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Effects of chronic caffeine exposure during adolescence and subsequent acute caffeine challenge during adulthood on rat brain serotonergic systems

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Abstract

Caffeine is the most commonly used drug in the world. However, animal studies suggest that chronic consumption of caffeine during adolescence can result in enhanced anxiety-like behavioral responses during adulthood. One mechanism through which chronic caffeine administration may influence subsequent anxietylike responses is through actions on brainstem serotonergic systems. In order to explore potential effects of chronic caffeine consumption on brainstem serotonergic systems, we evaluated the effects of a 28-day exposure to chronic caffeine (0.3 g/L; postnatal day 28-56) or vehicle administration in the drinking water, followed by 24 h caffeine withdrawal, and subsequent challenge with caffeine (30 mg/kg; s.c.) or vehicle in adolescent male rats. In *Experiment 1*, acute caffeine challenge induced a widespread activation of serotonergic neurons throughout the dorsal raphe nucleus (DR); this effect was attenuated in rats that had been exposed to chronic caffeine consumption. In *Experiment 2*, acute caffeine administration profoundly

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decreased *tph2* and *slc22a3* mRNA expression throughout the DR, with no effects on *htr1a* or *slc6a4* mRNA expression. Chronic caffeine exposure for four weeks during adolescence was sufficient to decrease *tph2* mRNA expression in the DR measured 28 h after caffeine withdrawal. Chronic caffeine administration during adolescence did not impact the ability of acute caffeine to decrease *tph2* or *slc22a3* mRNA expression. Together, these data suggest that both chronic caffeine administration during adolescence and acute caffeine challenge during adulthood are important determinants of serotonergic function and serotonergic gene expression, effects that may contribute to chronic effects of caffeine on anxiety-like responses.

1. Introduction

Caffeine is the most commonly used psychoactive drug by teens and young adults (Gilbert 1984); it is estimated that caffeine is used by 30-50% of teens and young adults in the US, accounting for \$2.3 billion dollars of energy drink sales per year (Rath 2012). Although caffeine has beneficial effects on alertness and reducing fatigue (Glade 2010; Smith 2002a), it can also have negative consequences. Recent clinical studies have shown that some individuals become dependent on the drug and are not able to reduce consumption despite awareness of health problems associated with its use (Meredith et al. 2013). These findings have led the World Health Organization to recognize caffeine dependence as a clinical disorder, while Caffeine Use Disorder has been identified as a condition for further study in the *Diagnostic and Statistical Manual of Mental Disorders, 5th ed. (DSM-5)* (American Psychiatric Association 2013).

Evidence suggests that excessive caffeine consumption can exacerbate chronic psychiatric conditions, including anxiety disorders at any point in an individual's life (De Freitas and Schwartz 1979; Mikkelsen 1978; Tondo and Rudas 1991; Winstead 1976; Zaslove, Russell, and Ross 1991). Some features of caffeine dependence, including tolerance and withdrawal, have been associated with major depression, adult antisocial disorder, panic disorder, and generalized anxiety disorder (Bergin and Kendler 2012; Kendler, Myers, and Prescott 2007). Evidence furthermore suggests that caffeine dependence is a clinically meaningful disorder that affects a significant proportion of caffeine users (Meredith et al. 2013). Despite this, little is known about how chronic caffeine exposure alters the neural systems that regulate anxiety.

Animal studies suggest that the long-term consequences of chronic caffeine intake are dependent on the developmental stage at which the first dose is given. In 2011, Rhoads et al., showed that during the withdrawal phase (24-48 hours after the last dose) after a 2-week period of exposure to caffeine (1 mg/ml in drinking water), rats responded with decreased baseline movement compared to caffeine-naïve rats, but only in adolescents (i.e., postnatal day (P) = 28 at the beginning of 2 week caffeine exposure). Likewise, motor responses to a challenge dose of caffeine (30 mg/kg, i.p.) were reduced after chronic caffeine consumption and withdrawal, but again, this effect was observed in adolescent, but not adult rats, suggesting increased tolerance to the effects of caffeine in adolescents. Evidence also suggests that chronic caffeine consumption triggers behavioral and neurochemical alterations in adolescent rats that lead to increases in anxiety-like behaviors (Ardais et al.

2014). Consistent with the hypothesis that caffeine consumption during adolescence may have long-term effects on anxiety-like behavior, a recent study by O'Neill et al., (2016) revealed that adolescent caffeine consumption (0.3 g/L in drinking water) for four weeks, followed by one week of withdrawal, led to increased anxiety-like behavior, compared to vehicle controls, as measured in a number of anxiety tests. In contrast, these anxiogenic effects of chronic caffeine consumption and withdrawal were not observed in adult rats (O'Neill et al. 2016). These data suggest that chronic caffeine exposure during adolescence may have the potential to negatively impact mental health later in life.

One mechanism through which caffeine may exert its effects on anxiety-related behavior is through the modulation of serotonergic systems. Serotonergic systems are involved in regulating myriad physiological and behavioral responses (Abrams et al. 2004; Frazer and Hensler 1999; C. A. Lowry et al. 2008; C. Lowry, Evans, and Gasser 2008; Lucki 1998). Of note, serotonergic systems are involved in controlling physiological and behavioral responses to anxiogenic stimuli (Hale, Shekhar, and Lowry 2012; Maier and Watkins 1998, 2005). Caffeine is a non-selective adenosine receptor antagonist that exerts largely excitatory effects throughout the brain (Fredholm et al. 1999; Rosin et al. 1998). Studies on glutamatergic synapses indicate that adenosine receptors are present on the presynaptic terminal (A1, A2a receptors); post-synaptic cell (A1, A2a receptors); as well as nearby astrocytes (A2a, A2b, A3 receptors) (Björklund et al. 2008; Ciruela et al. 2006; Rau, Ariwodola, and Weiner 2014; Shen et al. 2013). Despite a low abundance of adenosine receptors in the serotonergic raphe nuclei, both acute and chronic caffeine increase cellular activity within the raphe nuclei (Abrams et al. 2005; Nehlig et al. 1986; Singewald and Sharp 2000). Furthermore, caffeine's blockade of inhibitory adenosine A1 receptors at presynaptic terminals is a primary mechanism by which caffeine increases the release of various neurotransmitter systems including serotonin in terminal regions (Feuerstein, Hertting, and Jackisch 1985; Okada et al. 2002). It is possible that caffeine produces alterations in the serotonergic system in the production and maintenance of an anxiety-like phenotype, and that this phenotype can lead to chronic exaggeration of anxiety-like behavioral responses.

An acute withdrawal syndrome emerges within 24-48 h of withdrawal from chronic caffeine consumption that is associated with various physical and subjective symptoms (Juliano and Griffiths 2004; Smith 2002b). Previous studies have demonstrated that acute caffeine withdrawal produces alterations in brain serotonin concentrations (Haleem et al. 1995; Khaliq et al. 2012). We were therefore interested in identifying what alterations might arise in the serotonergic raphe nuclei following chronic caffeine administration, 24 hr withdrawal and caffeine re-exposure. To explore the effects of chronic caffeine administration during adolescence on brainstem serotonergic systems, we administered caffeine for 28 days in the drinking water, followed by 24 h withdrawal and subsequent challenge with caffeine or vehicle. Brainstem serotonergic systems within the dorsal raphe nucleus (DR) were evaluated by assessment of immediate-early gene expression (c-Fos) immunostaining in serotonergic and non-serotonergic neurons, and expression of genes thought to be important in controlling serotonergic neuronal function, i.e., *tph2*, encoding tryptophan hydroxylase (Tph2), the rate-limiting enzyme in the biosynthesis of brain serotonin, *slc22a3*, encoding Slc22a3, also known as organic cation transporter 3 (OCT3), a sodium independent, low

affinity, high capacity serotonin transporter expressed in serotonergic neurons, *htr1a*, encoding the serotonin (5-HT)_{1A} receptor, an inhibitory serotonergic autoreceptor, and *slc6a4*, encoding Slc6a4, also known as the serotonin transporter, a presynaptic, sodium dependent, high affinity, low capacity serotonin transporter. As previous studies have implicated both the dorsal raphe nucleus and median raphe nucleus in anxiety-like behavioral responses (Abrams et al. 2005; Lowry and Hale 2010), we investigated gene expression in the DR and median raphe nucleus (MnR), but also in the supramedian serotonergic cell group (B9), and pontomesencephalic reticular formation (PMRF).

2. Materials and methods

2.1 Animals

Juvenile male Sprague Dawley® rats (Envigo, Cat. No. 002, Indianapolis, IN, USA) were received on postnatal day (pnd) 21, randomly assigned to treatment groups and pair housed with *ad libitum* food (Teklad 22/5 rodent diet Cat. No. 8640, Envigo Indianapolis, IN, USA) and tap water. Adolescence in rodents consists of early adolescence [prepubescent or juvenile, pnd 21–34], middle adolescence (periadolescent, pnd 34–46), and late adolescence (pnd 46–59) time periods (Spear 2000). Consequently, rats were in the juvenile period (pnd 21) upon arrival and initial treatment with caffeine or vehicle in the drinking water, and late adolescence at the time of caffeine or vehicle challenge and euthanasia (pnd 57). All experimental procedures were conducted during the light period of a 12 h light/12 h dark cycle. Rats were housed in high-temperature polycarbonate cages (45 cm L × 25.2 cm W × 14.7 cm D; R20 series cage, Ancare, Bellmore, NY, USA). All procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals*, Eighth Edition (Institute for Laboratory Animal Research, The National Academies Press, Washington, DC, 2011) and were approved by the University of Colorado Boulder Institutional Animal Care and Use Committee. All possible efforts were made to minimize the number of rats used and their suffering.

