Effects of Adolescent Caffeine Consumption on Anxiety and Stress in Adulthood

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Abstract

Caffeine is the most widely used psychoactive substance in the world. Although adolescents are the most rapidly increasing group of caffeine consumers, few studies have been done on the effects of caffeine on the still-developing adolescent brain. Since caffeine has been associated with stress reactivity and anxiety behavior, this study examined the effect of adolescent caffeine consumption on these measures in adulthood using a rat model. Male Sprague Dawley rats were exposed to caffeine (0.3 g/L) for the duration of their adolescence (postnatal days 28-56). Once they were adults and had withdrawn from caffeine for a week (after postnatal day 60), they were exposed to either an elevated plus maze to measure anxiety behavior, or underwent a pedestal stress test. From the pedestal stress test, in situ hybridization was used to measure c-fos mRNA in the basolateral amygdala (BLA), paraventricular nucleus (PVN), and bed nucleus of the stria terminalis (BST), and immunoassays were performed to determine corticosterone (CORT) levels in the blood plasma. Animals that consumed caffeine in their adolescence spent significantly less time in the open arms of the elevated plus maze in adulthood, indicating heightened anxiety. Accordingly, animals that consumed caffeine in their adolescence had heightened BLA activity in response to the pedestal stress. These animals also as adults displayed higher basal CORT levels and blunted CORT levels in response to a stressor, implying a dysregulation of their stress response system. PVN activity was higher basally, potentially explaining the rise in basal CORT release. The BST activity had no significant results. Although it is unclear how caffeine is causing these changes in behavior, neuronal activity and neuroendocrine measures, it is apparent that the consumption of caffeine in adolescence can have persistent unwanted effects on anxiety behavior and stress reactivity in adulthood.
Introduction

Caffeine is consumed by a huge number of people. In the United States, the average amount of caffeine consumed is 168 mg/person/day (Fredholm et al., 1999), and the number of caffeine consumers has been steadily increasing (Frary et al., 2005). Interestingly, the amount of caffeine consumers who are adolescents has been increasing the fastest. Over the past 30 years the amount of adolescent caffeine consumers has grown 70% (Harnack et al., 1999). The adolescent brain is still undergoing biological growth and change, and it is unclear how caffeine may impact the brain during this developmental period. Surprisingly few studies have looked at the effects of caffeine in the adolescent brain. The studies in this thesis are designed to assess the effects of adolescent caffeine consumption on subsequent behavioral, neuroendocrine and brain responses to stress and anxiety.

In adolescence, the brain is still actively developing. The adolescent stage of development is conserved across species (Spear, 2004). The limbic system controls motivation and emotion, and matures earlier in adolescence (Gladwin et al., 2011). Additionally, the prefrontal cortex that mediates decision-making is not yet fully developed (Steinberg, 2005). This leads to a sensitive period in brain development where emotional systems are strong, but lack the capacity to be fully modulated by critical thinking coming from the prefrontal cortex. While brain systems are still being formed in adolescence, changes in receptor density or neuronal firing produced by caffeine could have long-lasting effects on the functioning of these systems.

Caffeine is a nonselective adenosine receptor antagonist, meaning it blocks the actions of adenosine on neurons. Adenosine is a neurotransmitter that is widely expressed throughout the brain and it interacts with two main classes of adenosine receptors – A1 (inhibitory) and A2A
(excitatory). These two classes are expressed in different brain regions. $A_1$ receptors present throughout the brain but are most highly expressed in the hippocampus and the cortex (Fredholm et al., 1999). $A_{2A}$ receptors are highly expressed in the nucleus accumbens and striatum (Rosin et al., 1998). Adenosine has various modulatory effects in the brain, especially on the release of other neurotransmitters. Typically, adenosine causes neural inhibition. So when an antagonist such as caffeine is applied, the inhibition is stopped and brain activity increases (Fredholm et al., 1999). This is what fundamentally makes caffeine a stimulant.

Caffeine has a multitude of behavioral and physiological effects in both the body and the brain. Caffeine can cause a rise in systolic blood pressure (Daniels et al., 1998) and locomotor activity (Holtzman, 1983). Caffeine can also increase learning ability and memory consolidation and retention (Temple, 2009). While there are not many health risks associated with caffeine as it is normally consumed, extremely high doses can have deleterious effects and persistent caffeine use has the ability to cause dependence and withdrawal (Fredholm et al., 1999).

