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Effect of Withdrawal from Chronic Adolescent Caffeine Consumption on Anxiety Behaviors and the Serotonin System

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Abstract

Caffeine is a frequently used psychoactive substance and consumption by children and adolescents continues to rise. Here, we examine the effects of withdrawal from chronic adolescent caffeine consumption on anxiety-related behaviors and the serotonin system, specifically the dorsal raphe nucleus. Adolescent male rats consumed caffeine (0.3 g/L) for 28 days from postnatal day 28 (P28) to P55 and control rats consumed water. Behavioral testing for anxiety-related behavior began in adulthood (P62) 24 hours after removal of caffeine. Adolescent caffeine consumption augmented anxiety-related behavior in the elevated plus maze and social interaction tests. Gene expression of *tryptophan hydroxylase 2* (*tph2*), the rate-limiting enzyme involved in serotonin synthesis, and *solute carrier family 6 neurotransmitter transporter member 4* (*slc6a4*), the gene encoding the serotonin transporter, were used as markers of serotonin transmission and their expression was evaluated in different subregions of the raphe. Expression of *tph2*, but not *slc6a4*, was decreased in all dorsal raphe subregions following withdrawal from adolescent caffeine consumption. Caffeine challenge decreased *tph2* expression in vehicle-treated rats in the DRD and *slc6a4* expression in caffeine-treated rats in the DRI. Together, these findings suggest that adolescent caffeine consumption and withdrawal may alter some aspects of the serotonin system, although their functional relationship to the emergence of withdrawal-induced anxiety is not clear.

*Keywords*: caffeine, withdrawal, anxiety, dorsal raphe nucleus, serotonin, *tph2, slc6a4,*
Introduction

Caffeine is a naturally occurring compound found in coffee, tea, soft drinks, energy drinks, chocolate, and over-the-counter pills. It is the most widely used psychostimulant in the world (Rath, 2012, Warzak et al., 2011). Its consumption has increased in recent years, especially among children and adolescents (Ahluwalia and Herrick, 2015, Frary et al., 2005, Temple, 2009), with about 30-50% of adolescents in the United States consuming caffeine on a regular basis (Rath, 2012). However, the effects of chronic caffeine consumption and withdrawal during adolescence are relatively unknown.

Caffeine consumption has been linked to anxiety disorders in adults, despite low to moderate consumption being considered relatively safe. For example, oral caffeine administration triggers panic attacks in adults diagnosed with panic disorder, social anxiety disorder, and performance social anxiety disorder (Nardi et al., 2009). Animal studies also report caffeine-induced anxiety in adult rats, shown in the elevated plus maze, light dark box, and open field box (El Yacoubi et al., 2000 and Noschang et al., 2009). Tolerance develops to the anxiogenic effects of caffeine following chronic caffeine administration, and withdrawal shows a re-emergence of anxiety 48 hours after chronic caffeine consumption in adult rats (Bhattacharya et al., 1997).

There also appears to be a relationship between anxiety and caffeine consumption occurring during adolescence. Adolescence is a period of development when many endogenous and environmental factors can affect the maturation process (Arain et al., 2013; Wahlstrom et al., 2010). 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). Therefore, there is considerable evidence suggesting that caffeine consumption can influence anxiety behaviors in both adults and adolescents, although the mechanisms are still unclear.

Caffeine is known to act on the adenosine system in the brain. Adenosine is an endogenous purine that has a general inhibitory effect on the central nervous system. It is involved in many physiological processes, such as energy transfer, signal transduction, neuromodulation, and vasodilation. Caffeine’s psychostimulant effects are mediated primarily through non-selective antagonism of adenosine A₁ and A₂A receptors (Ferre, 2008). Adenosine A₂A receptors are coupled to Gₛ proteins, whereas A₁ receptors are coupled to G₁ proteins that stimulate and inhibit the adenylyl cyclase/cAMP cascade, respectively (Nehlig et. al., 1992; Fredholm, et. al., 1999). Caffeine also increases intracellular levels of cAMP by inhibiting the enzyme phosphodiesterase (Arnaud, 1987). Caffeine’s effects on the adenosine system also influence the dopamine system in two ways. First, caffeine blocks adenosine A₁ receptors on dopamine terminals in the nucleus accumbens and prefrontal cortex to increase dopamine release in these areas (Solinas et al., 2002). Caffeine also facilitates dopamine receptor activity by removing adenosine receptor antagonism of dopamine receptors (Ferre, 2008). These actions of caffeine are primarily responsible for the arousal and psychostimulant effects of caffeine.

