Prenatal caffeine damaged learning and memory in rat offspring mediated by

ARs/PKA/CREB/BDNF pathway

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Short title: Prenatal caffeine & learning and memory in offspring

Summary

Prenatal exposure to caffeine can cause developmental problems. This study determined chronic influence of prenatal caffeine at relatively higher doses on cognitive functions in the rat offspring. Pregnant Sprague-Dawley rats (4-month-old) were exposed to caffeine (20mg/kg, twice a day) for whole pregnancy from gestational day 4. Fetal and offspring body and brain weight was measured. Learning and memory were tested in adult offspring with Morris water maze; Learning and memory-related receptors were measured. The exposure to prenatal caffeine not only caused fetal growth restriction, but also showed long-term effects on learning and memory in the offspring. The caffeine offspring exhibited longer escape latency and path length in navigation testing. The number of passing the target was significantly reduced in those offspring. The expression of adenosine A_1 and A_{2A} receptors, nuclear PKA C_α, C_β subunits, and pCREB were significantly increased in the fetal and neonatal brain, and suppressed in the hippocampus of the adult offspring. The expression of BDNF and TrkB were reduced regardless of various ages. The results suggest that intrauterine programming dysfunction of adenosine receptors and the down-stream of cAMP/PKA/pCREB system may play an important role in prenatal caffeine induced cognition disorders in the adult offspring.

Keywords: Caffeine; spatial cognition; PKA; fetal growth restriction; adenosine receptors

1. Introduction

Since the concept of fetal origins of adult health/diseases was introduced about 30 years ago (Barker and Osmond, 1986), extensive evidence has been accumulated to demonstrate that fetal adaptation to adverse factors or uterine environments may result in programming of long-term health problems after birth (Kuang et al. 2014; Li N et al. 2015). Caffeine, a xanthine alkaloid, widely existed in coffee or tea, is consumed by 68-74% of pregnant women at an average intake of 125-193 mg/day (Frary et al. 2005). Chronic caffeine exposure in gestation can accumulate the chemical in the fetal brain (Wilkinson and Pollard, 1993), causing low birth weight or risk of premature birth (Brent et al. 2011). Moreover, prenatal exposure to caffeine was reported to affect neurobehavioral development in the offspring. Low dose of approximately 10mg/kg/day caffeine administration led to decreased locomotor activity in both juvenile and adult rat offspring following prenatal caffeine exposure (Hughes et al. 1991; Zimmerberg et al. 1991). However, Olga Björklund et al. reported that there was no functional deficit in the Morris water maze by low dose of prenatal caffeine in the offspring (Soellner et al. 2009). The effects of caffeine should be assessed depending on the doses used. Notably, addictive users and heavy drinkers of coffee are common. Whether high concentrations of prenatal caffeine exposure could impact on long-term learning and memory in adulthood is worth for investigation.

The hippocampus plays important roles in brain functions, including learning and memory (Lever et al. 2002). Caffeine acts by inhibiting adenosine receptor (ARs) A_1 and A_{2A} in the brain (Fredholm et al. 2005). The primary second messenger of adenosine receptors is adenylate cyclase (AC), which catalyzes ATP into cyclic adenosine monophosphate (cAMP). cAMP-dependent protein kinase A (PKA) plays pivotal roles in the consolidation of spatial and non-spatial long-term memories. The

dissociated catalytic (C) subunits of PKA could phosphorylate cAMP response element binding protein (pCREB) on serine 133 when cAMP sequential and cooperative bind to the regulatory (R) subunits. pCREB modulate transcription of LTP-related genes (Abel and Nguyen, 2008), such as brain-derived neurotrophic factor (BDNF). Thus, PKA signaling is important for the hippocampus-dependent memory. BDNF is essential for growth, survival, and neuronal cell differentiation (Huang and Reichardt, 2001). It is involved in the pathophysiology of Alzheimer's disease, Huntington's disease (Ma Q et al. 2015) and Parkinson's disease (Khalil et al. 2016). Through binding to neural receptor protein-tyrosine kinase-β (TrkB), BDNF/TrkB also represents a major player in learning and memory formation by the regulation of neuroplasticity (Panja and Bramham, 2014; Bekinschtein et al. 2014).

