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Prenatal caffeine intake differently affects synaptic proteins during fetal brain development

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ABSTRACT

Caffeine is the psychostimulant most consumed worldwide. However, little is known about its effects during fetal brain development. In this study, adult female Wistar rats received caffeine in drinking water (0.1, 0.3 and 1.0 g/L) during the active cycle in weekdays, two weeks before mating and throughout pregnancy. Cerebral cortex and hippocampus from embryonic stages 18 or 20 (E18 or E20, respectively) were collected for immunodetection of the following synaptic proteins: brain-derived neurotrophic factor (BDNF), TrkB receptor, Sonic Hedgehog (Shh), Growth Associated Protein 43 (GAP-43) and Synaptosomal-associated Protein 25 (SNAP-25). Besides, the estimation of NeuN-stained nuclei (mature neurons) and non-neuronal nuclei was verified in both brain regions and embryonic periods. Caffeine (1.0 g/L) decreased the body weight of embryos at E20. Cortical BDNF at E18 was decreased by caffeine (1.0 g/L), while it increased at E20, with no major effects on TrkB receptors. In the hippocampus, caffeine decreased TrkB receptor only at E18, with no effects on BDNF. Moderate and high doses of caffeine promoted an increase in *Shh* in both brain regions at E18, and in the hippocampus at E20. Caffeine (0.3 g/L) decreased GAP-43 only in the hippocampus at E18. The NeuN-stained nuclei increased in the cortex at E20 by lower dose and in the hippocampus at E18 by moderate dose. Our data revealed that caffeine transitorily affect synaptic proteins during fetal brain development. The increased number of NeuN-stained nuclei by prenatal caffeine suggests a possible acceleration of the telencephalon maturation. Although some modifications in the synaptic proteins were transient, our data suggest that caffeine even in lower doses may alter the fetal brain development.

1. Introduction

Caffeine is one of the most consumed psychostimulant present in our diet in drinks like coffee, tea and cola soft drinks. The pharmacological actions of caffeine consist in blocking adenosine actions, a nucleoside that acts as a neuromodulator in the central nervous system (CNS). Adenosine controls neurotransmitter release, neuronal excitability and circadian rhythm through its metabotropic receptors A_1, A_{2A}, A_{2B} and A_3 (Cunha, 2001; Fredholm et al., 2005). The major effect of caffeine at the concentrations relevant to daily intake is the antagonism of A_1 and A_{2A} receptors (Fredholm et al., 2005). It is well documented the beneficial effects of caffeine on cognitive functions in different ages in adult animals (Angelucci et al., 1999; Costa et al., 2008a; for review see Cunha and Agostinho, 2010). Chronic caffeine prevents memory disturbances associated with aging (Arendash et al., 2006; Costa et al., 2008b; Prediger et al., 2005; Sallaberry et al., 2013) and experimental models of Alzheimer's disease (Arendash et al., 2009; Dall'Igna et al., 2007; Espinosa et al., 2013).

Regarding effects of caffeine during brain development epidemiological studies have associated caffeine intake and birth outcomes, such as low birth weight, intrauterine growth retardation and miscarriage (Brent et al., 2011; Bakker et al., 2010; Giannelli et al., 2003). However, the effects of caffeine intake during fetal and even postnatal brain development have been poorly investigated (for review see Porciúncula et al., 2013; Temple, 2009). Caffeine enters into all tissues of the fetus (Bracken et al., 2003) since it can easily cross biological membranes including the placental barrier and fetal brain (Arnaud, 1987). Some experimental studies in rodents revealed that caffeine administered during

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pregnancy reduced the number of somites and extent of the neural tube in the post-implantation embryo, suggesting intrauterine growth retardation (Jacombs et al., 1999). Besides, caffeine in pregnant females decreased the immunocontent of metabotropic glutamate receptors of group I in the brain in fetuses and pregnant rats (León et al., 2005), and also influenced serotonergic system in chick embryo, increasing the contents of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) (Li et al., 2012). A recent study assessing the effect of moderate caffeine intake during pregnancy on mice pups resulted in delayed migration of cortical γ-aminobutyric acid (GABA) neurons, an increased susceptibility to seizures, and deficits in spatial memory when assessed in adulthood (Silva et al., 2013).