If caffeine can cause dependence, it must have long-term effects in the brain. Chronic caffeine exposure increases $c-fos$ expression in various brain regions, such as the nucleus accumbens and the hippocampus (Svenningsson et al., 1996; Nakajima et al., 1989). $C-fos$ is an immediate early gene that is expressed very rapidly in neurons in response to an environmental experience and is thus used as an experimental tool to identify neurons within brain regions that had increased activity to a given experience. $C-fos$ expression leads to significant biological changes in neurons. The $c-fos$ mRNA encodes the Fos protein that dimerizes with the Jun protein to form the AP-1 complex (Chiu et al., 1988). The AP-1 complex serves as a transcription factor that can then increase the transcription of other proteins that are needed in response to an environmental experience, such as proteins involved in cell proliferation, neuroregeneration, or
neurodegeneration (Wagner, 2001). Therefore, caffeine-induced c-fos expression indicates that caffeine has activated neurons and is causing neurons to undergo adaptive changes (Svenningsson et al., 1996). Additionally, caffeine affects receptor expression. For example, studies have shown that caffeine can increase adenosine A₁ receptors, decrease β-adrenergic receptors, and promote intracellular Ca^{2+} release (Hawkins et al., 1988; Jacobson et al., 1996; Daly et al., 2007). All of these changes were observed in adult animals and it is unclear whether they also occur in adolescent animals. If so, it is likely that caffeine could affect the development and functioning of certain brain structures.

Caffeine consumption has been associated with anxiety, especially when consumed at high doses. For example, caffeine can trigger panic attacks in patients with anxiety disorders (Uhde et al., 1984). Caffeine can also increase corticosterone (CORT) levels in rats (Patz et al., 2006). Corticosterone is equivalent to the molecule cortisol in humans, which is released in response to stress (Dickerson et al., 2004). CORT is released by the adrenal gland, but results from the activation of the hypothalamic-pituitary-adrenal (HPA) axis.

The HPA axis is a stress response system that allows the brain to coordinate a neuroendocrine response in preparation for fight or flight. There are multiple structures that provide input to activate the HPA axis by stimulating the paraventricular nucleus of the hypothalamus (PVN). The hippocampus stimulates the PVN with glutamate. The bed nucleus of the stria terminalis (BST) activates the PVN with either glutamate or corticotrophin-releasing hormone (CRH). The amygdala also activates the PVN and provides a strong link between brain structures regulating anxiety behavior (amygdala) and the structure regulating stress response (hypothalamus). The PVN releases CRH, which subsequently activates the anterior pituitary. The anterior pituitary releases adrenocorticotropic hormone (ACTH) that causes the adrenal cortex to
release glucocorticoids (e.g. CORT) (Tsigos and Chrousos, 2002). Interestingly, if rats are exposed periodically to a stressor throughout their adolescence, they exhibit blunted hormonal responses to stress in adulthood (Goliszek et al., 1996). This indicates that during adolescence, animals are particularly sensitive to the effects of stress.

We conducted a series of experiments to address whether chronic caffeine administration throughout adolescence alters anxiety behavior, the neuroendocrine stress response, and brain activation patterns in adulthood. Anxiety-like behavior was determined by measuring the exploration of an anxiety-provoking elevated plus maze. Additional experiments exposed animals to an elevated pedestal stress challenge. Then, an enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of CORT present in blood plasma. Finally, brain slices were processed for c-fos mRNA expression in various brain regions using in situ hybridization. The hypothesis is that chronic caffeine consumption throughout adolescence will increase anxiety behavior, the neuroendocrine stress response, and brain activation in response to a stressor in adulthood.

Methods

Animals

Male Sprague Dawley rats (Charles River) were housed two to a cage with food and water available ad libitum. All experiments took place during the light period of a 12-hour light/dark cycle. All procedures were conducted in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Colorado Boulder.
Caffeine Consumption

Figure 1: Rats arrived on postnatal day 21, the first day they could be weaned from their mothers. After a seven-day adjustment period, the rats were exposed to a single bottle containing caffeine (0.3 g/L). They consumed caffeine for a 28-day period. The caffeine-containing bottle was replaced with water on postnatal 56. After an additional 7 days without caffeine, they were subjected to a pedestal stress test.