2.2 Caffeine consumption procedure

Seven days after arrival, caffeine-consuming rats were given access to a single bottle containing caffeine (Fisher Scientific, Cat. No. 01728, Waltham, MA, USA) dissolved in tap water (0.3 g/L) for 28 days based on previously published procedures (Ardais et al. 2014; O'Neill et al. 2015). Adolescent caffeine consumption procedures occurred continuously between pnd 28–pnd 56. Caffeine and water consumption were recorded by measuring the amount of fluid consumed throughout the procedure by each pair of rats. Following 28 days of caffeine exposure, the caffeine solution was replaced with water for 24 hours until a final challenge dose of caffeine (30 mg/kg, i.p.) was given on pnd 57 and tissue was collected either 90–120 min or 4 hours later for immunohistochemistry or *in situ* hybridization histochemistry, respectively.

2.3 Study design

An a priori power analysis was completed using G*Power 3.1 software (Faul et al. 2007). Based on previous work it was estimated that using an effect size (f) of 0.6 with alpha error probability of 0.05, a beta error probability of 0.95, 1 degrees of freedom (overall model), 4

groups, and one covariate, the total sample size needed for the model is 39. Therefore, we used a 2×2 experimental design with $n = 10$ per group. Treatment consisted of either drinking water alone (H₂O) or caffeine (Caff; 3 mg/L) in the drinking water for 28 days, followed by a 24-hour washout period, then acute challenge with either vehicle (Veh) or caffeine (30 mg/kg, i.p.). The four treatment groups were therefore H₂O/Veh, H₂O/Caff, Caff/H₂O, Caff/Caff. Rats were euthanized 90-120 minutes (*Experiment 1*) or 4 hours (*Experiment 2*) later for analysis using immunohistochemistry, or *in situ* hybridization histochemistry, respectively (Figure 1). In situ hybridization histochemistry and immunohistochemistry were used in conjunction to understand differential gene expression changes and neuronal activation at a high spatial resolution. The high spatial resolution allows us to quantify differential activation and expression changes in specific subpopulations of 5-HT neurons within the DR.

2.4 Tissue processing for immunohistochemistry

Ninety to 120 minutes after i.p. caffeine administration, rats were deeply anesthetized three at a time with sodium pentobarbital (60 mg/kg, i.p.) followed by transcardial perfusion with roughly 150 mL ice-cold 0.05 M phosphate-buffered saline, then roughly 250 mL ice-cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Brains were post-fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer overnight (12-16 hours), rinsed twice for 12 hours each time in 0.1 M sodium phosphate buffer, and finally placed into cryoprotectant solution (30% sucrose in 0.1 M sodium phosphate buffer, pH 7.4) until they sank. Brains were then blocked caudal to the mammillary bodies (approximately -5.0 mm with respect to bregma) into forebrain and hindbrain pieces using a rat brain matrix (RBM-4000C, ASI Instruments, Warren, MI, USA), snap-frozen in isopentane cooled with dry ice, and stored at -80 °C until sectioning. Brains were sectioned using a Leica cryostat (Model CM1900, Leica GmbH, Wetzlar, Germany) at 30 microns and collected as 6 alternate sets of sections and placed into 1.5 mL of cryoprotectant solution (30% ethylene glycol/20% glycerol/0.05 M sodium phosphate buffer, pH 7.4) in 24-well tissue culture plates and stored at -20 °C until processed for immunohistochemical staining. Sections were collected for the DR spanning from -9.86 mm bregma to -7.04 mm bregma.

2.5 Antibodies

The brains were sectioned and processed for immunohistochemical staining of tryptophan hydroxylase (sheep anti-tryptophan hydroxylase (*Tph2*) antibody (Cat. No. T8575, Sigma-Aldrich®, St. Louis, MO, USA) as a marker of serotonergic neurons. This antibody has been characterized previously, and has been shown to bind both Tph1 and Tph2 isoforms (Hale et al. 2011). C-Fos (rabbit anti-c-Fos antibody, Cat. No. PC-38, Lot No. D00119667, CalBiochem®, San Diego, CA, USA), used as a marker of neuronal activation in the DR has previously been characterized and has been shown to bind specifically to amino acids 4-17 of the human c-Fos protein (Rinaman et al. 1997). Although we have used this antibody extensively in previous studies (Bouwknicht et al. 2007; Gardner et al. 2005; Hale et al. 2006, 2008, 2011; Hale, Johnson, et al. 2010; Hollis et al. 2006; Johnson et al. 2011, 2005; Kelly et al. 2011; C. A. Lowry et al. 2008; Lukkes et al. 2012; Spannuth et al. 2011; Spiga et al. 2006; Staub, Evans, and Lowry 2006; Staub, Spiga, and Lowry 2005; de Vries et al. 2005) it is no longer available and therefore we tested the suitability of the recommended

replacement using our standardized staining procedure. Therefore, a second set of sections was stained for c-Fos using a different anti-c-Fos antibody (rabbit anti-c-Fos antibody (Cat. No. ABE457, Lot No. 2441976, EMD Millipore, Billerica, MA, USA) and Tph. In addition, a third set of sections was stained for FosB/delta FosB (Cat. No. sc-7203, Lot. C239, Santa Cruz Biotechnology, Santa Cruz, CA, USA see (Grande et al. 2004; Hiroi et al. 1997, 2002; Hiroi and Graybiel 1996)), a marker of chronic activation.

2.6 Immunohistochemical staining procedure

Immunohistochemistry for c-Fos and FosB was conducted on free-floating tissue in 12-well tissue culture plates in 1.0 mL of solution and gently shaken on an orbital shaker throughout the staining process. Unless stated otherwise, tissue was washed for 15 min in-between steps. Tissue was rinsed twice in 0.05 M phosphate-buffered saline (PBS) then placed in 0.05 M PBS containing 1% hydrogen peroxide (H₂O₂) for 15 min. Tissue was then rinsed twice with 0.05 M PBS containing 0.8% Triton™ X-100 (0.05 M PBST). One set of sections was incubated at 4 °C for 60 hours with rabbit anti-c-Fos primary antiserum (1: 1000, Cat. No. PC-38, Lot No. D00119667, CalBiochem®, San Diego, CA, USA); another set of sections was incubated with a second rabbit anti-c-Fos primary antiserum (1:1000, Cat. No. ABE457, Lot No. 2441976, EMD Millipore, Billerica, MA, USA), and a third set was incubated with a FosB polyclonal antibody (1:500, Cat. No. sc-7203, Lot. C239, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.8% PBST containing 0.1% bovine serum albumin (BSA) and 4% normal donkey serum (Cat. No. S30-100ML, Millipore Corporation, Billerica, MA USA). After 60 hours, tissue was washed twice in 0.05 M PBS followed by incubation with biotinylated donkey anti-rabbit IgG (1:400, Cat. No. 711-065-152, Lot No. 86149, Jackson Immunolabs, West Grove, PA, USA) in 0.05 M PBST for 90 min. Tissue was then washed twice in 0.05 M PBS followed by incubation with an avidin-biotin-peroxidase complex (Elite® ABC reagent, 1:500 avidin, 1:2000 biotin, Cat. No. PK-6100, Vector Laboratories, Burlingame, CA, USA) in 0.05 M PBS for 90 min. Tissue was then washed twice in 0.05 M PBS, then placed in a peroxidase substrate solution (SG substrate, Cat No. SK4700, Vector Laboratories, diluted as recommended by the vendor) in 0.05 M PBS for 10 min. After the chromogen reaction, tissue was then immediately washed twice in 0.05 M PBS, then placed in 0.05 M PBS containing 1% H₂O₂ for 15 min. Tissue was then washed twice in 0.05 M PBST followed by a 60 h incubation in sheep anti-Tph (1:2500, Cat. No. T8575, Sigma-Aldrich®, St. Louis, MO, USA) in 0.05 M PBST containing 4% normal donkey serum at 4 °C. After 4 nights, tissue was then washed twice in 0.05 M PBST followed by a 90-min incubation in biotinylated rabbit anti-sheep IgG (1:200; Cat. No. PK-6016, Vectastain® Elite®, Vector Laboratories) in 0.05 M PBS. After incubation, tissue was washed twice in 0.05 M PBST followed by incubation with an avidin-biotin-peroxidase complex (Elite® ABC reagent, 1:250 avidin, 1:1000 biotin, Cat. No. 6100, Vector Laboratories) in 0.05 M PBST for 90 min. Tissue was washed twice in 0.05 M PBST followed by an optimal incubation in 0.01% 3-3'-diaminobenzidine tetrahydrochloride (DAB, Cat. No. D9015; Sigma-Aldrich®) in 0.05 M tris-buffered saline (TBS) containing 0.0066% H₂O₂. Once optimal staining was achieved, the tissue was rinsed twice in 0.05 M PBS to stop the reaction. Brain sections were stored in 0.1 M sodium phosphate buffer with 0.01% NaN₃ at 4 °C until tissue mounting. Brain sections were then rinsed in 0.1 M sodium phosphate buffer and then rinsed in 0.15% gelatin diluted in distilled H₂O, then mounted on

glass microscope slides (VistaVision™ UniMark microscope slides, Cat. No. 16005-106, VWR International, Aurora, CO, USA), dehydrated through an alcohol series and cleared with xylenes. Slides were mounted with cover slips using mounting medium (Entellen® mounting medium, Cat. No. RT14082, EM Sciences, Hatfield, PA, USA).

