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Early exposure to caffeine affects gene expression of adenosine receptors, DARPP-32 and BDNF without affecting sensibility and morphology of developing zebrafish (*Danio rerio*)

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ABSTRACT

Adenosine receptors are the most important biochemical targets of caffeine, a common trimethylxanthine found in food and beverages. Adenosine plays modulatory action during the development through adenosine receptors and their intracellular pathways activation. In this study, we aimed to evaluate if caffeine gave to zebrafish in the very first steps of development is able to affect its direct targets, through the adenosine receptors mRNA expression evaluation, and latter indirect targets, through evaluation of the pattern of dopamine and cAMP-regulated phosphoprotein and brain-derived neurotrophic factor (BDNF) mRNA expression. Here, we demonstrate that zebrafish express adenosine receptor subtypes (A1, A2A1, A2A2 and A2B) since 24 h post-fertilization (hpf) and that caffeine exposure is able to affect the expression of these receptors. Caffeine exposure from 1 hpf is able to increase A1 expression at 72–96 hpf and A2A1 expression at 72 hpf. No alterations occurred in A2A2 and A2B expression after caffeine treatment. DARPP-32, a phosphoprotein involved in adenosine intracellular pathway is also expressed since 24 hpf and early exposure to caffeine increased DARPP-32 expression at 168 hpf. We also evaluate the expression of BDNF as one of the targets of adenosine intracellular pathway activation. BDNF was also expressed since 24 hpf and caffeine treatment increased its expression at 48 and 72 hpf. No morphological alterations induced by caffeine treatment were registered by the check of general body features and total body length. Assessment of tactile sensibility also demonstrated no alterations by caffeine treatment. Altogether, these results suggest that caffeine is able to affect expression of its cellular targets since early phases of development in zebrafish without affect visible features. The up-regulation of direct and indirect targets of caffeine presents as a compensatory mechanism of maintenance of adenosinergic modulation during the developmental phase.

1. Introduction

Adenosine, as a natural constituent of intra and extracellular medium, plays multiple effects on a diversity of organs and cells. At intracellular medium, adenosine plays a homeostatic role while at extracellular medium adenosine can acts as a modulator of cell differentiation, proliferation and activity. Extracellular adenosine induces its intracellular effects through activation of P1 receptors subtypes (A1, A2A, A2B, and A3) which has a different patterns of tissue expression, distinct ligand binding properties and G protein coupling, well described in mammals and in lesser extension in other vertebrates and invertebrates (Burnstock, 2007). A1 and A3 adenosine receptors are coupled to inhibitory G proteins whereas A2A and A2B are linked to excitatory ones (Linden, 2001). The signal pathways triggered by adenosine receptors involve control of phosphatases and kinases activities, ion channels function and gene expression (Fredholm and Svenningsson, 2003).

During the complex phase of initial development, adenosine plays differential role from that observed in adults. While its increase during brain ischemia in adult mammals promotes neuroprotection through A1 adenosine receptor, in neonates a lack of its effects was showed (Rudolphi et al., 1992; Åden et al., 2001). Immature A2A

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adenosine receptors knockout mice developed increased neurological damage after hypoxic ischemia, suggesting a beneficial role for adenosine during hypoxic ischemia (Ådén et al., 2003). In opposite way, adult mice receiving A2A adenosine receptor antagonist and adult A2A adenosine receptors knockout mice exhibited adenosine neuroprotective effects (Monopoli et al.., 1998; Chen et al., 1999). Also, during prenatal life, the adenosinergic system is the dominant humoral regulator of cardiac function, and activation of adenosine A1 receptors may result in cardiac hypoplasia (Rivkees et al., 2001; Zhao and Rivkees, 2001).

Caffeine, an antagonist of adenosine receptors, has been exhaustively studied on the basis of its effects during the entire body development. Caffeine administered to rats in moderate to high doses (30–60 mg/kg daily) throughout gestation resulted in altered blood flow through placenta and subsequent smaller offspring in uterus and at postnatal life (Momoi et al., 2008; Tye et al., 1993). More subtle effects of caffeine during developmental phase can be demonstrated by delayed in neural tube closure, increase in brain-derived neurotrophic factor levels (BDNF) and controversial effects on adenosine receptor expression (Ådén et al., 2000; Wilkinson and Pollard, 1994). As no animal models can exactly predict a response of human body, there are few consistent studies relating human caffeine consume during gestation and negative developmental outcomes (CARE, 2008).

