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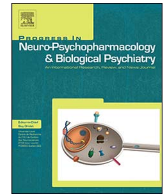

Sex differences in the effects of pre- and postnatal caffeine exposure on behavior and synaptic proteins in pubescent rats

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ABSTRACT

Few studies have addressed the effects of caffeine in the puberty and/or adolescence in a sex dependent manner. Considering that caffeine intake has increased in this population, we investigated the behavioral and synaptic proteins changes in pubescent male and female rats after maternal consumption of caffeine. Adult female Wistar rats started to receive water or caffeine (0.1 and 0.3 g/L in drinking water; low and moderate dose, respectively) during the active cycle at weekdays, two weeks before mating. The treatment lasted up to weaning and the offspring received caffeine until the onset of puberty (30–34 days old). Behavioral tasks were performed to evaluate locomotor activity (open field task), anxious-like behavior (elevated plus maze task) and recognition memory (object recognition task) and synaptic proteins levels (proBDNF, BDNF, GFAP and SNAP-25) were verified in the hippocampus and cerebral cortex. While hyperlocomotion was observed in both sexes after caffeine treatment, anxiety-related behavior was attenuated by caffeine (0.3 g/L) only in females. While moderate caffeine worsened recognition memory in females, an improvement in the long-term memory was observed in male rats for both doses. Coincident with memory improvement in males, caffeine increased pro- and BDNF in the hippocampus and cortex. Females presented increased proBDNF levels in both brain regions, with no effects of caffeine. While GFAP was not altered, moderate caffeine intake increased SNAP-25 in the cortex of female rats. Our findings revealed that caffeine promoted cognitive benefits in males associated with increased BDNF levels, while females showed less anxiety. Our findings revealed that caffeine promotes distinct behavioral outcomes and alterations in synaptic proteins during brain development in a sex dependent manner.

1. Introduction

Caffeine is a psychostimulant drug, which at doses regularly consumed by population exerts its primary effects via non-selective antagonism of adenosine A_1 and A_{2A} receptors (Einother and Giesbrecht, 2013; Fredholm et al., 1999). The reasons for high prevalence of caffeine consumption by adult population include its ability to promote arousal, increase vigilance and improve attention and mental performance (Chen et al., 2014; Fredholm et al., 1999; Knight et al., 2004; Temple, 2009).

The consumption of caffeine before pregnancy has been estimated at 97% of women and approximately 68% will persist during this period (Chen et al., 2014; Doepker et al., 2016; Frary et al., 2005; Knight et al., 2004). Caffeine and its metabolites can easily cross placenta barrier and cellular membranes, including from the fetal brain (Nehlig and Derbry, 1994; Parsons and Neims, 1981; Soellner et al., 2009; Yu et al., 2016). Given that the hepatic enzyme systems CYP1A2 that metabolizes caffeine develop its expression and activity at postnatal periods, pregnant women and their fetuses are naturally vulnerable to potential harmful effects of caffeine (Leeder, 2001). Among the documented consequences of exposure to high amounts of caffeine during prenatal are spontaneous abortion, prematurity and low birth weight (Fernandes et al., 1998; Hoyt et al., 2014; Weng et al., 2008).

Experimental studies have also revealed alterations in synaptic proteins essential for brain maturation by chronic exposure of caffeine during fetal and early postnatal brain development (Mioranzza et al.,

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2014; Sahir et al., 2000; Silva et al., 2013; for review see Porciúncula et al., 2013; Temple, 2009). Of particular importance, caffeine consumed during embryonic period altered brain-derived neurotrophic factor (BDNF) and its precursor form (proBDNF) levels in both hippocampus and cortex (Mioranzza et al., 2014). As a neurotrophin synthetized from proBDNF, BDNF can be either cleaved intracellularly and secreted in a mature form (mBDNF), or secreted as proBDNF and then cleaved extracellularly to mBDNF (reviewed in Lessmann et al., 2003). The activation of p75 or TrkB receptors by proBDNF or mBDNF, respectively, often results in opposite actions in terms of cell survival or cell death (Teng et al., 2005; Pang et al., 2004; Woo et al., 2005). BDNF signaling pathway also participates of the synaptogenesis and mechanisms underlying learning and memory in adult animals (Alsina et al., 2001; Lu and Figurov, 1997).

The preventive effects of caffeine against age-related memory impairment have been associated with changes in the BDNF and related proteins in the hippocampus (Costa et al., 2008a,b; Sallaberry et al., 2013). Recently, caffeine was able to induce long-term potentiation (LTP) in an NMDA receptor-independent manner via increases in the BDNF secretion in a calcium-dependent manner, which is necessary for LTP maintenance through a TrkB receptor-mediated process (Lao-Peregrín et al., 2016). Other preclinical and even clinical studies have not reported long-term harmful effects of caffeine; thus it remains unclear how safe is caffeine consumption during pregnancy and brain development (Bakker et al., 2010; Linn et al., 1982; Lynch et al., 2008; Pollack et al., 2010; see recent comment Rutherford and Mayes, 2016; Savitz et al., 2008; Yu et al., 2016).

Of note, the vast majority of studies had been conducted with administrations to pregnant or lactating dams and the offspring undergoes testing at adulthood. In fact, few studies have addressed the effects of caffeine in the pubertal and/or adolescent period (Ardais et al., 2014; O'Neill et al., 2016). Importantly, sex differences in the effects of caffeine are still poorly investigated and some studies have already reported differences. For example, caffeine exposure at weaning worsened recognition memory in adult female rats while it increased locomotor activity in adult male rats (Ardais et al., 2016). While memory impairment was observed in both sexes in the adulthood after chronic prenatal caffeine exposure (Soellner et al., 2009), recognition memory was not affected in adolescent rats from both sexes exposed to caffeine, but males were less anxious than females in the light/dark test (Turgeon et al., 2016). Recently, a double blind placebo-controlled dose–response study showed that girls showed greater changes in subjective responses compared with boys after pre- and post-pubertal caffeine exposure, but they also varied as a function of pubertal stage and menstrual cycle phase (Temple et al., 2015).

As a part of normal brain development, synaptic density starts to increase during early childhood, peaking around puberty and declines across adolescence into adulthood (Glantz et al., 2007; Huttenlocher and Dabholkar, 1997). Given that every stage of the brain development relies on precisely orchestrated process, each period may suffer alterations from external agents that can dramatically change its structure and function, reverberating for a lifetime. The puberty is a period of intense synaptic remodeling and of high vulnerability to psychoactive substances. In the present study, we investigated the behavioral outcomes and synaptic proteins changes in pubescent rats from both sexes that received caffeine since pregnancy. We hypothesized that caffeine would affect the behavior and synaptic proteins crucial for brain development in a sex selective manner.