The rats arrived at 21 days after birth and had a 7-day adjustment period. Starting on postnatal day 28 (P28), half of the rats had access to a single bottle filled with caffeinated water (0.3 g/L), and the other half had a single bottle filled with normal water. Twice a week (every three or four days), all bottles were weighed to determine how much liquid was consumed. On the days the bottles were weighed, the body weight of each rat was also recorded. After 28 days of caffeine acquisition (P56), the animals had a week off of caffeine. By P60, the rats are considered adults.

Elevated Plus Maze

Eight rats (four water, four caffeine) were subjected to the elevated plus maze between P62-66. The elevated plus maze consisted of four arms (50 x 10 cm each) joined by a central platform (10 x 10 cm). Two arms are enclosed with 40 cm high walls with open ends, 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 five minutes. A stopwatch was used to record the amount of time
spent in the open arms. Being in an open arm was defined as more than half of the rat’s body being in the open arm.

**Pedestal Stress Challenge**

A separate set of 30 animals was used for the pedestal stress experiment. There were 15 rats exposed to caffeine and 15 exposed to water. In each group, nine rats underwent pedestal stress and the other six served as handled, home-cage controls. So, a total of 18 rats underwent the pedestal stress. The pedestal stress test occurred during the trough of the circadian corticosterone cycling. Rats were placed on a pedestal 60 cm elevated off the floor for 5 minutes. The pedestal is 27 cm², and has no protective borders aside from a small 1 x 1 cm rim around the square to keep the rats from falling off. Exposure to the pedestal is thought to represent a psychological stress that is sufficient to induce physiological indications of stress (Pace et al., 2005).

After the pedestal stress, rats were returned to their home cages. Thirty minutes after the pedestal stress began, the rats were sacrificed. Brain samples were immediately collected and flash frozen in a beaker of isopentane and dry ice for thirty seconds. They were then stored at -80°C. Trunk blood was collected in EDTA-coated tubes which were centrifuged at 2000 rpm for 10 min at 4°C. Then, blood plasma was pipetted into .5 mL microcentrifuge tubes and stored at -80°C (Babb et al., 2014).

**Corticosterone Immunoassay**

CORT levels in the blood plasma were analyzed using a commercially available kit (Cat. # 901-097; Arbor Assay, Ann Arbor, MI). The directions on the kit were followed. The plasma samples and CORT standards were incubated together in the provided 96-well plate. After incubating, unbound reagents were washed from the plates and a substrate added. An hour later,
the color intensity in each well was analyzed using a microplate reader, and the CORT concentration for each sample analyzed against a standard curve (Babb et al., 2014).

**In-situ Hybridization**

The regions of interest include the paraventricular nucleus (PVN), the basolateral amygdala (BLA) and the bed nucleus of the stria terminalis (BST). Each region was sliced into 12 µm slices on a cryostat. Slices were then mounted onto polylysine-coated slides and stored at -80°C. A radiolabeled riboprobe against c-Fos mRNA was generated by adding DNA, RNA polymerase, NTPs (ATP, GTP, CTP), and $[^{35}\text{S}]$-UTP to a buffer solution. Slides were fixed in 4% paraformaldehyde, acetylated in triethanolamine and acetic anhydride, and dehydrated through graded alcohol. The slides were plated with the riboprobe mixed with hybridization buffer and incubated overnight at 55°C.

The next day, slides were washed with RNase and rinsed in grades of standard saline citrate (SSC). The slides were incubated in a .1x SSC at 70°C for an hour and a half, cooled, and again dehydrated through graded alcohol. After drying, the slides were put into cassettes and exposed to x-ray film. The BST region was developed after 15 days, while the PVN and BLA regions were developed after 13 days (Day et al., 2005; Day and Akil, 1996).