The present study hypothesized that relatively higher doses of prenatal caffeine might show long-term impact on the development of learning and memory, probably by affecting related receptors and cAMP/PKA/CREB signaling factors, as well as BDNF/TrkB in the hippocampus of the offspring. The hypothesis was tested using different ages of the offspring rats (fetus, neonates, and young-adult).

2. Methods and materials

2.1 Experimental animals

All procedures and protocols were approved by the Institutional Animal Care and Use Committee and in accordance with the Guidelines for the Care and Use of Laboratory Animals.

Sprague Dawley rats (250-280 g) were obtained from Soochow University Experimental Animal Center. All rats were housed in a controlled environment of temperature 22°C and a 12h light/dark cycle, with free access to water and standard rat food. The day after mating was designated as gestational day (GD) 1 if vaginal plug was observed. Pregnant rats were randomly divided into two

groups. Caffeine group (16 mothers) was provided with caffeine (20mg/kg, Sigma-Aldrich, twice daily) via subcutaneous injection from GD 4 to 21, and physiological saline was used for the control group (16 mothers).

2.2 Offspring experiments

Pregnant rats (GD 21) were anesthetized with sodium pentobarbital (100 mg/kg; Hengrui Medicine, Jiangsu, China). After cesarean section delivery, fetal brain and body weight was measured (the litter size was 9 to 13, n=30 rats from 6 mothers/group). Natural delivery was allowed for other pregnant rats. All rats were raised in the same environment. Offspring (14-day-old, n=30 rats from 6 mothers/group) was anesthetized , brain and body weight was measured. The other male offspring was raised to 4 month old (n=30 rats from 6 mothers/group) for behavioral and biochemistry testing.

2.3 Spatial water maze

The Morris water maze (MWM) was used to assess spatial learning and memory as reported (Morris R. 1984). For water maze acquisition, the rats were trained to locate a submerged platform (26 cm in height and 10 cm in diameter) in a circular white pool (0.46 m, depth; and 1.2 m, diameter). Water was made opaque by addition of nontoxic white paint. The pool was divided into four quadrants. There were 4 acquisition trials each day with training lasting for 7 days and the position of the cues was not changed during testing. Each trial was started by placing a rat facing toward the wall. A trial was ended until the rat climbed on the hidden platform with all 4 paws or until 120 seconds elapsed. If the rats couldn't find the platform, they were guided to the platform and sit on it for 15 seconds before being removed and dried with a towel. In the end, the latency and path length for swimming to the platform were measured.

The retention test started at 24 h after the last day of the acquisition training. Rats were placed in

the pool facing the wall in a randomly determined quadrant and allowed to swim within a controlled time (120 s). The platform was not present, and retention measures during the probe test included time spent in the target quadrant and number of target approaches. Swim speed was also measured. All activities of rats in the testing were monitored, recorded, and analyzed using MT-200 water maze video tracking system (Taimeng, Chengdu, China).

2.4 ELISA analysis

Brain tissue was homogenized with 20% ethanol in phosphate buffer solution (PBS), then centrifuged at 3,000×g, 4°C for 5 min. Supernatants were collected for analysis using an ELISA kit (JIMIAN Industrial, Shanghai, China) following the manufacturer's protocol. Briefly, 50μl standard sample was added into the standard sample well, containing 40μl sample dilution and 10μl testing sample (final dilution is 5-fold). The antigen-coated wells were then incubated for 40 min at 37°C, following a five times' washing with buffer. Then, the antigen-coated wells were incubated with 50μl of HRP-Conjugate reagent for 30min at 37°C. The unbound antibodies were washed away with washing buffer, and then incubated with Chromogen Solution. After incubation for 15 min at 37°C in dark, 50μl of stop solution was added to each sample, and the absorbance at 450 nm was determined. Data were handled in a blind manner.