2.7 Cell counting

Four rostrocaudal levels of the DR were chosen for analysis (−7.46 mm, −7.82 mm, −8.18 mm, and −8.54 mm with reference to bregma). The subdivisions of the DR studied included the dorsal raphe nucleus, dorsal part (DRD), and dorsal raphe nucleus, ventral part (DRV) at −7.46 mm bregma, the DRD and DRV, and dorsal raphe nucleus, ventrolateral part (DRVl)/ventrolateral periaqueductal gray (VLPAG) at −7.82 mm and −8.18 mm bregma, and the dorsal raphe nucleus, caudal part (DRC), and dorsal raphe nucleus, interfascicular part (DRI) at −8.54 mm bregma (Figures 2–3). Rostrocaudal levels and anatomical divisions of the brainstem raphe complex were based on comparison of immunostained sections to a stereotaxic rat brain atlas (Paxinos and Watson 1998), and an atlas of tryptophan hydroxylase immunostaining in the rat DR (Abrams et al. 2004). Cell counting of c-Fos-immunopositive (+)/Tph+ c-Fos+/Tph-immunonegative, and total Tph+ neurons was performed. Cells were counted from both left and right sides of the DRVl/VLPAG and summed to give a total number of cells. All remaining cell counts were from midline subdivisions. Cell counts were performed using brightfield microscopy at a total magnification of 100×. If necessary, a total magnification of 400× was used to verify double labeling. The experimenter was blind to the treatment groups throughout cell counting.

2.8 Tissue processing for *in situ* hybridization histochemistry

Four hours after i.p. caffeine or vehicle administration, rats were removed from their cages, euthanized using rapid decapitation and the brain was quickly removed. The brains were blocked caudal to the mammillary bodies (approximately −5.0 mm with respect to bregma) into forebrain and hindbrain pieces using a razor blade and a rat brain matrix (RBM-4000C, ASI Instruments, Warren, MI, USA), snap-frozen in isopentane cooled with dry ice, and stored at −80 °C until sectioning. Brains were sectioned using a Leica cryostat (Model CM1900, Leica GmbH, Wetzlar, Germany) at 12 microns at 20 °C and collected in series of 7 alternate sets of sections between −7.244 mm and −8.672 mm bregma in order to include the entire DR, MnR, B9, and PMRF. Sections were then thaw-mounted onto VistaVision™ “HistoBond®” microscope slides (VWR Scientific, West Chester, PA, USA), and used in an *in situ* hybridization histochemistry assay with riboprobes (*tph2* and *slc22a3*), or oligonucleotide probes (*htr1a* and *slc6a4*).

2.9 Riboprobe-based *in situ* hybridization histochemistry (*tph2* and *slc22a3*)

Previously published methods were used for *in situ* hybridization histochemistry using riboprobes (Fox et al. 2017). Briefly, brains were sectioned into 12 µm-thick sections on a cryostat (Leica CM 1950, Leica Biosystems, Buffalo Grove, IL, USA), in a series of 7 sets of sections, thaw-mounted on HistoBond® slides (Cat. No. 16,004-406; VWR, West Chester, PA, USA) and stored at −80 °C. A [³⁵S]-UTP-labeled riboprobe was used for detection of *tph2* mRNA, i.e., a 462 base (1552-2013) antisense riboprobe complementary to the rat cDNA encoding Tph2 (*tph2*, NCBI Reference Sequence: NC_005106.4, from C.A. Lowry

(Gardner et al. 2009)). A [³⁵S]-UTP-labeled riboprobe was used for detection of *slc22a3* mRNA, i.e., a 1081 base (1313-2393) antisense riboprobe complementary to the rat cDNA encoding OCT3 (*slc22a3*, NCBI Reference Sequence: NM_019230.1, designed by M.R. Arnold and H.E.W. Day). Sense and antisense riboprobes were created by adding the T7 phage and clamp sequences (5'-GCGTAATACGACTCACTATAGGGAGA-3') to the 5' end of the forward and reverse primer sequence (Eckalbar et al. 2012). The OCT3 forward primer sequence was AAGTGCAATGGGAAACACCTCTCG and the reverse primer sequence was TGCCAGGCCTTGATATGTTTCATCC. Riboprobes were 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 hour, 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, USA) for 3 (*tph2*) or 21 (*slc22a3*) days prior to film development.

2.10 Oligonucleotide-based *in situ* hybridization histochemistry (*htr1a* and *slc6a4*)

Previously published methods were used for *in situ* hybridization histochemistry using oligonucleotide probes (Donner et al. 2018). To detect *htr1a* mRNA, two synthetic anti-sense oligonucleotides, one 49-base oligonucleotide (5'-ACG AAG TTC CTA AGC TGG TGC CTG CTC CCT TCT TTT CCA CCT TCC TGA C-3', Integrated DNA Technologies, Coralville, IA, USA) complementary to bases 810–858 of rat *htr1a* mRNA and one 47-base oligonucleotide (5'-GCC TCA CTG CCC CAT TAG TGC ACG GAG TCC CCA CCG CCC TGT TCT CA-3', Integrated DNA Technologies) complementary to bases 923–969 of rat *htr1a* mRNA, were used (Donner et al. 2018). To detect *slc6a4* mRNA, a synthetic 50-base antisense oligonucleotide (5'-ACT GCA GAG TAC CCA TTG GAT ATT TGG CTA GGC TCT GCC CTG TCC GCT GT-3', Integrated DNA Technologies), complementary to bases 207–256 of rat *slc6a4* mRNA (previously reported as bases 77–126; (Fujita et al. 1993; Hansen and Mikkelsen 1998) based on the published sequence of the rat serotonin transporter cDNA clone (Blakely et al. 1991) of *slc6a4* mRNA (*Rattus norvegicus* solute carrier family 6 (neurotransmitter transporter, serotonin), member 4, mRNA; GenBank® Accession no., NM_013034.3) was used as previously described (Donner et al. 2018; Gardner et al. 2009). The oligonucleotides were labeled at the 3' end with [³⁵S]-deoxyadenosine-5'-triphosphate (Cat. No. 5620001, MP Biomedicals, Santa Ana, CA, USA) using terminal deoxynucleotidyl transferase (20 U/µL, Cat. No. EP0161, Fermentas, Glen Burnie, MD, USA) for 1 h at 37 °C, cleaned using a QIAquick® nucleotide removal kit (Cat. No. 28304, Qiagen, Valencia, CA, USA), and used in an *in situ* hybridization histochemistry assay as described previously (Donner et al. 2018; Gardner et al. 2009). Air-dried slides were apposed to a BioMax® MR autoradiography film (Cat. No. 871 5187, Carestream Health, Rochester, NY, USA) 17 (*htr1a*) or 11 (*slc6a4*) days, respectively.

2.11 Image quantification

Autoradiographic images of the probe bound to *tph2* mRNA, encoding tryptophan hydroxylase 2, the rate-limiting enzyme in the synthesis of brain serotonin; *slc22a3* mRNA, encoding the corticosterone-sensitive, sodium independent, low affinity, high capacity serotonin transporter, organic cation transporter 3; *htr1a* mRNA, encoding the 5-HT_{1A} inhibitory autoreceptor, and *slc6a4* mRNA, encoding the sodium dependent, high affinity, low capacity serotonin transporter, were measured using a computer-assisted image analysis system. For each gene, all slides from the study were exposed to the same film. Analysis was performed on a PC using the publicly available NIH-developed image analysis software ImageJ (<https://imagej.nih.gov/ij/>). Virtual matrices in the shape of the respective DR subregions, as well as the MnR, B9, and PMRF were created, overlaid with the image, and the “mean gray value × area”, taking into account the area of only the above-threshold signal, was measured within each matrix. 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 value 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*, *slc22a3*, *htr1a*, *slc6a4*, for expression in the DR, MnR, B9, or PMRF was created by comparing the image of the tissue sections with a stereotaxic rat brain atlas (Paxinos and Watson 1998) and with *tph2* mRNA expression topography as reported by Gardner et al. (2009). According to Gardner et al. (2009) and Tph immunostaining (Abrams et al. 2004), each rostrocaudal level was further divided into respective subregions of the DR. Throughout all rostrocaudal levels, the values for each subdivision were then averaged, and all values per treatment group also summarized to display the overall expression in each subregion. Depending on the gene of interest 13 to 18 rostrocaudal levels were studied throughout the brainstem (see Figures 4, S1–S3). The subdivisions studied were summarized into the following anatomical subregions of the DR: dorsal raphe nucleus, caudal part (DRC), –8.336 mm to –8.672 mm from bregma; dorsal raphe nucleus, dorsal part (DRD), –7.244 mm to –8.252 mm from bregma; dorsal raphe nucleus, interfascicular part (DRI), –8.336 mm to –8.672 mm from bregma; dorsal raphe nucleus, ventral part (DRV), –7.244 mm to –8.504 mm from bregma; left and right dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray region (left and right DRVL/VLPAG), –7.748 mm to –8.504 mm from bregma; left and right supramedian serotonergic cell group (B9), –7.244 mm to –8.084 mm from bregma; median raphe nucleus (MnR), –7.244 mm to –8.672 mm from bregma; and pontomesencephalic reticular formation (PMRF) –7.244 mm to –7.496 mm from bregma. Regions of interest are defined in Figures 4, S1–S3.

