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Neonatal Caffeine Administration Causes a Permanent Increase in the Dendritic Length of Prefrontal Cortical Neurons of Rats

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KEY WORDS caffeine; neonatal; dendrites; Golgi-Cox stain; prefrontal cortex; pyramidal neurons

ABSTRACT We studied the morphological changes of the dendritic length of the pyramidal neurons of the prefrontal cortex (PFC) induced by the effect of chronic administration of caffeine in the neonatal rat. The caffeine (50 mg/kg, s.c.) was injected from day 1 after birth (P1) to day 12 (P12). The morphology of the pyramidal neurons of layer 3 of the PFC was investigated in these animals at two different ages, before puberty (P35) and after puberty (P70). Before the animals were sacrificed by using overdoses of sodium pentobarbital and being perfused intracardially with 0.9% saline, the locomotor activity in a novel environment was measured. The brains were then removed, processed by the Golgi-Cox stain, and analyzed by the Sholl method. The dendritic morphology clearly showed that the neonatal animals administered caffeine showed an increase in the dendritic length of the pyramidal neurons of the PFC when compared with the control animals at both ages. The present results suggest that neonatal administration of caffeine may in part affect the dendritic morphology of the pyramidal cells of this limbic structure and this effect persists after puberty and may be implicated in several brain processes. **Synapse 60:450–455, 2006.**

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid present in high concentrations in tea and coffee and also found in a number of beverages such as coca cola. Coffee is a beverage known all over the world with millions of people drinking it every day. A significant proportion of the effects of coffee are related to the actions of caffeine, the best-known pharmacologically active constituent of coffee. The reasons for people to consume caffeine are manifold. In addition, an important source of caffeine for children includes chocolate and soft drinks. Most caffeine is consumed at home, with the second preferred place of consumption at work. Especially at work, coffee is considered a pleasant occasion to break working hours (D'Amicis and Viani, 1993). This alkaloid is a behavioral stimulant. In the last decade, several reports have noted the beneficial effects of caffeine (for reviews see Fredholm and Svenningsson, 2003; Lorist and Tops, 2003), i.e., caffeine has demonstrated a neuroprotective effect in Parkinson disease (for review see Fredholm and Svenningsson, 2003). Evidence has shown the effects of caffeine on human cognition (for review see Lorist and Tops, 2003). The reports suggest that the effect of caffeine on cognition is because it increases attention (for review see Lorist and Tops, 2003), whereas EEG studies support the hypothesis that caffeine only acts as a stimulant (Siepmann and Kirch, 2002).

Attention shifting in the working memory system plays an important role in goal-oriented behavior (reading, reasoning, and driving) because it involves several cognitive processes, which may be regulated by the prefrontal cortex (PFC) (for reviews see Burke and Barnes, 2006; Naghavi and Nyberg 2005). The develop-

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ment of the PFC plays a critical role in the cognitive function, attention (Fuster, 2002). The role of the PFC in cognition is well-recognized when its connections are analyzed. The PFC is interconnected via glutamatergic projections (Goldman-Rakic et al., 1984) with the hippocampus and with other limbic cortexes via intracortical projections (Goldman-Rakic et al., 1984; Jay and Witter, 1991). Hippocampal neuronal activity exerts an important regulatory control on the layer-3 pyramidal neurons of the PFC (Finch et al., 1983; Jay and Witter, 1991; Lewis and Anderson, 1995; Sesack and Pickel, 1992; Sesack et al., 1989). The hippocampal to PFC synapses are modifiable synapses and may express different forms of plasticity in all cognitive processes (for review see Fuster, 2002).

The effect of caffeine consumption during pregnancy is under discussion (for review see Signorello and McLaughlin, 2004). Some epidemiologic studies have not observed association between caffeine intake during pregnancy and a risk factor for the fetus (for review see Signorello and McLaughlin, 2004). The effects of caffeine in the neonatal human or animal brain are unknown. We posed the question of whether caffeine exerts a positive or negative effect on the brain during development, especially on the pyramidal neurons of the PFC, a region related to the cognitive function. Our investigation was designed to assess whether the neonatal caffeine administration affects the dendritic length and spine density on pyramidal neurons of layer-3 of the PFC at both pre- and postpubertal ages. The results suggest an interesting and persistent effect of the neonatal administration of caffeine on these pyramidal cells.