For ELISA test of PKA C_α, C_β, and pCREB, nuclear extracts were prepared with the method below. In brief, brain tissue was homogenized in 10 volumes of buffer C (in mM): HEPES-NaOH (pH 7.9) 10, containing KCl 10, EDTA 1, EGTA 1, DTT 5, sodium fluoride (NaF) 10, sodium pyrophosphate 10, Na_3VO_4 1, sodium β-glycerophosphate 10, PMSF 1, pepstatin A 1.25 μg/ml, leupeptin 10 μg/ml and aprotinin 2.5 μg/ml. Following the addition of 10% Nonidet P-40 for a final concentration of 1%, the homogenates were centrifuged at 15,000 g for 5 min. Pellets were washed in

three volumes of buffer C, and centrifuged at 15,000 g for 5 min. The pellets were then suspended in

one volume of buffer D (in mM): HEPES NaOH (pH 7.9) 20, NaCl 400, EDTA 1, EGTA 1, DTT 5,

NaF 10, sodium pyrophosphate 10, Na_3VO_4 1, sodium β-glycerophosphate 10, pepstatin A 1.25 μg/ml,

leupeptin10 μg/ml, aprotinin 2.5 μg/ml, and PMSF 1, and centrifuged at 15,000g for 5 min. The

supernatant was stored at -80℃ as the nuclear extract.

2.5 Data analysis and statistics

Data were analyzed using the program Prism (GraphPad). The water maze data were analyzed

using two-way analysis of variance followed with Bonferroni post hoc test. Other data were analyzed

using t-test. Results were expressed as mean±S.E.M., and $P<0.05$ was considered statistically

significant.

3. Results

3.1 Body weight, brain weight, and litter size

No significant changes were observed in body weight between normal (CON) and prenatal

caffeine offspring (CAF) groups at 4 month old. The body weight of CAF offspring was

significantly reduced at GD21 and PD14 (Fig. 1a). Brain weight was reduced in the CAF at PD14

and 4 month old, while no significant difference was found at GD21 (Fig.1b). The treatment used

in the present study didn't induce significant differences in weight of the pregnant mothers

between the two groups. The number of pups per litter and their sex was similar between the

control and experimental groups (Table 1).

3.2 Spatial memory performance in the MWM

To assess whether exposure to caffeine in pregnancy affected behavioral functions in the

adult offspring, behavioral tests was conducted. Time to find the platform was getting shorter when the course went on for all rats. The CAF offspring exhibited longer escape latency and longer path length during testing in a hidden platform-learning phase (Fig. 2). The escape latency was significantly increased in the CAF offspring from day 1 to day 5 compared with the control, and the path length was significantly increased at day 1 to day 3 (Fig. 2a and b).

In the probe trial (retention test), there was no significant difference in the time spent in the target approaches, however, the number of target quadrant approaches was significantly less than that of the control (Fig. 2c and d).

3.3 Adenosine receptors and PKA pathway proteins in the fetal and neonatal offspring

3.3.1 The expression of adenosine A_1 and A_{2A} receptors

In contrast with that in the young adult brain, the protein expression of A_1 and A_{2A} receptors were increased in the CAF group at GD21 and PD14 (Fig. 3a). The ratio of A_{2A}/A_1 was significantly raised (Fig. 3b).

3.3.2 cAMP content, PKA subunits, and pCREB

Unlike the young adult offspring, there was an enhanced cAMP production in the CAF offspring at GD21 and PD14 (Fig. 3c), and the increased protein expression of nuclear PKA C_α and C_β (Fig. 3d). Additionally, the pCREB level was also augmented in CAF at both GD21 and PD14 (Fig. 3d).