The anxiogenic effects of caffeine also occur via the adenosine system, although these effects arise from interactions in other brain regions and other neurochemicals. In fact, caffeine pretreatment decreases the anxiolytic-like effect of adenosine (Kulkarni, et.
Chronic caffeine administration causes up-regulation of adenosine $A_1$ and $A_{2A}$ receptors in the brain (Hawkins et al., 1988; Johansson et al., 1997; Shi and Daly, 1999; Varani et al., 1999) and this corresponds with the development of tolerance to caffeine-induced anxiety (Bhattacharya et al., 1997; Holtzman, 1983) but withdrawal is associated with anxiety symptoms (Bruce 1990, Nutt 1990). Adenosine $A_1$ and $A_{2A}$ receptor knockout mice display more anxiety than wild-type mice (Ledent et al., 1997; Gimenez-Llort et al., 2002). This may be explained by the connection between adenosine and proopiomenalocortin (POMC). POMC is a precursor peptide of alpha-melanocyte-stimulating-hormone ($\alpha$-MSH) that modulates anxiety via the melanocortin 4 receptor (Chaki and Okuyama, 2005). Adenosine $A_{2A}$ receptor KO mice show increased $POMC$ mRNA expression and $\alpha$-MSH in the anterior pituitary and amygdala, respectively (Jegou et al., 2003) and the MC4 receptor in the amygdala mediates $\alpha$-MSH-triggered anxiety (Chaki et al., 2003; Kokare et al., 2005). Importantly, Bhorkar et al. (2014) propose that the adenosine system acts upstream of the melanocortin system to regulate anxiety behavior. Interestingly, the non-peptidic compound MCL0042 acts as an MC4 receptor antagonist and inhibits the serotonin transporter to produce anxiolytic and anti-depressant-like effects (Chaki et al., 2005).

The serotonin system has also been implicated in anxiety and anxiety disorders. The dorsal raphe nucleus (DRN) is the major source of serotonin innervation of forebrain structures and provides the anatomical basis for serotonin’s role in anxiety and other psychiatric disorders. Anxiogenic states are associated with increased serotonin levels in multiple brain areas, particularly under stressful conditions (Kawahara et al., 1993; Amat et al., 1998, 2004; Maier and Watkins, 2005; Mitsushima et al., 2006). However,
stimulation of serotonin receptors in the basolateral amygdala induces anxiolysis (Graeff and Zangrossi, 2010). It is thought that serotonin acts on 5-HT$_{2A}$ receptors in the amygdala to reduce anxiety-like behavior, and acts on 5-HT$_{2C}$ receptors to increase anxiety-like behavior. Therefore, the role of serotonin and anxiety depends on specific areas of the brain and the specific receptors involved.

Tryptophan hydroxylase 2 (TPH2) is the rate-limiting isoenzyme involved in serotonin biosynthesis and is found primarily in the raphe nucleus (Ichiyama, et. al., 1970). Increased TPH2 expression has been implicated in anxiety. For example, a chronic anxiety state led to increased $tph2$, but not $slc6a4$ expression, in the ventrolateral DR (DRVL), a region associated with panic-related responses, and the increased $tph2$ was correlated with increased anxiety behavior (Donner, et. al., 2012). Single-nucleotide polymorphisms (SNP) of the $tph2$ gene have been implicated in anxiety, but have differing results depending on the specific SNP analyzed. Berger, et. al. (2012) suggest the anxiety phenotype of specific SNPs may result from compensatory changes in the serotonergic system, such as 5-HT$_{1A}$ autoreceptor desensitization, rather than the reduced amount of serotonin tissue or transmission.

Solute Carrier Family 6 (Neurotransmitter Transporter), Member 4 (SLC6A4) is the gene encoding the serotonin transporter, SERT, which transports serotonin from the synaptic cleft back into the presynaptic terminal. Its expression may be determined by interactions between early life experience and stressful experience during adulthood (Gardner, et. al., 2009). There are also varying results associated with SNPs in the $slc6a4$ gene. Numerous studies have found no association between the serotonin transporter-linked polymorphic region of $slc6a4$ and panic disorder (Hamilton et. al., 1999; Olesen
et. al., 2005; Deckert et. al., 1997; Domschke et. al., 2006; Blaya et. al., 2007; Blaya et. al., 2010). However, recent studies suggest panic disorder may be associated with variation in the \textit{slc6a4} 3’ untranslated region [Strug et. al., 2010; Gyawali et. al., 2010], and that susceptibility to panic attacks in healthy subjects may be associated with variation in the serotonin transporter-linked polymorphic region [Maron et. al., 2004]. However, variants of the \textit{slc6a4} gene may be genetic predictors of anxiety (for review, see Hariri and Holmes, 2006; Hu et al, 2006; Lesch et al, 1996; Ozaki et al, 2003). Therefore, TPH2 and SLC6A4 are both implicated in anxiety, but TPH2 may be of more direct importance.

