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Research Report

Maternal caffeine exposure alters neuromotor development and hippocampus acetylcholinesterase activity in rat offspring

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ABSTRACT

The objective of this study was to evaluate the effects of maternal caffeine intake on the neuromotor development of rat offspring and on acetylcholine degradation and acetylcholinesterase (AChE) expression in the hippocampus of 14-day-old infant rats. Rat dams were treated with caffeine (0.3 g/L) throughout gestation and lactation until the pups were 14 days old. The pups were divided into three groups: (1) control, (2) caffeine, and (3) washout caffeine. The washout group received a caffeine solution until the seventh postnatal day (P7). Righting reflex (RR) and negative geotaxis (NG) were assessed to evaluate postural parameters as an index of neuromotor reflexes. An open-field (OF) test was conducted to assess locomotor and exploratory activities as well as anxiety-like behaviors. Caffeine treatment increased both RR and NG latency times. In the OF test, the caffeine group had fewer outer crossings and reduced locomotion compared to control, while the washout group showed increased inner crossings in relation to the other groups and fewer rearings only in comparison to the control group. We found decreased AChE activity in the caffeine group compared to the other groups, with no alteration in AChE

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transcriptional regulation. Chronic maternal exposure to caffeine promotes important alterations in neuromotor development. These results highlight the ability of maternal caffeine intake to interfere with cholinergic neurotransmission during brain development.

1. Introduction

Caffeine (1,3,7-trimethylxanthine) is one of the most widely consumed psychoactive substances worldwide, with approximately 2.5 billion cups (30 mL/cup) of coffee consumed per day (Heckman et al., 2010). This high intake is due to the acute benefits of caffeine in physiological, psychomotor, and cognitive performance (Einöther and Giesbrecht, 2013). However, caffeine is often consumed by childbearing-age women—around 98% of them consume caffeine regularly and 72% continue to consume it during pregnancy (James, 1991). Caffeine easily crosses the placental barrier and thus may affect the development of the fetus (Andersson et al., 2004). Newborns may also be exposed to caffeine in the postnatal period via breastfeeding (Nehlig and Debry, 1994; Santos et al., 2012).

It is a behavioral stimulant found mostly in many beverages and foods, and is not strongly avoided during pregnancy (da Silva et al., 2008; Fredholm et al., 1999). Chronic caffeine intake during pregnancy has been shown to induce a decrease in locomotor activity, learning abilities, and memory in adult rats (Soellner et al., 2009), disturbances in the renin–angiotensin system (Serapião-Moraes et al., 2013), changes in hippocampal acetylcholinesterase (AChE) after 1 g/L of caffeine intake (da Silva et al., 2008), and alterations in fetal brain development (Silva et al., 2013). In both humans and rats, caffeine is rapidly absorbed and crosses the placenta, thereby reaching the fetus. Fetal elimination of caffeine is limited because of a lack of P-450 cytochrome activity, resulting in increased caffeine half-life (Kirkinen et al., 1983; Knutti et al., 1982; Kot and Daniel, 2008). This is further complicated by the fact that caffeine metabolism is slower in pregnant women (Knutti et al., 1982).

Caffeine is a well established adenosine receptor antagonist (Ferre et al., 2008; Sawynok et al., 2010; Sawynok, 2011) that enhances the release of various neurotransmitters, such as dopamine (Fredholm et al., 1999) and acetylcholine (ACh). In addition, caffeine increases the response from dopaminergic receptors due to a negative interaction between adenosine and those receptors (Fredholm and Svenningsson, 2003). Caffeine can also affect ACh levels and its metabolism in the brain (Carter et al., 1995; Murray et al., 1982), and Ach in turn might be involved in the stimulant properties of caffeine (Acquas et al., 2002). In the prefrontal cortex, the cholinergic nerve terminals are involved in attentional processes (Sarter and Bruno, 2002), and ACh turnover is increased after the use of methylxanthines (Murray et al., 1982).

