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Effects of Prenatal Cocaine Exposure in the Prefrontal Cortex of the Rat

A Morphometric Evaluation

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Abstract

This work was undertaken in order to assess the organization of the prelimbic area of the medial prefrontal cortex of rats exposed prenatally to cocaine. Pregnant Wistar rats were assigned to the following groups:

1. Cocaine—60 mg/kg body wt/d sc, from gestational days 8–22;
2. Saline;
3. Pair-fed; and
4. Nonmanipulated.

Male offspring were perfused on postnatal days 14 and 30. Six brains per group and per age were embedded in celloidin to calculate the volumes of the prelimbic area; sections from the other six brains were embedded in resin and processed for electron microscopy. Using semithin sections (2 μm) of layers II–III and V–VI, the following parameters were calculated:

1. The fraction of the neuropil occupied by neurons (V_V);
2. The packing (N_A) density; and
3. The numerical (N_V) density.

Qualitative alterations consisted of dispersed profiles of degenerated neurons and dendrites in the medial prefrontal cortex. No significant differences were found in the gross morphometric parameters when the cocaine group was compared with the other groups. A high interanimal variation was shown in the prelimbic volumes of postnatal day (PND) 14 cocaine-treated rats, and a decrease in volumes was detected at PND30. Although there are some alterations in the main afferent cortical target area for dopaminergic input, its gross morphometric parameters do not seem to be sufficiently affected to account for the behavioral alterations referred to as being dependent on this brain region.

Index Entries: Prefrontal cortex; prelimbic area; cocaine; prenatal exposure; rat; morphometry.

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Introduction

Cocaine use among females of childbearing age has led to a significant increase in the number of fetuses exposed to the effects of this abused substance. Recent studies of both humans and animals have demonstrated the adverse effects of cocaine during critical periods of development (Gingras et al., 1992; Volpe, 1992). Cocaine interacts with brain neurotransmitter systems (Galloway, 1988; Dow-Edwards, 1989; Karoum et al., 1990; Akbari and Azmitia, 1992; Akbari, et al., 1992; Meyer and Dupont, 1993), leading to neurobehavioral, biochemical, and morphological alterations (Chasnoff et al., 1985; Church et al., 1988, 1990; Dow-Edwards, 1989; Spear et al., 1989; Silva-Araújo et al., 1991, 1993; Heyser et al., 1992; Johns et al., 1992).

Prenatal cocaine exposure has been reported to induce marked alterations in the functioning and organization of the dopaminergic systems (Galloway, 1988) and on dopamine receptors (Scalzo et al., 1990). Recently, Akbari and Azmitia (1992) described an increase in the tyrosine hydroxylase activity in the cingulate cortex after gestational exposure to cocaine. Moreover, some of the behavioral alterations described after gestational exposure to cocaine have been related to a delayed development of the dopaminergic systems (Spear et al. 1989).

In order to assess the effects of prenatal exposure to cocaine, we selected the prefrontal cortex and its prelimbic subarea for several reasons:

1. The prefrontal cortex (PFC) of the rat is the main cortical area receiving dopaminergic projections, which play a crucial role in its normal development (Van Eden and Uylings, 1985a,b; Van Eden et al., 1987);
2. In both humans and rodents, this brain area is involved in a wide range of cognitive and emotional functions (Brutkowski, 1965);
3. The prelimbic area of the medial prefrontal cortex has the highest density of dopaminergic fibers as compared with the other three medial prefrontal areas (Van Eden et al., 1987);
4. It has a well-defined structural organization, evaluated both in normal (Van Eden and Uylings, 1985a,b; Van Eden et al., 1987) and experimental conditions (Cadete-Leite et al., 1990; Madeira et al., 1990); and
5. Although a few recent studies focus on the effects of cocaine on the dopamine levels of the medial prefrontal cortex, no morphological support of these findings has been provided (Moghaddam and Bunney, 1989; Karoum et al., 1990; Maisonneuve et al., 1990).

Methods

Animals and Drug Administration

Male and female Wistar rats purchased from the Gulbenkian Institute of Science, Oeiras, Portugal, were paired for mating; the morning on which a sperm plug was found was designated as gestational day (GD) 1. Sperm-positive females were randomly assigned to the following experimental groups: cocaine-treated (CO), saline (S), pair-fed (PF), and nonmanipulated (C).