There is some evidence to suggest that adenosine may be an important modulator of the serotonin system and its control of anxiety. Thus, it has been shown that anxiogenic drugs, including caffeine, increase behavioral arousal and vigilance behaviors, which are associated with increases in c-Fos expression in the dorsal raphe nucleus dorsal part (DRD) and dorsal raphe nucleus, caudal part (DRC) [Abrams, et. al., 2005]. Interestingly, adenosine receptors can regulate the release of serotonin in the hippocampus through A\textsubscript{1} and A\textsubscript{2A} receptors [Okada, et. al., 2001]. The central adenosine A\textsubscript{1} receptors occur both on presynaptic terminals, where they inhibit the release of neurotransmitters such as serotonin, and postsynaptically where they suppress neuronal activity by opening potassium channels [Prince and Stevens, 1992]. It has also been shown that caffeine can decrease restraint-induced stress, which correlates with its ability to reduce serotonin levels in the hippocampus, and this effect is attributed to A\textsubscript{2A} receptors [Yamato, et. al., 2002]. Therefore, caffeine-induced anxiety may be due to
adenosine A₁ receptor antagonism leading to increases in serotonergic function, which has been implicated in anxiety (Lister, 1990, Nutt, 1990).

We are interested in how caffeine consumption during adolescence influences the expression of anxiety and the neurobiological systems that produce anxiety. The adolescent developmental period is characterized by many intrinsic neurobiological and hormonal changes that shape an organism’s behavior in adulthood. These changes generally serve the betterment of the individual by improving impulse control, decision-making, cognitive flexibility and motivational and emotional control. The serotonin system is considered to be relatively mature after postnatal day 21, although adolescence is still associated with important developmental changes. There is significant synaptic pruning in the basal forebrain of serotonergic neurons around puberty (Dinopoulos et al., 1997). The primary autoreceptor, serotonin receptor 1A, binding decreases progressively during adolescence (Dillon et al., 1991). In addition, SLC6A4 is expressed more widely during early development and becomes more tightly regulated during adolescence and into adulthood (Daws and Gould, 2011). Extrinsic factors can negatively impact these developmental processes, potentially altering neurobiological systems and corresponding behavioral patterns. The adolescent period is therefore considered to be a vulnerable developmental window that can be impacted by environmental insults, such as drug exposure. In particular, caffeine exposure may impinge on the serotonin raphe system to significantly change the neurobiological development of the serotonin system functioning and impact the future expression of anxiety and other behaviors associated with serotonin system.
The prevalence of caffeine and its relation to anxiety disorders make it an important area of study. It acts via the adenosine system to influence other systems, such as serotonin, to produce anxiety. Little is known about how adolescent caffeine consumption may alter the serotonin system to produce subsequent anxiogenic effects. Therefore, the goal of the present study is to explore the effects of chronic adolescent caffeine consumption on the serotonin system and related anxiety behaviors.

Materials and Methods

Rats and Housing

Male Sprague-Dawley rats (Charles River) were received on P21 and pair housed with *ad libitum* food and water. Separate groups 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 followed 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.

Caffeine Consumption

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. Adolescent caffeine consumption procedures occurred continuously between P28-P55. Age-matched control groups received water throughout the procedure. Caffeine and water consumption were
monitored throughout the procedure to determine whether there was a difference in consumption, and so the amount of caffeine consumed could be quantified. Following the 28-day caffeine consumption period, caffeine was removed and replaced with water for the remainder of the experiment. 24 hours after caffeine removal, a final dose of caffeine (30 mg/kg, ip) or saline was given to adolescent rats, and brain tissue was collected 4 hours following the injection. Behavioral testing was done 24 hours after the last caffeine consumption. The experimental timeline is shown in Figure 1.

![Experimental Timeline](image)

**Figure 1.** Experimental timeline showing the period of caffeine consumption from days postnatal day 28-55 (P28-P55). Administration of the final dose of caffeine or saline was given on P56, when tissue samples were collected and anxiety behavioral tests were conducted in separate cohorts of rats. Rats were sacrificed 4 hours following the injection.