As the very first periods of body development are less accessible in mammal models of developmental studies, design of studies using zebrafish has been more attractive. In fact, the use of zebrafish to access toxicological, neurochemical, genetic, and other approach on developmental phase has become more common. A2 adenosine receptors were detected in zebrafish embryos as a product of two genes for A2A adenosine receptor and one for A2B adenosine receptor sharing high amino acid identity to mammalian A2 adenosine receptors (Boehmler et al., 2009). Exposure of zebrafish to caffeine has been recently studied to contribute to this issue. Low to high concentration of caffeine in the living water of zebrafish embryos has been shown to promote shortening in body length, reduction of mobility, reduction of tactile sensitivity, misalignment of muscle fibers, cardiac arrhythmia and motor neuron defects (Chen et al., 2008; Rana et al., 2010).

In this study, we aimed to evaluate if caffeine gave to zebrafish in the very first steps of development is able to affect its direct targets, the adenosine receptors gene expression, and latter indirect targets, through evaluation of the pattern of dopamine and cAMP-regulated phosphoprotein (DARPP-32 and BDNF) gene expression. We also evaluated the effects of caffeine exposure on the tactile sensibility and morphological aspects. All these approaches together contribute to the establishment of zebrafish as a tool to evaluate the impact of adenosinergic modulation during critical periods of the development.

2. Material and methods

2.1. Animal maintenance and caffeine exposure

Zebrafish embryos were obtained from natural mating of adult zebrafish (wild type) breeds and maintained in an automated recirculating tank system. After the posture, eggs were collected and kept in water of maintenance (water from reverse osmosis plus marine salt) in an incubator at 28.5 °C on 14:10 light/dark cycle. In order to choose a dose of caffeine, an evaluation of three doses of caffeine (10, 50 e 100 μM) on locomotor activity (distance traveled and mean speed) was assessed in 7 days post-fertilization zebrafish during 5 min by commercial video tracking systems (ANY-maze, Stoelting Co., Wood Dale, IL). Fertilized eggs were collected and treated with the selected caffeine concentration of 100 μM or maintained in water of maintenance as control animal. Caffeine was diluted in the same maintenance water of control embryos. Caffeine-treated animals started their treatment at 1 h post-fertilization (hpf) until 24, 48, 72, 96 and 168 hpf on Petri dishes (30 embryos per dish). For those animals that were used after 72 hpf, caffeine solution was renewed on the third day of exposure by a new 100 μM caffeine solution. Relative mortality rate, hatching and larvae morphology were monitored. For molecular analysis we performed at least three different set of experiments for each age with a pool of 30 embryos each (Boehmler et al., 2009). For sensibility assessment we utilized 30 embryos per group (control and caffeine). All procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the Ethical Council from Pontificia Universidade Católica do Rio Grande do Sul (PUCRS) (Registration number CEP004/09).