2. Materials and methods

2.1. Animals

A total number of 187 divided into males and females were used (35 days old), which also included female Wistar rats (70 days old) mated within our colony at Federal University of Rio Grande do Sul. Animals were maintained under 12 h light-dark-cycle (lights on at 7:00 AM), at constant temperature (22 ± 1 °C) and with free access to food, water or caffeinated solution. The pups of the litters were used for this study and others. All experimental procedures were designed to minimize the number of animals used and their suffering and were approved by the Committee on Ethics of Animal Experiments of the Federal University of Rio Grande do Sul (CEUA - UFRGS - Protocol number 20332).

2.2. Caffeine treatment

Caffeine (0.1 or 0.3 g/L) was administered in the drinking water only during the active cycle (lights off at 7:00 PM), with doses regimen corresponding to low and moderate caffeine intake (0.1 or 0.3 g/L, respectively), acting selectively on adenosine receptors (Ardais et al., 2016; Fredholm et al., 1999). During the light cycle (lights on at 7:00 AM) tap water was available for all groups. Fifteen days before mating, the females were organized into three groups: water (control group), caffeine 0.1 g/L and 0.3 g/L (onset of treatment). At postnatal day 21 (PND21), the offspring of each litter was weaned and separated by sex, but continued to receive the same treatment from their respective progenitors up to the end of puberty (34 days old). After this separation (PND 21), the pups were kept at 3–4 rats per cage so that rats of the same litter, sex and treatment could be housed together. The timeline summarizes the schedule of administration and the subsequent behavioral and synaptic proteins levels analysis (Fig. 1).

2.3. Behavioral analysis

Behavioral analysis started when rats were 30 days old, which corresponds to the end of puberty and/or early adolescence (Quinn, 2005; Spear, 2000). All behavioral tests were conducted in a sound-attenuated room under low-intensity light (12 lx) and recorded by means of a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL). All procedures were carried out during the first period of the light cycle (7:00 to 12:00 AM), in which plasma levels of caffeine

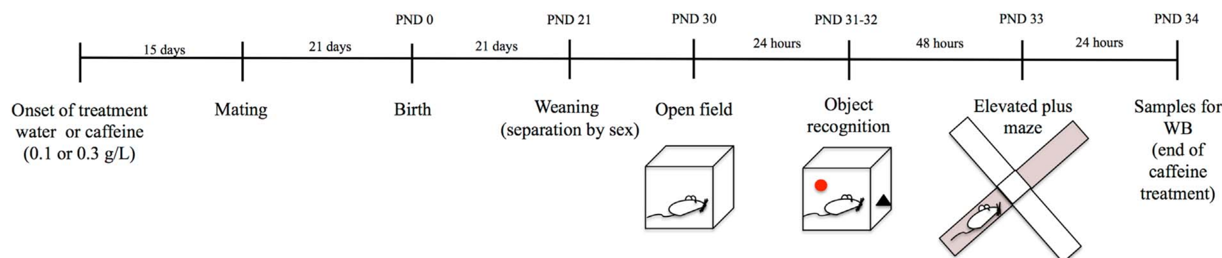

Fig. 1. Schematic overview of the experimental design. Fifteen days before mating female rats were divided into three groups: water (control group), caffeine 0.1 g/L and 0.3 g/L (onset of treatment). At postnatal day 21 (PND 21), the offspring from each litter was weaned, separated by sex and the same treatment from their respective dams was maintained up to the end of puberty (34 days old). Caffeine was available only during the active cycle of the animals (lights off 7:00 P.M). All behavioral tests were carried out between 7:00 A.M. and 12:00 P.M. WB—western blot.

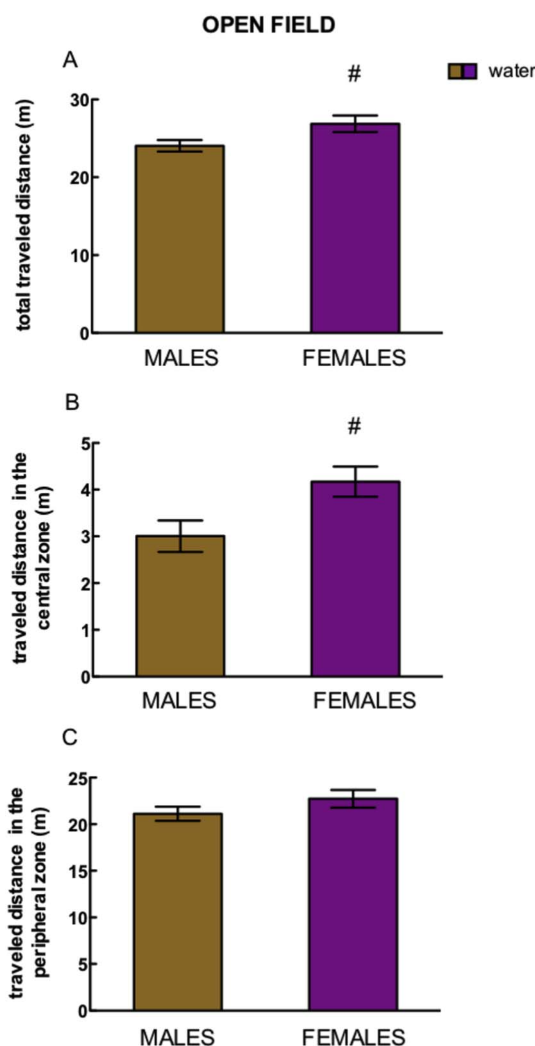

Fig. 2. Sex differences in the locomotor activity displayed by pubescent male and female rats receiving water. Panels show the traveled distance in meters (m) in each area of the open field apparatus during 10 min. (A) Total traveled distance); (B) traveled distance in the center; (C) traveled distance in the periphery. Data are means ± S.E.M (n = 20–25 animals per group). $\#P < 0.05$; (Unpaired t-test).