*In situ hybridization*

A [35S]- UTP-labeled oligonucleotide probe against *tph2* mRNA or riboprobe against *slc6a4* mRNA was generated using standard transcription methods (Donner et al. 2012). Tissue sections were briefly thawed at room temperature and fixed with 4% paraformaldehyde in 0.05 M phosphate buffered saline (PBS) for 10 min. Following two
washes in 0.05 M PBS, slides were placed into freshly prepared 0.25% acetic anhydride in 0.9% NaCl containing 0.1 M triethanolamine (TEA) for 10 min. Sections were then dehydrated through a graded series of alcohols, delipidated in chloroform, rehydrated through a second series of alcohols, and then allowed to air dry. Sections were hybridized at 55 °C overnight in 90 μl hybridization solution (1 X 10^6 cpm radioactively labeled probe per slide (about 0.4 μg/ml), 25 mM Tris, 40% deionized formamide, 500 μg/ml single-stranded salmon sperm DNA, 250 μg/ml transfer RNA, 1X Denhart’s solution, 4 mM EDTA, 5 mM sodium chloride, 10% dextran sulfate, pH 7.4). The next day, coverslips were removed before all slides were washed once in 2X standard sodium citrate (SSC) and three times in 1X SSC, for 10 min each. Slides were then incubated in ribonuclease. A solution (0.05 M Tris–Cl, 0.025 M EDTA, 0.5 M NaCl and 20 μg/ml RNase A) for 1 h at 37 °C. Slides were then washed in 1X SSC for 30 min at room temperature, and then in 1X SSC for 30 min at 60°C to remove nonspecific probe binding within the tissue. Lastly, slides were desalted in 0.5X and 0.1X SSC for 10 min each, and then gradually dehydrated in 50%, 70%, 90% and 100% ethanol containing 0.3 M ammonium acetate. After air-drying for about 20 min, all slides were apposed to the same Kodak BioMax autoradiography film (PerkinElmer, Waltham, MA) for region- and probe-appropriate times (1–3 weeks).

Image Quantification

Autoradiographic images of the probe bound to tph2 or slc6a4 mRNA together with 14-C-labeled standards were measured using a computer-assisted image analysis.
system. All slides from the study had been apposed to the same film, allowing use of a single set of 14-C-labeled standards for reference. Analysis was performed using the publicly available NIH-developed image analysis software ImageJ. Virtual matrixes in the shape of the respective dorsal raphe subregions were created, as shown in Figures 3 and 6, overlaid with the image, and the “optical density x area” within each matrix measured. All measurements were taken while blinded to the treatment groups. During the entire analysis a constant threshold function was applied, which determined the area that was actually measured within each matrix. Thus, all pixels with a gray density below threshold were automatically excluded. In addition, the individual background of each image was measured and subtracted from each value. A rostrocaudal analysis atlas for tph2 and slc6a4 expression in the dorsal raphe was created by comparing the image of the tissue sections with a stereotaxic rat brain atlas (Paxinos and Watson, 1998) and with tph2 or slc6a4 mRNA expression topography. Throughout all rostrocaudal levels, the values for each subdivision were then averaged, and all values per treatment group display the overall mRNA expression in the dorsal raphe. The bilateral DRVL/VLPAG was averaged across the right and left sides. The subdivisions studied were summarized into the following functional subregions of the DR: dorsal raphe nucleus, caudal part (DRC), dorsal raphe nucleus, dorsal part (DRD), dorsal raphe nucleus, interfascicular part (DRI), dorsal raphe nucleus, ventral part (DRV), left and right dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray region (left and right DRVL/VLPAG).
Elevated Plus Maze

Rats were tested in the elevated plus maze between P56-61, 24 hours after caffeine removal. 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, 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 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.

Social Interaction

Rats were evaluated in a social interaction test between P56-61, 24 hours after caffeine removal. Each rat was given 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 sniffing and grooming interactions.
Data Analysis

A two-way ANOVA was used to analyze the effects of caffeine consumption and caffeine challenge treatments. The independent variables were the four treatment groups, in which the rats either consumed caffeine or water, and whether they were challenged with caffeine or saline. The dependent variable was the expression of either TPH2 or SLC6A4. To measure the effect of adolescent caffeine consumption on anxiety behaviors, unpaired t-tests were used to compare the means of the two different consumption groups (caffeine and water). In all cases, significant interactions were followed by post-hoc analyses using Bonferroni correction and unpaired t-tests.

Results

Withdrawal from caffeine consumption increases adolescent anxiety behaviors

To determine if caffeine consumption had an effect of anxiety behaviors, two different anxiety-related behavioral tests were performed on different groups of rats 24 hours after removal of caffeine. Figure 2 shows that withdrawal from caffeine consumption significantly decreased percent time spent on the open arms and open arm entries of the elevated plus maze ($t_{18} = 10.36, p < .0001$; $t_{18} = 2.700, p = .0147$, respectively) compared to the control group. Withdrawal from caffeine consumption also significantly decreased the total amount of social interaction ($t_{15} = 2.350, p = .0329$)
compared with controls. These results indicate increased anxiety in the caffeine-consuming rats.