Starting on GD8 and continuing to GD22, pregnant rats were asssiged to CO group and were given sc injections of cocaine hydrochloride (Sigma, St. Louis, MO)—60 mg/kg body wt/d—in 0.9% saline divided into two equal doses injected at 8 AM and 6 PM. S and PF groups were given isovolumetric injections of saline throughout the same experimental period. Pair-feeding was performed by matching two females with the same time of pregnancy (Spear et al., 1989; Heyser et al., 1992); the saline-injected female was given the same amount of food on each day of gestation as had been consumed by the cocaine injected dam on the previous day. A group of pregnant rats that were not manipulated constituted the third control group (C). Selection of the dose was based on other experimental works (Church et al., 1988, 1990; Spear et al., 1989; Church and Overbeck, 1990).

Food and water were delivered ad libitum, except for the PF group. Records were kept on changes in maternal body wt gain and on food consumption (CO and PF groups) throughout

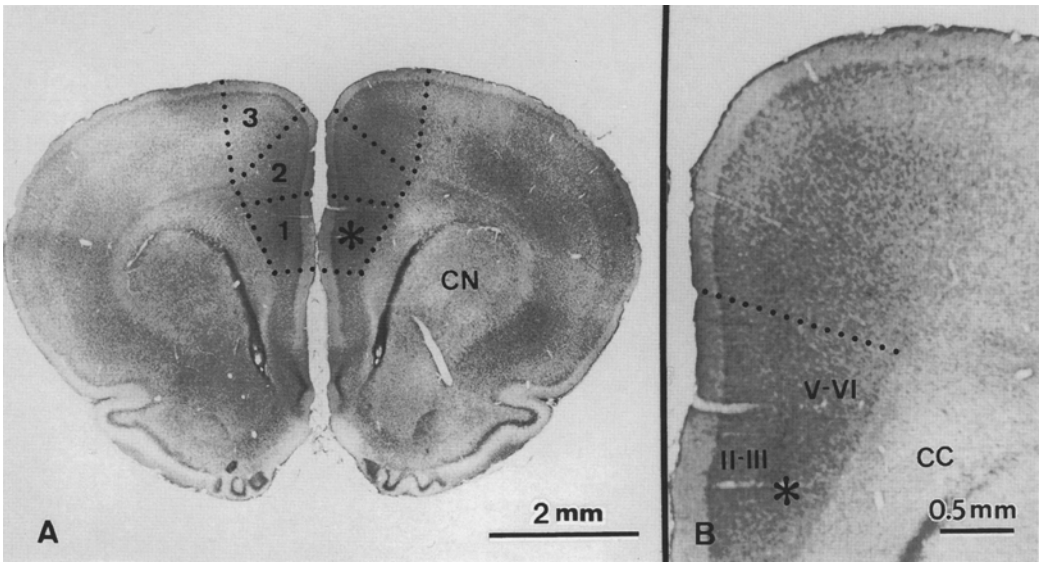

Fig. 1. **(A)** Light micrograph of a coronal section of a control rat brain. The subareas of the medial prefrontal cortex are represented: 1—prelimbic; 2—dorsal anterior cingulate; 3—medial precentral. **(B)** Higher magnification of the prelimbic cortex (*) with the cortical layers II–III and V– VI. CN—caudate nucleus; CC—corpus callosum. Celloidin. Cresyl violet.

gestation. The weight and sex of each pup were determinated after delivery of the entire litter (postnatal day 0—PND0); the litters were culled to eight pups (four males and four females). Rats were weaned on PND21. Offspring were weighed on PND1, PND2, and then on alternate days until the end of the experimental period (PND14 and PND30).

Tissue Processing

At PND14 and PND30, male offspring were anesthetized with ether and transcardially perfused following the techniques described by Palay and Chan-Palay (1974). In this experiment, the evaluations were restricted to male offspring owing to the known sex differences in the response to cocaine (Glick and Hinds, 1984; Dow-Edwards, 1989).

The fixative used for perfusion consisted of 1% glutaraldehyde/1% paraformaldehyde in 0.12*M* phosphate buffer at pH 7.4; after fixation, the brains were removed and weighed. From each experimental group (CO, S, PF, and C) in each age (PND14 and PND30) six brains (from three different litters) were embedded in celloidin.

From another six animals, brain sections of the PFC—identified according to Van Eden and Uylings (1985a,b)—were postfixed in cacodylate-buffered 0.1*M* 1% osmium tetroxide and dehydrated in ascending concentrations of ethanol followed by mixtures of resin/propylene oxide and embedded in synthetic resin. From each group and per age, a small sample of two to three brains was used for determination of the volumetric shrinkage factor owing to celloidin processment; this factor was calculated in the PFC area using the replicas of sections obtained after fixation and celloidin embedding (Van Eden and Uylings, 1985b).