3.3.3 BDNF/TrkB

The CAF offspring at GD21 and PD14 expressed lower BDNF and TrkB compared to that in the control (Fig. 3e).

3.4 Adenosine receptors and PKA pathway proteins in the young-adult offspring

3.4.1 The expression of adenosine A_1 and A_{2A} receptors

Both the expression of adenosine A_1 and A_{2A} receptors were decreased in the CAF at 4 month old (Fig. 4a), and the ratio of A_1/A_{2A} also was reduced (Fig. 4b).

3.4.2 cAMP content, PKA subunits, and pCREBA

Significant decrease in cAMP content was found in the hippocampus of the CAF offspring (Fig. 4c). The expression of PKA C_α and C_β protein was reduced (Fig. 4d). The expression of pCREB also was significantly decreased (Fig. 4d).

3.4.3 BDNF/TrkB

Both the expression of BDNF and TrkB were significantly reduced in the hippocampus of the CAF offspring (Fig. 4e).

4. Discussion

The present study determined the influence of chronic caffeine exposure during gestation on the hippocampus-regulated behavior in the young adult (4-month-old), neonatal (2-week-old) offspring as well as in the fetal rats, and found: (1) the learning and memory in the young adult offspring were damaged, (2) the expression of BDNF and TrkB was reduced in the adult, neonatal offspring, and fetuses exposed to prenatal caffeine, (3) ARs/PKA/CREB signaling pathway was down-regulated in the young adult hippocampus, while that was up-regulated in the CAF fetal and neonatal brain.

In the present study, relatively higher doses of caffeine caused low body weight in the fetus as that reported (Liu et al. 2012). Moreover, the neonatal weight at 2 weeks after birth was still lower than that of the control, while this difference disappeared in the adult offspring. These data

suggest that exposure to relatively higher caffeine during pregnancy not only hurt fetal development, but also influence neonatal growth. However, the body weight could become normal later if caffeine was not continued in postnatal life. An interesting phenomenon was noted regarding the impact of prenatal caffeine on the brain. Fetal brain weight was unchanged by caffeine in pregnancy, the weight was reduced in the brain of both neonatal and adult offspring, indicating long-term influence of relatively heavy caffeine in pregnancy could affect brain development, which might be caused by chronic damage induced by the caffeine that can easily pass the placenta and into the fetal body and accumulated in the brain. Why fetal brain weight could be rescued while postnatal brain weight was affected is worth of further investigations in future.

To understand whether and how the brain could be injured by relatively higher doses of caffeine, functional testing on the brain should be reasonable. The present study performed behavioral exams on the offspring. The experiments revealed that the CAF offspring exhibited longer path length toward the hidden platform, associated with an increase of escape latency, indicating damaged learning ability. In addition, the retention test showed the number of target approaches was significantly reduced, suggesting memory was injured. Previous study suggested that lower doses of caffeine had no significant effects on the Morris water maze functions in the offspring (Soellner et al. 2009). This study added new information regarding the chronic influence of relatively high doses of caffeine on learning and memory in the young adult offspring. The immediate question raised then was what was possible mechanisms underlying the functional changes in the offspring brain and their behavior. Although multiple causes could be involved in the mechanisms, the ARs/cAMP/pCREB/BDNF signaling pathway was considered in

the present study, due to a series of studies have shown the link between caffeine and learning/memory as well as those molecules in the brain (Abel et al. 2008; Fredholm et al. 1999).