Figure 2. Adolescent caffeine consumption increases anxiety-related behaviors 24 hours after caffeine removal. A) Rats that consumed caffeine spent less time on the open arms of the elevated plus maze. B) Rats that consumed caffeine enter the open arms less frequently when exposed to the elevated plus maze. C) Chronic adolescent caffeine consumption decreases social interaction. * indicates significance compared to controls (p< .05).
Expression of TPH2, but not SLC6A4, is decreased following adolescent caffeine consumption

To determine if caffeine had an effect on TPH2 expression within the raphe, its expression was evaluated in different raphe subregions. In the DRD, a two-way ANOVA revealed a significant interaction between consumption and challenge ($F_{1,32} = 4.763, p = .0365$) and main effect of challenge ($F_{1,32} = 34.13, p < 0.0001$), as shown in Figure 4A. However, there were no significant effects of consumption. Post-hoc analysis showed that the caffeine challenge significantly decreased TPH2 expression in both water- and caffeine-consuming groups ($t_{32} = 5.674, p < 0.0001$ and $t_{32} = 2.588, p < .05$, respectively). Caffeine consumption also significantly decreased TPH2 expression in vehicle treated rats ($t_{14} = 2.370, p = .0327$). In the DRV, there were no significant interaction or consumption effects, but there was a significant challenge effect ($F_{1,32} = 38.54, p < .0001$). As shown in Figure 4B, a caffeine challenge significantly decreased TPH2 expression in the DRV. Figure 5 shows that in the DRI, DRVL/VLPAG, and DRC, there were only significant challenge effects ($F_{1,32} = 20.96; F_{1,32} = 39.33; F_{1,31} = 45.01$, respectively, and $p < .0001$ for all three regions).
Figure 3. Neuroanatomical atlas of rat TPH2 gene expression used for analysis of dorsal raphe nucleus subregions. Shown (from top left to bottom right) are coronal brain sections of TPH2 mRNA expression from rostral (−7.244 mm bregma) to caudal (−8.672 mm bregma). Abbreviations: DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRVL; VLPAG, ventrolateral periaqueductal gray. Scale bar: 1 mm.
Figure 4. Adolescent caffeine consumption decreases TPH2 expression in the raphe nucleus. A) Caffeine challenge decreases TPH2 expression in both water- and caffeine-consuming groups and decreases TPH2 expression in vehicle treated rats in the dorsal raphe nucleus, dorsal part (DRD). B) Caffeine challenge significantly decreases TPH2 expression in the dorsal raphe nucleus, ventral part, (DRV). C) In situ hybridization pictures showing TPH2 expression in the DRD and DRV. From top left to bottom right: water/vehicle, water/caffeine, caffeine/vehicle, caffeine/caffeine. * indicates significance from respective vehicle group (p< .05). # indicates significance between control and caffeine consuming vehicle-treated groups (p< .05). ^ indicates a significant main effect of challenge (p< .05).
Figure 5. Adolescent caffeine consumption decreases TPH2 expression in the raphe nucleus. A) Caffeine challenge decreases TPH2 expression in the dorsal raphe nucleus, interfascicular part (DRI). B) Caffeine challenge decreases TPH2 expression in the dorsal raphe nucleus, caudal part (DRC). C) Caffeine challenge decreases TPH2 expression in the dorsal raphe nucleus, dorsolateral part and ventrolateral periaqueductal grey (DRVL/VLPAG). D) In situ hybridization pictures showing TPH2 expression in the DRI, DRC, and DRVL/VLPAG. From top left to bottom right: water/vehicle, water/caffeine, caffeine/vehicle, caffeine/caffeine. ^ indicates a significant main effect of challenge (p< .05).
To determine if caffeine had an effect on SLC6A4 expression within the raphe, its expression was evaluated in different raphe subregions. In the DRV, DRD, and DRC, there were no significant interaction, challenge, or consumption effects, as shown in Figure 8. However, in the DRI, a two-way ANOVA revealed significant interaction between consumption and challenge ($F_{1,31} = 4.249$, $p = .0477$) and a main effect of challenge ($F_{1,31} = 15.48$, $p = .0004$), but no significant consumption effects. Post-hoc analysis showed that the caffeine challenge significantly decreased SLC6A4 expression in the DRI in caffeine-consuming, but not water-consuming group, as shown in Figure 7A. Similarly, the DRVL/VLPAG showed a significant interaction effects ($F_{1,32} = 4.512$, $p = .0415$), but no main effects. Post-hoc analysis showed no significant effect of challenge within either consumption group. Unpaired t-tests revealed a significant decrease in SLC6A4 in the caffeine-consuming rats receiving a vehicle treatment ($t_{14} = 2.956$, $p = .0104$), as shown in Figure 7B.
Figure 6. Neuroanatomical atlas of rat SLC6A4 gene expression used for analysis of dorsal raphe nucleus subregions. Shown (from top left to bottom right) are coronal brain sections of SLC6A4 mRNA expression from caudal (-8.588 mm bregma) to rostral (-7.580 mm bregma). Abbreviations: DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRVL, dorsal raphe nucleus, ventrolateral part; VLPAG, ventrolateral periaqueductal gray. Scale bar: 1 mm.
Figure 7. Adolescent caffeine consumption increases SLC6A4 in the DRI, but not the DRC. A) No significant effects of consumption or challenge on SLC6A4 expression in the dorsal raphe nucleus, caudal part (DRC). B) Caffeine challenge decreases SLC6A4 expression in the dorsal raphe nucleus, interfascicular part (DRI) in caffeine-consuming, but not water-consuming group. C) In situ hybridization pictures showing SLC6A4 expression in the DRI and DRC. From top left to bottom right: water/vehicle, water/caffeine, caffeine/vehicle, caffeine/caffeine. * indicates significance from respective caffeine-consuming vehicle group (p<.05).
Figure 8. Adolescent caffeine consumption has no effect on SLC6A4 expression in the DRD, DRV, and DRVL/VLPAG. A) No significant effects of consumption or challenge on SLC6A4 expression in the dorsal raphe nucleus, dorsal part (DRD). B) Caffeine-consuming rats receiving vehicle treatment show decreased SLC6A4 expression in the dorsal raphe nucleus, ventrolateral part (DRVL) and ventrolateral periaqueductal grey (VLPAG). C) No significant effects of consumption or challenge on SLC6A4 expression in the dorsal raphe nucleus, ventral part (DRV). In situ hybridization pictures showing SLC6A4 expression in the DRD, DRV, and DRVL/VLPAG. From top left to bottom right: water/vehicle, water/caffeine, caffeine/vehicle, caffeine/caffeine. # indicates significance between control and caffeine-consuming vehicle groups (p< .05).
Discussion