Acetylcholine is a key neurotransmitter involved in cortical activation, attention, memory, learning, pain, control of motor tone and movement, and control of autonomic functions (Herlenius and Lagercrantz, 2004). The cholinergic system is known to be under development during gestation and early postnatal life (Abreu-Villacxa et al., 2011). Studies testing hippocampus-dependent learning and long-term memory showed that caffeine alters the neurogenesis of hippocampal cells in a dose-dependent manner (Han et al., 2007; Wentz and Magavi, 2009). Chronic maternal exposure to high-dose caffeine enhanced AChE activity (42%) without changes in the levels of AChE mRNA transcripts in 21-day-old rats (da Silva et al., 2008). It has also been shown that oral and intravenous administration of caffeine promotes ACh release in the hippocampus and prefrontal cortex (Acquas et al., 2002; Carter et al., 1995). Additionally, long-term consumption of caffeine can disrupt normal hippocampal neurogenesis in adult rats (Han et al., 2007).

Many clinical and experimental studies have been conducted on the behavioral and biological effects of caffeine use on pregnant women (Björklund et al., 2008; Brent et al., 2011; Loomans et al., 2012; Lorenzo et al., 2010; Sengpiel et al., 2013). The prenatal and perinatal periods are crucial and decisive for further ontogenetic development (Horn, 1987); thus, repeated caffeine intake may trigger several neurochemical processes related to anxiety, learning, and lifetime depression during those periods (Li et al., 2012). Considering the susceptibility of the immature brain to adenosine receptor activation and the neuromodulatory role of adenosine on the cholinergic system, our aim was to evaluate the effects of caffeine intake (0.3 g/L) by rat dams during gestation and lactation on the neuromotor development of the offspring and on AChE activity and expression in the hippocampus of 14-day-old rats.

2. Results

The intake of liquids by the dams and the food consumption and weight of the pups were not significantly different between groups (control, caffeine, and washout groups; data not shown).

2.1. *Neuromotor development*

2.1.1. *Righting reflex*

The caffeine and washout-caffeine groups had increased righting reflex latency compared to the control group (ANOVA/SNK, $F_{(2,40)}=6.06$, $p=0.005$; Fig. 1).

2.1.2. *Negative geotaxis*

Prenatal exposure to caffeine significantly affected the negative geotaxis reflex latency time (Fig. 2). Significant differences from the mean were noted over the testing period. Latency was significantly increased in the caffeine and washout-caffeine groups compared to the control group. At the end, the washout group showed a marginally significant improvement, with a

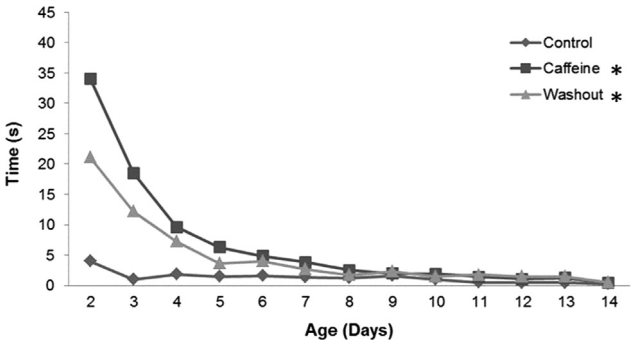

Fig. 1 – Effect of prenatal caffeine exposure on postural righting reflex latency time (s). Lines represent the mean ± SEM (n = 8–16). (*) Indicates a significant difference compared to the control group (ANOVA/SNK, $F_{(2,40)}$ = 6.06, p = 0.005).

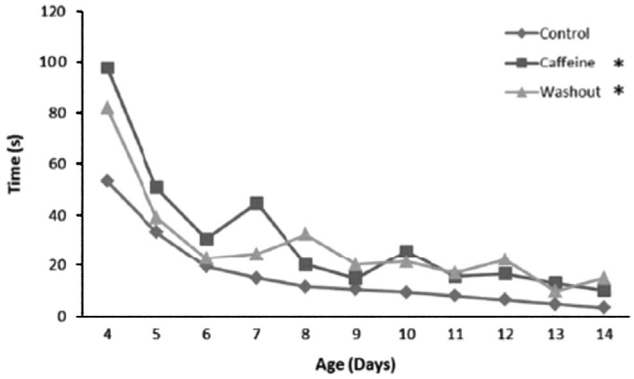

Fig. 2 – Effect of prenatal caffeine exposure on negative geotaxis latency time (s). Lines represent the mean ± SEM (n = 8–16). (*) Indicates a significant difference compared to the control group (ANOVA/SNK, $F_{(2,40)}$ = 5.64, p = 0.007).

small reduction in latency time compared to the control group (ANOVA/SNK, $F_{(2,40)}$ = 5.64, p = 0.007; Fig. 2).