2.2. Molecular analysis

After the treatment, three sets of animals were collected at 24, 48, 72, 96 and 168 hpf, frozen in liquid nitrogen and maintained in freezer at −80 °C at least 24 h prior to molecular analysis. Total RNA from zebrafish was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The purity of the RNA was spectrophotometrically quantified by calculating the ratio between absorbance values at 260 and 280 nm. Behind, all samples were adjusted to 160 ng/μl and cDNA species were synthesized with SuperScriptTM III First-Strand Synthesis SuperMix (Invitrogen, USA) following supplier's instructions. β-actin-PCR was performed as a cDNA synthesis control. PCR reactions were performed (total volume of 25 μl) using 0.4 μM of each primer indicated below and 1 U of platinum Taq DNA polymerase (Invitrogen, USA) in the supplied reaction buffer. PCR conditions for adenosine receptors (A1, A2A1, A2A2, and A2B), DARPP-32 and BDNF were as follows: initial 4 min denaturation step at 94 °C, 30 s at 94 °C, 30 s annealing step at 62 °C for adenosine receptors and DARPP-32 and 58 °C for BDNF, 1 min and 30 s extension step at 72 °C for 34 cycles and a final 10 min extension at 72 °C. 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 54 °C, 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C. The zebrafish sequence encoding to adenosine receptors, DARPP-32 and BDNF was retrieved from the GenBank database [A1 receptor: NM_001128584.1, A2A1 receptor: NM_001039815.1, A2A2 receptor: NM_001040036.1, A2B receptor: NM_001039813, DARPP-32: NM_001002538 and BDNF: NM_131595] and used for searching specific primers, which were designed using program Oligos 9.6. In order to confirm the primer specificity, each primer was compared with the zebrafish genome and it was able to recognize only its specific target sequence. No results retrieved for A3 adenosine receptors. Thus, the strategy adopted to construct the primers did not allow cross-amplification. The following set of primer was used: for A1: forward 5′-ACA AGA AGG TGT CCA GTC ATT CGC AAC C-3′; and reverse 5′-TAT CAG GAG GAG GCG GAG CTT CCT TGC-3′ for A2A1: forward 5′-AAA GTC AAC CGG TCT GGT CGC CGA AC-3′ and reverse 5′-AGA GCT GAT TTA ATA TGA AGC GGC GAG-3′ for A2A2: forward 5′-GTC CAA TGG AGG CAT GAC AGC GTC GTC G-3′ and reverse 5′-AGA TGA GTT GCT TGT TCT TAC GGG CAT C-3′ for A2B: forward 5′-GCC TCT CCT CAT CAT TGC TGG GCA TC-3′ and reverse 5′-CCT AAA AGT GAC TCT GAA CTC CCG AAT GC-3′ (Boehmler et al., 2009); for β-actin: forward 5′-GTC CCT GTA CGC CTC TGG TCG-3′; and reverse 5′-GCC GGA CTC ATC GTA CTC CTG-3′ (Rosemberg et al., 2007), for DARPP-32: forward 5′-CCC CAA GAA GAT TCA GTT TGC CGT TC-3′; and reverse 5′-GGC TCT GAG CTG TTG TGA TTG GGC CCC AC-3′and for BDNF: forward 5′-GCT TGA GGT GGA AGG GGA AGC GAC-3′; and reverse 5′-CCC GCC GTG CGG GGT CCG AG-3′. The amplification products were: A1 adenosine receptor — 312 bp, A2A1 adenosine receptor — 284 bp, A2A2 adenosine receptor — 364, A2B adenosine receptor — 343 bp, DARPP-32 — 375 bp, BDNF — 245 bp and β-actin — 678 bp. PCR products were submitted to electrophoresis using a 1% agarose gel and

the relative abundance of mRNA versus β-actin was determined by densitometry using freeware ImageJ 1.37 for Windows. From Image J analysis tools, we selected rectangles with fixed size to perform the quantification of optical density.

2.3. Morphological evaluation

Morphological evaluation was performed in larvae from 48 to 168 hpf under stereomicroscopy to search by body defects. Measure of body length was performed by photographical registration followed by analysis using the software Image J 1.37 for Windows. The body length was assumed as the distance of the center of an eye to tail bud.

2.4. Sensibility assessment

Tactile sensibility was accessed just in the free swimming larvae at 95–100 hpf from control and caffeine-treated groups. The tactile sensibility was evaluated by the count of larval escape from the center of a Petri dish containing water of maintenance (12 ml), where a thin brush was touched gently in the tail of the larvae. The length of escape was registered by the ability of the larvae to cross two concentric circles (2.5 mm and 6.35 mm). For sensibility protocol the registration of the response of thirty larvae per group receiving 30 stimuli with 05 s of intervals were conducted by a blinded observer (adapted from Chen et al., 2008).

2.5. Statistical analysis

Results of gene expression were shown as the mean ± SEM of optical density. To analyze the effect of caffeine on genes expression through the development we performed the two way analysis of variance considering age and treatment as a factor, followed by Bonferroni's Post-hoc analysis of means. Analysis of control group through ages by one way analysis of variance was used to indicate differences of gene expression during the development, which was showed by Tukey's Post-hoc analysis. Locomotor activity was analyzed by one way analysis of variance. Morphological aspects and tactile sensibility were analyzed by Student's *T* test. The significance level was established at $p<0.05$.

3. Results

3.1. Locomotor activity

Evaluation of larval locomotor activity showed no differences between doses tested ($p=0.7959$ to distance traveled and $p=0.6825$ to medium speed). The average of distance traveled was 0.431 m to the control group and, 0.431, 0.370, 0.424 m to the caffeine group at 10, 50 and 100 μm, respectively. The medium speed was 0.0018 m/s to the control group and 0.0017, 0.0019, 0.0020 m/s to caffeine group at 10, 50 and 100 μm, respectively. After that, all the experiments were conducted using 100 μM of caffeine.

