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ORIGINAL PAPER

Profile of Nucleotide Catabolism and Ectonucleotidase Expression from the Hippocampi of Neonatal Rats After Caffeine Exposure

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Abstract Nucleotides and nucleosides play an important role in neurodevelopment acting through specific receptors. Ectonucleotidases are the major enzymes involved in controlling the availability of purinergic receptors ligands. ATP is co-released with several neurotransmitters and is the most important source of extracellular adenosine by catabolism exerted by ectonucleotidases. The main ectonucleotidases are named NTPDases (1–8) and 5′-nucleotidase. Adenosine is a powerful modulator of neurotransmitter release. Caffeine blocks adenosine receptor activity as well as adenosine-mediated neuromodulation. Considering the susceptibility of the immature brain to caffeine and the need for correct purinergic signaling during fetal development, we have analyzed the effects of caffeine exposure during gestational and lactational periods on nucleotide degradation and ectonucleotidase expression from the hippocampi of 7-, 14- and 21-days-old rats. Nucleotides hydrolysis was assessed by colorimetric determination of inorganic phosphate released. Ectonucleotidases expression was performed by RT-PCR. ATP and ADP hydrolysis displayed parallel age-dependent decreases in both control and caffeine-treated groups. AMP hydrolysis increased with caffeine treatment in 7-days-old rats (75%); although there was no significant difference in AMP hydrolysis between control (non caffeine-treated) rats and 14- or 21-days caffeine-treated rats. ADP hydrolysis was not affected by caffeine treatment. Caffeine treatment in 7- and 14-days-old rats decreased ATP hydrolysis when compared to the control group (19% and 60% decrease, respectively), but 21-days-treated rats showed an increase in ATP hydrolysis (39%). Expression levels of NTPDase 1 and 5 decreased in hippocampi of caffeine-treated rats. The expression of 5′-nucleotidase was not affected after caffeine exposure. The changes observed in nucleotide hydrolysis and ectonucleotidases expression could promote subtle effects on normal neural development considering the neuromodulatory role of adenosine.

Keywords Caffeine · Ectonucleotidases · Neural development · Maternal caffeine intake · Nucleotides · Purinergic system

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Introduction

Development of the mammalian nervous system involves the interaction of several compounds such as neurotransmitters and neuromodulators [1]. Neurochemical changes during developmental period can be affected by external molecules, such as caffeine. Caffeine is the most widely consumed behaviorally active substance in the world [2]. The mechanism of action of caffeine at concentrations reflecting normal human consumption is the blockade of adenosine A_1 and A_{2A} receptors. This mechanism underlies most caffeine-related effects, including wakefulness [3] and prevention of age-associated recognition memory decline [4, 5]. Additional effects are observed after high concentrations of caffeine exposure, such as inhibition of cyclic nucleotide phosphodiesterases, sensitization to calcium of the cyclic adenosine diphosphate ribose-modulated

calcium-release channel, inhibition of inhibitory GABA-A and glycine receptors, and the enhancement of *N*-methyl-D-aspartic acid (NMDA) receptor neurotransmission [6].

Caffeine can pass through biological barriers and acts on the immature brain, altering several biochemical and behavioral parameters [7, 8]. Early caffeine exposure has long been a target of scientific research, mostly due to apnea treatment of very preterm infants [9]. However, exposure to caffeine in pre and postnatal period can be earlier and larger than therapeutic caffeine exposure if the normal intake of heavy consumers of coffee and caffeinated food and beverages is considered. Caffeine intake during pregnancy is not strictly avoided when compared to alcohol and tobacco restriction [10, 11].

The biochemical and behavioral effects achieved after early caffeine exposure seems to have some effects on the expression levels of adenosine receptors [12–14]. Further, agonism of adenosine receptors in early development has been shown to disrupt the normal development of the central nervous system [16, 17]. Thus, the control of adenosine availability seems to be important in early neural development.