MATERIALS AND METHODS

Pregnant Sprague–Dawley rats were obtained at gestational day 14–17 from our facilities (University of Puebla). Animals were individually housed in a temperature- and humidity-controlled environment on a 12–12 h light–dark cycle, with free access to food and water. The day following birth, litters with six male pups were grouped and each pup was assigned to either a caffeine (dissolved in 0.9% NaCl solution) administration (50 mg/kg s.c.) or saline administration (control). The caffeine or saline solution was injected every day in the morning (0800–0900) for 12 days. All experimental procedures were approved by the BUAP Animal Care Committee and met governmental guidelines (Mexican Council for Animal Care, Norma Oficial Mexicana NOM-062-ZOO-1999). All efforts were made to minimize animal suffering and to reduce the number of animals used. On P21, animals were weaned and grouped 2–3 animals per cage.

Three weeks (P35, $n = 8$–9 animals per group) and 8 weeks (P70; $n = 8$–9 animals per group) after finishing the caffeine injections, spontaneous locomotor activity of saline-control and caffeine administration rats was assessed for 60 min in 8-photocell activity boxes ($20 \times 40 \times 30$ cm^3) connected to a computer to count beam crosses (Tecnologia Digital, Mexico). All animals were tested between 0800 and 1000. Immediately after the activity measurements, the rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, ip) and perfused intracardially with 0.9% saline. The brains were removed and were processed with modified Golgi-Cox staining using procedures described by Gibb and Kolb (1998). The brains were first stored in the dark for 14 days in Golgi-Cox solution, then 3 days in 30% sucrose. Sections of 200-μm thickness in the coronal plane at the level of the PFC were obtained using a vibratome (Camden Instrument, MA752, Leicester, England). Sections were collected on cleaned, gelatin-coated microscope slides (four sections/slide) and stained with ammonium hydroxide for 30 min. They were immersed in Kodak Film Fixer for another 30 min, and then washed with water, dehydrated, cleared, and mounted using a resinous medium.

The Golgi-impregnated pyramidal neurons of layer-3 of the PFC (area Cg1 and 3, plate 7–9 of Paxinos and Watson, 1986, corresponding to Cg1 and prelimbic cortex of Paxinos and Watson, 1998) were readily identified by their characteristic triangular soma-shape, apical dendrites extending toward the pial surface, and numerous dendritic spines. The criteria used to select neurons for reconstruction was essentially as described previously (Flores et al., 2005; Martinez-Tellez et al., 2005; Silva-Gómez et al., 2003; Vega et al., 2004). Five neurons in each hemisphere (10 neurons per animal) were drawn using camera lucida at a magnification of 250× (DMLS, Leica Microscope) by a person blind to treatment conditions. Although the Golgi-Cox procedure has been extensively used to determine dendritic morphology, a caveat of the method is that only a small number of neurons are impregnated with the stain and that some spines hidden behind the dendritic shafts may not be counted. Nevertheless, given the random nature of these events, we consider that the neurons studied here are representative of the total population and that spine density measurements are relative. For each neuron, the three-dimensional basal dendritic tree, including all branches, was reconstructed in a two-dimensional plane and the dendritic tracing was quantified by Sholl analysis (Sholl, 1953). A transparent grid with concentric rings, equivalent to 10-μm spacing, was placed over the dendritic drawing and the number of branches intersecting each ring was used to estimate the total dendritic length (TDL) (Flores et al., 2005). Using a branching analysis, the branch order of the dendritic arborization was estimated by counting the number of ring intersections per every branch order for all the neurons. The density of dendritic spines was estimated by drawing at least 10-μm long segments from the terminal tips at high power (1000×) and counting the number of spines.