2.12 Statistical analysis

Analysis of in situ hybridization histochemistry and immunohistochemistry data

A detailed description of our statistical approach can be found in the Supplementary Materials section. Briefly, statistical analyses were conducted using the software package IBM Statistical Package for the Social Sciences (version 24.0, SPSS Inc., Chicago, IL, USA). A linear mixed effects model was used for the analysis of in situ hybridization

histochemistry and immunohistochemistry data. For in situ data, mean (corrected for background) gray value $\times$ area values for each rostrocaudal level of each DR subdivision, and, when relevant, B9, MnR, and PMRF regions, for each rat were generated for *tph2*, *htr1a*, *slc6a4*, and *slc22a3* gene expression. For immunohistochemistry data single and double labeled-immunoreactivity was measured for c-Fos (PC-38)/Tph, c-Fos (ABE457)/Tph, and FosB/Tph at four rostrocaudal levels of the DR. Two-tailed significance was set at $p < 0.05$. Post hoc pairwise comparisons were made with Fisher's least significant difference (LSD) test. Again, two-tailed significance was set at $p < 0.05$.

3. Results

3.1 Immunohistochemistry

3.1.1 c-Fos (PC-38)-immunoreactive (ir)/Tph2-ir neurons in the DR—Analysis of c-Fos (PC-38)-ir/Tph2-ir neurons using linear mixed model (LMM) analysis revealed a *chronic treatment* $\times$ *acute challenge* $\times$ *raphe subregion* interaction ($F_{12,28.1} = 4.210$, $p < 0.001$), a *chronic treatment* $\times$ *acute challenge* interaction ($F_{(1,32.0)} = 14.238$, $p < 0.01$), a main effect of chronic caffeine administration (Caff vs H₂O) ($F_{(1,32.0)} = 13.079$, $p < 0.01$), and a main effect of acute caffeine challenge (Caff vs Veh) ($F_{(1,32.0)} = 20.551$, $p < 0.001$) (Table 1). Based on this finding, *post hoc* analysis was conducted on each of the raphe subregions to determine significant pairwise comparisons.

Among rats exposed to chronic H₂O administration, acute caffeine challenge increased numbers of c-Fos (PC-38)-ir/Tph2-ir neurons within the DRD, DRV, and DRVL/VLPAG at all rostrocaudal levels studied (-7.46 , -7.82 , and -8.18 mm bregma (Figure 5,6). The effect of acute caffeine challenge was attenuated in rats that had received chronic caffeine administration prior to challenge. No effects of acute caffeine challenge were observed in the DRC or DRI, and no effects of chronic caffeine administration, by itself, were observed in any subregion of the DR.

3.1.2 c-Fos (PC-38)-ir/Tph2-immunonegative cells in the DR—Analysis of c-Fos (PC-38)-ir/Tph2-immunonegative cells using LMM analysis revealed a *chronic treatment* $\times$ *acute challenge* $\times$ *raphe subregion* interaction ($F_{27,39.0} = 4.168$, $p < 0.001$), a *chronic treatment* $\times$ *acute challenge* interaction ($F_{(1,34.8)} = 16.029$, $p < 0.001$), a main effect of chronic caffeine administration (Caff vs H₂O; $F_{(1,34.8)} = 9.596$, $p < 0.01$), and a main effect of acute caffeine challenge (Caff vs Veh; $F_{(1,34.8)} = 64.179$, $p < 0.001$) (Table 1). Based on this finding, *post hoc* analysis was conducted on each of the raphe subregions to determine significant pairwise comparisons.

Among rats exposed to chronic H₂O administration, acute caffeine challenge increased numbers of c-Fos (PC-38)-ir/Tph2-immunonegative cells within the DRD, DRV, and DRVL/VLPAG at all rostrocaudal levels studied (-7.46 , -7.82 , and -8.18 mm bregma) in addition to the DRC at -8.54 mm bregma (Table S1). The effect of acute caffeine challenge was attenuated in rats that had received chronic caffeine administration prior to challenge. No effects of acute caffeine challenge were observed in the DRI. Among rats exposed to an acute caffeine challenge, chronic caffeine treatment decreased numbers of c-Fos (PC-38)-ir/Tph2-immunonegative cells within the DRD at -7.46 mm bregma in addition to the DRV at

every rostrocaudal level studied. No effects of chronic caffeine administration, by itself, were observed in any subregion of the DR.

3.1.3 c-Fos (ABE457)-ir/Tph-ir and FosB-ir/Tph-ir immunohistochemistry results—Detailed c-Fos (ABE457)-ir/Tph-ir and FosB-ir/Tph-ir immunohistochemistry results can be found in the appendix.

3.2 *In situ* hybridization histochemistry

In situ hybridization histochemistry was used to evaluate the effects of chronic caffeine treatment and acute caffeine challenge using a 2 × 2 design on expression of genes important for control of serotonergic neurotransmission, including *tph2*, *slc22a3*, *htr1a*, and *slc6a4* mRNA.

3.2.1 *Tph2* mRNA expression—Overall, acute challenge with caffeine, in rats previously treated with either H₂O or caffeine in the drinking water, decreased *tph2* mRNA expression in all subregions of the DR, as well as in the MnR, B9, and PMRF serotonergic cell groups (Table S9; Figure S8). Analysis of *tph2* mRNA expression using LMM analysis revealed a *chronic treatment* × *acute challenge* × *rostrocaudal level* interaction ($F_{(16,289.9)} = 2.282, p < 0.01$), a *chronic treatment* × *acute challenge* interaction ($F_{(1,327.4)} = 11.024, p < 0.01$), an *acute challenge* × *rostrocaudal level* interaction ($F_{(17,283.6)} = 6.317, p < 0.001$), an *acute challenge* × *subregion* interaction ($F_{(7,202.4)} = 0.187, p < 0.001$), an *acute challenge* × *rostrocaudal level (subregion)* interaction ($F_{(56,269.0)} = 3.225, p < 0.001$), a main effect of chronic caffeine administration (Caff vs. H₂O) ($F_{(1,327.3)} = 16.357, p < 0.001$), and a main effect of acute caffeine challenge (Caff vs. Veh) ($F_{(1,327.4)} = 272.827, p < 0.001$) (Table S9). Based on these findings, secondary LMM were used to determine the effects of acute and chronic caffeine treatment within each subregion of the DR and within the MnR, B9, and PMRF serotonergic cell groups.

In order to assess the overall effects of treatment on *tph2* mRNA expression across the brainstem raphe nuclei, we analyzed the mean ± SEM *tph2* mRNA expression at each rostrocaudal level of the brainstem raphe nuclei studied, including all subregions at each rostrocaudal level (Figure 7A). We also analyzed the overall mean ± SEM *tph2* mRNA expression including data for all rostrocaudal levels of all subregions (Figure 7B). These analyses revealed a consistent pattern of effects of treatment on *tph2* mRNA expression across rostrocaudal levels of the brainstem raphe nuclei (Figure 7A), and across the entire the brainstem raphe complex (Figure 7B). Among rats with chronic H₂O administration, acute challenge with caffeine decreased *tph2* mRNA expression at all rostrocaudal levels investigated (Figure 7A,B). Likewise, among rats with chronic caffeine administration, acute challenge with caffeine decreased *tph2* mRNA expression at all rostrocaudal levels of the DR (Figure 7A,B). Chronic treatment with caffeine, by itself (i.e. Caff/Veh rats), decreased *tph2* mRNA expression at several rostrocaudal levels of the DR relative to H₂O/Veh control rats (Figure 7A,B). Figure 8 illustrates *tph2* mRNA expression using a single representative rat from each treatment group at −8.084 mm bregma (Figure 8).

3.2.2 *Slc22a3* mRNA expression—Overall, acute challenge with caffeine, in rats previously treated with H₂O in the drinking water, decreased *slc22a3* mRNA expression in all subregions of the DR studied. Acute challenge with caffeine, in rats previously treated with chronic caffeine in the drinking water, also decreased *slc22a3* mRNA expression at some rostrocaudal levels of the DRD, DRV, and DRI (Table S10; Figure S9). In some parts of the DRD, DRV, and DRVL/VLPAG, chronic administration of caffeine in the drinking water attenuated the ability of acute challenge with caffeine to decrease *slc22a3* mRNA expression (Table S10; Figure S9). Analysis of *slc22a3* mRNA expression using LMM analysis revealed a *chronic treatment* $\times$ *acute challenge* $\times$ *rostrocaudal level* interaction ($F_{(16,1086.6)} = 2.029, p < 0.01$), an *acute challenge* $\times$ *rostrocaudal level* interaction ($F_{(16,1086.6)} = 3.031, p < 0.001$), an *acute challenge* $\times$ *subregion* interaction ($F_{(4,312.0)} = 2.561, p < 0.05$), and a main effect of acute caffeine challenge (Caff vs. Veh) ($F_{(1,333.2)} = 48.700, p < 0.001$) (Table S10). Based on these findings, secondary LMM were used to determine the effects of acute and chronic caffeine treatment within each subregion of the DR.