Adenosine is a product of ATP catabolism and its concentration at extracellular milieu is controlled by the ectonucleotidases pathway and bidirectional transporters [18]. ATP can be released as a co-transmitter of dopamine, acetylcholine, noradrenaline, glutamate, γ-aminobutyric acid, and 5-hydroxytryptamine in CNS [19]. The sequential dephosphorylation produces adenosine that can modulate the release of several neurotransmitters and neuronal firing through P1 receptors [18]. The ectonucleotidase family seems to be the major way to control nucleotides and nucleosides availability and includes members of the ectonucleoside triphosphate diphosphohydrolases (E-NTPDase), ectonucleoside pyrophosphatase/phosphodiesterase (E-NPP), alkaline phosphatase and ecto-5′-nucleotidase [19, 20]. E-NTPDases are anchored to the plasma membrane and membranes of organelles [21]. The expression and activity of E-NTPDases and ecto-5′-nucleotidase have been documented in neural tissues during early neural development [22–24].

Disruption of normal ectonucleotidases activities has been documented in several situations and seems to be an important question since these effects can alter nucleotide availability. Acute caffeine administration is able to alter nucleotide hydrolysis and neurotransmitters release [25–27]. Caffeine acutely administered to adult rats promotes dose-dependently release of dopamine and acetylcholine from medial prefrontal cortex, while in chronic treatment, after motor tolerance, only acetylcholine release appears augmented [25]. Considering the co-release of these neurotransmitters and ATP, is reasonable that ATP also be released after caffeine exposure and that ectonucleotidases could adjust their activity or expression as a way to protect immature brain against neuronal overstimulation. To surround this hypothesis, we evaluated the effects of caffeine on the activity of nucleotide hydrolysis and expression of ectonucleotidases in hippocampus from rat pups in early postnatal life.

Materials and Methods

Chemicals

Caffeine, nucleotides (ATP, ADP and AMP), Green Malachite Base and Trizma base were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Animals

Thirty adult female Wistar rats (weighing approximately 220 g) and their pups were used for all experiments. Female rats were housed with male rats and had free access to food and drinking water. Male rats were removed from the cages after breeding. After evaluation of spermatozoa presence pregnant rats were divided into two groups according to the treatment they received: (1) control group, which received only tap water and (2) caffeine group, which received only a solution of 1.0 g/l caffeine diluted in tap water, replaced every 3 days. The treatment to dams was conducted during entirely gestation and lactation until pups achieve 7, 14 or 21 days of age. Pups were killed by decapitation at 7, 14, or 21 days of age, and the brain structures were separated on a cold surface. Procedures for the care and use of animals were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and were approved by our Institutional Ethics Committee (CEP06/02980 of the Pontifícia Universidade Católica do Rio Grande do Sul, Brazil).

Biological Samples

Slices of hippocampi were used to perform nucleotidase assays. The slices were cut transversely to 400 μm thickness by a McIllwain tissue chopper and maintained in a cold buffer (4°C) containing 115 mM NaCl, 3.0 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.0 mM $CaCl_2$ and 10 mM glucose under 95% O_2 and 5% CO_2.

To evaluate the gene expression pattern of E-NTPDase and 5′-nucleotidase, hippocampi from an additional set of animals (three per group) were separated from other brain structures and washed with RNAase-free water. Then, the hippocampi were frozen with liquid nitrogen and kept in a −80°C freezer until they were used for to RT-PCR assays.

Enzyme Assays

Nucleotidase activities were measured according to Bruno et al. [24]. Briefly, two slices of hippocampus were pre-incubated for 10 min at 37°C with 500 µl of saline-bicarbonate buffer (described above). The reaction was started by addition of 2.0 mM nucleotide (ATP, ADP or AMP). A 100 µl sample from the reaction was mixed with 10% trichloroacetic acid at a 1:1 ratio to stop the reaction. The inorganic phosphate released during the reaction was measured at 630 nm according to Chan et al. [28].

The protein concentration was measured after homogenization of slices used for enzymatic assays, along with serum bovine albumin as a protein standard, using the Coomassie blue method [29]. Specific enzyme activity was expressed as nmol of inorganic phosphate release per minute per microgram of protein. Differences between control and treated groups were expressed as a percentage of control group.