3.2. Morphological

There were no visible morphological alterations in caffeine-treated animals and the mortality was not statistically different between control and treated group ($p=0.81$). The results of body length evaluation also showed no differences between control and caffeine-treated groups (48 hpf — Control: 2.919 ± 0.02 mm, Caffeine: 2.964 ± 0.09 mm; 72 hpf — Control: 2.950 ± 0. 01 mm, Caffeine: 3.035 ± 0.07 mm; 96 hpf — Control: 3.381 ± 0.04 mm, Caffeine: 3.302 ± 0.01 mm; 168 hpf — Control: 3.600 ± 0.12 mm, Caffeine: 3.533 ± 0.04 mm).

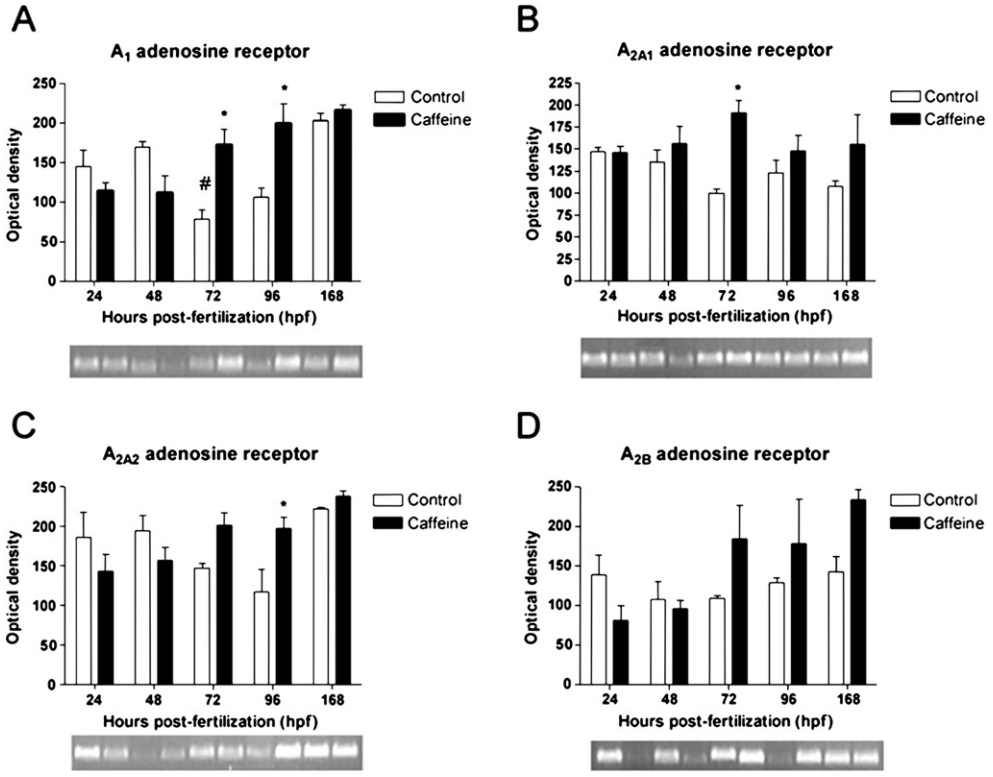

Fig. 1. Effect of caffeine treatment on zebrafish embryos from 1 hpf to 24, 48, 72, 96 and 168 hpf to adenosinergic receptors mRNA expression. (A) A1; (B) A2A1; (C) A2A2; (D) A2B; bars represent mean ± S.E.M of optical density ($n=3$). * represents significant difference at $p<0.05$ and ** at $p<0.01$ in relation to control at the same developmental phase. Two-way ANOVA was used, considering treatment and age as factors, and a Bonferroni test was used as a post hoc test. To evaluate differences through the ages tested in control group was used one-way ANOVA followed by Tukey's post-hoc test. # represents $p<0.05$. Representative gel from adenosinergic receptors mRNA expression from control and caffeine-treated groups follows the same order of bars from graphic.