2.2. *Open field test*

At P14, there were no differences between the groups in the latency to leave the first square (one-way ANOVA/Tukey, $F_{(2,46)}$ = 1.60, p > 0.05; Fig. 3a). There was a significant decrease in outer crossings and line crossings in the caffeine group compared to the control group (one-way ANOVA/Tukey, $F_{(2,46)}$ = 5.37, $F_{(2,46)}$ = 4.85, respectively, p < 0.05; Fig. 3b and f). The washout group was not different in outer crossings and locomotion in comparison to the caffeine and control groups (one-way ANOVA, p > 0.05). However, a significant increase in inner crossings was noted in the washout group compared to the control and caffeine groups (one-way ANOVA/Tukey, $F_{(2,46)}$ = 5.57, p < 0.05; Fig. 3c). We observed fewer rearings in the washout group relative to the control group (one-way ANOVA/Tukey, $F_{(2,46)}$ = 3.32, p < 0.05; Fig. 3d), and the caffeine group showed no difference from the other groups (one-way ANOVA, p > 0.05; Fig. 3d). No difference in grooming behavior was observed between groups (one-way ANOVA, p < 0.05; Fig. 3e)

2.3. *Determination of AChE activity*

The caffeine group showed significantly less hippocampal AChE activity at P14 compared to the control group (control: 39.20 ± 1.16 μmol SCh h^{-1} mg^{-1} protein; caffeine: 17.39 ± 8.40 μmol SCh h^{-1} mg^{-1} protein; one-way ANOVA/Tukey; $F_{(2,9)}$ = 4.51, p < 0.05; Fig. 4), which corresponded to approximately 56% less activity than the control. The washout group showed no difference from the other groups (one-way ANOVA, p > 0.05; Fig. 4).

2.4. *Gene expression analysis by quantitative real-time RT-PCR*

An RT-qPCR assay was conducted to determine whether alterations in AChE transcriptional regulation had occurred. The transcript levels of *ache* in the caffeine group were found to be unaltered when compared with those of the control group (one-way ANOVA, p > 0.05; Fig. 5), which indicates that decreased brain AChE activity is not directly related to transcriptional control.

3. Discussion

In the present study, we showed that chronic caffeine intake during gestation and breastfeeding impairs neuromotor development as reflected in increased latency of the righting reflex and negative geotaxis behavior accompanied by a reduction in exploratory locomotion activities of the rat offspring.

First, we exposed female rats to caffeine (0.3 g/L) in their drinking water during pregnancy and lactation to mimic routine daily caffeine consumption in humans. A previous study of a research group showed effects of maternal caffeine intake of 1 g/L (da Silva et al., 2008) and 0.3 g/L (Silva et al., 2013), as well as the dose of 0.3 g/L of caffeine diluted in water to produce blood levels in dams comparable to those in humans after the intake of ~3 cups of coffee (Bona et al., 1995; Björklund et al., 2008). Maternal intake of 0.3 g/L of caffeine leads to a serum concentration of 1 mM in the pups (Fredholm et al., 1999) and to a plasma level of 0.85 mg/L in 7-day-old rats (Adén et al., 2000)—similar values to those found in the umbilical cord of human neonates from mothers with moderate consumption of coffee (up to 3 cups per day) (Björklund et al., 2008).

The pups that received caffeine chronically exhibited an average delay in reflexes as evidenced by inferior performance in reflexive tests. Daily observations of reflexes (postural reflex and negative geotaxis) are sensitive indicators of early developmental stages of the newborn (Gibb and Kolb, 2005). Our results point to an impairment in the spatial patterns of reflex and in motor development. Maturation of reflexes in animals is synchronous with the growth and development of the central nervous system, which is markedly plastic to environmental interventions (Smart and Dobbing, 1971).