In the present study, the effects of withdrawal from chronic adolescent caffeine consumption on anxiety behaviors and the serotonin system were examined. Adolescent caffeine consumption increased anxiety-related behaviors in the elevated plus maze and social interaction tests 24 hours following the removal of caffeine. These results are consistent with other studies reporting that acute caffeine and caffeine withdrawal increases anxiety in adult rats, as shown in the elevated plus maze, light dark box, open field box, and social interaction test (El Yacoubi et al., 2000; Noschang et al., 2009; Bhattacharya, et. al., 1997). Interestingly, we recently reported that elevated anxiety persists at 7 days following adolescent caffeine consumption in adolescent animals, but not adult animals, indicating that adolescents who consume caffeine may be at more risk for anxiety disorders in adulthood (O’Neill et. al., 2016). Withdrawal from chronic caffeine also produces symptoms including locomotor suppression and decreased consummatory behaviors that develop 24 hours after caffeine removal and dissipate within 48 hours (Finn and Holtzman, 1986; Holtzman, 1983; Rhoads et al., 2011). Even though our results are consistent with previously published studies (El Yacoubi et al., 2000; Noschang et al., 2009; Bhattacharya, et. al., 1997), it is possible that performance on our anxiety tests at 24 hour withdrawal was confounded by the lethargic effect of caffeine withdrawal.

We also observed that caffeine consumption decreased the expression of tph2 in the DRD, but had no significant effects in other subregions of the DRN. Administration of a caffeine challenge decreased tph2 expression throughout the DR (e.g. DRD, DRV, DRI, DRC, and DRVL/VLPAG), and this response was unchanged by caffeine
consumption during adolescence. A caffeine challenge increased \textit{slc6A4} expression in the DRI, and caffeine-consuming rats receiving vehicle treatment show decreased SLC6A4 expression in the DRVL/VLPAG. However, there was no effect on \textit{slc6A4} expression in the DRC, DRD, DRV. Together, these findings suggest that both adolescent caffeine consumption and withdrawal, and acute administration of caffeine in caffeine-naïve animals may alter some aspects of the serotonin system, although their functional relationship to the emergence of withdrawal-induced anxiety is not clear.