In order to assess the overall effects of treatment on *slc22a3* mRNA expression across the brainstem raphe nuclei, we analyzed the mean $\pm$ SEM *slc22a3* mRNA expression at each rostrocaudal level of the brainstem raphe nuclei studied, including all subregions at each rostrocaudal level (Figure 9A). We also analyzed the overall mean $\pm$ SEM *slc22a3* mRNA expression including data for all rostrocaudal levels of all subregions (Figure 9B). These analyses revealed a consistent pattern of effects of treatment on *slc22a3* mRNA expression across rostrocaudal levels of the brainstem raphe nuclei (Figure 9A), and across the entire the brainstem raphe complex (Figure 9B). Among rats with chronic H₂O administration, acute challenge with caffeine decreased *slc22a3* mRNA expression at all rostrocaudal levels investigated (Figure 9A,B). Likewise, among rats with chronic caffeine administration, acute challenge with caffeine decreased *slc22a3* mRNA expression at all rostrocaudal levels of the DR (Figure 9A,B). Chronic treatment with caffeine, by itself (i.e. Caff/Veh rats), decreased *slc22a3* mRNA expression at two rostrocaudal level of the DR relative to H₂O/Veh control rats. Among rats that received an acute challenge of caffeine, chronic treatment with caffeine, (i.e. Caff/Caff rats) increased *slc22a3* mRNA expression at several rostrocaudal levels of the DR relative to rats receiving chronic H₂O treatment (i.e. H₂O/Caff rats) (Figure 9A,B). Figure S10 illustrates *slc22a3* mRNA expression using a single representative rat from each treatment group at -8.084 mm bregma (Figure S10).

3.3 Correlation between *tph2* and *slc22a3* mRNA expression

Pearson correlation revealed a positive correlation between *slc22a3* and *tph2* mRNA expression ($r^2 = 0.570; p < 0.001$) (Figure 10).

3.4 *Htr1a* and *Slc6a4* mRNA expression

Detailed *slc6a4* and *htr1a* mRNA expression results can be found in the appendix.

4. Discussion

Acute caffeine challenge activates serotonergic neurons and alters expression of serotonergic genes that control serotonergic signaling. The effects of acute caffeine challenge on serotonergic gene expression were evident throughout the DR, i.e., they were not restricted to specific rostrocaudal levels or subregions. Acute caffeine profoundly decreased *tph2* and *slc22a3* mRNA expression throughout the DR, with minimal or no effects on *htr1a* or *slc6a4* mRNA expression. Chronic caffeine exposure for four weeks during adolescence was sufficient to decrease *tph2* mRNA expression in the DR measured 28 h after caffeine withdrawal. Chronic caffeine administration during adolescence attenuated caffeine-induced activation of DR serotonergic neurons, as measured by c-Fos expression, but did not impact the ability of acute caffeine to decrease *tph2* or *slc22a3* mRNA expression. Although this study lacked the inclusion of females, developmental sex differences are likely to exist and need to be critically evaluated. Together, these data suggest that both chronic caffeine administration during adolescence and acute caffeine challenge during adulthood are important determinants of serotonergic function and serotonergic gene expression.

Effects of acute caffeine treatment on c-Fos expression in serotonergic neurons

Acute caffeine challenge activated serotonergic neurons, as measured by increased c-Fos expression, measured 90 min following caffeine administration. Acute caffeine challenge using a dose of 30 mg/kg, i.p., was sufficient to increase c-Fos expression in every subregion of the dorsal raphe nucleus studied, with the exception of the DRC and DRI subregions. This activation pattern was more widespread than that reported by Abrams et al., (2005). Abrams et al., using a dose of 50 mg/kg, i.p., reported a more restricted pattern of activation limited to the DRD and DRI subregions. These differences may be due to differences in the experimental design, as rats in the study by Abrams and colleagues were single-housed and habituated to the experimental room (36–48 h) before the experiment, whereas rats in the present study were pair housed and housed continuously in the same room. It is notable that consistent effects of 30 mg/kg and 50 mg/kg were observed in the DRD, at all rostrocaudal levels studied. The DRD, particularly its mid-rostrocaudal and caudal parts, often referred to as the dorsomedial DR (for a detailed discussion of the subregions of the DRD, see (Donner et al. 2018) has been considered an anxiety-related subregion of the DR (C. a. Lowry et al. 2008). A number of anxiety- and stress-related stimuli have been shown to activate the dorsomedial DR (but not other subsets of serotonergic neurons, such as those in the DRVl/VLPAG). This includes anxiogenic drugs such as the partial inverse agonist at the benzodiazepine allosteric site on the GABA_A receptor, N-methyl-9H-pyrido[5,4-b]indole-3-carboxamide (FG-7142) (Abrams et al. 2005), the non-selective 5-hydroxytryptamine 2C (5-HT_{2C}) receptor agonist *m*-chlorophenyl piperazine (*m*-CPP) (Abrams et al. 2005), anxiety-related neuropeptides (Hale, Stamper, et al. 2010; Staub et al. 2006, 2005), acoustic startle (Spannuth et al. 2011), and stress- or anxiety-related stimuli (Bouwknicht et al. 2007; Cordero et al. 2012; Gardner et al. 2005; Paul et al. 2011; Spiacci, Coimbra, and Zangrossi 2012). The selective activation of the dorsomedial DR may be due to activation of afferents that selectively impact dorsomedial DR serotonergic neurons. For example, overexpression of corticotropin-releasing hormone in the bed nucleus of the stria terminalis selectively alters corticotropin-releasing hormone type 2 receptors in the dorsomedial DR (Sink et al. 2013).

Tryptophan hydroxylase 2 mRNA expression varies in a diurnal fashion, with increased expression two hours into the active/dark phase, relative to two hours into the inactive/light phase (Donner et al. 2012, 2016), consistent with a role for serotonergic neurons in behavioral arousal and vigilance (Jacobs 1995; Jacobs and Fornal 1993, 1999; Rueter, Fornal, and Jacobs 1997). Recent studies suggest that increases in anxiety-like behavioral responses associated with inescapable stress are dependent on sensitization of serotonergic neurons specifically in the dorsomedial DR (Donner et al. 2018; Rozeske et al. 2011). Thus, activation of serotonergic neurons in the dorsomedial DR following acute caffeine challenge in the present study may be particularly relevant to the anxiogenic effects of caffeine, or increases in behavioral arousal and vigilance. The observation that chronic caffeine consumption for four weeks during adolescence prevented the effects of acute caffeine challenge on c-Fos expression in serotonergic neurons within the DR, including in the dorsomedial DR, suggests that caffeine consumption during adolescence results in tolerance to the effects of acute caffeine challenge in adulthood. These findings may relate to findings in humans that some caffeine consumers appear to develop tolerance to the negative effects, but not the positive effects, of caffeine, which may lead to increased caffeine reinforcement and intake (Griffiths, Bigelow, and Liebson 1989; Nehlig 1999). The adaptations of serotonergic neurons in the dorsomedial DR following chronic caffeine consumption may also be relevant to previous findings that the same chronic caffeine consumption protocol in adolescent rats, but not adult rats, results in development of a chronic anxiety-like state following a 7-day washout period (O'Neill et al. 2016).

Despite the fact that the effects of acute caffeine exposure on c-Fos expression in caffeine-naïve rats were consistently observed in the DRD serotonergic neurons in this, and a previous study, using a higher dose of caffeine (Abrams et al. 2005), the effects were much more widespread in the present study using 30 mg/kg caffeine, increasing c-Fos expression in all serotonergic cell groups excluding the DRC and DRI. This finding suggests that caffeine may have either direct, or proximal, effects on serotonergic neurons culminating in increased c-Fos expression, rather, or in addition to, activating afferent pathways to the DR, such as afferents from the BNST, which are thought to contribute to activation of anxiety-related serotonergic neurons in the dorsomedial DR (Donner et al. 2018). In support of such a mechanism, high concentrations of caffeine, spanning the dose of caffeine used in our study, have been shown to block cortical GABA_A receptor function in adult male CD-1 mice (Lopez et al. 1989). Such an effect, if also present in the DR, would be expected to result in a widespread activation of DR serotonergic neurons (Jolas and Aghajanian 1997), as observed in the current study. In addition, all subgroups of serotonergic neurons responded to chronic caffeine consumption with an attenuated response to acute caffeine administration, suggesting that all serotonergic neurons sensitive to the acute effects of caffeine respond to chronic caffeine administration with a degree of tolerance. An exception was the DRI serotonergic neurons, which did not respond to acute caffeine administration with increased c-Fos expression. Serotonergic neurons within the DRI have been described as Type II serotonergic neurons. Whereas Type I serotonergic neurons, present throughout the DR and MnR, show decreased firing during the inactive period of the sleep-wake cycle and are virtually silent during rapid eye movement (REM) sleep, Type II serotonergic neurons show no change in firing rate during the inactive period of the sleep-wake cycle and

are fully active during REM sleep (Rasmussen, Heym, and Jacobs 1984). Thus, the activity of Type II serotonergic neurons in the DRI seems to be independent of behavioral arousal and are insensitive to the effects of acute caffeine.