We found that prenatal and perinatal caffeine exposure reduced exploratory locomotion activity as shown by a decrease in outer crossings and total locomotion in the OF by the rat offspring. The stimulatory effects of caffeine on locomotion are significantly reduced after chronic caffeine consumption (Lorenzo et al., 2010). Our findings corroborate other studies suggesting that gestational and postnatal caffeine intake induce

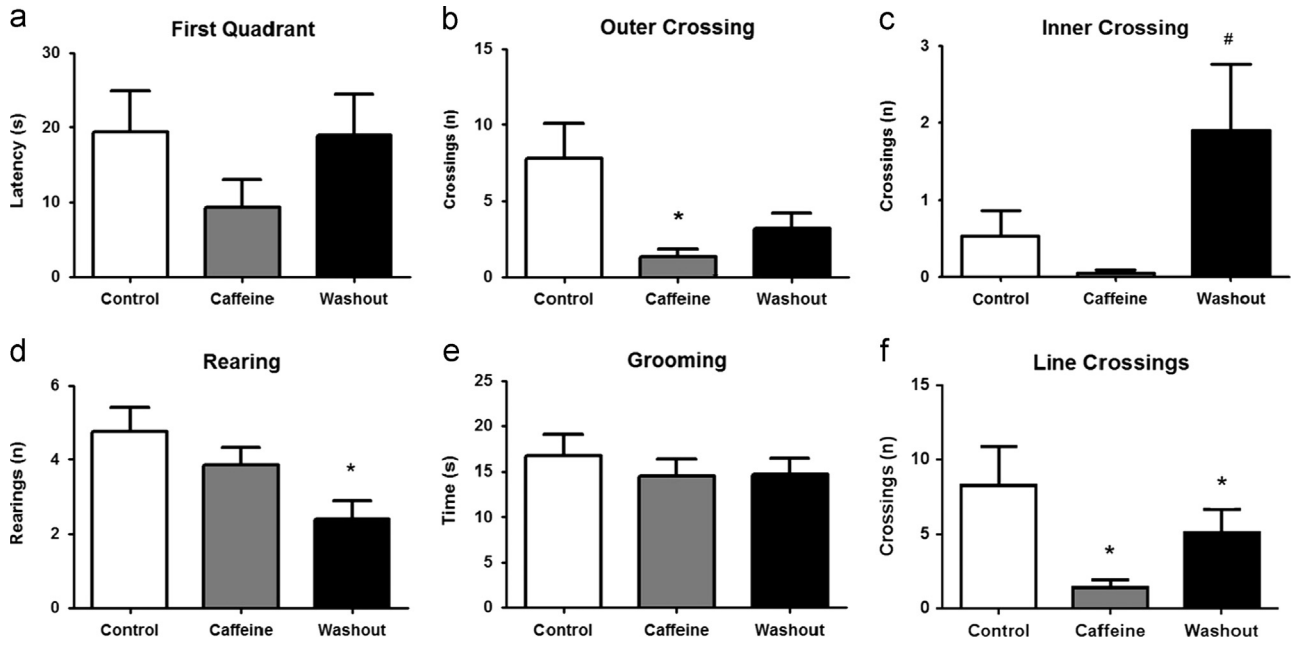

Fig. 3 – Effects of oral caffeine on (a) latency to leave the first square, (b) outer crossings, (c) inner crossings, (d) rearing, (e) grooming, and (f) line crossings in the open field test. Bars represent the mean ± SEM ($n = 10–22$). (*) indicates a significant difference compared to the control group and (#) indicates a significant difference from the caffeine and control groups (one-way ANOVA/Tukey, $p < 0.05$).

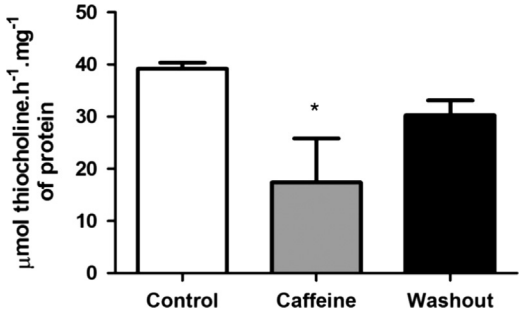

Fig. 4 – AChE activity in the hippocampus of rat offspring. Bars represent the mean ± SEM ($n = 4$). The specific enzyme activity is reported as μmol SCh h^{-1} mg protein $^{-1}$. (*) Indicates a significant difference from the control group (one-way ANOVA/Tukey; $p < 0.05$).