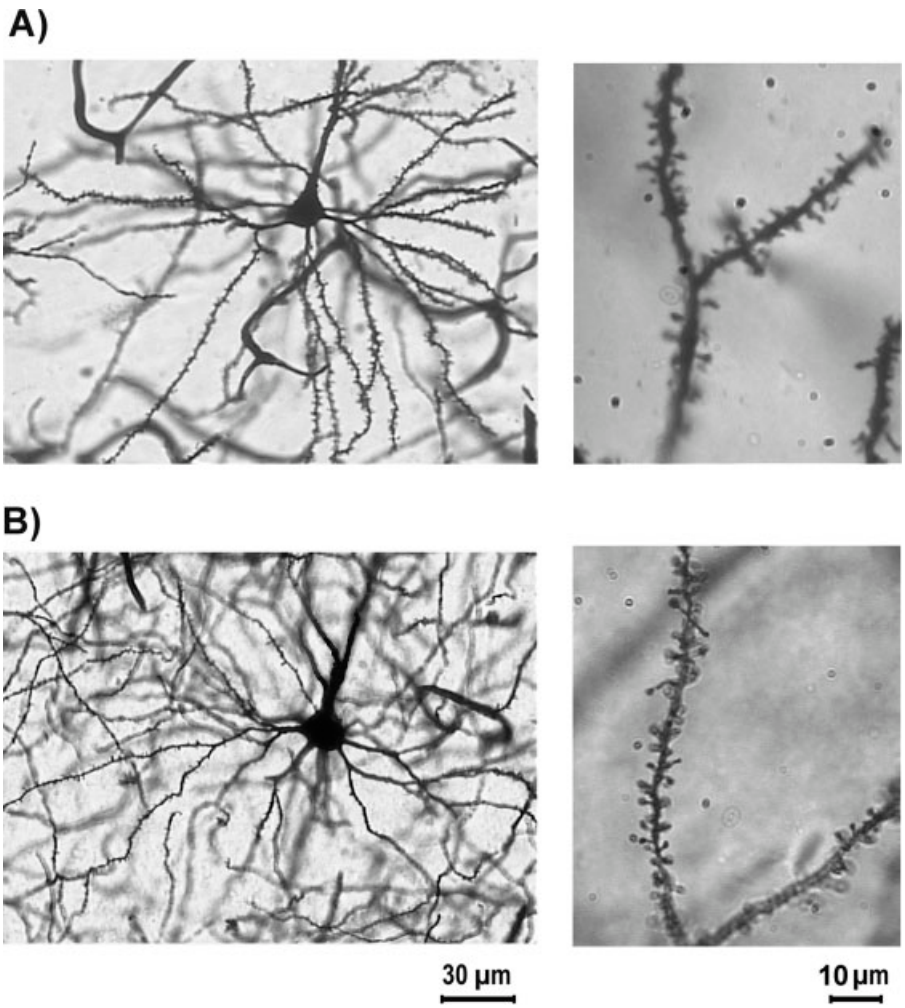

Fig. 1. Photomicrograph showing representative Golgi-Cox impregnated pyramidal neurons of the PFC from control animals (**A**) and animals with neonatal caffeine administration (**B**) at P35.

Data from the Sholl analyses and the spine densities were analyzed using two-way ANOVA, then by Newman-Keuls tests for post hoc comparisons, with age and neonatal caffeine administration as independent factors ($P < 0.05$ was considered significant).

RESULTS

Locomotor activity

The effect of the neonatal caffeine administration on spontaneous locomotor activity in a novel environment was not different between rats. However, both control animals and animals with neonatal caffeine administration initially show an active exploratory behavior in a novel environment, without changes in the locomotion when compared between groups at the same age, (P35: control: 1199 ± 63, caffeine: 1296 ± 122; P70: control: 930 ± 51, caffeine: 1043 ± 96).