Effects of acute caffeine treatment on c-Fos expression in non-serotonergic cells

The effects of acute caffeine treatment on c-Fos expression in non-serotonin cells largely parallels the effects of acute caffeine treatment on c-Fos expression in serotonergic neurons. For example, acute caffeine administration in caffeine naive rats induced a widespread increase in c-Fos expression in non-serotonergic cells in all subregions of the DR, excluding the DRI. A notable difference, however, is that increases in c-Fos expression in non-serotonergic cells following acute caffeine challenge were also observed in rats that had previously consumed caffeine in the drinking water for four weeks, particularly in the DRV subregion. Nevertheless, in nearly all subregions of the DR, increases in c-Fos expression in non-serotonergic cells following acute caffeine challenge were attenuated in rats that had previously consumed caffeine in the drinking water for four weeks. This suggests that tolerance to caffeine was evident in rats with adolescent caffeine consumption in both serotonergic and non-serotonergic cells throughout the DR.

Effects of acute caffeine treatment on FosB expression in serotonergic neurons

There were no effects of chronic caffeine consumption or acute caffeine challenge on FosB expression in serotonergic neurons, while effects on FosB expression in non-serotonergic cells were limited to a few subregions. No effects were observed in DRD serotonergic or non-serotonergic cells. Rats with a history of methamphetamine self-administration, with or without receiving methamphetamine on the test day, but not rats with a single acute administration of methamphetamine, respond with increased FosB in the DR (assessed specifically in the DRD) (Cornish et al. 2012), demonstrating that, despite the negative effects observed here, chronic drug administration is sufficient to increase FosB/ FosB in the DR, including the anxiety-related dorsomedial DR. Further studies are required to determine if the differences in the study by Cornish and colleagues and the present study are due to the different drugs used, methamphetamine versus caffeine, respectively, or are due to methodological differences.

Effects of acute caffeine treatment on tph2 mRNA expression in serotonergic neurons

Acute caffeine challenge induced a widespread and profound decrease in *tph2* mRNA expression at nearly every rostrocaudal level within each subregion of the DR. In contrast to effects of acute caffeine challenge on c-Fos expression in serotonergic neurons, which did not affect DRI serotonergic neurons, effects of acute caffeine challenge on *tph2* mRNA expression were evident in all subregions of the brainstem raphe complex studied, including the DRI, MnR, suprallemniscal serotonergic cell group and PMRF. Again, such widespread effects of acute caffeine challenge suggest that caffeine may act directly on brainstem serotonergic neurons, or proximate to these neurons, to globally affect the brainstem raphe serotonergic neuron population. The hypothesis that these widespread effects of caffeine in brainstem serotonergic neurons are dependent on blockade of GABA_A receptor signaling (Lopez et al. 1989), are consistent with a previous study demonstrating that acute challenge of rats with the partial inverse agonist at the benzodiazepine allosteric site on the GABA_A

receptor, FG-7142, which is potently anxiogenic (Evans and Lowry 2007), acutely activates dorsomedial DR serotonergic neurons (Abrams et al. 2005), and elevates serotonin metabolism in forebrain limbic structures (Evans et al. 2006), decreases *tph2* mRNA expression within all subregions of the DR, including the DRD, DRV, DRVL/VLPAG, and DRC, excluding the DRI (Lukkes et al. 2013). Together, the study by Lukkes et al., (2013) and the present study are remarkably consistent, in that drugs that have been shown to decrease GABA_A receptor signaling (i.e. FG-7142 and caffeine, respectively) both activate brainstem serotonergic neurons, particularly those in the anxiety-related dorsomedial DR (Abrams et al. 2005), and decrease *tph2* mRNA expression. These findings strongly suggest that there is an inverse relationship between serotonergic cellular activation and *tph2* mRNA expression, with acute increases in serotonergic cellular activation associated with decreases in *tph2* mRNA expression.

Although the mechanisms are not known, the inverse relationship between serotonergic cellular activation and *tph2* mRNA expression are consistent with the natural diurnal variation in serotonergic neuronal activity and *tph2* mRNA expression. For example, serotonergic neuronal activity and release are highest at the onset of behavioral activity associated with dark onset and the onset of the diurnal active phase (Jacobs 1995; Jacobs and Fornal 1993, 1999; Rueter et al. 1997). At the same time, *tph2* mRNA expression declines, and remains low throughout the active/dark phase of the diurnal cycle (Malek et al. 2007). Evidence suggests that this diurnal variation of *tph2* mRNA expression is driven by the circadian rhythm of corticosterone (Malek et al. 2007). Recent studies demonstrate that glucocorticoid receptors within the DR decrease *tph2* mRNA expression in mice (Vincent et al. 2018), which, if also the case in rats, may explain the decline in *tph2* mRNA expression in the early part of the active/dark phase of the diurnal behavioral activity rhythm, after the corticosterone surge (Malek et al. 2007). Of potential interest in this regard, acute caffeine exposure at the concentration used in our study (i.e., 0.3 g/L in the drinking water) increases plasma corticosterone concentrations in adolescent male rats (Patz et al. 2006), an effect that normalizes over the 28-day exposure to caffeine in the drinking water (O'Neill et al. 2016). However, an increase in corticosterone was detected at the circadian trough on day 29, 24 hours after the removal of caffeine from the drinking water (O'Neill et al. 2016). Also of potential interest, FG-7142, which also decreases *tph2* mRNA expression, increases plasma corticosterone concentrations (Evans et al. 2006). Thus, it is possible that decreases in *tph2* mRNA expression following acute caffeine challenge are due in part to caffeine-induced increases in corticosterone secretion, although this will need to be determined in future studies.

Effects of acute caffeine treatment on *slc22a3* mRNA expression in serotonergic neurons

Acute caffeine challenge induced a widespread decrease in *slc22a3* expression in a manner very similar to that observed for *tph2*, discussed above, at nearly every rostrocaudal level within each subregion of the DR. The possibility that *tph2* and *slc22a3* mRNA expression may be coregulated is supported by the finding that *tph2* and *slc22a3* mRNA expression levels were highly correlated. Thus, although little is known about *slc22a3* expression in DR serotonergic neurons, or its regulation, the current study provides some support for the hypothesis that *tph2* and *slc22a3* mRNA expression may be acutely co-regulated, perhaps by

corticosterone and DR glucocorticoid signaling. Slc22a3, also known as organic cation transporter 3 (OCT3), or extraneuronal monoamine transporter (EMT) is a low affinity, high capacity, sodium independent serotonin transporter, which also serves as a transporter for diverse organic cations, including norepinephrine, epinephrine, dopamine, and histamine (Koepsell, Schmitt, and Gorboulev 2003). To our knowledge, this is the first study to investigate in detail the topographical distribution of *slc22a3* mRNA expression in the dorsal raphe nucleus. However, several lines of evidence support the possibility that Slc22a3 is expressed in serotonergic neurons and therefore could function as a presynaptic serotonin transporter controlling serotonin reuptake from the synapse. First, early studies using *in situ* hybridization histochemistry described strong expression of *slc22a3* mRNA in the brainstem raphe nuclei (Amphoux et al. 2006; Lein et al. 2007). This was further supported by later studies using differential microarray analysis of gene expression in serotonergic and non-serotonergic neurons isolated from embryonic day 12.5 mice, showing increased expression of *slc22a3* in serotonergic neurons, relative to non-serotonergic neurons (Wylie et al. 2010). Most recently, Wyler and colleagues (2015) (Wyler et al. 2015) have shown using YFP+ Pet-1^{-/-} and wild type serotonergic neurons that expression of *slc6a4* and *slc22a3* in serotonergic neurons are under coordinate control by Pet-1, a critical transcription factor in the regulatory program that controls serotonergic neuronal development. The latter studies demonstrated that virtually all serotonergic neurons in the DR depend on Pet-1 for *slc22a3* expression. As with the effects of acute caffeine challenge on *tph2* mRNA expression, future studies are required to delineate the mechanisms through which acute caffeine challenge profoundly decreases *slc22a3* mRNA expression throughout the DR, and the physiologic and behavioral consequences of these changes in gene expression.

Effects of chronic caffeine treatment on *tph2* mRNA expression in serotonergic neurons

Chronic treatment with caffeine, by itself (i.e. Caff/Veh rats relative to H₂O/Veh rats), decreased *tph2* mRNA expression at several rostrocaudal levels of the DR, particularly in the DRD and DRV subregions. It is unclear if this effect is because rats exposed to chronic caffeine in the drinking water were given only a 24-h washout period such that residual caffeine was still present in low amounts, or if this effect is related to some aspect of caffeine withdrawal, and an associated anxiety-like state. Another possibility is the increased corticosterone levels detected at the circadian trough on day 29, 24 hours after the removal of caffeine from drinking water, as described in O'Neill et al., 2016 may have directly influenced *tph2* mRNA expression. These questions will require further study.