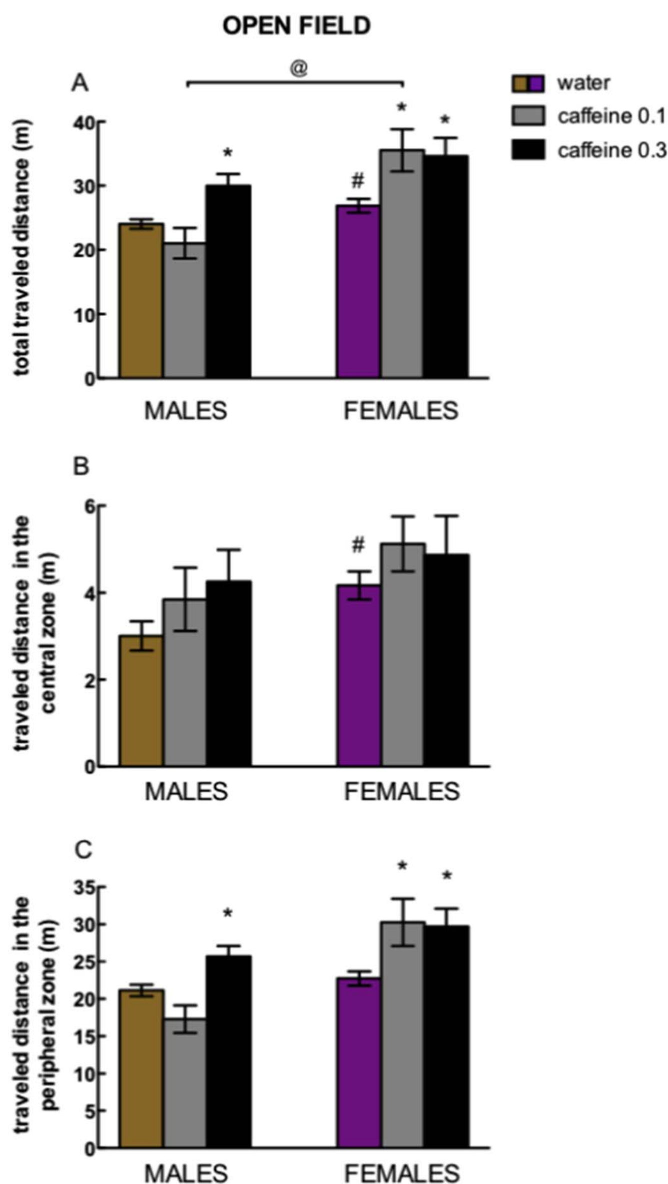

Fig. 3. Sex differences in the locomotor activity displayed by pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water. Panels show the traveled distance in meters (m) in the open field apparatus during 10 min. (A) Total traveled distance (m); (B) traveled distance in the center of the open field; (C) traveled distance in the periphery. Data are means ± S.E.M (n = 11–25 animals per group). $*P < 0.05$ – different from the water group (One-way ANOVA, Tukey's post hoc test). $\#P < 0.05$ (Unpaired t-test). $^{@}P < 0.0001$ (Two-way ANOVA, Tukey's post hoc test).

are still detected (Ardais et al., 2016).

2.4. Open field

The locomotor activity was analyzed in the open field test, as previously reported (Ardais et al., 2016). The apparatus consisted of a black-painted wooden box (50 × 50 cm) surrounded by 50 cm high walls and divided into two areas: center and periphery. Central zone was defined as a square area 20 cm from the wall. Each rat was placed in the center of open field and the traveled distance in the areas was recorded during a single session of 10 min. The experiments were conducted in a sound- attenuated room under low-intensity light (12 lx); activity was recorded with a video camera positioned above the arena and monitored in an adjacent room by an observer blind to the treatment of the animals. Locomotion was measured as the total distance traveled in meters, which was recorded with a video camera and analyzed by a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL).

2.5. Novel object recognition task

The object recognition test was carried out 24 h after an habituation session to open field apparatus, as previously described (Ardais et al., 2014). Rats first underwent a training session, in which two identical objects were placed near the two corners at either end of one side of the chamber. Rats were placed individually into the open field facing the center of the opposite wall and allowed to explore the objects for 5 min. The test session was performed 90 min and 24 h after training and two dissimilar objects were presented, a familiar and a novel one (Ardais et al., 2014; Bevins and Besheer, 2006). The exploration was defined by directing the nose to the object at a distance of at least 2 cm and/or touching the object with the nose or forepaws. The discrimination ratio was defined as: TN/(TN + TF), [TN = time spent exploring the novel object; TF = time spent exploring familiar object].

2.6. Elevated plus maze

The elevated plus maze, a pharmacologically validated apparatus

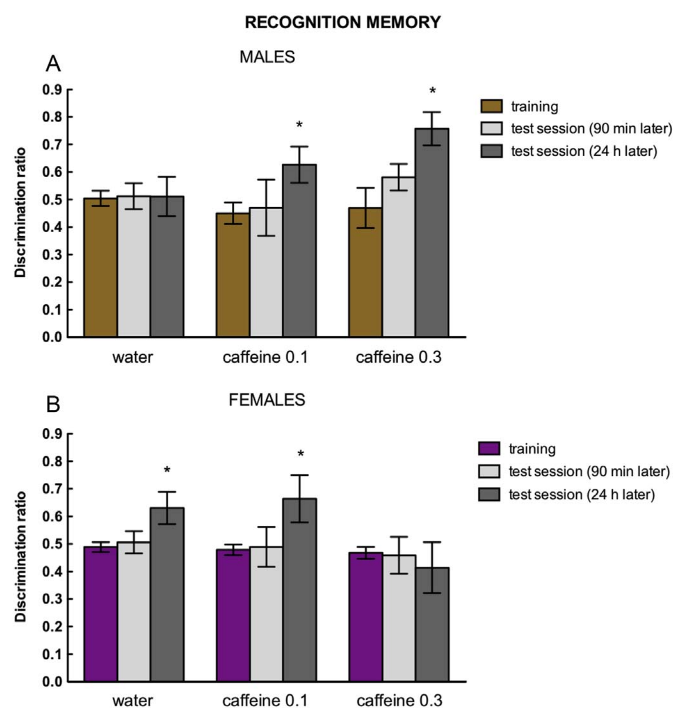

Fig. 4. Performance of the object recognition task for pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water. Panels show the discrimination ratio in the training (brown/purple bars), test session 90 min later (light grey bars) or 24 h later (dark grey bars). Data are means ± S.E.M. of the discrimination ratio (n = 10–24 animals). *P < 0.05 – differences between training and test sessions (Paired t-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for the measurement of anxiety in rodents (Johnston and File, 1991; Lister, 1987) was carried out as previously described (Ardais et al., 2014). The elevated plus-maze apparatus consisted of two open arms (30 cm × 5 cm) and two enclosed arms (30 cm × 5 cm × 10 cm), arranged so that the two arms of each type are positioned oppositely, being separated by a central platform (5 cm × 5 cm). The height of the maze was 70 cm, and the experiments were conducted under dim red light in a quiet room. Each rat was placed in the center of the apparatus facing an open arm. The number of entries and time spent in each apparatus zone (closed, open and central area) were recorded during one single session of 5 min. The entries were recorded when rats have entered with their four paws in each arm.