**Image Analysis**

Slide films were photographed and analyzed using Scion Image. A template was placed over the region of interest, and the signal and area values recorded (PVN used a 50x45 rectangle in the center, BST a 80x40 rectangle on either side, and the BLA a 50x50 circle on either side). The signal and area were multiplied, giving the integrated density value. This is the value that is used for statistical analysis.
**Data Analysis**

Total fluid consumption (ml/day) and body weights (g) were analyzed using a mixed design two-way ANOVA with treatment (water vs. caffeine) as a between subjects factor and days as a within subject factor. The amount of caffeine consumed (mg/kg/day) was analyzed using a one-way repeated-measure ANOVA across days. The effect of caffeine on percent time spent in the open arms was analyzed by an unpaired t-test. Integrated density was analyzed by a two-way ANOVA with treatment (water vs. caffeine) and stress (home cage vs. pedestal) as the factors. For the CORT assay, corticosterone (pg/dl) in the blood plasma was analyzed by a two-way ANOVA with treatment (water vs. caffeine) and stress (home cage vs. pedestal) as the factors. Significant interactions were followed by *a priori* and post hoc tests (unpaired t-test or Bonferroni’s comparisons). Statistical significance for all tests was set at $p < 0.05$.

**Results**

**Caffeine Consumption**

Throughout the caffeine drinking procedure (Figure 2), caffeine consumption (mg/kg/day), the volume of fluid consumed per day (ml/day), and body weights (g) were recorded. There were no significant group differences in the weights of the rats over the course of the caffeine consumption procedure. However both groups gained significant amounts of weight over the course of the procedure ($F_{(7,210)} = 1307, p < 0.0001$). Together, these indicate that caffeine consumption during adolescence did not adversely affect basic development processes (Figure 2A). However, the amount of caffeinated water ingested by the caffeine rats was consistently lower than the amount of normal water ingested by the control rats ($F_{(1,126)} = 5.183, p = 0.0352$). So although they drank less, their weights imply that this had no adverse
effect on their health. Total fluid consumption increased significantly for both groups over time \( (F_{(7, 126)} = 93.32, p < 0.0001) \) (Figure 2B). Caffeine consumption per the rats body weight decreased over time, even though the fluid consumption increased \( (F_{(7, 105)} = 32.08, p < 0.0001) \). This could be because the rats’ body weights increased at a faster rate than the increase their in fluid intake. The rats in the caffeine group on average consumed 29.8 mg/kg of caffeine per day (Figure 2C).

![Figure 2: A) This figure shows the average animal weight in the caffeine and water group per day. Rats were weighed every 3-4 days, and the change in weight averaged across the number of days. B) Shows the changes in bottle weight for each day. Bottles were weighed every 3-4 days, and the change in weight averaged across the number of days. This data is for both rats in the cage sharing the bottle. C) Shows the average amount of caffeine consumed by rats in the caffeine group per body weight (kg) per day.](image)

**Elevated Plus Maze**

We conducted the elevated plus maze to determine if caffeine consumption during adolescence would enhance anxiety-related behavior compared to water-consuming control rats. In an elevated plus maze experiment, anxiety-related behavior is reflected by the animal spending more time in the enclosed arms and less time in the open arms. The rats that consumed caffeine in adolescence showed a significant decrease in the amount of time they spent in the open arms of the elevated plus maze \( (t_{17} = 3.081, p = 0.0068) \). Since the rats that
consumed caffeine during adolescence spent less time in the open arms, this suggests they were more anxious than the control rats.

Figure 3: This figure shows the percent of time spent in open arms of the elevated plus maze for water and caffeine rats. * Significant from water, $p = 0.0068$.

**Corticosterone Levels**

Given that the performance on the elevated plus maze was suggestive of heightened anxiety, we wanted to next explore whether the physiological responses to a stressful stimulus similar to that of the elevated plus maze were also disrupted by adolescent caffeine consumption. CORT is a glucocorticoid hormone released from the adrenal gland when the HPA axis is activated in response to a stressful stimulus. Therefore, we would expect to see that when animals are exposed to either caffeine, stress, or both they would have higher CORT levels than the corresponding control. In this experiment, we found a significant effect of treatment ($F_{(1, 51)} = 6.49, p = 0.0139$) and stress ($F_{(1, 51)} = 92.93, p < 0.0001$). There was also a significant interaction effect ($F_{(1, 51)} = 21.56, p < 0.0001$). The interactive effects were further teased apart by $t$-tests. There were significant differences between stressed and unstressed water animals ($t_{26} =$...
10.53, \( p < 0.0001 \) and caffeine animals \((t_{25} = 3.360, p = 0.0025)\). So, in both groups, the pedestal stress caused a rise in CORT levels. Additionally, unstressed caffeine animals had higher basal CORT levels than water animals \((t_{36} = 2.821, p = 0.0077)\). However, contrary to what was expected, stress produced a significantly lower CORT response in caffeine animals compared with water animals \((t_{15} = 2.834, p = 0.0126)\).