Analysis of Gene Expression by Semi-Quantitative RT-PCR

Analysis of E-NTPDase (E-NTPDase 1, E-NTPDase 2, E-NTPDase 3, E-NTPDase 5 and E-NTPDase 6) and 5′-nucleotidase expression was carried out with a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. Total RNA was extracted from hippocampi with TRIzol reagent (Invitrogen Corporation, Carlsbad, USA) according to the manufacturer's instructions. Afterward, cDNA was synthesized with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) from 3 µg of total RNA and oligo dT, in accordance with the supplier's protocol. RT reactions were performed for 50 min at 42°C. cDNA (0.1 µl) was used as a template for RT-PCR, with specific primers for all enzymes analyzed (primer sequences described below). β-actin-PCR was performed as a control for cDNA synthesis. PCR reactions were performed (total volume of 25 µl) using 0.4 µM of each primer and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. Conditions for E-NTPDases PCR were as follows: initial 1 min denaturation step at 94°C, 1 min at 94°C, 1 min annealing step at 65°C (E-NTPDase 1, E-NTPDase 3, E-NTPDase 5, and E-NTPDase 6) or 66°C (NTPDase 2), 1 min extension step at 72°C for 35 cycles and a final 10 min extension at 72°C [30]. Conditions for β-actin PCR were as follows: initial 2 min denaturation step 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. PCR products were separated by electrophoresis with a 1% agarose gel. Band intensities were analyzed in a semi-quantitative manner using the software ImageJ. The following primers were used: E-NTPDase 1: Primer 1—5′-GAT CAT CAC TGG GCA GGA GGA AGG-3′, Primer 2—5′-AAG ACA CCG TTG AAG GCA CAC TGG-3′, E-NTPDase 2: Primer 1—5′-GCT GGG TGG GCC GGT GGA TAC G-3′, Primer 2—5′-ATT GAA GGC CCG GGG ACG CTG AC-3′, E-NTPDase 3: Primer 1—5′-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3′, Primer 2—5′-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3′, E-NTPDase 5: Primer 1—5′-TGG TGG TAA CCA AGA AGG GGA GAT GG-3′, Primer 2—5′-GCA GGT GAA AGG TGG CTC CCA AGG-3′; E-NTPDase 6: Primer 1—5′-GGC CTC TAC GAG CTG TGT GCC AGC AG-3′ Primer 2—5′-TCA GTA CCT TGT CCC CGG GAA AAC C-3′; 5′-Nucleotidase (CD73): Primer 1—5′-CCC GGG GGC CAC TAG CAC CTC A-3′, Primer 2—5′-GCC TGG ACC ACG GGA ACC TT-3′, β-actin: Primer 1—5′-TAT GCC AAC ACA GTG CTG TCT GG-3′, Primer 2—5′-TAC TCC TGC TTC CTG ATC CAC AT-3′. Semi-quantitative expression was showed as a rate of specific gene expression and constitutive gene expression. Differences between control and treated groups were expressed as a percentage of control group.

Statistical Analysis

The results were analyzed by two-way ANOVA, considering treatment and age as factors. For comparison of caffeine or water intake for dams in all groups, one-way ANOVA was used. Primary effects were further analyzed by Bonferroni post-hoc analysis. Statistical significance was set at $P < 0.05$. All data are presented as means ± SEM.

Results

In this study, we compared the nucleotide catabolism and expression of ectonucleotidases in rats receiving either caffeine or water during the gestational and lactational phases. The ingestion of liquid (water or 1 g/l of caffeine diluted in water) from rat dams showed no differences between groups ($P = 0.91$). No deaths occurred in either the control or caffeine-treated group.

Developmental differences on nucleotide catabolism were observed in control animals (Fig. 1). There was a decrease in ATP hydrolysis in control pup rats as they aged; this decrease was evident when comparing 7-days-old rats (104.1 nmolPi min^{-1} mg^{-1} of protein) to 14- and 21-days-old rats (28 and 60.5%, respectively) [F (2;12) = 140.6, $P < 0.0001$] (Fig. 1c). A declining profile of ADP hydrolysis was observed through the ages in control rats (Fig. 1b). ADP hydrolysis decreased 33% in 14-days-old rats in relation to 7-days-old rats (37.56 nmolPi min^{-1} mg^{-1} of protein), while 21-days-old rats presented 42% of decrease

[F (2;12) = 22.96, $P < 0.0001$]. No developmental differences in AMP hydrolysis were observed through the ages (Fig. 1a).

