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Maternal caffeine intake affects acetylcholinesterase in hippocampus of neonate rats

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Abstract

Transcriptional factors and signalling molecules from intracellular metabolism modulate a complex set of events during brain development. Neurotransmitter and neuromodulator synthesis and their receptor expressions vary according to different stages of brain development. The dynamics of signalling systems is often accompanied by alterations in enzyme expression and activity. Adenosine is a neuromodulator that controls the release of several neurotransmitters, including acetylcholine, which is an important neurotransmitter during brain development. Caffeine is a non-specific antagonist of adenosine receptors and can reach the immature brain. We evaluated the effects of rat maternal caffeine intake (1 g/L) on acetylcholine degradation and acetylcholinesterase expression from hippocampus of 7-, 14- and 21-day-old neonates in caffeine-treated and control groups. Caffeine was not able to change the age-dependent increase of acetylcholinesterase activity or the age-dependent decrease of acetylcholinesterase expression. However, caffeine promoted an increase of acetylcholinesterase activity (42%) without modifications on the level of acetylcholinesterase mRNA transcripts in 21-day-old rats. Considering the high score of phosphorylatable residues on acetylcholinesterase, this profile can be associated with a possible regulation by specific phosphorylation sites. These results highlight the ability of maternal caffeine intake to interfere on cholinergic neurotransmission during brain development.
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Keywords: Acetylcholinesterase; Adenosine; Caffeine; Neural development

1. Introduction

During neural development a correct and organized set of neurotransmitters and neuromodulators is essential to promote the stimuli needed. Neurotransmitters and neuromodulators affect formation of synaptic contacts, maturation of synapses and structural refinement of connectivity (Zhang and Poo, 2001). Acetylcholine is one of the major neurotransmitters in the brain involved in cortical activation, attention, memory, learning, pain, control of motor tone, locomotion and control of autonomic functions (Herlenius and Lagercrantz, 2004). Central cholinergic deficits are strongly related to neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases (Perry, 1988; Oda, 1999). In the brain of rodents, acetylcholine reaches mature levels around 8 weeks after birth. The rate of acetylcholine degradation, played by the enzyme acetylcholinesterase, increases in a time-dependent manner after birth and attains stability at 21 days of neonatal life (Berger-Sweeney and Hohmann, 1997; Lassiter et al., 1998; Mortensen et al., 1998).

Abbreviations: AChE, acetylcholinesterase; ACSCh, acetylthiocholine; DARPP32, dopamine-and cAMP-regulated phosphoprotein of 32-kd; DTNB, 5; 5′-dithiobis-2-nitrobenzoic acid; PKA, AMPc-dependent protein kinase; PKC, protein kinase C; RT-PCR, reverse transcriptase polymerase chain reaction.

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Central cholinergic neurons undergo strong adenosine modulation, mainly over ascending cholinergic projections to the thalamus and cortex. Adenosine acts via adenosine A_1, A_{2A}, A_{2B} and A_3 receptors. The activation of high affinity adenosine receptors, A_1 and A_{2A}, is able to inhibit or stimulate neurotransmitter release, respectively (Fredholm et al., 2005). At 18 days of fetal life, there is a pattern of adenosine receptor distribution similar to adult rats (Weaver, 1996). Ventriculomegaly, reduction in white matter volume and neuronal loss were detected after activation of adenosine receptors during the first 2 weeks of neonatal life, which suggests that adenosine is involved in neurodevelopment and neuronal death during early development (Rivkees et al., 2001; Turner et al., 2002).

The most widely known adenosine receptor antagonist is caffeine. Caffeine is a behavioral stimulant largely present in many beverages and foods and is not strongly avoided during pregnancy. The hydrophobic propriety of caffeine allows its passage through all biological barriers, including the fetal barrier. The behavioral effects of caffeine follow a biphasic dose-response pattern in humans and animals, with low doses acting as stimulant and high doses being depressant (Fredholm et al., 1999). The biochemical basis that underlies caffeine effects is the blockade of adenosine receptors, disrupting adenosine modulation. Oral and intravenous administration of caffeine promote acetylcholine release in hippocampus and prefrontal cortex, respectively (Carter et al., 1995; Acquas et al., 2002). Moreover, long-term consumption of caffeine is able to disrupt normal hippocampal neurogenesis in adult rats (Han et al., 2007). Since adenosine modulates a broad set of actions, the effects of caffeine during gestational and neonatal periods represent an important question in these phases of intense neuronal growth and connection. 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 maternal caffeine intake during gestational and lactational time on acetylcholinesterase (AChE) activity and expression from hippocampus of neonate rats.