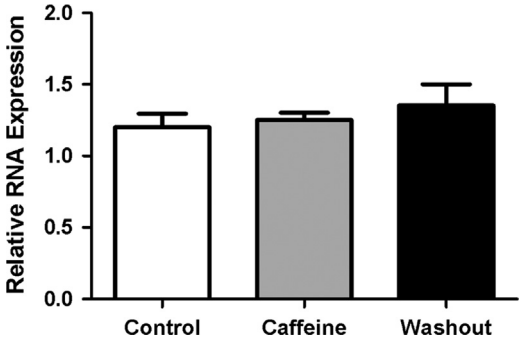

Fig. 5 – Relative AChE expression profile after caffeine exposure (0.3 g/L) in the hippocampus of rat offspring. Bars represent the mean ± SEM ($n = 4$). No differences found (one-way ANOVA/Tukey; $p > 0.05$).

tolerance to some of the behavioral effects of caffeine, such as locomotor activity stimulation (Holtzman and Finn, 1988; Lorenzo et al., 2010). In one study, rats treated chronically with caffeine became tolerant to the stimulant effects of caffeine on locomotor behavior; tolerance developed to the dopamine-related stimulant effect but not to the ACh-related stimulant effect of caffeine in the prefrontal cortex (Acquas et al., 2002).

Our results show that increased central ACh is likely associated with the diminished locomotion activity observed in the caffeine-treated pups. We can also suggest a possible alteration in the dopaminergic system, since acetylcholine and dopamine strongly interact and play an important role in motor control (Calabresi et al., 2006; Jensen et al., 2011). Decreased dopaminergic system activity has been shown to occur in parallel with cholinergic system hyperactivity, according to the classical clinical hypothesis (Dagaev et al., 2004). The release of ACh underlies the inhibitory control of the dopamine D2 receptors present in cholinergic interneurons; decreased dopaminergic activity leads to enhanced ACh release and consequently to over-stimulation of muscarinic receptors in the striatum (Alfaro et al., 2005; Threlfell et al., 2010). Therefore, given the interaction between ACh and dopamine, we can suggest that the increased Ach levels in the synaptic cleft resulting from diminished AChE activity could induce decreased dopaminergic activity and, consequently, reduced locomotor activity.

The effectiveness of chronic caffeine consumption has been demonstrated in the treatment of attention deficit hyperactivity disorder (ADHD) (Caballero et al., 2011; Pandolfo et al., 2013). Extracellular levels of adenosine can modulate dopamine release (Borycz et al., 2007; Gomes et al., 2009), since A1 and A2A receptors are located at dopaminergic terminals (Borycz et al., 2007; Gomes et al., 2009). Antagonistic adenosine/dopamine

interactions result in adenosine inhibiting several effects of dopamine in the basal ganglia (Caballero et al., 2011). Thus, adenosine receptor antagonists such as caffeine may become pro-dopaminergic agents in situations of dopamine/adenosine neurotransmission imbalance (i.e., hyperfunction of the adenosinergic and hypofunction of the dopaminergic systems), which may be implicated in ADHD.

Caffeine induces sensitization of locomotor activity (Cauli et al., 2003; Hsu et al., 2010; Simola et al., 2006), and it has been shown that low-dose caffeine intake by rats (equivalent to human caffeine intake) elicits behavioral plasticity (Ball and Poplawsky, 2011; Reagan-Shaw et al., 2007). The depressant effects of adenosine analogues were enhanced after chronic exposure to caffeine (Nikodijević et al., 1993), providing a possible behavioral effect correlated to the up-regulation of A1 adenosine receptors.

Furthermore, decreased hippocampal AChE activity in animals chronically exposed to caffeine, as observed in the present study, may be associated with antagonism of caffeine-sensitive adenosine receptors, since cholinergic transmission is regulated by adenosine (Forloni et al., 1986; Haubrich et al., 1981). This enables us to suggest that caffeine antagonism could lead to an increase in extracellular adenosine with consequent inhibition of subcortical cholinergic neurons known to promote arousal (Basheer et al., 2004; Porkka-Heiskanen et al., 2002), since ACh is a key brain neurotransmitter involved in cortical activation, attention, memory, learning, pain, control of motor tone and movement, and control of autonomic functions (Herlenius and Lagercrantz, 2004).