Nucleotide hydrolysis was affected by caffeine treatment (Fig. 1). Statistical analysis of AMP hydrolysis showed significant interaction between factors (age and treatment) [F (2;12) = 11.81, $P = 0.0015$]. Hydrolysis of AMP was statistically different between the caffeine-treated group and control group for 7-days-old rats only [F (1;12) = 22.73, $P < 0.001$] (Fig. 1a). At this age, AMP hydrolysis increased 75% in the caffeine group compared to the control group (7.163 nmolPi min^{-1} mg^{-1} of protein). For ADP hydrolysis, there were no significant interactions between factors [$P = 0.3024$]. Caffeine treatment did not interfere with ADP hydrolysis (Fig. 1b) [$P = 6{,}231$]. Statistical analysis of ATP hydrolysis from hippocampi of 7-, 14-, and 21-days-old rats showed a significant interaction between factors [F (2;12 = 49.38, $P < 0.0001$]. The caffeine-treated pup rats showed a decrease of 19 and 60% in ATP hydrolysis when compared to the control group in 7- (104.1 nmolPi min^{-1} mg^{-1} of protein) and 14-days-old rats (74.92 nmolPi min^{-1} mg^{-1} of protein), respectively [F (1;12) = 38.19, $P < 0.0001$] (Fig. 1c). However, 21-days-old rats receiving caffeine showed an increase of 39% in ATP hydrolysis when compared to the control group at the same age (41.07 nmolPi min^{-1} mg^{-1} of protein) [$P < 0.01$] (Fig. 1c).

Next, mRNA expression of NTPDases 1, 2, 3, 5, and 6 and 5′-nucleotidase from hippocampi of control and caffeine-treated groups were analyzed (Fig. 2). NTPDases 2, 3, and 6 did not show any modification in mRNA expression after caffeine treatment [for NTPDase 2, 3 and 6, $P = 0.1049$, $P = 0.3729$, $P = 0.2578$, respectively] (Fig. 2b, c and e). However, statistical analysis of NTPDase 1 and 5 showed a significant interaction between age and treatment in groups tested at the same age [F (2;12) = 7.346, $P = 0.0083$ and F (2;12) = 6.831, $P = 0.0105$, respectively]. The mRNA expression of NTPDase 1 from the caffeine-treated group decreased 36 and 42% in 14- and 21-days-old rats, respectively, when compared to the control group (0.9207 optical density) [F (1;12) = 32.93; $P < 0.01$ and $P < 0.001$, respectively] (Fig. 2a). NTPDase 5 mRNA expression decreased 30% in 21-days-old rats from the caffeine-treated group compared to the control group at the same age (1.092 optical density) [F (1;12) = 18.35, $P < 0.001$] (Fig. 2d). The 5′-nucleotidase mRNA expression was not affected by caffeine treatment at any of the ages evaluated [$P = 0.6474$] (Fig. 2f).