Dendritic morphology

The dendritic branching and density of dendritic spines on neurons of the PFC were measured by Golgi-Cox stain between neonatal caffeine administration and control rats. The maximum branch order, spine density, and TDL obtained were similar to our previous reports (Flores et al., 2005; Martinez-Tellez et al., 2005; Silva-Gomez et al., 2003; Vega et al., 2004). The Golgi-Cox impregnation procedure clearly filled the dendritic shafts and spines of layer-3 pyramidal neurons of the PFC (Fig. 1). As measured by Sholl analysis, a two-way ANOVA for the TDL showed there was a significant effect of the neonatal administration of caffeine ($F_{1,\ 30} = 44.75$, $P < 0.001$) with the interaction neonatal caffeine x age ($F_{1,\ 30} = 11.\ 52$, $P = 0.002$) and without effect by the age ($F_{1,\ 30} = 0.9$, $P = 0.35$). The post hoc test revealed that TDL was significantly longer in the pyramidal neurons from animals with neonatal caffeine administration at prepubertal ($P < 0.001$) and at postpubertal ($P < 0.05$) age with significant differences between these two groups ($P < 0.01$) with neonatal caffeine administration but not between the control groups (Fig. 2A). A two-way ANOVA for the analysis of the density of the dendritic spines revealed that there was a significant effect of the neonatal administration of caffeine ($F_{1,\ 30} = 5.9$, $P = 0.02$) with effect by the age ($F_{1,\ 30} = 8.88$, $P < 0.01$) and not with the interaction neonatal caffeine × age ($F_{1,\ 30} = 0.05$, $P = 0.8$). The post hoc test did

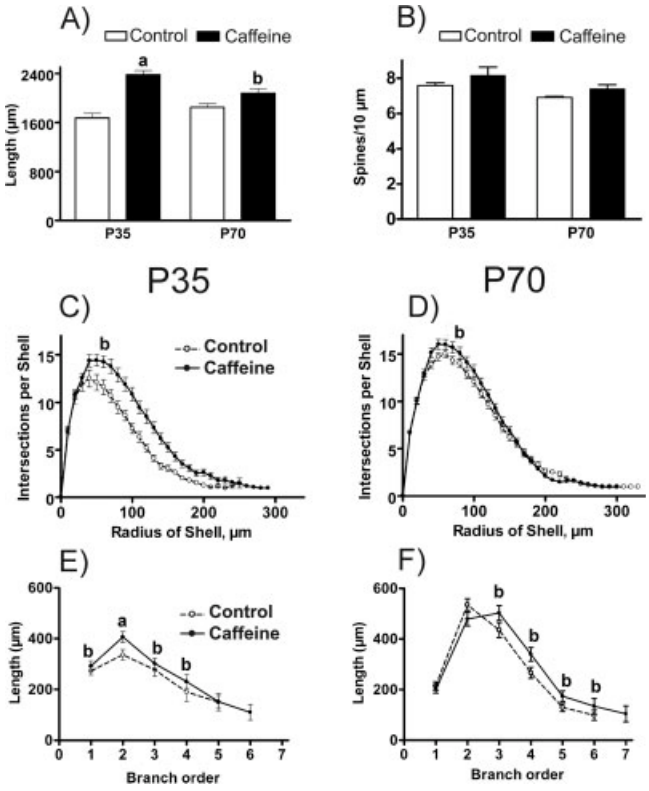

Fig. 2. Analysis of the pyramidal neurons in control animals and animals with neonatal caffeine administration ($n = 8$–9 animals per group). **A:** Total dendritic length. Rats with neonatal caffeine administration exhibit an increase in the total dendritic length. **B:** Spine density. **C:** Sholl analysis of intersection per shell at P35. **D:** Sholl analysis of intersection per shell at P70. The neonatal caffeine administration produced an increase in the number of intersections per concentric ring at both ages. **E:** Branch order at P35. **F:** Branch order at P70. The increase in the branch order by the neonatal caffeine administration was from the first to the fourth branch orders in the P35 animals, whereas at P70 the increase was from the third to sixth branch orders. a, $P < 0.01$ and b, $P < 0.05$.

not show differences between neonatal caffeine administration and control animals at both ages (Fig. 2B). However, the neonatal caffeine administration groups at both ages showed an apparent, but not statistically significant, trend towards an increased density of the dendritic spines compared with the control animals (P35: $P = 0.09$; P70: $P = 0.06$) (Fig. 2B).