Previous study demonstrated that the expression of adenosine A_1 receptors was significantly increased in the fetal rats after maternal caffeine treatments (Marangos et al. 1984; Etzel and Guillet 1994; Gaytan and Pasaro 2012). The present study showed an increased A_1 receptors in the fetal brain following exposure to caffeine in pregnancy, suggesting caffeine used in pregnant mothers could impact on certain receptors in the fetal brain. Compared to A_1 receptors, there were fewer studies on a relationship between prenatal caffeine and adenosine A_{2A} receptors in fetuses and offspring. Our study showed an increase in protein expression of A_{2A} receptors in the fetal brain, which was inconsistent with the minor effect on fetal A_{2A} receptors reported by Ulrika Ådén et al (Ådén et al. 2000). The difference could be due to different caffeine dosage used. In Ådén' study, the dose of caffeine given was mild to moderate (0.3g/L in drinking water, equivalent to about 2 cups a day). However, since addictive users often use significantly higher doses and some people drink more coffee daily, our study designed a protocol of 20 mg/kg caffeine, twice a day, for a total caffeine of 40 mg/kg/day in rats. Based on the dose-conversion correlation between humans and rats (human: rats = 1.00:6.17), 40 mg/kg/day roughly equivalent to 4 cups of coffee (a cup of coffee contains nearly 150 mg of caffeine on average) (Reagan-Shaw et al. 2008). The present study added new information that too much coffee intake in mothers or heavily using caffeine in pregnancy would stimulate more expression of both A_1 and A_{2A} receptors in the fetal brain.

The present study found that prenatal caffeine may affect the development of fetal brain via an increase of the drug binding site adenosine receptors. Activation of adenosine receptors initiates

the post-receptor signal transduction involved in cAMP/PKA/CREB, which was demonstrated to play an important role in learning and memory (Fredholm et al. 1999; Takeo et al. 2003; McGuire et al. 2005; Zhao et al. 1995). We then tested the down-stream targets of PKA pathway. Generally, adenosine receptor subtypes have been classically characterized by the adenylate cyclase effector system, which utilises cAMP as a second messenger. The A_1 receptors, coupled with G_i proteins, inhibit adenylate cyclase, leading to a decrease in cellular cAMP levels, while the A_{2A} receptors, coupled to Gs proteins and activate adenylate cyclase, leading to an increase in cellular cAMP levels (Poulsen et al. 1998). Although both A_1 and A_{2A} receptors were increased by prenatal caffeine, the expression of cAMP, however, was increased in the fetus following maternal treatments of caffeine. This could be caused by the increased ratio of A_{2A}/A_1, making intracellular cAMP increased. PKA is a tetrameric protein composed of two regulatory subunits and two catalytic subunits (C_α, C_β and C_γ). Among the three catalytic subunits, C_α and C_β are localized preferentially in the hippocampus (Takeo et al. 2003). cAMP binds to PKA regulatory subunits, therefore releasing catalytic subunits, permitting phosphorylase activity of PKA. The present study demonstrated that both the nuclear protein of C_α and C_β were increased at GD21 in the fetuses exposed to maternal caffeine, which could be due to the augmented up-stream second messenger——the cellular cAMP level. Those results suggested that the increased expression of pCREB in nucleus, since the liberation and subsequent translocation of PKA-C subunits into the nucleus, determined the stoichiometry and kinetics of CREB phosphorylation (Hagiwara et al. 1993). pCREB is a core component of the molecular switch, converting short-term memory to long-term memory, and nuclear pCREB acts as a transcription factor regulating BDNF in response to drugs. Protein analysis showed that pCREB was significantly increased in the brain of both the

fetus and neonatal offspring at postnatal 2 weeks, indicating pCREB signal was more active following prenatal exposure to higher doses of caffeine. Interestingly, pCREB protein expression in the hippocampus of the adult offspring was turned to the opposite direction, significantly decreased. There must be inner mechanisms underlying that the increased pCREB during early developmental periods was turned into the decreased level at the late life stage, which deserves new investigations. Our interpretation for the finding is that prenatal insults such as higher doses of caffeine caused acute responses in the fetal and neonatal brain via activation of pCREB. Over-activity of pCREB and related signaling may induce damage in the brain as chronic consequences such as the suppressed expression found in the hippocampus. Notably, previous studies (Kuang et al. 2014) showed prenatal insults induced apoptosis in the offspring brain.