2.7. Immunoblotting

24 h after the end of behavioral tests, rats were sacrificed under anesthesia. The hippocampi and cerebral cortex were dissected out and immediately homogenized in a 5% SDS solution containing a protease and phosphatase inhibitor cocktail (Sigma, São Paulo/SP, Brazil), and frozen at − 20 °C. After defrost, the protein content was determined using the bicinchoninic acid assay (BCA, Pierce, São Paulo, Brazil). The extracts were diluted at a final protein concentration of 2 μg/μL in sample buffer and either 20 μg (cerebral cortex) or 50 μg (hippocampus) for GFAP and SNAP-25 and 80 μg of protein for proBDNF and BDNF were applied along with pre-stained molecular weight standards (Bio-Rad, São Paulo, Brazil) for SDS-PAGE analysis using 8 or 12% running gel at a 4% concentrating gel. After electro-transfer, membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin (BSA) for 1 h. The nitrocellulose membranes (Amersham, São Paulo, Brazil) were then incubated overnight at 4 °C with rabbit anti-GFAP antibody (1:2000; Sigma), rabbit anti-SNAP-25 antibody (1:5000; Sigma), mouse anti-proBDNF (1:2000; Abcam, São Paulo, Brazil) or mouse anti-BDNF (1:1000; Santa Cruz Biotechnologies, São Paulo, Brazil). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and developed with chemiluminescence ECL kit (Amersham, São Paulo, Brazil). Densitometric analyses were performed using NIH ImageJ software. β-Tubulin was used as loading control and was quantified using a mouse anti-β-tubulin antibody (1:4000; Santa Cruz Biotechnologies, São Paulo, Brazil), as described above.

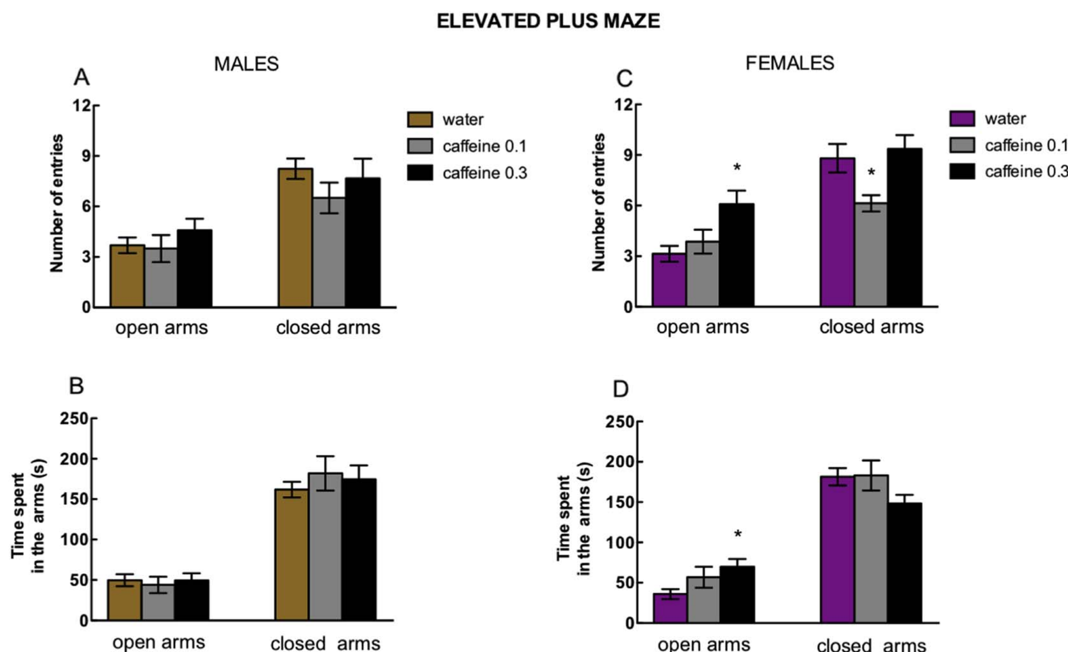

Fig. 5. Anxiety-related behavior in the elevated plus maze displayed by pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water. (A and B) – number of entries and time spent in the open and closed arms for pubescent male rats; (C and D) – number of entries and time spent in the open and closed arms for pubescent female rats. Data are represented as means ± S.E.M. of the time spent in seconds (s) (n = 11–25 animals). *P < 0.05 – differences between water and caffeine groups (One-way ANOVA, Tukey's post hoc test).

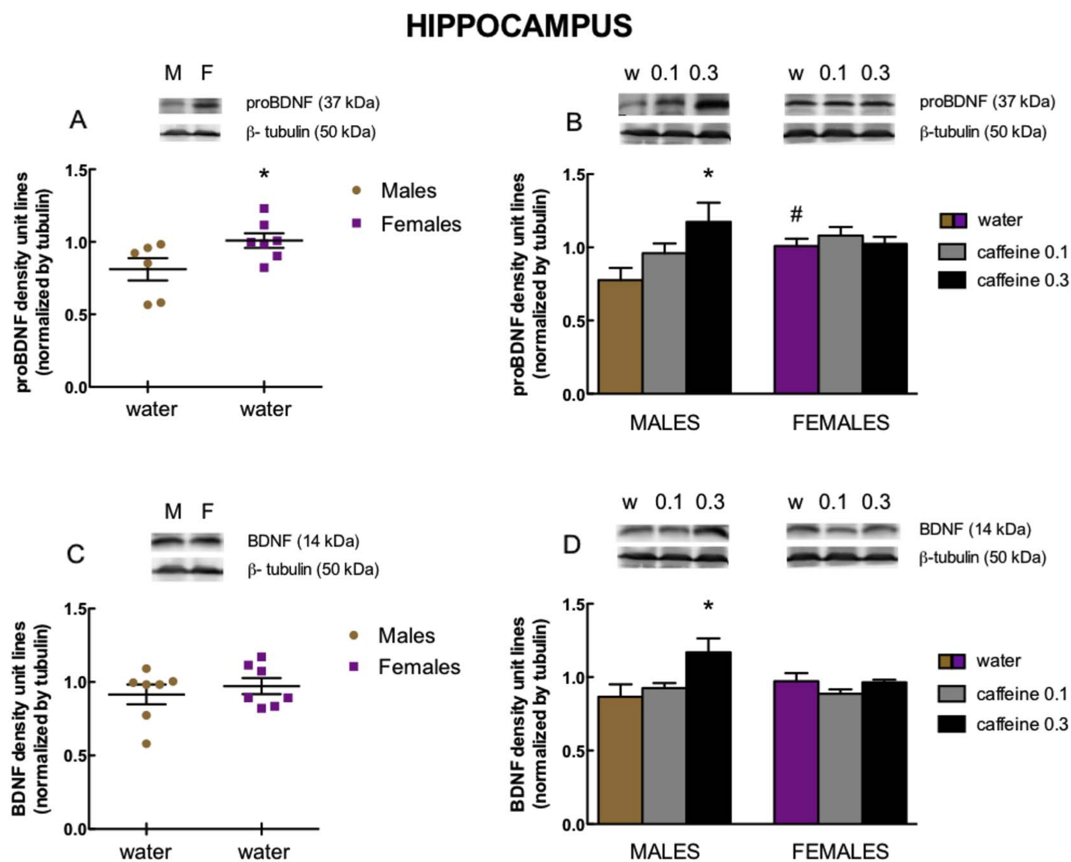

Fig. 6. The immunocontent of proBDNF and BDNF in the hippocampus from pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water. (A and C) – sex differences in the proBDNF and BDNF levels from hippocampus of water groups; $^{*}P < 0.05$; (Unpaired - *t*-test). (B and D) – proBDNF and BDNF levels from hippocampus of water and caffeine-treated groups. Data are represented as means ± S.E.M ($n = 6$–7 animals per group) of density unit lines (normalized by β-tubulin). At the top of each graphic are representative bands for all proteins. $^{*}P < 0.05$ – different from the water group (One-way ANOVA, Tukey's post hoc test). $^{\#}P < 0.05$; (Unpaired - *t*-test).