Before considering the relationship of the behavioral and neurobiological changes we observed, it is important to address a possible limitation of the study. The dose of caffeine administered in our model exceed the average dose of caffeine consumed by human adolescents. The average amount of caffeine consumed in children and adolescents is 100-400 mg per day (Frary et al., 2005; Rudolph et al., 2014; Temple, 2009). The rats in the present study consumed about 30 mg/kg/day, which is comparatively higher. However, human studies are often not adjusted for body weight. Rats also metabolize caffeine faster than humans, suggesting that a lower dose in humans could produce the same effect (Arnaud, 1987; Bonati et al., 1982). Therefore, it is difficult to compare drug dosing between the two species. In addition, caffeine clearance rates of adolescents are faster in early adolescence compared with adults (Bienvenu et al., 1990; Ginsberg et al., 2002; Latini et al., 1980). It is unclear whether these dosing differences factor into our results and interpretations since withdrawal of caffeine consumed at lower caffeine doses also produces anxiogenic responses (El Yacoubi et al., 2000; Noschang et al., 2009; Bhattacharya, et. al., 1997).
We observed that withdrawal from caffeine consumption decreased \textit{tph2} expression in vehicle-treated rats in the DRD, suggesting that acute withdrawal is associated with decreased serotonin. This notion is consistent with findings showing that repeated caffeine administration significantly increases brain serotonin levels and its withdrawal significantly decreases serotonin levels (Khaliq et al., 2012). Historically, decreased serotonin activity has been associated with anxiety disorders given the relative effectiveness of selective serotonin reuptake inhibitors in the treatment of anxiety disorders (Gross and Hen, 2004). However, it is unclear how accurately changes in \textit{tph2} gene expression reflect serotonin levels and whether they accurately correspond with anxiety since other studies show an inverse relationship of TPH2 expression with anxiety.

For example, increases in TPH2 in the DRVL/VLPAG are also associated with increased anxiety (Donner, et. al., 2012 and Paul et. al., 2014). In addition, TPH2 knockout animals exhibit decreased anxiety-like behavior (Mosienko, et. al., 2012 and Gutknecht, et. al., 2015). Furthermore, our terminology of “caffeine consumption” refers to the 28-day caffeine administration plus 24 hours withdrawal, and therefore, it is not possible to determine if the effects of caffeine consumption on \textit{tph2} mRNA in the DRD are due to the caffeine or withdrawal from the caffeine. Thus, the relationship between the increased anxiety behavior and decreased \textit{tph2} expression observed in our studies remains unclear.

Interestingly, a caffeine challenge also decreased \textit{tph2} expression, an effect observed in both control and caffeine-consuming adolescents in each region of the DRN. This suggests that acute caffeine may also decrease serotonin. Acute administration of caffeine is known to produce anxiogenic effects during adolescence (Ardais et. al., 2014), raising further questions about whether TPH2 expression is associated with the
expression of anxiety-related behavior. It is unclear how caffeine may produce these decreases acutely, although TPH2 has highly flexible gene expression that is modulated by numerous internal and external environmental factors including the biological clock, stressors, and endogenous hormones (Chen and Miller, 2013). Furthermore, in an extensive review, Feenstra and van der Plasse (2010) suggest that only in animal studies in which either serotonin synthesis rate is already decreased or serotonin utilization is increased has the effect of tryptophan depletion on serotonin release been demonstrated.

We also observed that a caffeine challenge increased slc6a4 expression in the DRI of caffeine-consuming animals, and caffeine consumption decreased basal slc6a4 expression in the DRVL/VLPAG. Similar to TPH2, it is unclear how these changes in the serotonin transporter gene correspond to the caffeine withdrawal-induced anxiety. For example, numerous studies have found no association between the serotonin transporter-linked polymorphic region of SLC6A4 and panic disorder (Hamilton, et. al., 1999; Olesen et. al., 2005; Deckert et. al., 2007; Domschke et. al., 2006; Blaya et. al., 2007; Blaya et. al., 2010). In contrast, rats exposed to both a developmental and adulthood stressor had increased slc6a4 mRNA expression in all regions except the DRI (Gardner et. al., 2009). These discrepancies may be explained by the topographical organization of the serotonin system since different subregions within the DRN differentially affect anxiety behavior based on differing afferents and efferents in the brain. For example, neurons in the DRD and DRC are known to facilitate anxiety-like responses, whereas neurons in the DRVL/VLPAG suppress fear and panic-like responses and control presympathomotor neurons in the spinal cord, and neurons in the DRI are associated with stress resilience and anti-depressant effects (Hale et. al., 2012; Fox and Lowry, 2013). It
is difficult to relate the challenge effect on the DRI to anxiety, because the anxiety behavioral tests did not include a caffeine challenge. In addition, DRI serotonin neurons are sensitive to stimulation of sensory afferents of a number of different modalities, including auditory, visual, immune, and thermal stimuli (Hale and Lowry, 2011). The consumption effect in the DRVL/VLPAG may be explained by its role in the locomotor system, which may explain the decreased locomotion observed in the anxiety tests. In addition, Abrams et. al. (2005) observed that caffeine and a benzodiazepine receptor partial inverse agonist produced different c-fos immunoreactivity in non-serotonin cells compared to serotonin cells of the DRVL/VLPAG, suggesting that other mechanisms in those regions were at play. Furthermore, DRI serotonin neurons are co-activated with DRVL/VLPAG serotonin neurons following injection of mice with LPS (Hollis et al. 2006), suggesting effects in these regions may have affected one other. Therefore, the relationship between slc6a4 expression and anxiety remains unclear.

