety-related behavior in adulthood
- Withdrawal from adolescent caffeine consumption increases blood corticosterone levels in adulthood
- Adolescent caffeine consumption decreases adrenal gland sensitivity in adulthood
- Adolescent caffeine consumption increases basal *c-fos* expression in the paraventricular nucleus of the hypothalamus in adulthood
- Adolescent caffeine consumption increases basal *Crf* expression in the central nucleus of the amygdala in adulthood

Figure 1.

Adolescent caffeine consumption does not produce significant alterations in fluid consumption or body weight outcomes over the course of the caffeine consumption procedure. A) Male Sprague-Dawley rats received caffeine or water for 28 days beginning on P28. Behavioral testing, neuroendocrine measures, and tissue collection occurred after 7 days in the absence of caffeine. B) Caffeine-consuming adolescent rats gained weight equivalently to control rats that had *ad libitum* access to water. C) The volume of fluid consumed by the two groups was equivalent throughout the procedure. D) The amount (mg/kg) of caffeine consumed was assessed throughout the procedure and resulted in a progressive decrease in caffeine intake over the course of the procedure. $n = 15/\text{group}$

Figure 2.

Adolescent caffeine consumption, but not adult caffeine consumption, increases anxiety-related behaviors. A) Rats that consumed caffeine during adolescence spend less time on the open arms (left) and enter the open arms less frequently (right) when exposed to the elevated plus maze. $n = 9-10/\text{group}$. B) Rats exposed to caffeine during adulthood show no differences in time spent on or entries into the open arms of the elevated plus maze. $n = 8/\text{group}$. C) Caffeine consumption during adolescence decreases time spent in the center of an open field chamber (left), but does not change overall locomotion (right). $n = 4/\text{group}$. D) Caffeine consumption during adulthood has no effect on open field behavior (left) or total locomotion (right). $n = 8/\text{group}$. E) Chronic caffeine consumption during adolescence decreases social interaction in adulthood. $n = 8-10/\text{group}$. F) Chronic caffeine consumption during adulthood has no effect on social interaction. $n = 8/\text{group}$. E) * significant from water controls ($p < 0.05$)

Figure 3.

Increased anxiety-related behavior was observed in both adolescent and adult animals during the last week of caffeine consumption. A) Rats consuming caffeine during adolescence spend less time on the open arms (left) when tested on the elevated plus maze during the last week of caffeine consumption. $n = 20/\text{group}$. B) Rats consuming caffeine during adulthood also spend less time on the open arms (left) and enter the open arms less frequently (right) when tested on the elevated plus maze during the last week of caffeine consumption. $n = 9-10/\text{group}$. * significant from water controls ($p < 0.05$)

Figure 4.

Adolescent caffeine consumption produces HPA axis dysregulation. A) Basal plasma corticosterone (CORT) levels across the course of the caffeine consumption paradigm. Caffeine consumption initially increases basal CORT, but rats appear to become tolerant to the HPA-axis activating effects of caffeine by day 14. Twenty-four h after the removal of caffeine rats show increased basal CORT. $n = 8-10/\text{group}$ B) Basal CORT measures at the circadian trough and peak following a 7 day washout period without caffeine. Rats that consumed caffeine exhibit persistent CORT elevations at the circadian trough, but no differences in peak CORT levels. $n = 7-8$ C) Plasma adrenocorticotropin hormone (ACTH) levels were decreased in rats that consumed caffeine during adolescence following pedestal stress ($t_{26} = 2.474$, $p < 0.05$, $n = 7-8$). D) Plasma CORT levels were also decreased in adolescent caffeine consuming rats following pedestal stress ($t_{26} = 4.323$, $p < 0.001$, $n = 7-8$). E) A peripheral injection of ACTH (1 mg/kg) induced significantly higher levels of plasma CORT in water control rats compared to rats that consumed caffeine during adolescence. $n = 8-10/\text{group}$ * significant from respective water control group ($p < 0.05$), ‡ significant from Day 1 ($p < 0.05$), ^ significant from respective No Stress condition ($p < 0.05$), # significant ($p < 0.001$) from plasma CORT levels at the circadian trough

Figure 5.

Chronic caffeine consumption during adolescence alters basal *c-fos* mRNA expression and *c-fos* mRNA expression in response to a psychological stressor. A) Stress increases *c-fos* mRNA expression in the PVN. Caffeine consumption during adolescence enhanced basal expression of *c-fos* mRNA in the PVN, but did not alter stress-induced *c-fos* expression. $n = 6-9/\text{group}$ B) Pedestal stress increases in *c-fos* mRNA expression in the central amygdala (CeA) of control rats, but not rats that consumed caffeine during adolescence. $n = 5-9/\text{group}$. C) Pedestal stress increases *c-fos* mRNA in the basolateral amygdala (BLA) in both water and caffeine consuming groups. No Stress: $n = 6-9/\text{group}$. D) Pedestal stress has no effect on *c-fos* mRNA in the bed nucleus of the stria terminalis anteroventral portion (BSTav). No Stress: $n = 5-9/\text{group}$. E) Pedestal stress increases *c-fos* mRNA in the PFC in both water and caffeine consuming groups. No Stress: $n = 6-9/\text{group}$. F) Both adolescent caffeine and water groups show a stress-induced increase in *c-fos* mRNA expression in the nucleus accumbens shell (NAc shell). No Stress: $n = 6-9/\text{group}$. * significant from respective water control group ($p < 0.05$), # significant main effect of stress ($p < 0.05$)

Figure 6.

Adolescent caffeine consumption produces increases *Crf* mRNA expression in the CeA. A) Both caffeine and water groups show the same levels of *Crf* mRNA in the PVN. $n = 9-16/\text{group}$. B) Chronic caffeine consumption increases *Crf* mRNA expression in the CeA No Stress: $n = 5-8/\text{group}$. C) There is no effect of adolescent caffeine consumption on *Crf* mRNA expression in the PFC. $n = 6-9/\text{group}$. D) *Crf* mRNA expression in the BSTav is not altered by caffeine consumption during adolescence. * significant from water controls ($p < 0.05$)