3.3. Tactile sensibility

The tactile sensibility evaluation compared the response of control and caffeine-treated animals to a touch on tail. The total entrances on the first (41% and 38% for control and caffeine treated, respectively, $p = 0.5$) and the second circle (12% for both groups, $p = 0.94$) did not differed between control and caffeine-treated group.

3.4. Molecular analysis

Caffeine treatment of zebrafish embryos was also conducted on the way to assess effects on the expression of direct and indirect targets, considered adenosine receptors and DARPP-32 and BDNF, respectively.

3.4.1. Evaluation of adenosine receptors expression

We observed that all adenosine receptors are already present at 24 hpf (Fig. 1). The evaluation of adenosine receptors expression in control group through the ages showed that A1 adenosine receptor decreases this expression at 72 hpf (46% in relation to 24 hpf of control group, $p<0.05$). Adenosine receptors expression from whole body of zebrafish embryos was altered by caffeine exposure (Fig. 1). A1 adenosine receptor mRNA expression was higher in caffeine-treated group than in control group at 72 and 96 hpf (117% and 87%, respectively; $F(1; 20) = 5.124$, $p<0.01$) (Fig. 1A). A2A1 adenosine receptor mRNA expression was also increased at 72 hpf in caffeine-treated group in relation to control group (92%; $F(1; 20) = 13.70$, $p<0.01$) (Fig. 1B). A2A2 and A2B adenosine receptors mRNA expression were unaltered by caffeine during the evaluated period ($F(1; 20) = 1.482$; $F(1; 20) = 2.991$, respectively) (Fig. 1C and D).

3.4.2. Evaluation of DARP-32 and BDNF expression

DARPP-32 and BDNF were also detected at 24 hpf. No developmental differences were observed in DARPP-32 and BDNF expression through the period evaluated in control group. However, when embryos were treated with caffeine we observed increase on DARPP-32 mRNA expression at 168 hpf (51%; $F(1; 20) = 6.243$, $p<0.01$) and on BDNF mRNA expression at 48 and 72 hpf (94% and 44%, respectively; $F(1; 20) = 13.77$, $p<0.01$) (Figs. 2 and 3, respectively).

4. Discussion

In the present study, we described that embryos of zebrafish exposed to caffeine display a pattern of up-regulation of direct and indirect targets of caffeine as a response to the adenosinergic block, without affecting visible features of zebrafish body and tactile sensitivity.

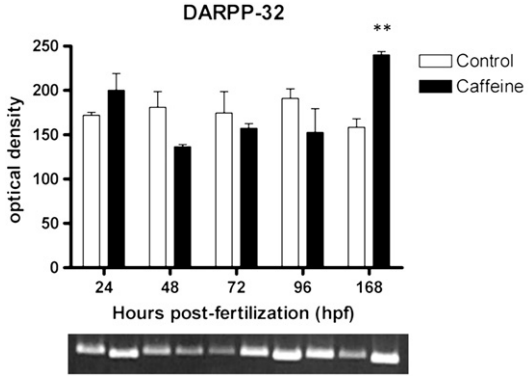

Fig. 2. Effect of caffeine treatment on zebrafish embryos from 1 hpf to 24, 48, 72, 96 and 168 hpf to DARPP-32 mRNA expression. Bars represent mean ± S.E.M of optical density ($n = 3$). ** represent significant difference at $p<0.01$ in relation to control at the same developmental phase. Two-way ANOVA was used, considering treatment and age as factors, and a Bonferroni test was used as a post hoc test. Representative gel of DARPP-32 mRNA expression from control and caffeine-treated groups follow the same order of bars from the graphic.

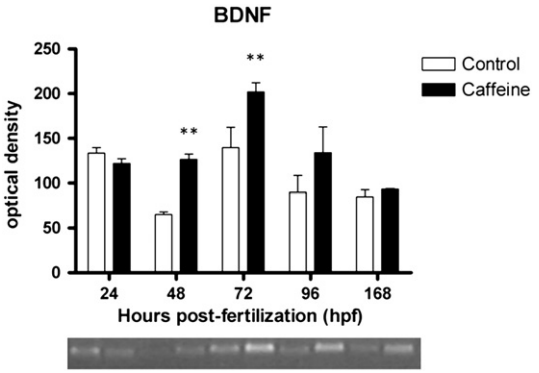

Fig. 3. Effect of caffeine treatment on zebrafish embryos from 1 hpf to 24, 48, 72, 96 and 168 hpf on BDNF mRNA expression. Bars represent mean ± S.E.M of optical density ($n = 3$). ** represent significant difference at $p<0.01$ in relation to control at the same developmental phase. Two-way ANOVA was used, considering treatment and age as factors, and a Bonferroni test was used as a post hoc test. Representative gel of BDNF mRNA expression from control and caffeine-treated groups follow the same order of bars from the graphic.