2. Material and methods

2.1. Chemicals

Caffeine, 5,5′- dithiobis-2-nitrobenzoic acid (DTNB) and acetylthiocholine iodide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All others chemicals were of analytical grade.

2.2. Animals

The female Wistar rats were allocated with male rats with free access to food and drinking water. They were divided in two groups related with treatment received: (1) control group, which received tap water and (2) caffeine group, which received 1.0 g/L caffeine diluted in tap water. The male rats were removed from the cage after breeding, which was confirmed by the presence of spermatozoids in vaginal mucus samples. Thirty male and female rat pups were used to perform all experiments. The 7-, 14- and 21-day-old pups were killed by decapitation and the structures were separated on a cold surface. Procedures of care and use of animals were adopted according to the regulations of National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by our Institutional Ethics Committee (CEP06/02980 of the Pontifícia Universidade Católica do Rio Grande do Sul, Brazil).

2.3. Biological samples

Total homogenates were prepared for AChE assays. The hippocampus was homogenized on ice in 5 volumes of a buffered solution containing 320 mM Sacarose, 5.0 mM Hepes and 0.1 mM EDTA, pH 7.5, in a motor driven Teflon–glass homogenizer.

To perform the extraction of total RNA, the hippocampus was washed with free RNAase water and frozen with liquid nitrogen until the experimental day.

2.4. Enzyme assays

AChE activity was measured by the method of Ellman et al. (1961). Briefly, 5–10 μg of hippocampus homogenate (protein concentration of 0.6–0.8 mg/ml) were incubated in a solution composed by DTNB and potassium phosphate buffer (DTNB final concentration 1 mM, pH 7.5) at the proportion of 1:4. The preincubation time was 2 min at 25 °C and the enzyme reaction was initiated by addition of 8.0 mM acetylthiocholine (ACSCh). Substrate hydrolysis was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2 min (30 s intervals). AChE activity was expressed as micromole of thiocholine released per hour per milligram of protein. Controls to determine non-enzymatic hydrolysis of ACSCh was performed by incubation of ACSCh, DTNB and potassium phosphate at the same proportion and concentration described above without addition of hippocampal homogenate.

Protein concentration was measured by the Commassie blue method using serum bovine albumin as standard (Bradford, 1976).

2.5. Analysis of gene expression by semi-quantitative RT-PCR

The analysis of acetylcholinesterase expression was carried out by a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. The hippocampi from 7-, 14- and 21-day-old rats were isolated for total RNA extraction with Trizol reagent (Invitrogen) in accordance with the manufacturer instructions. The cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) from 3 μg of total RNA and oligo (dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 42 °C. cDNA (0.1 μl) was used as a template for PCR with the specific primer for acetylcholinesterase. β-actin-PCR was performed as a control for cDNA synthesis. PCR reactions were performed (total volume of 25 μl) using a concentration of 0.4 μM of each primer indicated below and 200 μM and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. Conditions for acetylcholinesterase PCR were as follows: initial 2 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step at 55 °C, 1 min extension step at 72 °C for 35 cycles and a final 7 min extension at 72 °C (Jamal et al., 2007). Conditions for β-actin PCR were as follows: initial 1 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step at 58.5 °C, 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C. The amplification products were: acetylcholinesterase 785 bp; β-actin – 210 bp. PCR products were submitted to electrophoresis using a 1% agarose gel. Bands intensities were analyzed by Kodak 1D v.3.5.4 software. The following set of primers were used: for acetylcholinesterase: forward 5′-GAC TGC CTT TAT CTT AAT GTG-3′; and reverse 5′-CGG CTG ATG AGA GAT TCA TTG-3′; for β-actin: forward 5′-TAT GCC AAC ACA GTG CTG TCT GG-3′; and reverse 5′-TAC TCC TGC TTC CTG ATC CAC AT-3′.