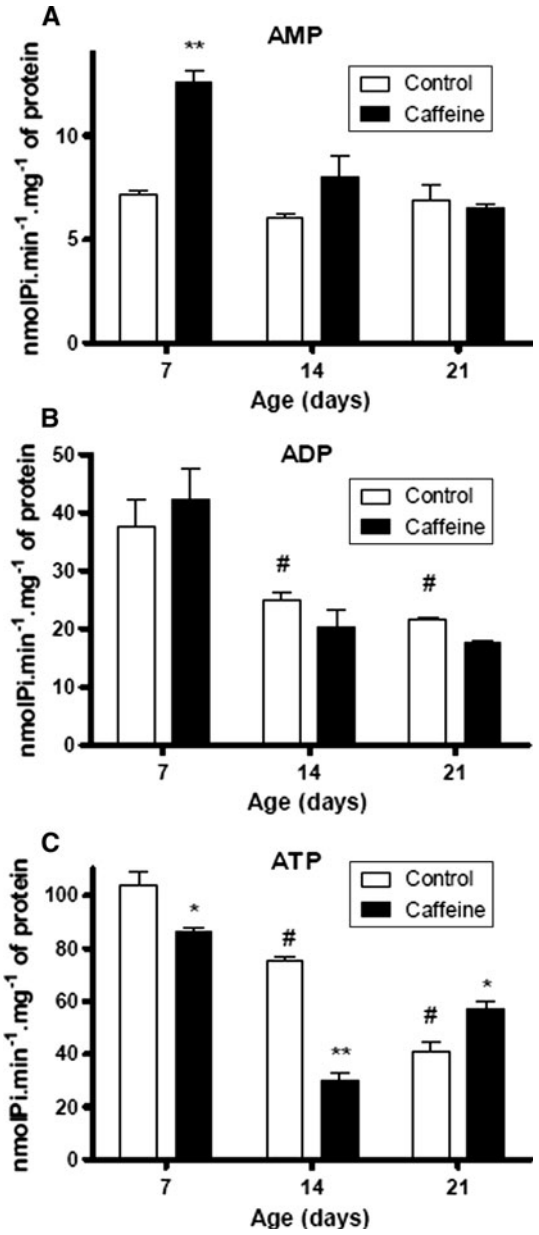

Fig. 1 Effect of caffeine treatment during gestational and lactational period on nucleotide hydrolysis from hippocampi of 7-, 14- and 21-days-old rats. **a** AMP; **b** ADP; and **c** ATP. *Bars* represent mean ± S.E.M. *Significant difference at $P < 0.01$ and **At $P < 0.001$ in relation to control rats at the same age. #Significant difference at $P < 0.0001$ between 14- and 21-days-old rats and 7-days-old rats in the control group. The data are from at least three different experiments. The data are from Two-way ANOVA was used considering treatment and age as factors, and a Bonferroni test was used as a post hoc test

Discussion

Prospective, case control and experimental studies indicate that chronically administered caffeine has beneficial effects against a number of neurological disorders, such as stroke, Alzheimer's and Parkinson's diseases [31–38]. However, when we consider early chronic exposure to caffeine, either as a therapeutic drug or food, the effects on the adenosinergic tonus must be carefully evaluated, considering the physiological environment of the immature brain [9, 11].