Figure 4: This figure shows the picograms/deciliter of CORT in trunk blood plasma for each group. * Significant from other treatment in same stress condition; # Significant from same treatment in other stress condition.

C-fos expression changes

To further delineate how caffeine consumption during adolescence may contribute to the disruptions on elevated plus maze and neuroendocrine measures, we sought to identify neuronal activity patterns using c-fos expression. When a neuron is highly activated, the immediate early gene c-fos is transcribed and provides a measure of active neurons (Hoffman et al., 1993). Therefore, we used in situ hybridization to analyze c-fos mRNA levels in various brain regions that have been implicated in anxiety and stress responses. The value for the amount of c-fos is
the integrated density. So the higher the value of integrated density, the more active the brain structure was.

**Basolateral Amygdala (BLA)**

The basolateral amygdala (BLA) was analyzed because of its role in anxiety and emotional memory (Maren et al., 1999). Since caffeine rats showed more anxiety behavior in the elevated plus maze, we would expect that caffeine rats in reaction to pedestal stress would show a higher BLA response than the control. The region that was quantified for the BLA is shown in Figure 5.

Figure 5: This diagram shows the brain region that was quantified for the BLA. The circle indicates the areas the integrated density values were taken from (50 pixel diameter circle). A value was taken from each side.

There was a significant effect of stress ($F_{(1, 26)}= 16.33, p= 0.0004$). There was no significant effect of treatment. There was also a significant interaction effect ($F_{(1, 26)}= 6.92, p= 0.0142$).
Caffeine animals that were exposed to stress had higher BLA activation than caffeine animals that had no stress ($t_{13} = 4.610, p = 0.0005$). There is also a significant difference between caffeine and water animals that received stress ($t_{16} = 2.861, p = 0.0113$). Rats that consumed caffeine during adolescence had more BLA activation following pedestal stress than the water-consuming rats suggesting that they had significantly more anxiety to the same stressor, consistent with the elevated plus maze results.

Figure 6: A) This figure shows the integrated density of the BLA c-fos for each group. * Significant from other treatment in same stress condition; # Significant from same treatment in other stress condition. B) This figure shows an example slice from each group.

**Paraventricular Nucleus (PVN)**

The paraventricular nucleus (PVN) is the part of the hypothalamus that begins the HPA axis cascade. It is activated by stressful stimuli and natural body rhythms, and eventually leads to the release of CORT into the bloodstream. Since the CORT experiment resulted in caffeine rats having a lessened CORT response to a stressor than water rats, we wanted to analyze the PVN to identify whether changes in the CORT response was at the level of the PVN or further downstream. The region that was quantified in the PVN is shown in Figure 7.
Figure 7: This diagram shows the brain region that was quantified for the PVN. The rectangle indicates the area the integrated density value was taken from (50x45 pixel rectangle).

In the PVN, we found a significant effect of stress \( (F_{(1, 26)} = 36.36, p < 0.0001) \). There was also a significant interaction effect \( (F_{(1, 26)} = 4.39, p = 0.0461) \). Stress caused a rise in PVN activation for water animals \( (t_{13} = 5.181, p = 0.0002) \) and for caffeine animals \( (t_{13} = 3.160, p = 0.0075) \). There is also a significant difference between water and caffeine animals with no stress \( (t_{10} = 2.226, p = 0.05) \). Thus, in the absence of stress, the caffeine animals had more PVN activity than water animals. A slight decrease can be seen in the caffeine animals PVN activation to a stressor from the water animals. However since it is not significant, no conclusions can be drawn from the
parallel.

Figure 8: A) This figure shows the integrated density of the PVN $c$-fos for each group. * Significant from other treatment in same stress condition; # Significant from same treatment in other stress condition. B) This figure shows an example slice from each group.