2.8. Statistical analysis

Data were analyzed by using Two-way or One-way ANOVA followed by Tukey Multiple Comparison post hoc test when appropriated. Unpaired *t*-test was used to compare differences between sexes. Paired *t*-test was used to compare training and test sessions within groups in the object recognition task. Data are expressed as means ± SEM and differences were considered for $P < 0.05$. Graph pad prism version 6.0 was the software used for statistical analysis and graphics.

3. Results

3.1. Open field analysis

Sex differences in the water group were found for total traveled distance ($t = 2.183$; $P < 0.05$) and traveled distance in the central zone ($t = 2.511$; $P < 0.05$) (Fig. 2). In male rats, both total traveled distance [F (2,46) = 7.123; $P < 0.01$] and distance traveled in the peripheral zone were increased by caffeine at 0.3 g/L [F (2,46) = 8.949; $P < 0.001$] (Fig. 3). In female rats, both doses of caffeine also caused a similar increase in the total traveled distance [F (2,53) = 5.026; $P < 0.01$] and in the peripheral zone [F (2,53) = 4.722; $P < 0.05$] (Fig. 3). Two-way ANOVA revealed effects of sex [F (1,99) = 19.32; $P < 0.0001$]; treatment [F (2,99) = 6.003; $P = 0.0035$] and interaction [F (2,99) = 4.914; $P = 0.0092$]) on total traveled distance.

3.2. Novel object recognition task

Recognition memory was assessed by the novel object recognition task. The discrimination ratio between training and test session was similar in male rats that received water. Female rats receiving water showed difference in the discrimination when long-term memory was evaluated ($t = 2.125$; $P < 0.05$). In male rats, caffeine improved long-term memory at low (0.1 g/L: $t = 3.015$; $P < 0.05$) and moderate doses (0.3 g/L: $t = 3.349$; $P < 0.01$) (Fig. 4). No alteration was found in the object recognition task performance for female rats that consumed caffeine (0.1 g/L), while female rats receiving caffeine (0.3 g/L) presented impairment in the long-term memory (Fig. 4).

3.3. Elevated plus maze

The effects of low (0.1 g/L) and moderate (0.3 g/L) doses of caffeine on anxiety-related behavior were evaluated in the elevated plus maze. No differences between sexes were found in all parameters analyzed (Fig. 5). Caffeine was devoid of effects in male rats (Fig. 5). In female rats, caffeine at 0.3 g/L increased in both the time spent [F (2,45) = 3.341; $P < 0.05$] and the number of entries in the open arms [F (2,45) = 5.343; $P < 0.01$]. Caffeine at 0.1 g/L decreased the number of entries in the closed arms in female rats [F (2,44) = 4.496; $P < 0.05$] (Fig. 5).

3.4. Immunodetection of proteins in the hippocampus and cerebral cortex

3.4.1. BDNF and proBDNF

Sex differences in the water groups were found for proBDNF levels, with female rats presenting an increase in both hippocampus ($t = 2.222$; $P < 0.05$) and cerebral cortex ($t = 2.643$; $P < 0.05$) (Figs. 6A and 7A). Males that received caffeine (0.3 g/L) presented increased levels of proBDNF and BDNF in the hippocampus [F (2,14) = 3.882; $P < 0.05$; F (2,14) = 4.621; $P < 0.05$, respectively], while

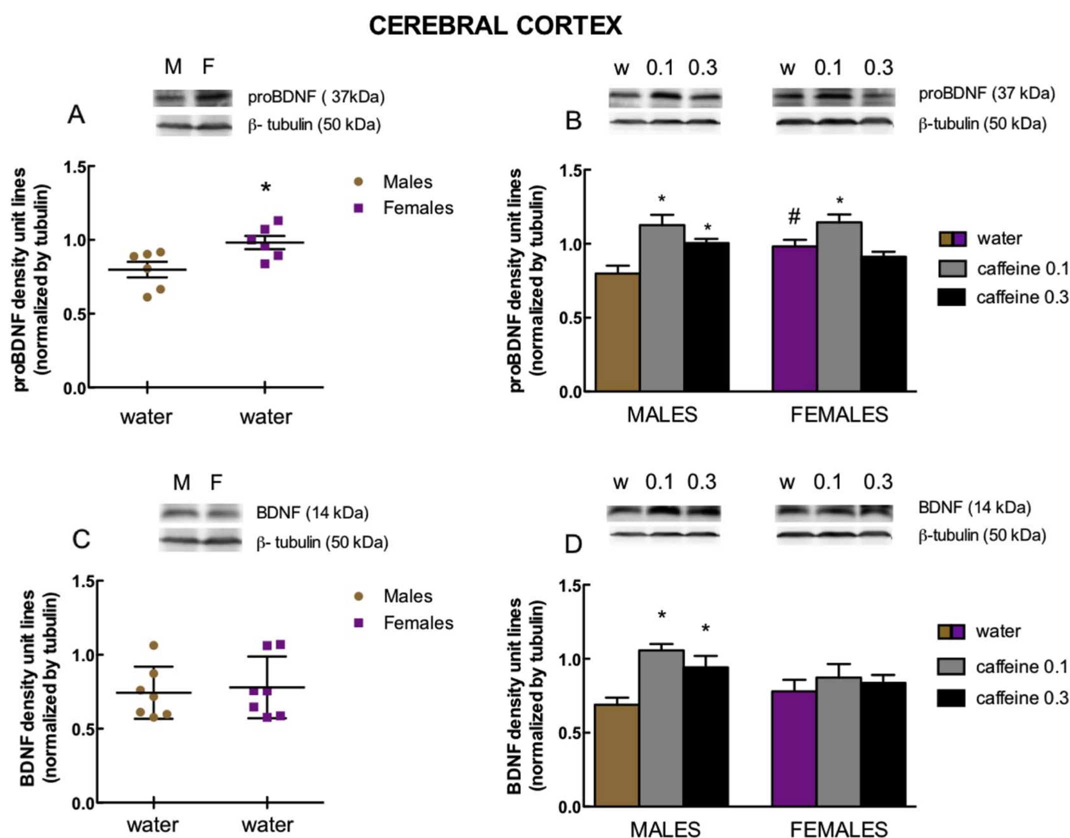

Fig. 7. The immunocontent of proBDNF and BDNF in the cerebral cortex from pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water. (A and C) – sex differences in the proBDNF and BDNF from hippocampus of water groups; $*P < 0.05$; (Unpaired - *t*-test). (B and D) – proBDNF and BDNF levels from cortex of water and caffeine-treated groups. Data are represented as means ± S.E.M (n = 6–7 animals per group) of density unit lines (normalized by β-tubulin). At the top of each graphic are representative bands for all proteins. $*P < 0.05$ – different from the water group (One-way ANOVA, Tukey's post hoc test).