Caffeine administration is a stressor and other systems in the body are likely to be affected by chronic consumption, such as the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is believed to impact the pathogenesis of stress-related disorders, such as anxiety, and acute caffeine administration increases HPA activity (Nicholson, 1989 and Patz et. al, 2006). In addition, 24 hour withdrawal from caffeine increases corticosterone, a stress-related hormone released during HPA activation (O’Neill, et. al., 2016). Caffeine-induced activation of the prelimbic cortex can lead to several pro-anxiety responses. Reduced GABA signaling from the prelimbic cortex increases immunoreactive corticotrophin-releasing factor positive (irCRF+) neuron activity in the bed nucleus of the stria terminalis (BNST). CRF neurons originating in the BNST
activate CRFR\(_2\) receptors in the DRD which cause excitation and lead to increased 5-HT\(_{2C}\) receptor activation in the BLA, which increases anxiety behavior (Hermann et al., 1997; Fox et al., 2013). The CRF derived from the BNST can be disinhibited by caffeine-induced activation in the prelimbic cortex. Furthermore, serotonin neurons in the DRD and DRC co-express CRF (Donner et. al., 2016). Consequently, the interactions between CRF and serotonin neurons may play important roles in stress-related psychopathology associated with chronic psychosocial stressors including generalized anxiety, anxiety associated with drug withdrawal, and stress-induced drug relapse (Lowry et al., 2000).

Similarly, corticosterone can facilitate the serotonin system. For example, chronic glucocorticoid exposure, such as corticosterone, is anxiogenic and increases TPH2 expression in the DRD and DRV (Donner et. al., 2016). In addition, chronic stress and elevated levels of corticosterone increases serotonin receptor activity in the hippocampus (Tokarski et. al., 2014).

Interestingly, corticosterone can also influence serotonin neuron activity by blocking the organic cation transporter 3 (OCT3). OCT3 expression on serotonin neurons provides an alternate mechanism to clear serotonin from the synapse. Corticosterone blockade of the OCT3 may result in the accumulation of serotonin in the synaptic cleft and ultimately result in altered serotonergic neurotransmission in the DRN (Gasser et al., 2009). Therefore, future studies should examine the effect of chronic adolescent caffeine consumption on OCT3. SLC22A3 is the gene encoding OCT3 and would be another useful target to study, because it is on virtually all serotonin neurons in the DRN (Wyler et al., 2015). Serotonin neurons are also tightly regulated by the serotonin autoreceptor, 5HT\(_{1A}\). 5HT\(_{1A}\) produces negative feedback on serotonin neuronal cell bodies via
potassium channel opening that is G_{i/o}-dependent. 5-HT_{1A} knockouts display increased anxiety in the open field test and the elevated plus maze (McDevitt et al., 2011b, Rozeske et al., 2011). Therefore, it is conceivable that 5-HT_{1A} may be regulated by chronic caffeine consumption and/or withdrawal to produce the heightened anxiety.

Future studies should also consider sex because anxiety disorders are twice as prevalent in women as men (Pigott, 2003). Sex-specific functions of serotonin have previously been reported (Bethea et al. 1998; Rubinow et al. 1998; Goel and Bale 2010; Hall and Steiner 2013). In addition, sex differences in anxiety- and depression-related behaviors were shown in TPH2 knockout mice (Gutknecht, et. al. 2015). Previous studies also show that chronic estrogen treatment increases tph2 mRNA in the DRC, and this increase was associated with decreased anxiety (Hiroi, et. al. 2011). Therefore, this research can help explain other disorders associated with the serotonin system, or focus on specific components of the DRD in developing medications for different types of anxiety disorders. It can also be used to help explain the lasting effects of chronic adolescent caffeine consumption into adulthood and help to develop medications specific to adolescent anxiety.

Conclusions

The results of this study indicate that withdrawal from chronic adolescent caffeine consumption modulates anxiety behaviors and the serotonin system. This suggests that caffeine consumption during adolescence may increase susceptibility to the development of anxiety-related disorders, although it is not clear whether this susceptibility is due to alterations in the serotonin system. Since caffeine consumption is increasing among children and adolescents (Ahluwalia and Herrick, 2015; Frary et al., 2005; Temple,
2009), it is important to enhance awareness of the potentially harmful effects of consuming caffeine during adolescence.

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