The present study measured the protein expression of BDNF and its specific receptor, TrkB, in the fetus and offspring. We found that the expression of BDNF and TrkB in the brain were significantly reduced in the fetus, neonatal and adult offspring. There could be several possibilities underlying the suppressed BDNF and TrKB in the fetal and neonatal offspring. First, there may exist other pathways regulating the expression of BDNF without involvements of pCREB; Second, the increased pCREB caused signaling imbalance in the body, which may directly or indirectly affect expression of other molecules such as BDNF. This study presents what were exactly observed, and provides information that is worth further investigated.

The BDNF/TrkB receptor complex plays critical roles in developmental processes, regulation of neuro-, glio-, and synaptogenesis, neuroprotection, and controlling of short- and long-lasting synaptic interactions that influence mechanisms of memory and cognition (Foltran and Diaz. 2016; Sasi et al. 2017; Messaoudi et al. 2002; Gruart et al. 2006; Whitlock et al. 2006). Mariga A et al.

reported that reduction of BDNF in the cultured rat hippocampal neurons was related to decreased expression of the genes that are functionally linked to vesicular trafficking and synaptic communication (Mariga et al. 2015). The pattern of changes in gene expression was similar to the profile observed in Alzheimer's disease and cognitive impairment. Similarly, prenatal caffeine caused learning and memory dysfunction in the young-adult offspring, as well as suppressed BDNF and TrkB in the brain, indicating an increased susceptibility to cognitive impairment and Alzheimer's disease.

The similar pattern of expression of adenosine receptors and the down-stream targets of cAMP/PKA/CREB signaling in the fetus and offspring was noted in the present study. The expression of adenosine A_1 and A_{2A} receptors, and the cAMP/PKA signaling were significantly enhanced in the brain of the fetus and two-week-old offspring, while the same molecules were degraded in the adult hippocampus later, accompanied by an impairment in learning and memory in adulthood. The over-activity might cause damage, inducing the suppressed signaling in the brain. In addition, it can be considered that prolonged agonist exposure of G protein-coupled receptors could result in a progressive loss of the receptors (down-regulation), whereas too much antagonist exposure might cause increased receptor levels (Böhm et al. 1997). Gestational exposure to higher doses of caffeine may inhibit actions of adenosine in the fetal brain as acute responses, and over responses may alter the caffeine binding sites—adenosine receptors and its post-receptor pathways, causing persistent alterations in the brain.

In summary, the present study showed that high doses of caffeine used in pregnancy not only injured learning and memory capabilities in the offspring, but also caused an over-activity of up-regulated ARs/cAMP/CREB signaling in the fetal and young rat brain. Importantly, that

over-activities in the brain eventually was tuned into long-term damage of the signaling pathway in the hippocampus evidenced as suppressed expression of the key signaling in regulations of the cognitive functions. To the best of our knowledge, this was the first demonstration of the influence and possible mechanism of higher doses of prenatal caffeine on fetal, neonatal and adult brain associated with injured learning and memory. The novel data generated may offer new ideas for early prevention and treatments for learning disability resulted from abnormal development during early life stages.

Conflict of interest

The authors declare no conflict of interest.

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Legends

Fig.1 The brain (a) and body weight (b) in the offspring (for GD21, n=60 from 6 litters each group; for PD14, n=50 from 5 litters each group; for PD120, n=30 from 5 litters each group). * $P<.05$; ** $P<.01$ vs. the control.

Fig.2 The effect of prenatal caffeine exposure on behavior performance in the 4-month-old offspring. Escape latency (a) and path length (b) in MWM. Time spent in target approaches (c) and target quadrant (d) in the retention test. n=10 each group. * $P<.05$, ** $P<.01$, *** $P<.001$ vs. the control.

