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MATERNAL CAFFEINE INGESTION DURING GESTATION AND LACTATION INFLUENCES RESPIRATORY ADAPTATION TO ACUTE ALVEOLAR HYPOXIA IN NEWBORN RATS AND ADENOSINE A_{2A} AND $GABA_A$ RECEPTOR mRNA TRANSCRIPTION

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Abstract—Caffeine is a widely used psychostimulant freely crossing the placental barrier. At the doses usually absorbed, it acts as an antagonist of both A_1 and A_{2A} adenosine receptors. Pregnant women are generally not advised to limit their caffeine consumption and thus expose their progeny to the drug during the whole of gestation and lactation. The possibility that such caffeine exposure may have long-term consequences on brain development has led to several behavioral investigations on animal models. Despite the crucial role played by adenosine receptor systems in neonatal breathing control, few studies *in vitro* have been concerned with the consequences of maternal caffeine absorption on breathing, and none in the unrestrained intact animal. The present investigation analyzed the influence of caffeine exposure via placental and milk transfer on resting ventilation and on the response to moderate alveolar hypoxia of 0 to 2-day-old newborn rat (P0–P2) together with the possible underlying mechanisms. Dams absorbed caffeine (46±3 mg/kg/day) via drinking fluid (0.2 g/L) throughout gestation, in conditions mimicking moderate human consumption. Caffeine exposure did not significantly affect basal respiratory parameters. In contrast, it attenuated both the early increase and the secondary decrease in ventilation triggered by moderate alveolar hypoxia (11% O_2 inhaled). The abolition of Fos protein expression evoked by hypoxia suggested that caffeine exposure may decrease the activity of O_2-sensing peripheral chemoreceptor pathway. From real-time PCR data, those functional alterations were associated to increases in A_{2A} adenosine receptor and $\alpha 2$ $GABA_A$ receptor subunit mRNAs in the medulla. This indicates that, even at moderate doses, maternal caffeine consumption may induce a series of subtle developmental alterations that may affect modulation of breathing control in the neonate in pathological situations such hypoxia.

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Abbreviations: CAF, newborns exposed to caffeine; CGVL, ventrolateral divisions of the central gray; CT, cycle threshold; LC, locus coeruleus; NTSc, nucleus tractus solitarius; PB, parabrachial area; rRNA, ribosomal RNA; VMS, ventral medullary surface; V_T, tidal volume.

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It is commonly acknowledged that caffeine is the most widely consumed psycho-active drug, mainly used in the adult as a general stimulant and secondarily for its analgesic and catabolic properties (Nehlig et al., 1992). Pregnant women are generally not warned against possible risks of caffeine consumption although it freely crosses placental and blood–brain barriers and thus accumulates in the fetal nervous system at critical periods of development (Tanaka et al., 1984). In fact, the relation between maternal caffeine consumption and infant development is still a matter of debate. Notably, several clinical investigations concluded that prenatal caffeine exposure may be associated with a higher risk of intra-uterine growth retardation and sudden infant death, this effect being more apparent in infants from women who smoked (Ford et al., 1998; Klebanoff et al., 2002).

The impact of long-term caffeine and other methylxanthines of therapeutic interest on newborn physiology has been investigated in animal models, together with the mechanisms involved, to overcome confounding environmental and social variables encountered in clinical studies on substance abuse. Rodent models of both neonatal therapy for apnea prevention and prenatal exposure revealed that caffeine has protracted consequences on higher brain function (Guillet, 1990; Nehlig and Debry, 1994; Guillet and Dunham, 1995; Fisher and Guillet, 1997; Tchekalarova et al., 2005, 2007).

Most studies on the underlying mechanisms have focused on the deregulation of adenosinergic systems. Indeed, at doses relevant to daily caffeine intake in humans, the antagonism of endogenous adenosine at A_1 and A_{2A} receptors may be the only mechanism of action of caffeine (Fredholm et al., 1999). Moreover, although both receptor subtypes develop at different rates, they are expressed early in rat ontogeny (E14) (Weaver, 1993, 1996; Aden et al., 2000) and they considerably increase in number and efficacy after birth (Marangos et al., 1984; Johansson et al., 1997).

Surprisingly, few studies have been concerned with the developmental consequences of caffeine exposure on breathing until recently, although the major contribution of adenosine to neonatal breathing has long been demonstrated (Runold et al., 1986). Prenatal caffeine dose-dependently increased the incidence of apnea in adult rats

(Tye et al., 1993), while neonatal caffeine affected both resting ventilation and hypercapnic chemoreflex, in males only (Montandon et al., 2006). The latter alterations coincide with a disruption of adenosinergic modulation of breathing mediated by both A_1 and A_{2A} receptors (Montandon et al., 2007). Investigations have also been conducted on brainstem–spinal cord preparations of variable rostro-caudal extent, to assess the impact of prenatal caffeine exposure on the activity of the network responsible for respiratory rhythmogenesis in the neonate. They did show respiratory alterations, but led to different conclusions as regards the possible deregulation of A_1 receptor expression, suggesting that caffeine may interfere with the development of adenosinergic systems at multiple sites (Herlenius et al., 2002; Bodineau et al., 2003; Saadani-Makki et al., 2004). Furthermore, there is evidence that chronic caffeine consumption affects the number not only of target adenosine receptors but also of benzodiazepine binding sites, although these data are somewhat controversial (Nehlig et al., 1992). This raises the issue of the consequences of caffeine exposure via maternal consumption in the intact animal and the molecular mechanisms involved.