Caffeine exposure did not alter AChE mRNA levels in rat hippocampus. The release of ACh is depressed at the level of the cholinergic nerve terminal by the activation of endogenous adenosine at A1 receptors (Jackisch et al., 1984); the density of A1 receptors is higher in the hippocampus than in other areas of the brain (Murphy and Snyder, 1982; Sasaki et al., 2011), and the effects of adenosine on ACh turnover are greatest in the hippocampus (Murray et al., 1982). We noted that low-dose caffeine (0.3 g/L) reduced AChE activity while a previous study (da Silva et al., 2008) showed that maternal chronic exposure to high-dose caffeine (1 g/L) enhanced AChE activity (42%) without altering the AChE mRNA transcripts in 21-day-old rats.

In addition, electrophysiological studies have shown that the hippocampus is particularly sensitive to the inhibitory effects of adenosine and its analogues (Dunwiddie et al., 1981; Schulz et al., 2012). The A1 subtype plays a neuroprotective role as it reduces the release of excitatory neurotransmitters (Dunwiddie and Masino, 2001). Previous studies have shown that chronic caffeine treatment down-regulated adenosine receptors in pregnancy and neonatal life (Léon et al., 2002, da Silva et al., 2008). Adenosine A1 receptors are down-regulated by adenosine analogues via a post-translational mechanism (Ramkumar et al., 1991). We postulate that the process leading to the inhibition of AChE activity probably involves a post-transcriptional or post-translational modulation of this enzymatic activity (Brito et al., 2012).

It should be emphasized that the effects of caffeine are exerted during the first postnatal days, a period when sensory neurological activities are refined and connections are established (Sale et al., 2007). Perinatal ontogenesis is characterized by intensive cell differentiation and receptor formation (Slotkin, 1998), and the use of drugs during this period may interfere with gene expression during the predetermined course of developmental processes and thus disorganize them. These disturbances at the cellular or subcellular level are not evident at birth, but become apparent during further development and maturation in the form of various neuro-psycho-behavioral deviations (Rokyta et al., 2008). One study showed the loss of A1 receptors with aging (Meyer et al., 2007); the authors suggested that such loss could underlie the pathophysiological mechanisms of several neurological diseases. In this context, it is worth noting that caffeine half-life is increased during the neonatal period (Aranda et al., 1979) due to the relative immaturity of some demethylation and acetylation pathways (Carrier et al., 1988). Hence, caffeine accumulation could trigger several neurochemical processes during fetal brain development involved in disorders such as learning impairment, anxiety, and lifetime depression (Li et al., 2012).

We need to consider few limitations in the present study: we were not able to assess the consumption of milk by the pups, however to minimize this question we standardized 8 pups per dam (Silveira et al., 2010, 2011) and avoiding milk intake dispute between the pups. And, based our results, the weight of pup rats was similar between the groups; we can suggest that no difference in milk intake was observed. In addition, the literature provides evidence that the handling of pups can produce stress on them and their mothers; however, in the present study, all animals were subjected to the same assessment protocols during the developmental phase, including the control group. At present, in line with a previous study by our group, our primary intention was to understand the alterations caused by maternal caffeine intake during the gestational and lactational periods, which are characterized by major brain development; furthermore, the animals were handled only in the specific testing days and for the time necessary to make the assessments.

Repeated prenatal and perinatal caffeine exposure at moderate doses may disturb neuromotor development, as indicated by the delay in reflexive and behavioral tests, leading to reflex losses related to spatial evaluation and locomotion associated to decreased AChE activity. Considering that caffeine is the most extensively consumed psychoactive substance, our findings highlight an important factor for translational information.

4. Statistical analyses

Data were expressed as mean ± standard error of the mean (SEM). A repeated-measures ANOVA was used, followed by the Bonferroni test (for time) and the Student-Newman–Keuls (SNK) test (for group) to evaluate righting reflex and negative geotaxis. One-way ANOVA, followed by the SNK test, was used for different analyses as needed. Results were considered statistically different at $p<0.05$.