Since caffeine is a non-selective antagonist of A_1 and A_{2A} adenosine receptors and coupling of these receptors to G-proteins occurs postnatally in cortex and hippocampus (Adén et al., 2000; Gaytan et al., 2006), most experimental studies with caffeine during brain development have investigated the impact of caffeine on these receptors at postnatal stages or during adulthood (Adén et al., 2000; Gaytan and Pasaro, 2012; Guillet and Kellogg, 1991). However, it remains to be investigated if the patterns of the development of synapses during intrauterine can be modified by caffeine intake. The effects of caffeine administration on proteins, hormones, signaling pathways and especially neurotrophic factors that regulate the development of the CNS during intrauterine life are still little investigated. Thus, our study aimed to evaluate the effects of administration of different doses of caffeine during pregnancy on proteins involved in the formation and maturation of synapses, namely: (i) the neurotrophin brain-derived neurotrophic factor (BDNF) and its receptor TrkB; (ii) two components of synapse (axons and nerve terminals): Growth Associated Protein 43 (GAP43) and Synaptosomal-associated protein 25 (SNAP-25); (iii) the major morphogen agent Sonic hedgehog (*Shh*). In addition, an estimation of the number of neuronal and non-neuronal cells was assessed in the cortex and hippocampus in fetuses from embryonic state 18 and 20 (E 18 and E 20) exposed to caffeine throughout intrauterine life.

2. Materials and methods

2.1. Animals and caffeine treatment

Female Wistar rats (2 months old) were obtained from the Central Animal House of our Department. They were maintained in standard cages under a standard dark–light cycle (lights on between 7 A.M. and 7 P.M.), at a room temperature of 22 ± 2 °C. All experimental procedures were performed according to the Ethical committee of Universidade Federal do Rio Grande do Sul (Proc. N° 20332) in compliance with Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care.

Adult female Wistar rats were treated with caffeine in drinking water (0.1, 0.3 and 1.0 g/L, corresponding to low, moderate and high intake, respectively) (Fredholm et al., 1999), during the active period (dark cycle) in weekdays, two weeks before mating. Although 1 mg/mL of caffeine is considered high consumption of caffeine, it yielded a plasma concentration of less than 60 μM in adult rats (Costenla et al., 2010; Duarte et al., 2009). At the beginning of light cycle all experimental groups received tap water. The presence of vaginal plug was considered as embryonic day 0 (E0). The same caffeine treatment continued during pregnancy up to embryonic days 18 or 20 (E18 or E20, respectively), when the dams were sacrificed under anesthesia. The fetuses were weighed and whole cortex and hippocampus were immediately dissected out at 4 °C for Western Blot analysis; or the whole brain were immersed in 4% paraformaldehyde in 0.1 M PBS for fixation of the brain tissue to determine total number of neuronal and non-neuronal cells in cortex and hippocampus by the isotropic fractionator. The litter size and number of implantations were also analyzed.

The moderate dose of 0.3 g/L caffeine was chosen considering the plasma concentration of caffeine in rat dams being this dose similar to that found in blood of human mothers drinking three to four cups of coffee per day (Adén et al., 2000). The other two concentrations were chosen to reach approximately 3 times lower and 3 times higher concentrations comparing with the moderate dose (0.1 g/L and 1.0 g/L, respectively), in order to have a wide range of caffeine intake. Female rats received caffeine solutions only during the dark cycle, which is their active period, to mimic the pattern of caffeine consumption in humans. During the light cycle, caffeine solutions were replaced by water *ad libitum*. All experimental groups consumed similar amounts of caffeinated-water and drinking water. Although we did not quantify the plasma levels of caffeine, the chosen treatment regimens are thought to correspond to a low, moderate and high caffeine intake in humans, with effects believed to be mainly operated through antagonism of adenosine receptors (Fredholm et al., 1999).