**Bed Nucleus of Stria Terminalis (BST)**

We also analyzed the bed nucleus of the stria terminalis (BST), as it is a relay site between the BLA and PVN and could possibly explain differences in activation between these structures for the same groups. The region that was quantified in the BST is shown in Figure 9. However the BST had no significant differences in activation between the groups (Figure 10). Interestingly, although not statistically significant, in the no stress condition the caffeine animals had lower BST activation than water animals.
Figure 9: This diagram shows the brain region that was quantified for the BLA. The rectangle indicates the areas the integrated density values were taken from (80x40 pixel rectangle). A value was taken from each side.

Figure 10: A) This figure shows the integrated density of the BST c-fos for each group. There were no significant effects. B) This figure shows an example slice from each group.
Discussion

These studies illustrate the powerful effects that adolescent caffeine consumption can have on anxiety and stress systems in the brain into adulthood. Our results demonstrate that when the rats that consumed caffeine were exposed to an elevated plus maze, they spent significantly less time in open arms implying heightened anxiety. Additionally, rats that consumed caffeine during adolescence had higher basal levels of both PVN activity and CORT levels compared to the control. When the animals were exposed to pedestal stress, the animals that consumed caffeine during adolescence had heightened BLA activity, blunted CORT release, and a trend towards blunted PVN activity. Together, these findings suggest that adolescent caffeine consumption impaired both basal and stress-induced neuroendocrine functioning in adulthood.

Acute caffeine is known to activate the HPA axis. Thus, low dose injections of caffeine have been shown to increase CORT release (Patz et al., 2006). It isn’t entirely clear how caffeine activates the HPA axis. This could occur by some sort of connection with the PVN, which releases CRH. Experiments have shown that when CRH release is blocked, there is no CORT response. Therefore, caffeine could be interacting directly with the PVN or with hypothalamic afferent structures to cause this change in PVN activity and CORT release (Patz et al., 2006; Nicholson et al., 1989). So far, there is not much information that describes how caffeine could directly modulate the PVN. An afferent region caffeine interacts with is the hippocampus. Caffeine causes the activation of hippocampal CA1 pyramidal neurons (Uneyama et al., 1993). Since caffeine activates the hippocampus which connects to the PVN, a change here could hypothetically change PVN activity. There is little information on caffeine’s effect on any other afferent structures (See Figure 11). The exact region caffeine interacts with to cause a rise in PVN activity could be explored with future experiments.
EFFECTS OF CAFFEINE ON ANXIETY AND STRESS

Given that acute caffeine activates the HPA axis (Patz, 2006), it seems likely caffeine is acting as a stressor. Thus chronic consumption of caffeine, and activation of the HPA axis, may be similar to chronic stress conditions. Our findings are consistent with other findings that demonstrate chronic stress causes an increase in basal CORT in animal models (Mizoguchi et al., 2001). Additionally, caffeine’s stimulant effects could be acting as a stressor by altering sleep/wake patterns. Since the animals are exposed to caffeine at all hours, drinking caffeine-containing water throughout the day may disrupt their sleeping patterns producing more bouts of
wakefulness. Caffeine has been shown to induce a dose-dependent increase in waking (Schweirin et al., 1996). This change in the sleep cycle could affect the activity of the HPA axis. For example, it has been demonstrated that awakenings and the offset of sleep are both accompanied by secretion of CORT (Balbo et al., 2010). Since caffeine is releasing CORT, and CORT causes more waking and less sleeping, the caffeine-consuming rats could be living under the stressful condition of disrupted sleep patterns.

The animals were exposed to pedestal stress to determine if chronic caffeine consumption in adolescence affected the response rats exhibited to an acute novel stressor. When rats in the water or the caffeine group were exposed to pedestal stress, there was an increase in PVN activity and a corresponding increase in CORT levels from the unstressed animals. This confirms that an acute stressor activates the PVN and causes a CORT response. Unexpectedly, the CORT analysis showed that in the stress group caffeine rats had a significantly lower level of stress-induced CORT than water rats. The PVN c-fos data illustrates a trend that correlates with the CORT result, although this was not statistically significant. A similar effect was seen in an experiment in which mice were selectively bred to be highly anxious. When these high anxiety mice were exposed to an acute stressor, such as pedestal stress, there was a blunted CORT response (Sotnikov et al., 2014). It is plausible that caffeine consumption during adolescence rendered these rats more anxious (as seen by the heightened BLA activity under unstressed conditions) so it follows that they display this same blunted CORT response. A reason this could occur is that their HPA axis compensated and adjusted to the higher basal levels by blunting CORT release to prevent damage due to an excess of it in the body (Fries et al., 2005). This could potentially occur through an upregulation of the glucocorticoid receptor in the PVN.
(Sotnikov et al., 2014), although this explanation does not fit with our result of heightened basal CORT.