Two other measured values obtained from the Sholl analysis, the intersection per shell and the length per branch order, gave information about of the quality of the dendritic arborization. The analysis of intersection per radius of shell also showed that the neonatal caffeine administration rats showed significantly more dendritic arborization ($P < 0.05$) when compared to the control animals at both ages (Figs 2C and 2D). The branch-order analysis suggests that P35 rats with neonatal caffeine administration exhibit more arborization at the levels of the first to fourth order compared to the controls (second order $P < 0.01$, rest of orders $P < 0.05$), whereas that P60 rats with neonatal caffeine administration show more arborization at the levels of the third order and higher ($P < 0.05$) (Figs. 2E and 2F).

DISCUSSION

Our major aim was to investigate the consequences of the neonatal caffeine administration on the development of the basilar dendritic structural morphology of layer-3 pyramidal cells of the PFC. We determined that neonatal caffeine administration causes a persistent increase in the dendritic length in layer-3 pyramidal neurons of the PFC without significant changes in the density of the spines. Interestingly, this permanent change in the dendritic length did not alter the locomotor activity in response to a mild stress, such as a novel environment. Our findings contribute to the current knowledge of the effects of caffeine on the limbic areas and cellular structures involved in the underlying basis of the cognitive function. Thus, these data suggest that changes in dendritic arborization caused by the neonatal caffeine administration may be linked in part to the increase of the cognitive function reported in children and rats (Bernstein et al., 1994; Elkins et al., 1981; Prediger et al., 2005; Rapoport et al., 1981).

Interestingly, several studies have shown a variety of effects of caffeine consumption in children and preadolescents, although it is surprising that so few studies have been specifically addressed to the effects on this population. At low doses, an increased performance in attention tests has been noted in children, whereas at high doses an increased motor activity has been reported in children (for reviews see Castellanos and Rapoport, 2002; Nawrot et al., 2003). More caffeine also caused an improvement in a manual dexterity test (Bernstein et al., 1994). In preadolescent boys it has been reported that a high dose of caffeine produced an increase in motor activity with an increase in speech rate, whereas low doses produced a reduction in reaction time in a vigilance test, and a reduced number of errors in a sustained-attention-measure test (Elkins et al., 1981; Rapoport et al., 1981). Other effects of caffeine in children caused their being nervous, anxious, fidgety, jittery, and restless and experiencing hyperactivity and difficulty sleeping (for reviews see Castellanos and Rapoport, 2002; Nawrot et al., 2003). However, all these studies only included the effects of one dose of caffeine, whereas for chronic caffeine effects only attention to the symptoms associated with caffeine withdrawal have been studied (for reviews see Castellanos and Rapoport, 2002; Nawrot et al., 2003). Only one report suggests that caffeine consumed for 2 months between ages 12 and 24 months did not alter the cognitive development (Engle et al., 1999). There is not enough information about the effects of chronic caffeine administration during early age on the morphological development of the cortex that may be present at puberty. However, mostly all children

at an early age start to consume chocolate and soft drinks, both important sources of caffeine. Our results suggest that the early consumption of caffeine plays a critical role in the development of the cortical neurons.

In the rat, the first 2 weeks after birth is a critical period in the development and synaptogenesis because of the stimulation of the outgrowth and maturation of neurons (Hindley et al., 1997; Kalb and Agostini, 1993; Roskams et al., 1994; Truman et al., 1996; Williams et al., 1994), in part corresponding to the development of the human brain in utero. For example, the interconnection between the hippocampus and amygdala with the PFC in the rat is after P7 (Bouwmeester et al., 2002a,b; Lipska, 2004) and in the human this happens after the fifth gestational month (for reviews see Ulfig et al., 2003; Weinberger and Lipska et al., 1995). Therefore, our data may in part have an implication about the consumption of caffeine during pregnancy.