A first consideration of these results is concerned about the actual caffeine concentration reached in zebrafish embryos during the caffeine treatment. The rationale to use 100 μM (19.4 μg/ml) of caffeine was based on the absence of locomotor effects and on available literature, especially in regard to the fact that this concentration is unable to promote significant embriotoxicity, effects on heart beat, blood circulation, number of somites, hatching, tactile sensibility and other phenotypic features (Chen et al., 2008; Selderslaghs et al., 2009). Zebrafish is not able to eat until around 7 days post-fertilization, considering the late development of its mouth and the diminishment of yolk ball. Therefore, the major way to expose this animal to caffeine is by diluting caffeine in maintenance water or injecting the drug. The presence of chorion until around 48 hpf appears as a barrier to drug exposure. We started the exposure of zebrafish to caffeine at 1 hpf up to 168 hpf (7 days) with natural dechorionation. However, it is reasonable that caffeine does not reach the same concentration inside the eggs during the chorionated phase. Here, the majority of the effects detected on gene expression appeared after the stage of loss of chorion, suggesting that these effects were only reached when the full concentration added was completely absorbed by embryos without the barrier of chorion.

The expression of selected genes of zebrafish showed that only adenosine A1 receptors had developmental differences along the evaluated period for the control group. In mammals, a transient expression of purinergic receptors through development was described and appears to be common between purinergic receptors (Cheung and Burnstock, 2002; Cheung et al., 2003, 2005; Crain et al., 2009). In several brain areas of rodents, the expression of A1 adenosine receptor is possible from early embryonic days such as embryonic day 14 (Ådén et al., 2000). During embryonic and post-natal life, the expression of A1 adenosine receptors also increases reaching the adult pattern in some brain areas at embryonic day 21 (Ådén et al., 2000).

Considering that zebrafish embryos offer the possibility to evaluate early stages inaccessible in mammal studies, they appear as a good tool to evaluate common drugs, such as caffeine, used even in periods of pregnancy. Here, we observed that zebrafish embryos at 72 hpf produce a compensatory adjustment of adenosine receptors expression after their block by caffeine. Up-regulation of adenosine receptor after the exposure to an antagonist is a known event described to occur in low intensities in a variety of species (Ådén et al., 2000; Léon et al., 2002). In zebrafish embryos, the effects on studied gene expression appear to be selective to A1 and A2A1 adenosine receptors probably by the high affinity of these receptors for caffeine

(Fredholm et al., 1999). However, A2A adenosine receptors are the highest affinity receptors for caffeine in rodents and humans, while in zebrafish we do not have this information. Additionally, zebrafish have two clones of A2A adenosine receptor with high similarity to the human A2A adenosine receptor (Boehmler et al., 2009). In our study, these clones presented a different response to caffeine exposure, which could be an indication of differential potency of caffeine to block these receptors. The lack of effects in A2B adenosine receptor expression after caffeine exposure could indicate a similarity to A2B adenosine receptor from rodents and humans, for which caffeine shows a low potency to block (Fredholm et al., 1999).

Now we turn to a component of adenosine intracellular pathway, the DARPP-32, which was also investigated on the base of caffeine exposure in embryos of zebrafish. DARPP-32 is known to be a multifunctional regulator of protein kinases and phosphatases targets of a variety of drugs such as alcohol, nicotine and caffeine (Svenningsson et al., 2005). This study is one of the first studies on DARPP-32 expression in zebrafish. The increase of DARPP-32 expression after caffeine treatment is complementary to adenosine receptors up-regulation. However, this effect should receive more attention since DARPP-32 activation is shared by other systems of cell communication such as serotoninergic, glutamatergic and dopaminergic systems that could be affected by caffeine effects on DARPP-32 up-regulation. In fact, there is a growing body of evidence showing that caffeine biochemical effects could be involved in drug addiction, since that caffeine pre-exposure seems to increase the sensibility to nicotine, methylphenidates, opiates and cocaine, a mechanism that seems to involve DARPP-32 (Horger et al., 1991; Schenk et al., 1994; Shoaib et al., 1999; Ribeiro et al., 2003; Boeck et al., 2009).