Effects of acute caffeine treatment on *htr1a* and *slc6a4* mRNA expression

In contrast to the effects of acute and chronic caffeine administration on *tph2* and *slc22a3* mRNA expression, discussed above, there were minimal or no effects of treatment on *htr1a* and *slc6a4* mRNA expression. These findings demonstrate that effects of acute and chronic caffeine administration on gene expression in DR serotonergic neurons are not due to a nonselective, global dysregulation of serotonergic gene expression. There are several potential explanations for the observation that *slc6a4* and *htr1a* are not affected by caffeine administration, while *slc22a3* is strongly affected by this drug. One potential explanation is differential endocrine control of *slc22a3* mRNA expression. For example, chronic caffeine administration from puberty in rats increases the concentrations of testosterone and

dihydrotestosterone, and increases androgenic signaling (Sarobo et al. 2012). Meanwhile, previous studies have identified *slc22a3* as an androgen-responsive gene (Bolton et al. 2007). Novel mechanisms underlying epigenetic regulation of *slc22a3* expression have also been described (Chen et al. 2013; Nie et al. 2012; Sleutels et al. 2003; Wu 2016; Zhang et al. 2014). The mechanisms through which acute and chronic caffeine administration alter *tph2* and *slc22a3* mRNA expression, but not *htr1a* and *slc6a4* mRNA expression, as well as the consequences for physiological and behavioral function, deserve further study.

Sex differences

The US National Institute of Mental Health reports that the lifetime prevalence of anxiety disorders is roughly 60 percent higher in women than men (Kessler et al. 2005). In addition, the onset, severity, clinical course, and treatment response of anxiety disorders differ significantly in women (Pigott 2003). While our investigation adds to the few studies aimed at quantifying the effects of caffeine during adolescence, it fails to address potential sex differences. Though the outcomes of previous studies lack agreement in their overall findings, the data suggest the adenosine system during development is a highly complex modulatory system that varies depending on sex. It has been shown, for example, that caffeine exposure from conception to weaning worsened object recognition memory in female rats, but not male rats, when tested during adulthood (Ardais et al. 2016). Conversely, the same exposures to caffeine during development increased locomotor activity in adult male rats, but not female rats, when tested during adulthood (Ardais et al. 2016). Additionally, studies have revealed sex differences in responses to an adenosine A2A/A1 receptor antagonist on motivation to self-administer cocaine in rats, increasing the motivation for cocaine in females, but not males (Doyle et al. 2012). One potential mechanism that may underlie these sex differences is the region-specific control of microglia by adenosine A2A receptors. It has been shown that chronic blockade of adenosine A2A receptors, which are core regulators of microglia morphology and physiology, for three weeks during adulthood ameliorated the cognitive deficits, but not the anxiety-like behavior, in adult females that had been exposed *in utero* to the synthetic glucocorticoid dexamethasone (Duarte et al. 2018). This mechanism is attractive because microglia have notable sex differences that are found at each stage of life, see (Lenz and McCarthy 2015) for a thorough review. The hypothesis that neuroinflammation specifically in the dorsal raphe nucleus, which shows sex differences in stress responsivity (Donner and Lowry 2013; Howerton, Roland, Fluharty, et al. 2014), may be important in sex-dependent behavioral dysregulation is supported by the findings that induction of neuroinflammation in the dorsal raphe nucleus, but not medial prefrontal cortex, results in increased stress-induced locomotion and responsivity, as well as reduced risk-aversion/fearfulness (Howerton, Roland, and Bale 2014). However, more work needs to be carried out in order to determine the extent to which microglia play a role in the development and the associated sex differences of the adenosine system, serotonergic systems, and stress-related behavioral dysregulation.

4. Conclusions

Together, these data suggest that both chronic caffeine administration during adolescence and acute caffeine challenge during adulthood are important determinants of serotonergic function and serotonergic gene expression. Future studies are warranted to determine the mechanisms through which chronic caffeine administration during adolescence and acute caffeine challenge during adulthood alter serotonergic neuronal activity and gene expression, as well as physiological and behavioral function relevant to drug seeking, drug taking, and drug withdrawal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

Aq	cerebral aqueduct
B9	supralemniscal serotonergic cell group
Caff	caffeine
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	dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray
mlf	medial longitudinal fasciculus
MnR	median raphe nucleus
PMRF	pontomesencephalic reticular formation
s.c.	subcutaneous
i.p.	intraperitoneal

Veh vehicle

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Highlights

Acute caffeine administration induces a widespread activation of serotonergic neurons

Acute caffeine administration decreases *tph2* and *slc22a3* mRNA expression

Chronic caffeine consumption for four weeks decreased *tph2* mRNA expression

Acute effects of caffeine on *tph2* and *slc22a3* mRNA expression persisted after adolescent caffeine consumption

Figure 1.

Experimental timelines for **A**, *Experiment 1* and **B**, *Experiment 2*. Rats from both *Experiment 1* and *Experiment 2* were pair housed and allowed to habituate in home cages for one week before the beginning of the experimental protocol. After habituation rats were randomly divided into 2 groups. One group received caffeine in their drinking water (0.3 g/L) and the other group received tap water only. The horizontal black bar below the timeline indicates the period during which caffeine was present in drinking water. After 28 days of caffeine in the drinking water or control conditions, the rats received a 24-hour washout period followed by either a vehicle or caffeine challenge (30 mg/kg, i.p.). **A**) In *Experiment 1*, rats were anesthetized and perfused with fixative in preparation for immunohistochemical staining of c-Fos or FosB and tryptophan hydroxylase in the dorsal raphe nucleus, 90-120 min after the caffeine or vehicle challenge. **B**) In *Experiment 2*, rats were euthanized 4 hours after the caffeine or vehicle challenge and the brains were frozen in preparation for *in situ* hybridization histochemistry for analysis of *tph2*, *slc22a3*, *htr1a* and *slc6a4* mRNA expression in the brainstem raphe nuclei.

Figure 2.

Low magnification photomicrographs illustrating c-Fos (A-D, Cat. No. PC-38, CalBiochem®; E-H, Cat. No. ABE457, EMD Millipore) and tryptophan hydroxylase (Tph) (Cat. No. T8575, Sigma-Aldrich®) immunostaining from different rostrocaudal levels of the rat dorsal raphe nucleus (DR) in *Experiment 1*. C-Fos immunostaining can be identified by a dark blue/black nuclear stain. Tryptophan hydroxylase-immunoreactive (ir) neurons and dendrites can be identified by a red/brown cytoplasmic stain. The rostrocaudal levels selected for analysis were A,E) –7.46 mm bregma, B,F) –7.82 mm bregma, C,G) –8.18 mm bregma, and D,H) –8.54 mm bregma. The subdivisions of the DR that were analyzed at each rostrocaudal level are delineated by dashed lines. Abbreviations: Aq, cerebral aqueduct; 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, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; mlf, medial longitudinal fasciculus. Scale bar, 500 μ m.

Figure 3.

Low magnification photomicrographs illustrating FosB (Cat. No. sc-7203, Santa Cruz Biotechnology) and tryptophan hydroxylase (Tph) (Cat. No. T8575, Sigma-Aldrich®) immunostaining from different rostrocaudal levels of the dorsal raphe nucleus (DR) in *Experiment 1*. FosB immunostaining can be identified by a dark blue/black nuclear stain. Tryptophan hydroxylase-immunoreactive (ir) neurons and dendrites can be identified by a red/brown cytoplasmic stain. The rostrocaudal levels selected for analysis were A) -7.46 mm bregma, B) -7.82 mm bregma, C) -8.18 mm bregma, and D) -8.54 mm bregma. The subdivisions of the DR that were analyzed at each rostrocaudal level are delineated by dashed lines. Abbreviations: Aq, cerebral aqueduct; 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, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; mlf, medial longitudinal fasciculus. Scale bar, 500 μ m.

Figure 4.

Atlas of *tph2* mRNA expression using *in situ* hybridization histochemistry. Displayed (from top left to bottom right of each panel) are 18 representative coronal sections (12 μ m) that were used to measure *tph2* mRNA expression in all subdivisions of the brainstem dorsal raphe nucleus (DR), median raphe nucleus (MnR), suprallemniscal serotonergic cell group (B9), and pontomesencephalic reticular formation (PMRF), from rostral (–7.244 mm bregma) to caudal (–8.672 mm bregma). A–R) Photomicrographs are autoradiographic images revealing the localization of hybridized 35 S-labeled ribonucleotide probe complementary to *tph2* mRNA. Dashed lines indicate the subregions of the DR used in the analysis and numbers in the lower left hand corner of each panel indicate the rostrocaudal level. Abbreviations: Aq, cerebral aqueduct; B9, suprallemniscal serotonergic cell group; 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, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; mlf, medial longitudinal fasciculus; MnR, median raphe nucleus; PMRF, pontomesencephalic reticular formation. Scale bar: 500 μ m.

Figure 5.