Quantitative Analysis

The morphometric evaluations were performed in the prelimbic arlea of the agranular medial prefrontal cortex of the rat (Fig. 1A and B). In order to obtain an overview of the whole extent of the prelimbic cortex, the total volume was screened, and the volumetric, packing,

and numerical densities of neurons in layers II–III and V–VI were evaluated.

Volume of the PFC

The forebrain (brain minus cerebellum, olfactory lobes, and brain stem) was separated from the remainder of the brain at the occipital pole (Madeira et al., 1990). For volumetric determinations, the right hemispheres of six animals (two from three different litters) were used. The brains previously embedded in celloidin were sectioned at 60 μm (nominal thickness) in a sliding microtome, always with the same knife. Sectioning was performed serially in the coronal plane. These sections were stained with cresyl violet, mounted on glass slides, and covered with Histomount and a coverslip.

The boundaries of the PFC and its subareas—medial precentral, prelimbic, dorsal, and ventral cingulate (Van Eden and Uylings, 1985a,b)—were all drawn by the same investigator with the aid of a camera lucida at a final magnification of ×32 (Fig. 1A,B). The first section of the prelimbic cortex to be drawn was the one in which the rostral part of the corpus callosum could be first identified (Van Eden and Uylings, 1985a,b; Madeira et al., 1990), followed by each alternate section. The area of each drawing was measured with the aid of a MOP-Videoplan. The volume of each subarea, between each pair of sections, was calculated using the formulae (Van Eden and Uylings, 1985b)

$$V = [Ai + (Ai + 1)\ 1] / 2 \times T \quad (1)$$

where Ai is the profile area of the prelimbic cortex, Ai + 1 is the profile area at the next level, and T is the distance between corresponding surfaces. Total volumes were then calculated by adding the volumes of all the levels representing the prefrontal layers (Van Eden and Uylings, 1985b). Data presented are referred to as the volumes of the right prefrontal cortical areas.

The correction for tissue shrinkage was made in another set of animals from the four different groups (2–3 animals/group), according to Van Eden and Uylings (1985b); this determination replicated other experiments performed in the laboratory of the Institute of Anatomy in other experimental paradigms (Madeira et al., 1990; Tavares and Silva, 1993); as mentioned by other authors, determination of the shrinkage of the prelimbic cortex as a single area would not be possible to perform (Madeira et al., 1990).

Morphometry of the Prelimbic Area

Semithin sections (2 μm) were obtained, stained with toluidine blue, and mounted on glass slides. For each animal, layers II–III and V–VI of the prelimbic cortex from four pairs of two sequential sections were photographed with a ×20 objective; a final magnification of ×300 was used in photographic prints. Using these prints and the method of the point counting (Weibel, 1979), the neuronal volumetric density (V_V)—fraction of the neuropil occupied by neurons—and the number of neurons per unit surface area (N_A) were calculated. For the counts of the nuclear profiles, the rule of the "forbidden" line (Gundersen, 1977) was applied.

The numerical density (number per unit volume of reference area) of neurons in layers II–III and V–VI neurons was determined in the prelimbic area of the right hemisphere by applying the dissector method (Sterio, 1984). Three blocks were selected at random from the whole extent of the prelimbic area; after identification of the prelimbic area, the sections were cut perpendicularly to the pial surface. The physical dissector requires two parallel sections separated by a known distance; these sections are referred to as the "reference" and the "look-up" section (Sterio, 1984; Braendgaard and Gundersen, 1986). Nuclear profiles were used to define the cellular profiles in each test area; the number of nuclear profiles found in the counting grid and not in the "look-up" section represents the number of cells per unit volume within the area of the counting grid and the distance between the two sections. From each pair of sections, two dissectors were performed by using in turn the "reference" and the "look-up" section. A total of 16 dissectors

Table 1
Maternal and Litter Variables

	CO $n = 21$[a]	S $n = 10$	PF $n = 10$	C $n = 22$
Maternal weight gain (g) GD1–GD22[c]	82.0 (28.9)[b]	100.3 (19.6)	97.0 (27.6)	97.5 (27.7)
Gestational length (d)	22.5 (0.6)	22.6 (0.5)	22.3 (0.5)	22.5 (0.6)
Litter size	12.0 (4.0)	11.6 (2.5)	12.2 (2.8)	12.2 (2.7)
Male pups per litter	6.4 (2.5)	5.3 (2.0)	6.4 (2.5)	6.1 (2.5)
Female pups per litter	5.6 (2.4)	6.3 (2.4)	5.8 (1.1)	6.1 (2.0)
Pup weight (PND0)[e]	5.0 (0.9)[f] $n = 42$[d]	5.8 (0.7)[g] $n = 20$	5.6 (0.6)[h] $n = 20$	4.9 (0.6)[i] $n = 44$

[a] n = Number of litters.
[b] Mean (SD).
[c] GD—gestational day.
[d] n = 2 males/litter.
[e] PND—postnatal day.
[g] and [h] Different from [f] and [i], $p < 0.05$.