no differences were found for both brain regions in female rats (Fig. 6B and D). In addition, both tested doses of caffeine increased proBDNF and BDNF in the cortex of male rats [F (2,16) = 8.919; $P < 0.01$; F (2,15) = 10.59; $P < 0.01$, respectively] (Fig. 7B and D). In female rats, only the lowest dose of caffeine increased proBDNF in the cerebral cortex [F(2,16) = 6.917; $P < 0.01$], with no effect in the BDNF levels (Fig. 7B and D).

3.4.2. GFAP and SNAP-25

The GFAP levels were not modified either by sex or caffeine treatment (Fig. 8). In addition, SNAP-25 levels were similar in both sexes in the hippocampus and cerebral cortex (Fig. 9A and C). Caffeine (0.3 g/L) increased SNAP-25 levels in the cerebral cortex from female rats [F (2,15) = 8.554; $P < 0.01$] (Fig. 9D).

4. Discussion

In this study, pubescent male and female rats exposed to caffeine since prenatal period were evaluated on locomotor activity, recognition memory and anxiety-like behavior. In parallel, a set of synaptic proteins was assessed in the hippocampus and cerebral cortex.

4.1. Locomotor activity

Rodents at different ages tend to avoid central areas of a new environment and explore preferentially perimeters of a novel environment, a behavior called *thigmotaxis* (Bogdanov et al., 2013; Lamprea et al., 2008; Treit and Fundytus, 1988). Based on this premise, the time spent and traveled distance in the central zone are used as an indicative of anxiety behavior, while the same parameters in the periphery are interpreted as locomotor activity (Prut and Belzung, 2003). Thus, pubescent females showed hyperlocomotion and attenuated anxiety in the open field, which is in line with other reports at different ages (Ardais et al., 2016; Brown and Nemes, 2008; Frye and Walf, 2002; Hiroi and Neumaier, 2006; Hughes and Beveridge, 1990; Simpson and Kelly, 2012).

Caffeine usually shows biphasic effects on locomotion in adult animals, with lower doses promoting increases and higher doses decreasing the locomotor activity (El Yacoubi et al., 2000; Fisone et al., 2004; Marin et al., 2011; Wise, 1988). The acute effects of low-moderate doses of caffeine are via adenosine receptors antagonism (Fredholm et al., 1999), preferentially to the blockade of A2A receptors since caffeine did not trigger hyperlocomotion in $A_{2A}R$ knockout mice (Ledent et al., 1997; El Yacoubi et al., 2000). Some studies have also shown that chronic exposure to caffeine leads to tolerance to its stimulatory actions (Svenningsson et al., 1999), specially for higher doses (Holtzman and Finn, 1988; Lau and Falk, 1995). It can be noted that hyperlocomotion in females was observed at both doses, while in males only at moderate dose, suggesting that pubescent males may have developed tolerance to caffeine earliest than females. In fact, hyperlocomotion after pre and postnatal exposure to caffeine was found only for adult males (Ardais et al., 2016). Besides, caffeine exposure in early life periods may have great impact on locomotion since adolescent male rats exposed to its only in this period maintained similar distance in two days exposure of open field apparatus, evidencing an atypical locomotor behavior (Ardais et al., 2014). Thus, pre and postnatal caffeine exposure caused hyperlocomotion in the puberty in a sex and dose dependent manner, but females seem to be less responsive to hyperlocomotion by caffeine over time (Ardais et al., 2016; Caravan et al., 2016). Based on these findings, the effects of caffeine in the locomotor activity change across ages for both sexes.