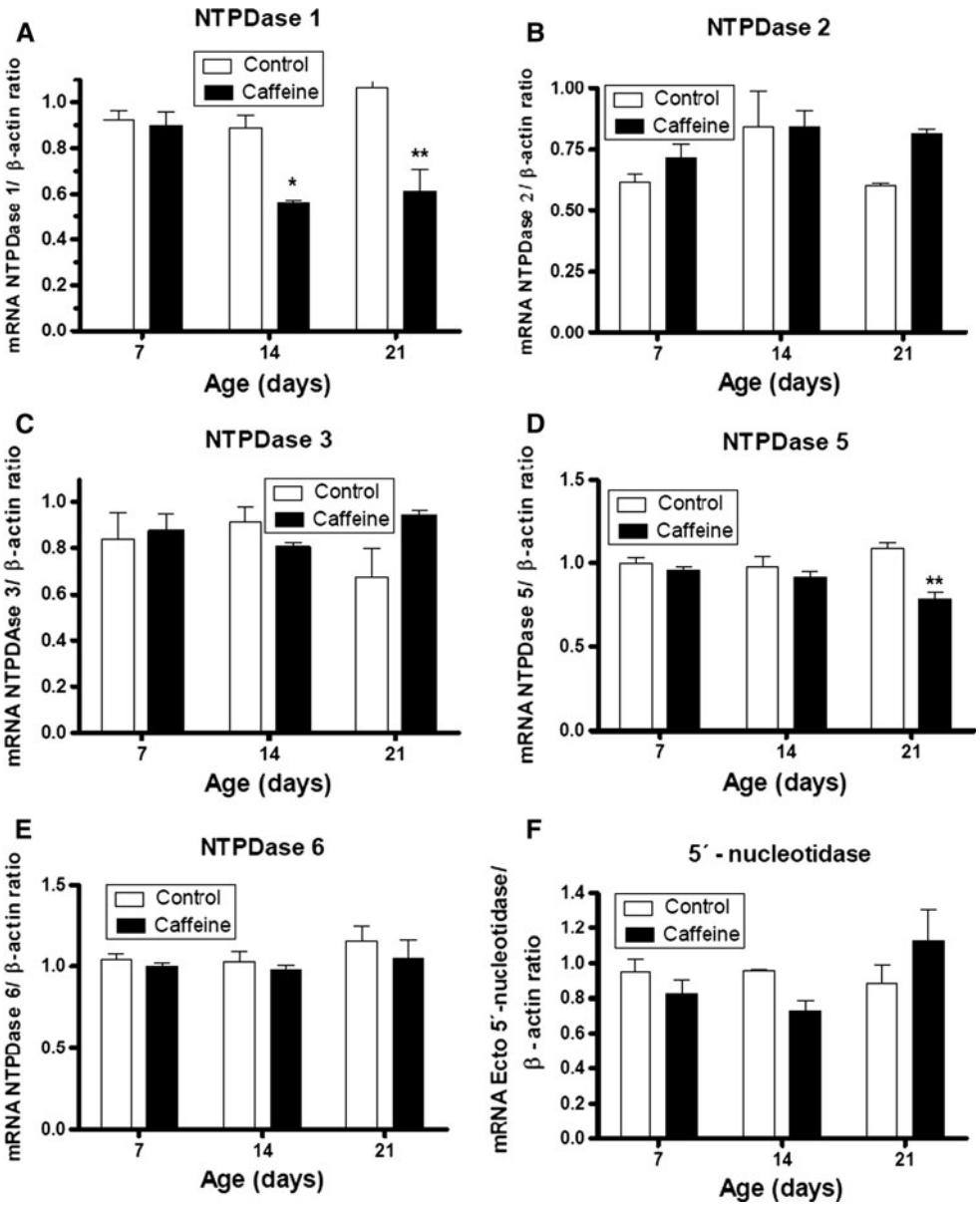

Fig. 2 Effect of caffeine treatment during gestational and lactational periods on NTPDase 1, 2, 3, 5 and 6 and 5′-nucleotidase mRNA transcript levels from hippocampi of 7-, 14- and 21-days-old rats. **a** NTPDase 1; **b** NTPDase 2; **c** NTPDase 3; **d** NTPDase 5; **e** NTPDase 6 and **f** 5′-nucleotidase. The expected amplification products were: E-NTPDase 1: 543 bp, E-NTPDase 2: 331 bp, E-NTPDase 3: 267 bp, E-NTPDase 5: 392 bp, E-NTPDase 6: 304 bp, 5′-Nucleotidase: 405 bp, β-actin: 210 bp. *Bars* represent mean ± S.E.M of optical density. *Significant difference at $P < 0.01$ and **At $P < 0.001$ in relation to control rats at the same age. The data are from at least three different experiments. Two-way ANOVA was used, considering treatment and age as factors, and a Bonferroni test was used as a post hoc test

Caffeine was administered to rat dams during their entire gestational and lactational periods at a concentration of 1 g/l. At this concentration, we can mimic high human exposure to caffeine, which is similar to the ingestion of approximately 230 mg/person/day (normal consumption is equal to 70–76 mg/person/day). Similar caffeine levels have been used to mimic heavy consumers of caffeine in previous rat studies [8, 14, 15, 39–41]. Gasior et al. [42] determined that caffeine plasma concentrations in adult rats treated with 1 g/l of caffeine reached 5.95 μg/ml (30 μM). Here, we evaluated the pups from rats receiving 1 g/l of caffeine. Considering the immature liver of pups and that caffeine freely crosses the placenta, caffeine concentrations in fetus can be near to those in the plasma of rat dams.

Purine nucleotides perform several roles in cellular homeostasis, neurotransmission and neuromodulation [19]. The reduction in ATP and ADP degradation in hippocampal slices of control rats could be an evidence of a natural response to the reorganization and refinement of the central nervous system, such as those that occur to several neurotransmitter systems during neonatal life [1, 39]. Despite the clear nucleotide hydrolysis profile observed, mRNA levels of nucleotidases did not reflect this same pattern of decrease through the ages.

The control of nucleotide concentrations by ectonucleotidases is important for the regulation of nucleotide and nucleoside availability to purinoreceptors P2X, P2Y and P1, and for the considerable number of trophic effects

exerted on neurons and glial cells, including cell proliferation, cell differentiation, axonal growth, and synthesis and release of trophic factors [19, 43–45]. In previous studies [27], we verified that chronic caffeine treatment (1 g/l for 14 days) did not affect nucleotide hydrolysis in synaptosomes of hippocampi or striatum of adult rats, reinforcing the differences in susceptibility to caffeine action between mature and immature brains. These differences in caffeine susceptibility can be related to the transient expression and functionality of adenosine receptors during early development [12].