(Braendgaard and Gundersen, 1986) were performed per layer and per animal.

Statistical Analysis

All data are presented as mean and standard deviation. The maternal and litter data were analyzed using ANOVA. For the quantitative parameters of the prelimbic cortex, owing to the number of animals used (6 rats/group), a nonparametric analysis of variance (Kruskal-Wallis test) was used; whenever significant differences were detected, the Mann-Whitney U-test was used for pairwise comparisons (Conover, 1980).

Results

Maternal and Litter Variables

Maternal weight gain was determined by calculating the increase in body weight from GD1 to GD22. The analysis of variance indicated no significant differences in maternal weight gain among the different groups (F [3,63] = 1.76, $p > 0.05$) (Table 1). Treatment had no influence on gestational length as well (F [3,63] = 0.77, $p > 0.05$) (Table 1). No females died during treatment.

There were no significant differences in litter size (litter size was based on the number of live births) (F [3,63] = 0.10, $p > 0.05$), and in the male to female ratio (F [3,65] = 0.51 and F [3,63] = 0.33, $p > 0.05$, respectively) (Table 1). There were significant differences in body weight at PND0 (F [3,126] = 9.72, $p < 0.05$), and in body weight gain between PND1 and PND14 (F [3,126] = 13.3, $p < 0.05$). Saline pups were heavier than C and CO pups at PND0; the body weight gain (PND1–14) was lower in the C group than in the remainder (Table 2). This difference was diluted if body weight gain was calculated throughout the whole experimental period (Table 3).

No significant differences were found in the weights of the cerebella on PND14 and PND30 and in brain weight at PND14. Brain weights were significantly different between S and other groups at PND30 (F [3,59] = 4.74, $p < 0.05$).

Qualitative Observations

The organization of the PFC in the different groups was similar to that described in previous works (Van Eden and Uylings, 1985a;

Table 2
Body, Brain, and Cerebellar Weights in Groups of Rats Exposed Prenatally to Cocaine and Respective Controls (Postnatal Day 14)

	CO	S	PF	C
Body weight (g)	30.5 (5.7)[a] $n = 42$[b]	31.4 (2.8) $n = 20$	30.0 (4.0) $n = 20$	28.6 (4.7) $n = 44$
Body weight gain (PND1–PND14)[d]	24.4 (3.6) $n = 42$	23.8 (3.5) $n = 20$	25.6 (4.6) $n = 20$	20.4 (3.1)[c] $n = 44$
Brain weight (mg)	907 (70) $n = 14$	905 (52) $n = 14$	876 (70) $n = 13$	877 (58) $n = 21$
Cerebellar weight (mg)	147 (15) $n = 14$	148 (17) $n = 14$	145 (16) $n = 13$	148 (23) $n = 21$

[a]Mean (SD).
[b]n = number of animals.
[c]Different from CO, S, PF, $p < 0.05$.
[d]PND—postnatal day.

Table 3
Body, Brain, and Cerebellar Weights in Groups of Rats Exposed Prenatally to Cocaine and Respective Controls (Postnatal Day 30)

	CO	S	PF	C
Body weight (g)	103.4 (16.4)[a] $n = 42$[b]	97.9 (24.1) $n = 20$	103.2 (13.9) $n = 20$	94.8 (14.9) $n = 36$
Body weight gain (PND1–PND14)[d]	101.1 (17.8) $n = 42$	95.8 (18.5) $n = 20$	99.4 (14.9) $n = 20$	97.5 (12.1)[c] $n = 36$
Brain weight (mg)	1175 (69) $n = 19$	1257 (68)[c] $n = 12$	1154 (67) $n = 11$	1167 (89) $n = 17$
Cerebellar weight (mg)	211 (16) $n = 19$	224 (24) $n = 12$	208 (13) $n = 11$	229 (45) $n = 17$

[a]Mean (SD).
[b]n = number of animals.
[c]Different from CO, S, PF, $p < 0.05$.
[d]PND—postnatal day.

Cadete-Leite et al., 1990; Madeira et al., 1990) (Figs. 1A,B and Fig. 2).