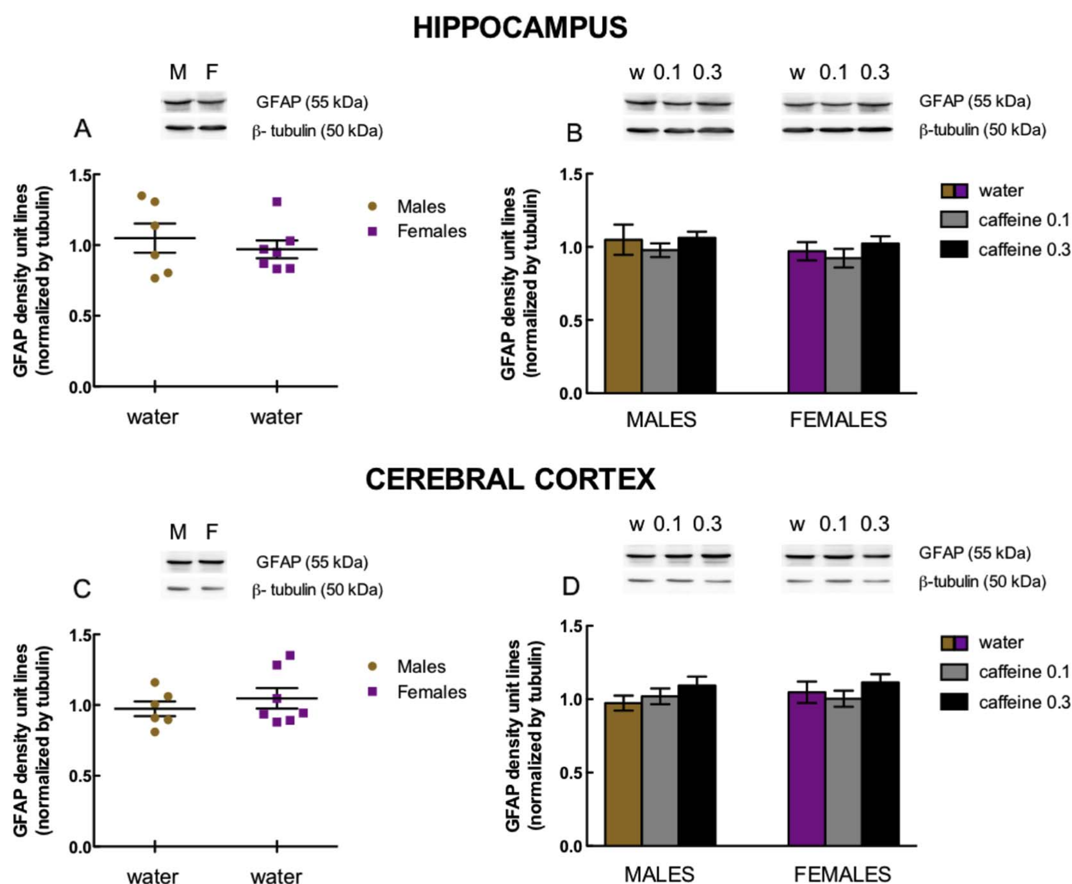

Fig. 8. The immunocontent of GFAP in the hippocampus and cerebral cortex from juvenile male and female rats treated with caffeine (0.1 and 0.3 g/L). Sex differences in the GFAP levels from hippocampus (A) and cortex (C) of water groups. GFAP levels from hippocampus (B) and cortex (D) of water and caffeine-treated groups. Data are represented as means ± S.E.M (n = 6–7 animals per group) of density unit lines (normalized by β-tubulin). At the top of each graphic are representative bands for all proteins.

4.2. Anxiety-related behavior

It is relatively established that female rodents are less anxious and fearful than males (Archer, 1975; Brown and Nemes, 2008; see recent comments in Shansky and Woolley, 2016), and this pattern of emotionality may already be observed in earlier periods of brain development. Pubescent females were less anxious than males in the open field, but both sexes showed similar behavior in the elevated plus maze, as previously noted (Estanislau and Morato, 2006). Pre and postnatal moderate caffeine attenuated anxiety in the elevated plus maze only in pubescent females, with no effect observed for both sexes in the open field. Interestingly, only adult female rats exposed to low, moderate and high caffeine since prenatal period were less anxious in the open field, and at high dose both sexes showed attenuated anxiety in the elevated plus maze (Ardais et al., 2016). In the same study, caffeine treatment interrupted at weaning did not alter anxiety behavior in the adulthood of both sexes in the elevated plus maze. Apart from differences in the responsiveness to apparatuses, the classical anxiogenic effects of caffeine seem to be more evident in later periods of brain development (Bhattacharya et al., 1997; Noschang et al., 2009; Pechlivanova et al., 2012). For example, male rats receiving caffeine during throughout adolescence period have already showed an exacerbation of anxiety at low and moderate doses (Ardais et al., 2014; O'Neill et al., 2016).

4.3. Recognition memory

Another important sex difference was found for recognition memory. We have already reported that adolescent male rats had poor performance in the object recognition task (Ardais et al., 2014). In this study, pubescent females presented a better performance in the object recognition task for long-term memory (LTM), and both sexes displayed worsened recognition memory when short-term memory (STM) was evaluated. Age differences have been described for the object recognition task, with younger rats presenting a decrement compared to adults (Anderson et al., 2004; Reger et al., 2009), probably due to the relatively slow maturation at this time of brain development of the neural circuits involved in recognition memory (Bachevalier and Beauregard, 1993). Furthermore, it has been demonstrated that sex hormones exert influence in distinct brain areas involved in learning and memory (Duarte-Guterman et al., 2015; Hamson et al., 2016; Sánchez-Andrade and Kendrick, 2011).

Of particular importance, caffeine differently affected memory according to the sex. Pubescent males were most favored by improvements in the recognition memory, similar to previous findings in adolescent male rats (Ardais et al., 2014). Of note, caffeine differently affected memory according to the sex. Pubescent males were most favored by improvements in the recognition memory, similar to previous findings in adolescent male rats (Ardais et al., 2014). This could be associated to increases in cortical and hippocampal BDNF and proBDNF levels, once that caffeine was described to alter signaling of this neurotrophin by inducing LTP and promoting its secretion, both events implicated in learning and memory processes (Lao-Peregrín et al., 2016).

Pubescent female rats presented a worsened recognition memory at moderate dose. During early stages of development, a great number of functional changes occur in the brain as a result of remodeling of the synaptic circuits. Since cytochrome P450 activity is low in neonates, exposure to caffeine exposure in this period could be altering these processes. Likewise, memory impairment in females could be also related to increase in the SNAP-25 levels in the cortex, which