Fig.3 The expression of adenosine A1, A2A receptors and cAMP/PKA/CREB/BDNF/TrKB cascade protein in the brain of fetuses and 14-days-old offspring. a, protein expression of A1 and A2A receptors. b, Ratio of A2A/A1 receptor. c, cAMP content of hippocampus from the two group. d, protein expression of PKA subunits (Cα and Cβ) and pCREB. e, protein expression of BDNF and TrkB. n=8-12 each group. * $P<.05$, ** $P<.01$, *** $P<.001$ vs. the control
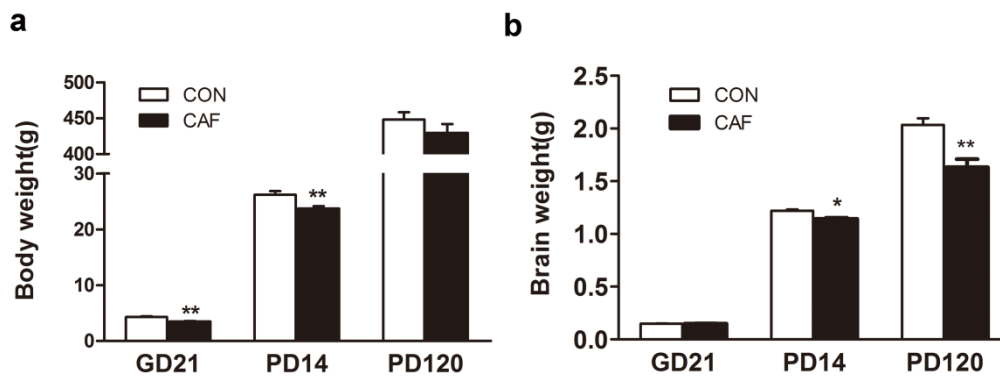
Fig.4 The expression of adenosine A1, A2A receptors and cAMP/PKA/CREB/BDNF/TrKB cascade protein in the hippocampus of young-adult offspring. a, protein expression of A1 and A2A receptors . b,

ratio of A2A/A1 receptor. c, cAMP content of the hippocampus. D, protein expression of PKA subunits

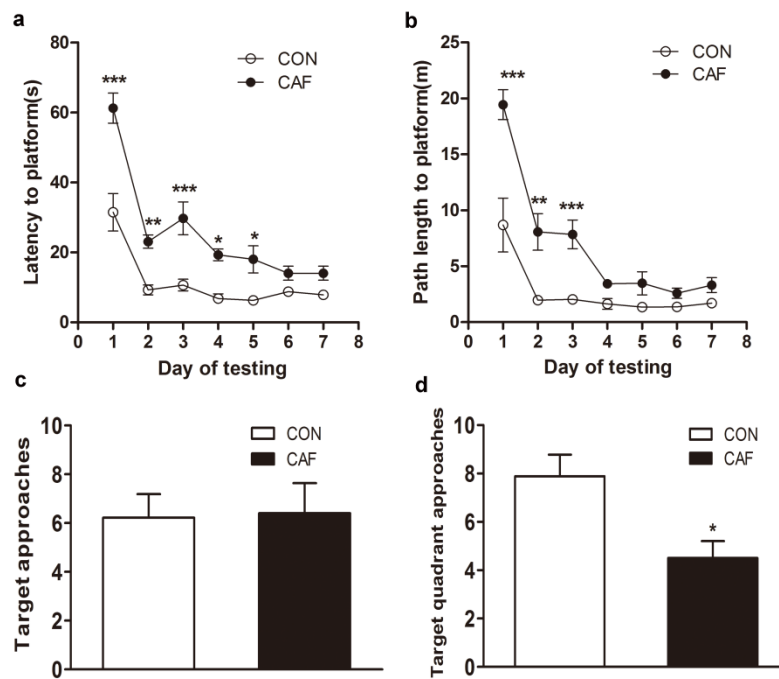(Cα and Cβ) and pCREB. e, protein expression of BDNF and TrkB. n=12 each group. * $P<.05$, **