5. Experimental procedures

5.1. *Animals*

Adult female Wistar rats (mean weight, ~220 g) at 90 days of age were obtained from the institutional breeding facility (CREAL,

ICBS, UFRGS, Porto Alegre, Brazil). Vaginal lavage was performed in all female rats to verify the estrous cycle. In their fertile phase, females were housed with a fertile male overnight. In the following morning, the males were removed and vaginal smears collected. Mating was confirmed by the presence of sperm in the vaginal smears. The sperm positive day was counted as day 0 of pregnancy (G0). All pregnant females were housed in individual laboratory-grade polypropylene cages (49 × 34 × 16 cm). At birth, the litters were culled to 8 pups per dam according to Silveira et al. (2010, 2011), with minor modifications. However, only the males were assessed in the study. All animals were maintained in a standard 12/12 h light/dark cycle (lights on at 0700 h and off at 1900 h) in a controlled environment (22±2 °C). The animals had access to water (or caffeine) and standardized pellet food ad libitum. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (CEUA-Hospital de Clínicas de Porto Alegre, protocol No. 110034) in compliance with the Laboratory Guide for the Care and Use of Animals (The National Academies Press, Eighth Edition, 2011). Animal handling and all experiments were performed in accordance with international guidelines for animal welfare and measures were taken to minimize animal pain and discomfort.

5.2. Chemicals

Caffeine, Trizma base, ethylenedioxy-diethylene-dinitrilo-tetraacetic acid (EDTA), ethylene glycol bis (beta amino ethylether)-N,N,N0,N0-tetraacetic acid (EGTA), sodium citrate, Coomassie Blue G, bovine serum albumin, acetylthiocholine, and 5,50-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Trizol reagent, Platinum1 Taq DNA Polymerase, and SYBR1 Green were purchased from Invitrogen (Carlsbad, CA, USA). ImProm-IITM Reverse Transcription System was purchased from Promega (Madison, USA). All other reagents used were of analytical grade.

5.3. Experimental design

On G0, the rat dams were allocated into three groups according to the treatment received during gestation and lactation via drinking water: (1) control group, receiving only water; (2) caffeine group, receiving only a 0.3 g/L solution of caffeine diluted in water to produce blood levels comparable to those found in humans after consumption of ~3 cups of coffee (Bona et al., 1995; Björklund et al., 2008), and (3) washout-caffeine group, receiving the same solution of caffeine diluted in water until the seventh postnatal day (P7). Evidence from the literature showed that rats at postnatal day 8 (P8) exhibit similar neurological development to a human newborn, and rats aged 2–3 weeks are similar to human 1-year-old infants (Fitzgerald and Anand, 1993). Considering that, the purpose of the washout group was to mimic an intervention up to the stage at which the rats exhibit a similar neurological development to that of a human neonate.

5.4. Development of neuromotor reflexes

5.5.1. Righting reflex (RR)

The RR test was carried out from P2 to P14. The pups were placed in a supine position and the latency time to turn over their longitudinal axis to restore a normal prone position was measured. This was considered fully achieved when the pups turned 180° around their longitudinal axis and their four paws were in contact with the plane surface within the observed 120 s time frame.

5.5.2. Negative geotaxis (NG)

The NG test consists of a postural reaction bringing the animal to face upwards when it is placed on a sloped surface facing downwards. Testing was conducted from P4 to P14. The rat pups were placed on a 35-cm-long inclined platform (45° slope) facing downslope. The animals were expected to turn around 180° to face upwards and climb up the board with their forepaws reaching the upper edge of the board. The test was considered negative if the pups did not succeed in this task within the observed time frame of 120 s.