As one of the major contributors for the correct development, especially to survival and growth of neurons, BDNF gene expression was also verified in this study. In rodents, it was shown that caffeine stimulates CREB (Ca^{2+}/cAMP response element binding)-dependent transcription genes, especially BDNF (Connolly and Kingsbury, 2010). Here, we observed that caffeine was also able to increase BDNF expression of caffeine-treated animals. While we did not find any gross body defects in zebrafish with augment of BDNF expression, the reduction of BDNF protein has been suggested to be one of the most contributors for developmental defects in zebrafish model of Huntington's disease (Diekmann et al., 2009).

Regarding to morphological analysis, we did not observe differences on body length of caffeine-treated zebrafish embryos that could be a result of the caffeine concentration used in our experiments. It has been reported that zebrafish embryos exposed to caffeine have decreased heart rate, an effect delayed by adenosine and partially performed by antagonist of adenosine receptor (Rana et al., 2010). Zebrafish exposed to high caffeine concentrations leads to shorter body length as well as dose and time dependent disorganized muscle fibers alignment (Chen et al., 2008; Boehmler et al., 2009).

Considering that subtle defects could have occurred and were not detected, we also observed the response of caffeine-treated zebrafish embryos to a tactile stimulus. However, we did not detect differences between control and caffeine-treated zebrafish groups in our experimental conditions. Effects on tactile sensibility have been demonstrated in high caffeine concentration exposure and can be associated to malformation of muscle fiber alignment (Chen et al., 2008).

5. Conclusion

In conclusion, adenosine receptors, DARPP-32 and BDNF are early expressed in zebrafish and are sensitive to caffeine exposure. The up-regulation of direct and indirect adenosine targets could be related to a compensatory mechanism that promotes the adenosinergic tonus during developmental phase, keeping the normal development of body length and response to stimulus. Such information contributes to the investigation about the participation and susceptibility of the adenosinergic system during early development.

Conflict of interest statement

No conflicts of interest exist.