$P<.01$, *** $P<.001$ vs. the control.
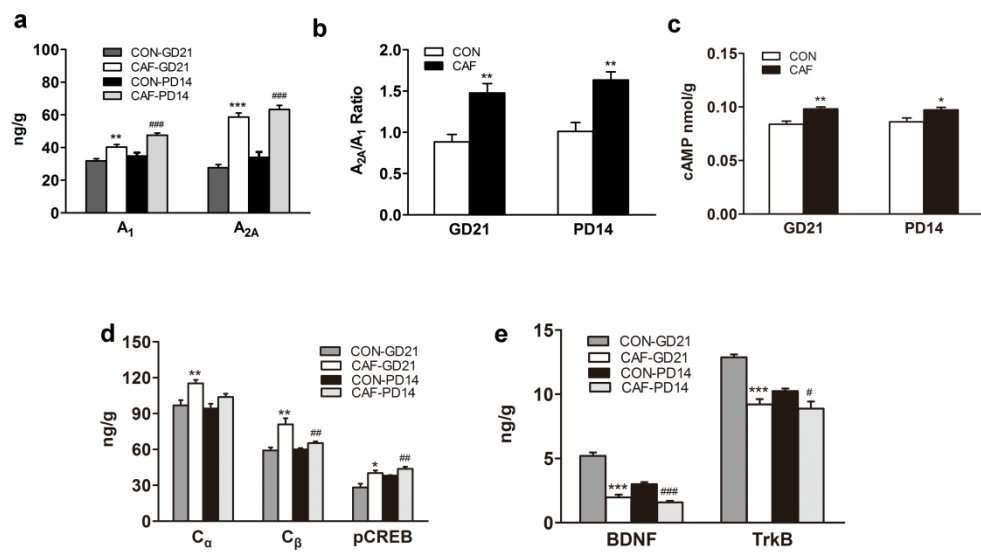
Figure 1

Figure 2
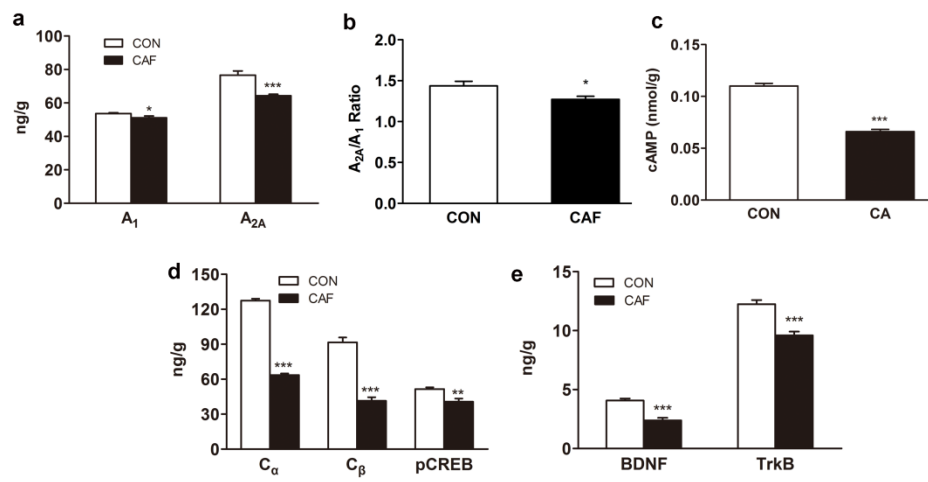
Figure 3

Figure 4

Table 1. The effect of prenatal caffeine on weight gain of mothers, number of pups per litter and their sex

Treatment	Weight gain of mothers	Number of pups per litter	Sex	
			Male	Female
Control	79.04±4.719	10.78±1.63	5.2±0.37	5.6±0.51
Caffeine	76.54±5.345	10.29±2.16	5.2±0.39	5.6±0.34

n=60-62 rats from 6 mothers