2.2. SDS-PAGE (sodium dodecyl sulfate-polyacrilamide) immunoblotting

Hippocampus and whole cortex were dissected out from E18 or E20 (*pool* of two fetuses per litter, considering one litter as one subject), were immediately homogenized in 5% SDS with a protease and phosphatase inhibitor cocktail (Sigma, São Paulo/Brazil) and frozen at −70 °C. After defrost, the protein content was determined by Bicinchoninic acid assay using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). Sample extracts were diluted to a final protein concentration of 2 μg/μL in SDS-PAGE buffer. Aliquots corresponding to 20–40 μg protein for hippocampus and whole cortex samples were separated by SDS-PAGE (12% running gel with a 4% concentrating gel) and electro-transferred to nitrocellulose membranes. After blocking with Tris-buffered saline 0.1% Tween-20 (TBS-T) containing 3% bovine serum albumin for 1 h, the membranes were incubated for 24 hours at 4 °C with the following primary antibodies: mouse anti-BDNF (1:500, Sigma, São Paulo/Brazil); rabbit anti-TrkB (1:1000, Abcam, São Paulo/Brasil); rabbit anti-GAP-43 (1:2000, Sigma, São Paulo/Brazil); mouse anti-Shh (1:500, Abcam, São Paulo/Brazil); or rabbit anti-SNAP-25 (1:1000, Sigma, São Paulo/Brazil). The membranes were then washed with TBS-T, incubated with horseradish peroxidase conjugated secondary antibody for 1 h at room temperature and developed with chemiluminescence ECL kit (Amersham, São Paulo/Brazil). Membranes were re-probed for β-tubulin immunoreactivity. The autoradiographic films were scanned and densitometric analyses were performed using public domain NIH Image Program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Band intensity was normalized to β-tubulin as a loading control to assess protein levels. The data used in statistical analysis were obtained from the ratio of the protein studied and β-tubulin density unit lines.

2.3. Quantification of neuronal and non-neuronal nuclei

We used the isotropic fractionator method to estimate total number of cells and neurons, as previously described (Herculano-Houzel and Lent, 2005). Whole cortex and hippocampus from the fixed brain were weighed and mechanically dissociated in a saline solution with 0.1% Triton X-100 and turned into an isotropic suspension of isolated nuclei. The total number of nuclei in suspension – and therefore the total number of cells in the original tissue – was estimated by determining the density of nuclei in small aliquots stained with the fluorescent DNA marker DAPI (4,6-diamidino-2-phenylindole dihydrochloride), under the microscope with a 40× objective, using a hemocytometer for quantification. Neuronal nuclei from an aliquot of the suspension were selectively immunolabeled overnight, at room temperature, with mouse monoclonal anti-NeuN antibody (Millipore, MAB377) at a dilution of 1:200 in PBS. After washing the nuclei in PBS, they were incubated for 2 h at room temperature with AlexaFluor 488 anti-mouse IgG secondary antibody (Invitrogen, Biogen São Paulo/Brazil), at a dilution of 1:500 in PBS in the presence of 10% normal goat serum. The neuronal fraction in each sample was estimated by counting NeuN-labeled nuclei in at least 500 DAPI-stained nuclei. Image analysis and quantification were manually performed and also confirmed by using software from Nikon Eclipse E600. The results were presented as number of cells per mg tissue.

2.4. Statistical analysis

Statistical analysis was performed by using One-way ANOVA and Newman–Keuls *post hoc* test. The statistical significance was considered for $P < 0.05$.

3. Results

3.1. Reproductive data

The litter size and the number of implantations were not affected by chronic caffeine, as shown in Table 1. The fetal weight was decreased by 16% in embryos (E20) from dams that received caffeine at the highest dose (1.0 g/L) (Table 1).

3.2. The impact of different caffeine doses during pregnancy on BDNF signaling and on important proteins for synaptic development

Prenatal caffeine intake differently affected proteins crucial to fetal brain development in two distinct embryonic stages in whole cortex and hippocampus. At E18 in hippocampus, high caffeine