Graphs illustrating numbers of c-Fos/tryptophan hydroxylase- (Tph-) immunostained neurons in subregions of the dorsal raphe nucleus in *Experiment 1*. Closed bars represent the numbers of c-Fos-immunoreactive (ir) (Cat. No. PC-38, CalBiochem®)/Tph-ir neurons within each subregion. Open bars represent the total numbers of Tph-ir neurons within each rostrocaudal level of each subregion sampled. Graphs are arranged in vertical columns according to rostrocaudal level in mm from bregma and represent the bregma levels that were analyzed. The four vertical columns are -7.46 mm bregma, -7.82 mm bregma, -8.18 mm bregma, and -8.54 mm bregma. Values indicate mean + standard error of the mean (SEM). 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, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray. H₂O/Veh = drinking water/vehicle ($n = 8$); H₂O/Caff = drinking water/caffeine (30 mg/kg, i.p.) ($n = 12$); Caff/Veh = caffeine in the drinking water (0.3 mg/L)/vehicle ($n = 10$); Caff/Caff = caffeine in the drinking water (0.3 mg/L)/caffeine (30 mg/kg, i.p.) ($n = 10$). Significance was set at $p < 0.05$, Fisher's Least Significant Difference test. * indicates a difference between H₂O/Veh and H₂O/Caff ($p < 0.05$), ** indicates $p < 0.01$, *** indicates $p < 0.001$. + indicates a difference between H₂O/Caff and Caff/Caff ($p < 0.05$), ++ indicates $p < 0.01$, +++ indicates $p < 0.001$.

Figure 6.

Photomicrographs illustrating c-Fos (Cat. No. PC-38, CalBiochem®)/tryptophan hydroxylase (Tph) immunostaining in the dorsal raphe nucleus (–8.18 mm bregma) in representative rats from each treatment group. Photomicrographs illustrate immunostaining in rats exposed to A) drinking water/vehicle, B) drinking water/caffeine (30 mg/kg, i.p.), C) caffeine in the drinking water (0.3 mg/L)/vehicle, and D) caffeine in the drinking water (0.3 mg/L)/caffeine (30 mg/kg, i.p.) conditions. Black boxes in (A–D) indicate regions displayed at higher magnification in (E–H). Black boxes in (E–H) indicate regions displayed at higher magnification in (I–L). Dashed lines in (E–H) delineate the dorsal raphe nucleus, dorsal part (DRD). Black arrowheads indicate c-Fos-immunoreactive/Tph-immunoreactive neurons (brown cytoplasmic staining with blue/black nuclear staining). Abbreviations: Aq, cerebral aqueduct; DRD, dorsal raphe nucleus, dorsal part. Scale bar: A–D, 500 μ m; E–H, 100 μ m; I–L, 25 μ m.

Figure 7.

Effects of chronic adolescent caffeine consumption in the drinking water (0.3 g/L) or drinking water alone, followed by 24 h withdrawal and caffeine challenge (30 mg/kg, i.p.) or vehicle, on *tph2* mRNA expression throughout the brainstem raphe nuclei, measured 4 h following challenge. A) Mean $\pm$ SEM *tph2* mRNA expression at each rostrocaudal level of the brainstem raphe complex studied. The right y-axis is the percentage of the original sample size represented at each rostrocaudal level within each region. B) Overall mean $\pm$ SEM *tph2* mRNA expression within the brainstem raphe complex studied, including all rostrocaudal levels of all subregions. H₂O/Veh, drinking water/vehicle ($n = 8$); H₂O/Caff, drinking water/caffeine (30 mg/kg, i.p.) ($n = 10$); Caff/Veh, caffeine in the drinking water (0.3 mg/L)/vehicle ($n = 8$); Caff/Caff, caffeine in the drinking water (0.3 mg/L)/caffeine (30 mg/kg, i.p.) ($n = 10$). (AAA) $p < 0.001$, H₂O/Caff versus H₂O/Veh; (BB) $p < 0.01$, (BBB) $p < 0.001$ Caff/Caff versus Caff/Veh; (C) $p < 0.05$, (CC) $p < 0.01$, (CCC) $p < 0.001$ H₂O/Veh versus Caff/Veh groups; Fisher's Least Significant Difference test; Abbreviations; GV, gray value, *tph2*, tryptophan hydroxylase 2; Caff, caffeine; Veh, vehicle.

Figure 8.

Effects of chronic adolescent caffeine consumption in the drinking water (0.3 g/L) or drinking water alone, followed by 24 h withdrawal and caffeine challenge (30 mg/kg, i.p.) or vehicle, on *tph2* mRNA expression at -8.084 mm bregma of the brainstem raphe nuclei, measured 4 h following challenge. Dashed lines indicate the subregions of the dorsal raphe nucleus (DR), median raphe nucleus (MnR), or B9 used in the analysis. A-D) Autoradiograms illustrating *tph2* mRNA expression in representative rats from each treatment group. A) H₂O/Veh = drinking water/vehicle ($n = 8$); B) Caff/Veh = caffeine in the drinking water (0.3 mg/L)/vehicle ($n = 8$); C) H₂O/Caff = drinking water/caffeine (30 mg/kg, i.p.) ($n = 10$); D) Caff/Caff = caffeine in the drinking water (0.3 mg/L)/caffeine (30 mg/kg, i.p.) ($n = 10$). Abbreviations: B9, supramedian serotonergic cell group; DRD, dorsal raphe nucleus, dorsal portion; DRV, dorsal raphe nucleus, ventral part; DRV/VLPAG, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; MnR, median raphe nucleus; B9, supramedian serotonergic cell group. Scale bar: 500 μ m.

Figure 9.

Effects of chronic adolescent caffeine consumption in the drinking water (0.3 g/L) or drinking water alone, followed by 24 h withdrawal and caffeine challenge (30 mg/kg, i.p.) or vehicle, on *slc22a3* mRNA expression throughout the brainstem raphe nuclei, measured 4 h following challenge. A) Mean $\pm$ SEM *slc22a3* mRNA expression at each rostrocaudal level of the brainstem raphe complex studied. The right y-axis is the percentage of the original sample size represented at each rostrocaudal level within each region. B) Overall mean $\pm$ SEM *slc22a3* mRNA expression within the brainstem raphe complex studied, including all rostrocaudal levels of all subregions. H₂O/Veh, drinking water/vehicle ($n = 8$); H₂O/Caff, drinking water/caffeine (30 mg/kg, i.p.) ($n = 10$); Caff/Veh, caffeine in the drinking water (0.3 mg/L)/vehicle ($n = 8$); Caff/Caff, caffeine in the drinking water (0.3 mg/L)/caffeine (30 mg/kg, i.p.) ($n = 10$). (A) $p < 0.05$, (AA) $p < 0.01$, (AAA) $p < 0.001$, H₂O/Caff versus H₂O/Veh; (B) $p < 0.05$, (BB) $p < 0.01$, (BBB) $p < 0.001$ Caff/Caff versus Caff/Veh; (C) $p < 0.05$, (CC) $p < 0.01$, H₂O/Veh versus Caff/Veh groups; (D) $p < 0.05$, (DD) $p < 0.01$, (DDD) $p < 0.001$, H₂O/Caff versus Caff/Caff groups, Fisher's Least Significant Difference test; Abbreviations; GV, gray value, *slc22a3*, solute carrier family 22 member 3, organic cation transporter 3; Caff, caffeine; Veh, vehicle.

Figure 10.

Effects of chronic adolescent caffeine consumption in the drinking water (0.3 g/L) or drinking water alone, followed by 24 h withdrawal and caffeine challenge (30 mg/kg, i.p.) or vehicle. Each data point represents a single animal's overall average *tph2* mRNA expression versus *slc22a3* mRNA expression, measured 4 h following challenge, collapsed into a single value for the following regions: 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, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray. Abbreviations: GV, gray value, *slc22a3*, solute carrier family 22 member 3, organic cation transporter 3; *tph2*, tryptophan hydroxylase 2; Caff, caffeine; Veh, vehicle.

Table 1.

Linear mixed model analysis results for c-Fos (PC-38)-ir/Tph-ir neurons and c-Fos (PC-38)-ir/Tph-immunonegative cells

Model	Source	Test statistic	p-value
PC38			
Overall analysis c-Fos (PC-38)-ir/Tph-ir neurons			
First order factor analytic covariance structure			
	Chronic treatment (Caff vs. H ₂ O)	$F_{(1,32.0)} = 13.079$	0.001 **
	Acute challenge (Caff vs Veh)	$F_{(1,32.0)} = 20.551$	<0.001 ***
	Raphe subregion	$F_{(12,28.1)} = 11.311$	<0.001 ***
	Chronic treatment * Acute challenge	$F_{(1,32.0)} = 14.238$	0.001 **
	Chronic treatment * Acute challenge * Raphe subregion	$F_{(12,28.1)} = 4.210$	<0.001 ***
Overall analysis c-Fos (PC-38)-ir/Tph-immunonegative cells			
Unstructured covariance structure			
	Chronic treatment (Caff vs. H ₂ O)	$F_{(1,34.8)} = 9.596$	0.004 **
	Acute challenge (Caff vs Veh)	$F_{(1,34.8)} = 64.200$	<0.001 ***
	Raphe subregion	$F_{(9,36.2)} = 47.554$	<0.001 ***
	Chronic treatment * Acute challenge	$F_{(1,34.8)} = 16.029$	<0.001 ***
	Chronic treatment * Acute challenge * Raphe subregion	$F_{(27,39.0)} = 4.168$	<0.001 ***

¹The subregions used for this study included: 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, dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray; mlf, medial longitudinal fasciculus.

**
p<0.01.

p<0.001.