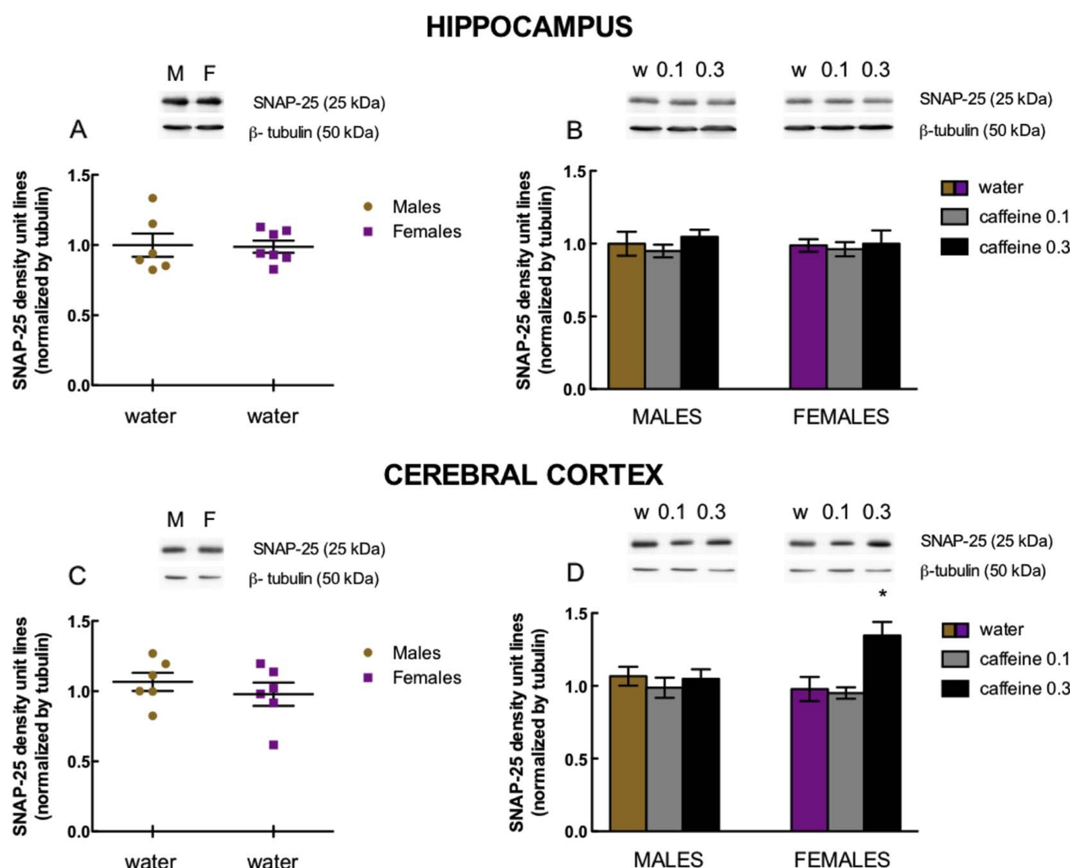

Fig. 9. The immunocontent of SNAP-25 in the hippocampus and cerebral cortex from pubescent male and female rats treated with caffeine (0.1 and 0.3 g/L) or water. Sex differences in the SNAP-25 levels from hippocampus (A) and cortex (C) of water groups. SNAP-25 levels from hippocampus (B) and cortex (D) of water and caffeine-treated groups. Data are represented as means ± S.E.M (n = 6–7 animals per group) of density unit lines (normalized by β-tubulin). At the top of each graphic are representative bands for all proteins. *$P < 0.05$ – different from the water group (One-way ANOVA, Tukey's post hoc test).

corroborates with previous data from other studies reporting memory impairment and increased SNAP-25 levels or overexepression (Cao et al., 2013; McKee et al., 2010). Furthermore, caffeine could be precluding the neuronal loss and dendritic pruning which occur, in a physiologic manner, in this female rats development period (Koss et al., 2014; Willing and Juraska, 2015). Moreover, adult females exposed since prenatal period have also showed impairment in the recognition memory when caffeine treatment was interrupted at weaning (Ardais et al., 2016). In the same study, recognition memory was not affected in adult male and female rats exposed continuously to caffeine since prenatal period (Ardais et al., 2016). It becomes increasingly clear that beyond sex differences, there is a time window of brain development in which caffeine affects permanently some types of memory. For example, adult male and female rats exposed to caffeine either gestation and/or lactation showed impaired memory in different tasks (Silva et al., 2013; Soellner et al., 2009). Furthermore, sexual dimorphism in the adult brain are originated from effects of the sexual hormones during specific time windows of development, which include the late embryonic period to the first postnatal weeks, revealing that the influence of sex hormones occurs even before birth (Colciago et al., 2015).

4.4. Synaptic proteins levels

Caffeine has altered the levels of BDNF in association with behavioral outcomes at different ages (Ardais et al., 2014; Costa et al., 2008a, 2008b; Sallaberry et al., 2013). Thus, in order to find some association between behavioral outcomes and sex differences by caffeine treatment, BDNF and related proteins were analyzed in the hippocampus and cerebral cortex. BDNF is synthesized as the proneurotrophin proBDNF (Lu et al., 2005) and its signaling is essential for recognition memory (Callaghan and Kelly, 2012; Greenberg et al., 2009).

Regarding the sex differences, pubescent female rats presented increased proBDNF, but not BDNF, suggesting that the proneurotrophin was not necessarily converted into the mature form. These differences between pro- and BDNF have been particularly evident in females during brain development, in which higher levels of proBDNF gradually decrease, while BDNF increases after puberty (Harte-Hargrove et al., 2013; Yang et al., 2009). Importantly, estrogen may regulate BDNF expression via an estrogen-sensitive response element in the BDNF gene (Sohrabji et al., 1995).

Coincident with the increase in both proBDNF and BDNF in the hippocampus, caffeine restored recognition long-term memory in pubescent male rats. It is plausible that both events are associated since BDNF signaling is essential for recognition long-term memory (Callaghan and Kelly, 2012, 2013; Greenberg et al., 2009), an association between both events might have occurred. Importantly, proBDNF is not only an inactive precursor of BDNF, but a signaling protein with specific functions.

The expression of GFAP abruptly increases until PND 6, then stabilized but increased progressively until PND 48 in the hippocampus (Kim, 2011). Caffeine during postnatal period promotes decreases in the GFAP at different ages during development of the hippocampus and neocortex (Desfrere et al., 2007), which also include decreases in these brain areas from adolescent male rats treated only during this period (Ardais et al., 2014). Differently from adult males, but not females, pre and postnatal caffeine treatment did not modify GFAP in both brain

areas (Ardais et al., 2016), suggesting that GFAP may not be involved in these behavioral outcomes in the puberty.

The synaptosomal protein of 25 kDa (SNAP-25) is a crucial component of the ternary soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, the minimal machinery required for vesicle exocytosis. There is substantial evidence that alterations in the SNAP-25 levels are associated with cognitive impairment, hyperactivity and exacerbation of anxiety-related behaviors (Braida et al., 2015; Hess et al., 1996; Kataoka et al., 2011). Since moderate caffeine increased cortical SNAP-25 only in pubescent female rats, this increase might be associated with worsened recognition memory and less anxiety behavior, two behavioral outcomes found exclusively in females. Although most studies collectively suggest that reduced levels of SNAP-25 may contribute to the cognitive impaiment and anxiety, memory impairment was observed in young adult rats after overexepression of SNAP-25 by infusion of a recombinant adeno-associated virus vector (McKee et al., 2010). While pubescent male rats treated with caffeine showed normal SNAP-25 levels, adolescent male rats showed exacerbated anxiety and decreased cortical SNAP-25 after caffeine treatment (Ardais et al., 2014). Additionally, both sexes that received caffeine since prenatal period showed attenuated anxiety in the adulthood at high dose of caffeine, but only males had increased levels of cortical SNAP-25 (Ardais et al., 2016).

4.5. Conclusions

Over the last years, there is a growing body of evidence pointing to the impact of caffeine consumption at different phases of brain development. While pubescent male rats were more benefited to the cognitive improvements afforded by caffeine than females, the attenuated anxious effects of caffeine were observed in females. In fact, females seem to be more vulnerable in drugs responses than males (Anker and Carroll, 2011; Becker and Hu, 2008). Our study tried to contribute for a better knowledge about the effects of caffeine in the puberty, and also to be in line with the rationale for incorporating Sex as a Biological Variable (SABV) in current investigations (Shansky and Woolley, 2016). The knowledge of the impact of this psychostimulant, according to the sex in the immature brain is crucial to establish the safety dose.

Author contribution

A.P. Ardais and L.O. Porciúncula designed the study. C. Sallaberry, A.P. Ardais, A.S. Rocha, M.F. Borges, G.T. Fioreze, S. Mioranzza, F. Nunes, N. Pagnussat and P.H.S. Botton performed behavioral experiments, caffeine treatments and neurochemical analysis. C. Sallaberry, A. P. Ardais and L.O. Porciúncula analyzed data and wrote the manuscript. All authors contributed and approved the final manuscript.

Declaration of interest

The authors declare no conflict of interest.

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