Adolescent caffeine consumption produced robust behavioral differences in elevated plus maze. Rats that consumed caffeine spent less time in the open arms of the maze, indicating heightened anxiety. The amygdala is a comprised of many subregions that orchestrate emotional processing and emotional learning. The BLA, in particular, has been found to be the region where emotional memories are modulated (Maren et al., 1999). We observed significantly higher stress-induced levels of $c$-fos in the BLA of rats that consumed caffeine during adolescence. All of this suggests that caffeine has an effect in the brain that causes the BLA to have a larger reaction to stress, potentially producing heightened anxiety. Interestingly, acute caffeine has been shown to activate interneurons within the BLA (Hale et al., 2010). So, since the BLA has been implicated in anxiety (Davidson, 2002) and caffeine activates the BLA, chronic caffeine consumption during adolescence may produce enduring effects in the BLA that leads to heightened sensitivity producing more anxiety in response to a stressful situation.

The BLA is also positioned to modulate the PVN through connections with both the BST and the central nucleus of the amygdala (CeA) (Herman et al., 2003). The CeA serves as the primary output of the amygdala and sends excitatory information directly to the PVN. The CeA also has indirect communication to the PVN through excitatory and inhibitory connections with the BST. The BST is a complex structure consisting of different subnuclei that differentially signal the PVN (Choi et al., 2007). The BLA and CeA can therefore bidirectionally modulate the output of the BST. Thus, the complex circuitry and communication between these structures makes interpretations of our $c$-fos expression data difficult.
 Nonetheless, we suspect that caffeine consumption during adolescence has rendered the BST dysfunctional. Since the BLA and PVN are showing different responses to the same stressor, and typically the BLA activates the PVN, there is a possibility there is a change in function of the structure that connects the two—the BST (Herman et al., 2003). We were looking specifically at the anteroventral BST, which normally excites the HPA axis (Choi et al., 2007). Supportive of a dysfunctional BST is that we did not observe the characteristic stress-induced increase in BST activity. However, the water animals did not show any change in BST activation in response to a stressor (possibly because the stressor was too mild).

It is important to note that our behavioral, neuroendocrine and brain changes are observed following the consumption of caffeine for the duration of their adolescence. This implies that caffeine could be causing a change during the development of the brain that changes its response to subsequent stressful stimuli. Caffeine can have many long-term effects on the brain, such as changing receptor density (Hawkins et al., 1988). If changes are occurring in the vulnerable adolescent brain, it is possible that there is a developmental effect contributing to our results. An interesting study would be to identify different stages throughout adolescent development that may be particularly sensitive to the changes in anxiety and stress responses that we observe.

Chronic caffeine consumption is associated with withdrawal symptoms. Therefore, it is possible that the heightened anxiety is a manifestation of caffeine withdrawal. It has been demonstrated that after chronic caffeine administration in adolescence, rats develop signs of withdrawal that do not appear after chronic caffeine administration in adulthood (Rhoads et al., 2011). When withdrawing from opiates, rats have shown heightened anxiety in these periods of withdrawal (Aston-Jones and Harris, 2004). Since caffeine causes withdrawal and withdrawal
can raise anxiety, we cannot exclude the possibility that the rise in anxiety behavior could be due to a withdrawal effect.

In conclusion, we found that chronic caffeine consumption in adolescent rats leads to higher basal CORT release and PVN activity. In response to a stressor, chronic caffeine consumption leads to heightened BLA activity and anxiety behavior, but blunted CORT release. Although more studies need to be done, these results imply that the consumption of caffeine in adolescence can have unwanted effects on stress reactivity in adulthood.
References


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