The strong decrease in ATP hydrolysis in hippocampal slices from the caffeine-treated 14-days old rats presented here could be related to the decrease in NTPDase 1 mRNA. NTPDase 1 is an important enzyme in the control of ATP/ADP levels, since it shows an equal preference for hydrolyzing these nucleotides [20]. Despite the observation that ADP hydrolysis seemed to be unaltered in hippocampi of caffeine-treated rats, NTPDase 5 mRNA expression was reduced in 21-days-old caffeine-treated rats. The contribution of the reduction in NTPDase 5 mRNA for the nucleotide hydrolysis registered here seems to be minimal since NTPDase 5 is an intracellular nucleotidase and shows a preference for hydrolyzing diphosphate nucleotides. Additionally, slice hippocampal preparations may be poor in intracellular enzymes, since we performed washing steps prior to the nucleotide hydrolysis assessment. Although the expression of such ectonucleotidases had been altered, it was difficult to identify the enzyme contributing to the decreased ATP hydrolysis, considering the multiple contributions of the many cells contained in the hippocampal slices. Alteration in mRNA does not reflect changes in protein content. Even more, it has been shown that protein content of NTPDases, especially NTPDase 1, has no direct relation to NTPDase activities [46]. Caffeine acutely given to rats has been reported to be able to promote release of neurotransmitters, such as Glutamate and Dopamine [26]. However, chronic caffeine treatment has been shown to not keep the stimulus of neurotransmitter release, especially dopamine and glutamate, an effect reported as a response to A1 adenosine receptor up-regulation [25, 47]. In this way, it is possible an adjustment of ATP hydrolysis and NTPDase 1 expression with respect to changes in ATP availability after chronic treatment, considering the co-release of these neurotransmitters and ATP as well as the ability of glutamate to evoke ATP release [48, 49].

The caffeine-treated rats maintained the profile of ATP hydrolysis decrease in 7- and 14-days-old rats, but 21-days-old rats displayed an increase in ATP hydrolysis when compared to the control group. This difference could be a response to the adenosine receptors expression in the third week of post-natal life which reaches similar expression of adult rat brain [50]. If the blocking effect of caffeine on the adenosinergic receptors is complete from the third week of postnatal life, it is reasonable that effects such as the release of glutamate, acetylcholine and dopamine could occur as previously showed in adult brain [25, 26]. It was shown that glutamate can stimulate ATP hydrolysis from hippocampal slices from 20- to 23-days-old rats but not before this age [24], which could be related to the augment of ATP hydrolysis showed here in 21-days-old rats treated with caffeine.

The increased AMP hydrolysis from hippocampal slices of 7-days-old rats in the caffeine-treated rats could contribute to neural effects of caffeine on neonatal phase. In developing mice, neonatal caffeine exposure (from postnatal day 3 to 10) promoted a decrease in hippocampal and striatal astrocytogenesis until postnatal day 15, and this effect did not last to the others ages evaluated (postnatal day 20 and 40) [51]. This result could be related to an indirect effect of caffeine on enzyme activity, by altering the level of allosteric species, for example, H^+, inorganic phosphate, Mg^{2+}, ADP or ATP [52]. The increased 5′-nucleotidase activity appears to be not persistent, since AMP hydrolysis from 14- to 21-days-old caffeine-treated animals were not different than the control group, a pattern that can be related to the development of caffeine tolerance and restoration of adenosine tonus. The discrepancy between AMP hydrolysis activity and mRNA expression of 5′-nucleotidase could be a result of the abundance of ecto-5′-nucleotidase during early development, which is related to functions other than its catalytic activity, such as cell adhesion molecule [23].

Conclusion

In summary, caffeine altered nucleotides hydrolysis from hippocampus of rats verified from the first 3 weeks of postnatal life, which could be a result of adjustment in adenosine receptors expression and nucleotide availability after chronic caffeine exposure, and also, related to differential expression of nucleotidases and adenosine receptors during the intense phase of development until reaching the adult patterns. Such changes promoted by maternal consume of caffeine could have broad influence on the process of brain development and provide rationale for the investigation of subtle effects of caffeine and their relation to later neurochemical patterns.

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