Hence, we tested the hypothesis that maternal caffeine ingestion may influence the basal respiratory pattern, the response to moderate alveolar hypoxia and the development of adenosine and benzodiazepine receptor systems in unrestrained 0 to 2-day-old newborn rats (P0–P2). Moderate hypoxia is a common consequence of apneas and bradypnea in neonates, especially when they are premature (Martin and Abu-Shaweesh, 2005). When it is sustained, hypoxia evokes a biphasic response consisting of an initial hyperventilation triggered by the activation of peripheral O_2-sensing chemoreceptors followed by a respiratory depression triggered by central mechanisms. The secondary depression is dependent on adenosinergic modulation and its importance in the newborn reflects the immaturity of breathing control systems (Simakajornboon and Kuptanon, 2005). In parallel to measurements of respiratory parameters, immunohistochemical detection of Fos, the protein product of the immediate early gene *c-fos*, has been conducted to define more precisely the impact of caffeine exposure on neuronal activity within the brainstem relays of breathing control pathways in both resting and hypoxic conditions. Indeed, as a metabolic marker, the accumulation of Fos in neuronal nuclei previously led to detailed mapping of neuronal populations engaged in the adaptation to hypoxia in the developing mammal (White et al., 1994; Breen et al., 1997; Nitsos and Walker, 1999; Horn et al., 2000; Berquin et al., 2000; Bodineau et al., 2001; Voituron et al., 2006). Finally, quantitative real-time polymerase chain reaction has been used to monitor the transcription levels of genes encoding for not only A_1 and A_{2A} adenosine receptors but also α1 and α2 subunits of the $GABA_A$ receptor. These subunits are the most abundant constituent of the benzodiazepine binding site in the CNS (McKernan and Whiting, 1996) and they exhibit development-dependent expression throughout areas of the brain (Laurie et al., 1992).

EXPERIMENTAL PROCEDURES

Ventilatory variables, Fos protein expression and receptor gene expression were studied in separate groups of 0- to 2-day-old Sprague–Dawley rats born in our local animal care facility, with the approval of the University Council. Adequate measures were taken to minimize pain or discomfort and to limit the numbers of animals used, in accordance with the European Communities Council Directive (86/609/EEC).

Maternal caffeine administration

To mimic the most common situation in humans, caffeine was delivered to dams in drinking water (Sigma-Aldrich, France; 0.2 g/L) from the mating day onwards, according to previous studies (Bodineau et al., 2003; Saadani-Makki et al., 2004; Bodineau et al., 2006). Control dams received drug-free tap water. The drinking fluids were exchanged every 2 days and individual daily water intakes were calculated from the second week of gestation onwards.

Ventilation and temperature measurements

Breathing frequency (f), tidal volume (V_T) and minute ventilation ($\dot{V}_E$) were measured in unrestrained newborns by whole body plethysmography using routine procedures (Cayetanot et al., 2001). The 60 mL recording chamber was maintained at temperatures close to thermoneutrality (32–33 °C) (Mortola and Dotta, 1992). Newborns were kept in the chamber for a maximum of 75 min, to avoid stress related to isolation or dehydration, and were returned to the dam at the end of the experiments.

The chamber was ventilated with either humidified air (control normoxia) or a calibrated mixture of 11% O_2 in N_2 for hypoxic challenges. The chamber was sealed every 5 min for 20–30 s recording runs. The signals related to cyclic pressure changes induced by animal's ventilation and by injection of air for volume calibration (25 μL) were amplified, digitized and stored for off-line analysis via Spike 2 data acquisition system (Cambridge Electronic Design, Cambridge, UK). At every time point, the respiratory parameters were averaged from a series of at least 6 to 10 respiratory cycles. Pressure changes were converted to V_T according to Bartlett and Tenney (1970). As variables to be included to the barometric equation, atmospheric pressure at the time of experiment was monitored from local weather report and the water vapor pressures at the chamber and body temperatures were calculated. Rectal temperature was monitored via a flexible implantable thermoprobe (outer diameter 0.6 mm) left in place throughout the recording schedule in separate subsets of newborn rats, maintained under the conditions used for ventilation measurements (duration, temperature, O_2 fraction inhaled).

Fos protein expression evoked by caffeine exposure and/or hypoxia: immunohistochemistry and quantification

Fos immunohistochemistry was conducted on transverse brainstem sections (40 μm-thick) from newborn rats exposed or not to caffeine *in utero*, under normoxia or following 2 h under hypoxia (11% O_2 in N_2). The procedures of stimulation, kill and Fos detection were the same as those previously reported (Berquin et al., 2000). Briefly, rat pups were kept in the nest with their dam throughout stimulation to minimize unspecific Fos expression related to novelty or stress reactions. Pups used for quantifying basal Fos expression under the current environmental conditions were removed and killed just before starting hypoxic stimulation, routinely delivered between 14:00 and 17:00 h. Newborns were immediately removed for kill at the end of the stimulation period.

The animals were killed with an overdose of sodium pentobarbital (100 mg/kg i.p.). They were then transcardially perfused