Dendritic growth and the density of spines on the dendrites are related to the degree of connectivity and afferent activity (McAllister, 2000). Further, changes in the cortical excitability during early development produces alterations in dendritic spines (Friedlander et al., 1982; Globus and Scheibel, 1967; Guillery, 1973; Heumann and Rabinowicz, 1982; McMullen and Glaser, 1988; Wiesel and Hubel, 1963). Cortical excitability is mainly mediated by glutamate as the neurotransmitter and the glutamate by itself may control the spine creation and destruction at glutamatergic synapses (McAllister, 2000). In agreement with our dendritic spine density results, a recent report suggests that caffeine did not alter the cortical excitability (Orth et al., 2005). By comparison with our results, cocaine-sensitized adult rats have an enhanced total basilar dendritic length with an increase in the number of dendritic spines in the pyramidal neurons of the PFC (Robinson and Kolb, 1999). However, in these cocaine-sensitized animals, the morphological changes in the dendrites were correlated with an increase in the locomotor activity. The cocaine-sensitized effect may in part be caused by an indirect effect of the dopamine (DA) that modulates the glutamate transmission (Robinson and Kolb, 1999). In addition, a recent report demonstrated that in utero exposure to cocaine also produces an increase in the TDL of the PFC without changes in the somatosensory cortex (Lloyde et al., 2003).

The mechanism by which caffeine may cause an increase in the dendritic length of the pyramidal neurons of the PFC is not yet clear. However, consumption of caffeine, at the dose size we have used, is primarily related to its actions to block adenosine receptors (Fredholm et al., 1999; Phillis et al., 1991; Shi et al., 1993). The effects of caffeine in the brain are mediated particularly by its antagonistic actions at the A_1 and A_{2A} subtypes of the adenosine receptors (for review see Lorist and Tops, 2003). Adenosine A_1 receptors are present in almost all brain areas, but the cerebral cortex has the highest levels (Fastbom et al., 1987; Goodman and Snyder, 1982), whereas adenosine A_{2A} receptors are mainly present in the DA-rich regions of the brain, such as the striatum and tuberculum olfactorium, but not in the cerebral cortex (for review see Lorist and Tops, 2003). In addition, the blockade of A_{2A} receptors in striatopallidal neurons is crucial for the stimulatory action of caffeine. There is ample evidence that an intact dopaminergic neurotransmission is necessary for caffeine to be stimulatory (Ferre et al., 1992). Therefore, the effect of caffeine on neonates may be in part be caused by an adenosine A_1 blockade or an indirect effect via DA interaction. Other mechanisms of action of caffeine are the inhibition of phosphodiesterase and mobilization of intracellular calcium, but these effects require higher concentrations of caffeine (for review see Lorist and Tops, 2003). It is well known that neural cells after embryonic day 18 (E18) are sensitive to caffeine, and recent results suggest that after birth a differentiation of Ca^{2+} release channels, which regulate the Ca^{2+} internal (i) stores, are present in the cortical neurogenesis (Maric et al., 2000). Spinal cord neurons express caffeine-sensitive Ca^{2+} i stores only in the more mature neurons. Perhaps, the blocking of the adenosine receptors together with the inhibition of the mobilization of intracellular calcium stores may in part be correlated with the morphological data reported here. Our results add to a growing body of literature showing that changes in the dendritic arborization without changes of the dendrite spines may be caused by the effect of neonatal caffeine administration. Our results are more evidence that neonatal caffeine administration produces changes in the brain, and more studies are needed to understand what the physiological effect of these changes are.

In summary, our results are the first evidence showing significant dendritic morphological changes in the PFC at pre- and postpubertal animals with neonatal caffeine administration. Our results add to a growing body of literature about the effect of caffeine on the brain. It is possible that dendritic changes in the pyramidal neurons of the PFC, by altering synaptic inputs and organization, may contribute to the cognitive changes reported in caffeine consumers, such as attention and spatial learning (for reviews see Castellanos and Rapoport, 2002; Nawrot et al., 2003).

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