2.6. Statistical analysis

The results were analyzed using GraphPad Prism® software by two-way analysis of variance (ANOVA), considering treatment and age as factors. Main effects were further analyzed by multiple comparisons of means using Bonferroni Post-test. Student *T* test was used to define the differences between control and caffeine group. For comparison of caffeine or water intake from dams in all groups, one-way ANOVA was used. For comparison of rat weight in the same age between different treatment groups (caffeine and control), Student

T test was used. Statistical significance was attributed as $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$. All data are presented as mean ± S.E.M.

3. Results

The intake of liquid by the rat mothers was not significantly different between groups (control group 168.09 ± 27.48 ml/day/mg and caffeine group was 166.21 ± 33.65 ml/day/kg of body weight; $P = 0.91$). No death occurred during caffeine treatment and weights of pups at each age were no significantly different between treated and untreated groups (data not shown).

Caffeine treatment of dams promoted distinct alterations in acetylcholinesterase activity and expression in hippocampal preparations from 7-, 14- and 21-day-old rats. Age and treatment factors exhibited a significant interaction on acetylcholinesterase activity [F (2;24) = 3.40, $P < 0.0001$].

Compared to their respective 7-day-old groups, AChE activity significantly increased in 14-day-old rats [$P < 0.05$ for control animals and $P < 0.01$ for caffeine-treated animals] (Fig. 1) and in 21-days-old rats [$P < 0.001$], for both control and caffeine-treated groups (Fig. 1).

Caffeine treatment in 7- and 14-day-old rats did not modify acetylcholine degradation [$P = 0.08$ and 0.99, respectively] (Fig. 1). However, caffeine-treated group presented an increase of acetylcholine degradation when compared to control (42%) at 21-day-old [$P < 0.01$] (Fig. 1).

Next, we evaluated the relative expression of AChE by semi-quantitative RT-PCR. A decreasing pattern throughout the ages tested from control and caffeine-treated animals was observed [F (2;12) = 3.89, $P < 0.001$] (Fig. 2A and B). No significant interaction between age and treatment was detected. A significant age-dependent decrease of the mRNA expression was detected in both groups. In the control group, a decrease of 43 and 44% in mRNA expression was detected when 7-day-old rats were compared to 14- and 21-day-old rats, respectively [$P < 0.001$ and $P < 0.01$, respectively]. The group treated with caffeine presented a 30% mRNA expression decrease when comparing 7-day-old rats to 14- and 21-day-old rats [$P < 0.05$].

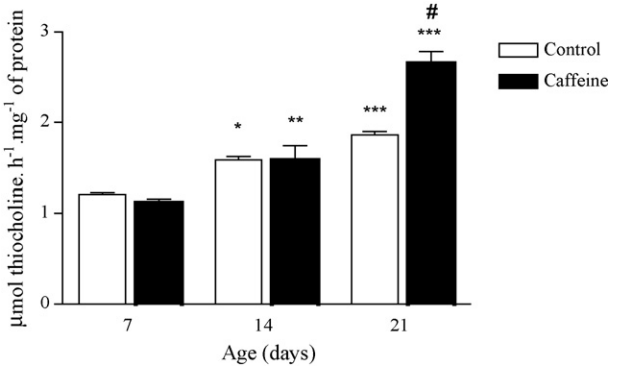

Fig. 1. Effects of caffeine maternal intake on hippocampal AChE activity in 7-, 14- and 21-day-old neonate rats. Bars represent mean ± S.E.M. ($n = 5$). *Represents significant difference at $P < 0.05$, **at $P < 0.01$ and ***at $P < 0.001$ in relation to 7-day-old rats in the same experimental group (control or caffeine). Two-way ANOVA was used considering treatment and age as factors and Bonferroni test was used as *post hoc* test. # Represent significant difference at $P < 0.01$ between control and caffeine group at the same age. Student *T* test was used to define the differences between control and caffeine group.