5.5. Open-field test (OF)

Exploratory and locomotor activity were assessed on P14 and performed in a varnished wooden cage measuring 60 × 40 × 50 cm with glass lining on the inside. The floor was covered with linoleum divided into 12 squares of 13 × 13 cm delimited by dark lines. Each animal was placed gently in the left-side back corner of the cage and then released to freely explore the surroundings for 5 min (Bianchin et al., 1993; Carlini et al., 2002). The number of line crossings by each animal was counted as locomotor activity (Roesler et al., 1999). The latency to leave the first square was considered a measure of anxiety (Britton and Britton, 1981; Lister, 1990). Rearing was defined as the moment when a rat rose up on its hind legs, which ended when one or both forepaws touched the floor again (Wells et al., 2009), and was assessed as exploratory activity (Silveira et al., 2005). Grooming was defined as licking/washing of head and body and evaluated as a biological function of caring for the body surface (Spruijt et al., 1992). The trial started immediately after the rat was placed in the environment for scoring purposes. In this test, the animal was recorded as entering a new area when all four paws crossed the boundary into an adjacent square. The following measures were taken during the 5-min testing sessions: (1) number of line crossings (i.e., horizontal activity) and outer and inner crossings in the OF; (2) latency to leave the first square; (3) grooming (time in seconds), and (4) number of rearings (i.e., vertical activity). The box was cleaned between trials.

5.6. Sample collection

At P14, the pups were killed by decapitation and the hippocampi were extracted and placed on a cold surface. The samples were freshly used in enzymatic assays or immediately frozen in liquid nitrogen according to experiment protocol.

5.7. Protein determination

Protein was measured by the Coomassie Blue method (Bradford, 1976) using bovine serum albumin as the protein standard.

5.8. *Determination of AChE activity*

The hippocampus was homogenized on ice in 60 volumes (v/w) of Tris–citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) using a Potter–Elvehjen-type glass homogenizer. The rate of hydrolysis of acetylthiocholine iodide (0.88 mM) was determined in a final volume of 300 μL with 10 mM phosphate buffer (pH 7.5) mixed with 2.0 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). Samples containing 5 μg of protein were added to the reaction medium specified above and preincubated for 10 min at 25 °C. The hydrolysis of acetylthiocholine iodide was monitored in a microplate reader by the formation of thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) (Ellman et al., 1961). Controls without the homogenate preparation were performed in order to determine the non-enzymatic hydrolysis of the substrate. AChE activity was expressed as μmol thiocholine (SCh) h^{-1} mg $protein^{-1}$. All experiments were performed in quadruplicate.

5.9. *Gene expression analysis by quantitative real-time RT-PCR (RT-qPCR)*

Total RNA was isolated using the Trizol® reagent (Invitrogen, Carlsbad, California, USA) in accordance with the manufacturer's instructions. Total RNA was quantified by spectrophotometry and cDNA was synthesized from 1 μg of total RNA using the ImProm-II™ Reverse Transcription System (Promega), following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-stranded cDNA synthesis. Reactions were done in a volume of 25 μL using 12.5 μL of diluted cDNA (1:50 for *Hprt*1, *Rlp*13*α*, and *ache*), containing a final concentration of 0.5 M betaine (Sigma-Aldrich), 0.2 × SYBR® Green I (Invitrogen), 100 μM dNTP, 1 × PCR Buffer, 3 mM $MgCl_2$, 0.25U Platinum-® Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primers. The PCR cycling conditions were as follows: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing, and 15 s at 72 °C for elongation. At the end of the cycling protocol, a melting-curve analysis was included and fluorescence measured from 60 °C to 99 °C. Relative expression levels were determined using the 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). Efficiency per sample was calculated with the aid of the LinRegPCR 11.0 software (http://LinRegPCR.nl); the stability of the reference genes *Hprt*1 and *Rlp*13*α* (*M-value*, and optimal number of reference genes according to the pairwise variation, *V*) was analyzed using the GeNorm 3.5 software (http://medgen.ugent.be/genorm/). Relative RNA expression levels were determined by the $2^{-\Delta\Delta CT}$ method.

Conflict of interest

There was no financial relationship between any of the authors or any commercial interest in the outcome of this study.

Contributors

This work was carried out in collaboration between all authors. Author ACS carried out the design of the study, and performed the experimental assays and statistical analysis; authors AS, LFM, KMC, LWK, CO and VLS carried out the experimental assays and statistical analysis; authors RSS, MRB, CDB and WC participated in the design of the study; author ILST coordinated the study, performed the statistical analysis, and helped to draft the manuscript. All authors read and approved the final manuscript.

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