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References

Åden U, Leverin AN, Hagberg H, Fredholm BB. Adenosine A1 receptors in the immature rat brain and heart. Eur J Pharmacol 2001;426:185–92.
Ådén U, Herlenius E, Tang L-Q, Fredholm BB. Maternal caffeine intake has minor effects on adenosine receptor ontogeny in the rat brain. Pediatr Res 2000;48(2):177–83.
Ådén U, Halldner L, Lagercrantz H, Dalmau I, Ledent C, Fredholm BB. Aggravated brain damage after hypoxic ischemia in immature adenosine A2A knockout mice. Stroke 2003;34:739–44.
Boeck CR, Marques VB, Valvassori SS, Constantino LC, Rosa DV, Lima FF, et al. Early long-term exposure with caffeine induces cross-sensitization to methylphenidate with involvement of DARPP-32 in adulthood of rats. Neurochem Int 2009;55:318–22.
Boehmler W, Petko J, Woll M, Frey C, Thisse B, Thisse C, et al. Identification of zebrafish A2 adenosine receptors and expression in developing embryos. Gene Expression Paterns 2009;9:144–51.
Burnstock G. Purine and pyrimidine receptors. Cell Mol Life Sci 2007;64:1471–83.
CARE Study Group. Maternal caffeine intake during pregnancy and risk of fetal growth restriction: a large prospective observational study. BMJ 2008;337:a2332.
Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, et al. A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. J Neurosci 1999;19:9192–200.
Chen YH, Huang YH, Wen CC, Wang YH, Chen WL, Chen LC, et al. Movement disorder and neuromuscular change in zebrafish embryos after exposure to caffeine. Neurotoxicol Teratol 2008;30:440–7.
Cheung KK, Burnstock G. Localization of P2X3 receptors and coexpression with P2X2 receptors during rat embryonic neurogenesis. J Comp Neurol 2002;18:368–82.
Cheung KK, Ryten M, Burnstock G. Abundant and dynamic expression of G protein-coupled P2y receptors in mammalian development. Dev Dyn 2003;228:254–66.
Cheung KK, Chan WY, Burnstock G. Expression of P2X purinoceptors during rat brain development and their inhibitory role on motor axon outgrowth in neural tube explant cultures. Neuroscience 2005;133:937–45.
Connolly S, Kingsbury TJ. Caffeine modulates CREB-dependent gene expression in developing cortical neurons. Biochem Biophys Res Commun 2010;397(2):152–6.
Crain JM, Nikodemova M, Watters JJ. Expression of P2 nucleotide receptors varies with age and sex in murine brain microglia. J Neuroinflammation 2009;6(24):1-16.
Diekmann H, Anichtchik O, Fleming A, Futter M, Goldsmith P, Roach A, et al. Decreased BDNF levels are a major contributor to the embryonic phenotype of huntingtin knockdown zebrafish. J Neurosci 2009;29(5):1343–9.
Fredholm BB, Svenningsson P. Adenosine–dopamine interactions: development of a concept and some comments on therapeutic possibilities. Neurology 2003;61:S5–9.
Fredholm BB, Bättig K, Holmén J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 1999;51(1):83-133.
Horger BA, Wellman PJ, Morien A, Davies BT, Schenk S. Caffeine exposure sensitizes rats to the reinforcing effects of cocaine. Neuroreport 1991;2:53–6.
Léon D, Albasanz JL, Ruíz MA, Fernadéz M, Martín M. Adenosine A1 receptor down-regulation in mothers and fetal brain after caffeine and theophylline treatments to pregnant rats. J Neurochem 2002;82:625–34.
Linden J. Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. Annu Rev Pharmacol Toxicol 2001;41:775–87.
Momoi N, Tinney JP, Liu LJ, Elshershari H, Hoffmann PJ, Ralphe JC, et al. Modest maternal caffeine exposure affects developing embryonic cardiovascular function and growth. Am J Physiol Heart Circ Physiol 2008;294:H2248–56.
Monopoli A, Lozza G, Forlani A, Mattarelli A, Ongini E. Blockade of adenosine A2A receptors by SCH 58261 results in neuroprotective effects in cerebral ischaemia in rats. Neuroreport 1998;9:3955–9.
Rana N, Moond M, Marthi A, Bapatla S, Sarvepalli T, Chatti K, et al. Caffeine-induced effects on heart rate in zebrafish embryos and possible mechanisms of action: an effective system for experiments in chemical biology. Zebrafish 2010;7(1):69–81.
Ribeiro JA, Sebastião AM, Mendonça A. Participation of adenosine receptors in neuroprotection. Drugs News Perspect 2003;16:80–6.
Rivkees SA, Zhao Z, Porter G, Turner C. Influences of adenosine on the fetus and newborn. Mol Genet Metab 2001;74:160–71.
Rosemberg DB, Rico EP, Guidoti MR, Dias RD, Souza DO, Bonan CD, et al. Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (*Danio rerio*). Life Sci 2007;81(21–22):1526–34.
Rudolphi KA, Schubert P, Parkinson FE, Fredholm BB. Neuroprotective role of adenosine in cerebral ischaemia. Trends Pharmacol Sci 1992;13:439–45.

Schenk S, Valadez A, Horger BA, Snow S, Wellman PJ. Interactions between caffeine and cocaine in tests of self-administration. Behav Pharmacol 1994;5:153–8.
Selderslaghs IW, Van Rompay AR, De Coen W, Witters HE. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. Reprod Toxicol 2009;28(3):308–20.
Shoaib M, Swanner LS, Yasar S, Goldberg SR. Chronic caffeine exposure potentiates nicotine self-administration in rats. Psychopharmacology (Berl) 1999;142:327–33.
Svenningsson P, Nairn AC, Greengard P. DARPP-32 mediates the actions of multiple drugs of abuse. AAPS J 2005;7:E353–60.
Tye K, Pollard I, Karlsson L, Scheibner V, Tye G. Caffeine exposure in utero increases the incidence of apnea in adult rats. Reprod Toxicol 1993;7:449–52.
Wilkinson JM, Pollard I. In utero exposure to caffeine causes delayed neural tube closure in rat embryos. Teratog Carcinog Mutagen 1994;14(5):205–11.
Zhao Z, Rivkees SA. Inhibition of cell proliferation in the embryonic myocardium by A1 adenosine receptor activation. Dev Dyn 2001;221:194–200.