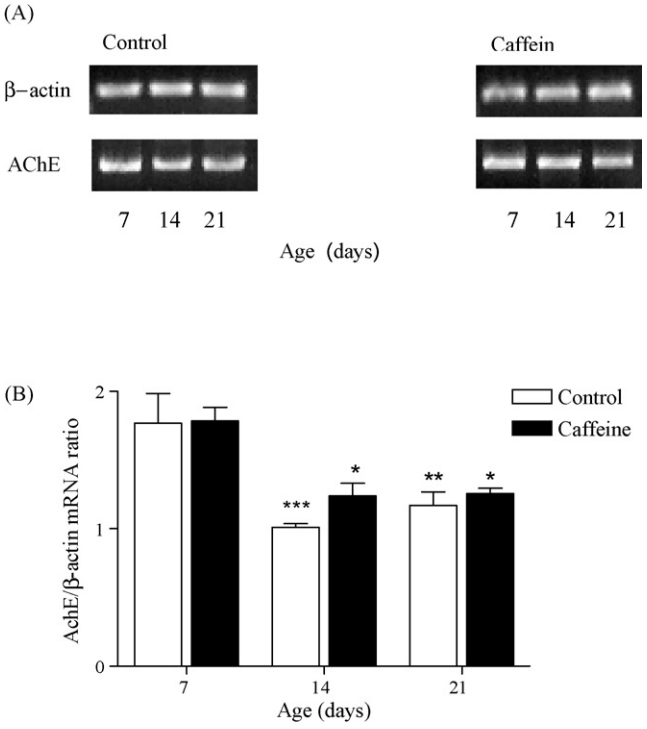

Fig. 2. Effects of caffeine maternal intake on hippocampal AChE mRNA transcription in 7-, 14- and 21-day-old neonate rats. (A) Representative electrophoresis of AChE and β-actin mRNA; (B) relative optical density presented as the average of AChE/β-actin mRNA ratios from three independent experiments. Bars represent mean ± S.E.M. *Represents significant difference at $P < 0.05$, **at $P < 0.01$ and ***at $P < 0.001$ in relation to 7-day-old rats in the same experimental group (control or caffeine). Two-way ANOVA was used considering treatment and age as factors and Bonferroni test was used as *post hoc* test.

4. Discussion

The time period of treatment tested here corresponds to the entire gestational time plus approximately the first 3 years of human life. It comprises an important period of neurodevelopment for both species when the diet comes from maternal sources (blood or milk) or management.

The sources of caffeine to fetuses and newborns depend on maternal dietary lifestyle. Here we intended to represent a non-toxic human consumption of caffeine by administering 1 g/L caffeine as it has been performed by other authors (Johansson et al., 1993; Karcz-Kubicha et al., 2003; Da Silva et al., 2005; León et al., 2005). Evidence about the ability of caffeine to cross blood–brain barrier shows that caffeine is several times better than its metabolites (Svenningsson et al., 1999). Maternal caffeine intake leads to low serum levels of caffeine in rat pups. Fredholm et al. (1999) described that 0.3 g/L of maternal caffeine intake results in a caffeine concentration of 1 μM in the serum of pups. Adén et al. (2000) used 0.3 g/L to treat dams and found 0.85 mg/L of caffeine in the plasma of 7-day-old rats.

Caffeine-mediated actions are mostly attributed to antagonism of high affinity adenosine A_1 and A_{2A} receptors. During

brain development adenosine A_1 and A_{2A} receptors are present at birth, whereas a prominent density and coupling to G-protein seems to occur at the beginning of post-natal life (Rivkees, 1995; Johansson et al., 1997). Adenosine A_1 mRNA is highly expressed in hippocampus during embryonic life, being a target of caffeine exposure (Weaver, 1996).

The disrupting impact of adenosine modulation during brain development has been considered (Guillet and Kellog, 1991; Weaver, 1996; Fredholm et al., 1999; Adén et al., 2000). Ventriculomegaly and white matter loss are consequences of adenosine A_1 receptor activation during the early postnatal life (Turner et al., 2002).

Cholinergic signalling system comprises the early set of neurotransmission systems present at brain development (Herlenius and Lagercrantz, 2004). In the present study we showed that caffeine was able to affect the age-dependent increase of AChE activity. This profile of enzymatic activity was accompanied by an age-dependent decrease of AChE mRNA transcript levels. A wide variety of signal molecules plays a mediator role for transcription and translation through a mechanism of negative feedback loop (Salgado et al., 2001; Keseler et al., 2005). This mechanism seems to adjust the transcription according to the availability of such signal molecules, which are proteins, enzyme products, or other molecules related to the action of the protein encoded by the gene considered (Khishna et al., 2006). This phenomenon at the interface of genetic and metabolic networks could explain the concomitant increase of AChE activity and the decrease of AChE mRNA levels in hippocampus of neonate rats.

Here caffeine was able to increase the activity of acetylcholine degradation in 21-day-old rats. Differential effects of caffeine on hippocampal AChE activity can be a reflex of temporal and regional changes that occur on the purinergic and cholinergic systems in the hippocampus during the first 3 weeks of postnatal life in rats. Lassiter et al. (1998) proposed that hippocampal AChE activity at 7-day-old rats is localized primarily to intrinsic neurons of the subicular complex, CA1, CA3, CA4 and dentate gyrus using enzymatic and histochemical approach. This pattern changed at 17-day-old rat hippocampus and AChE increased in the CA2 and dentate gyrus (Lassiter et al., 1998). Differential expression of adenosine receptors presents CA1 and CA3 as the first subregions of adenosine A1 receptor expression (Etzel and Guillet, 1994).

Caffeine can promote a wide set of actions on various neurotransmitter systems, since adenosine neuromodulation is blunted. Thus, caffeine interferes on the phosphorylation/desphosphorylation cascade triggered by adenosine (for review, see Fredholm et al., 2005). This imbalance could affect AChE activity, since it has been demonstrated that AChE and butyrylcholinesterase from other sources are clearly regulated by a post-translational event (Robitzki et al., 1997; Keller et al., 2001). In vitro studies with recombinant human AChE showed a 10-fold increase in AChE activity after phosphorylation promoted by AMPc-dependent protein kinase (PKA), but not by protein kinase C (PKC) or Casein Kinase II (Grifman et al., 1997). We observed that AChE from rats presents a high-predicted score of possible PKA phosphorylation sites (Ser88 and Thr280) according to analysis performed in NetPhosk, a kinase-specific prediction of protein phosphorylation site tool. Therefore, the increase on AChE activity could be attributed to possible changes in phosphorylation state.

Caffeine is recognized to increase the phosphorylation of Thr75 from dopamine-and cAMP-regulated phosphoprotein of 32-kd (DARPP32), an important mediator of phosphoprotein (Svenningsson et al., 2005). This effect is particularly important for caffeine actions on adenosine A_{2A} receptors from striatum. Considering the low distribution of adenosine A_{2A} receptors and the high distribution of adenosine A_1 receptors in hippocampus, a prominent antagonist action of caffeine on the inhibitory effects triggered by adenosine A_1 receptors could be considered. PKA inhibition is one of the responses induced by adenosine A_1 receptor activation (for review, see Fredholm et al., 2005). Thus, PKA phosphorylation is a possible candidate to perform the activation of AChE activity observed after caffeine treatment.

It has been demonstrated that caffeine is able to enhance the release of neurotransmitters, such as glutamate, dopamine and acetylcholine (Acquas et al., 2002; Solinas et al., 2002). In addition, chronic caffeine treatment promotes intermittent acetylcholine release from prefrontal cortex even after tolerance (Acquas et al., 2002). The increase in acetylcholine degradation promoted by caffeine in 21-day-old rats could present a physiological role, in order to counteract the increased acetylcholine availability observed after chronic treatment.

In a physiological scenario, important questions are raised: What are the consequences of AChE alterations promoted by caffeine in hippocampus of young rats? Could these alterations promote modifications that remain until adult life? Could these alterations be related to mechanisms of adult neuropathologies? These are open questions in regard to the broad effects of caffeine, the wide range of human caffeine consumption as well as the time and phase of caffeine exposure.

In summary, caffeine maternal treatment increased hippocampal AChE activity in 21-day-old pups, with no effect on mRNA expression. This profile suggests that caffeine exerts direct effects on AChE, such as phosphorylation mediated by PKA.

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