In the CO group (1–2 animals/litter), it was possible to detect dispersed profiles of degenerated neurons (Figs. 3 and 4) and dendrites in the prelimbic neuropil (Fig. 5). These animals were excluded from the quantitative evaluations, and only those not presenting gross morphological changes were used for determination of morphometric parameters.

Quantitative Results

Volumes of the Prelimbic Area

There were no significant differences in the shrinkage correction factors of the prefrontal pole of the brains among groups, although higher values were determined in PND14 animals (skrinkage correction factor: 2.80–2.55) when compared with PND30 (shrinkage cor-

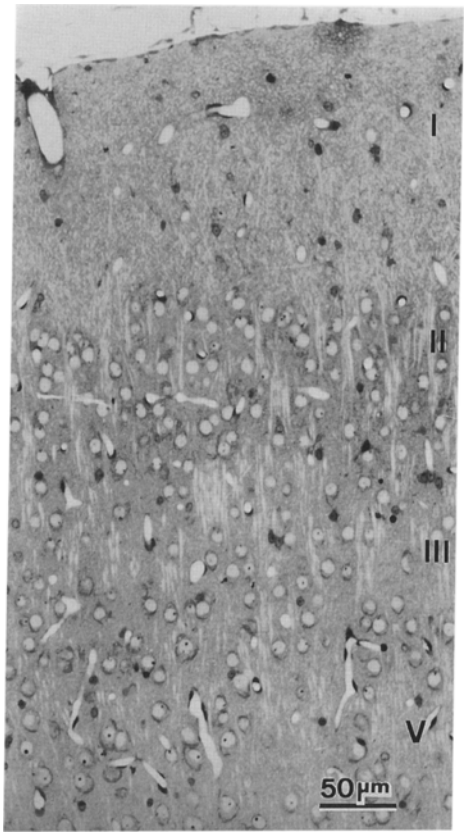

Fig. 2. Light micrograph of a semithin section from the prelimbic area of the medial prefrontal cortex of a control rat. Cortical layers: I, II, III, V. Toluidine blue.

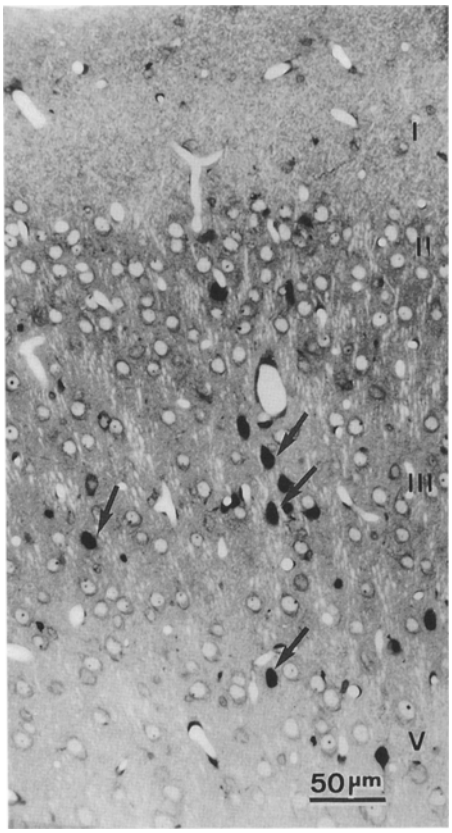

Fig. 3. Light micrograph of the medial prefrontal cortex of a PND14 cocaine-treated rat. Dark degenerated neurons can be depicted (arrows). Cortical layers I, II, III, V. Toluidine blue.

rection factor 2.50–2.40); it has been already demonstrated that shrinkage correction factors were higher in younger animals than in adults (Van Eden and Uylings, 1985b).

In the CO group, the volumes of the prefrontal cortex showed a high interanimal variation; the volume of the prelimbic cortex was smaller in C and S groups compared with PF and CO groups, although no significant differences were detected (Fig. 6). From PND14 to PND30, there was a reduction in C and S groups and an increase in PF and CO groups.

Volumetric Density of Prelimbic Neurons

No significant differences were detected in the volumetric densities of layers II–III and V–VI neurons on PND14 and on PND30. There was a significant age-dependent reduction in the volumetric density of the prelimbic neurons from PND14 to PND30 ($p < 0.05$), not found in the CO group in layer V–VI ($p > 0.05$) (Fig. 7).

Packing Density of Prelimbic Neurons

No differences were detected in the packing density of prelimbic neurons of layers II–III and layers V–VI on PND14 and on PND30. With the exception of layer V–VI of the CO group, the other experimental groups, in both layers, presented a significant decrease in the number of neurons per unit surface area between PND14 and PND30 ($p < 0.05$) (Fig. 8).

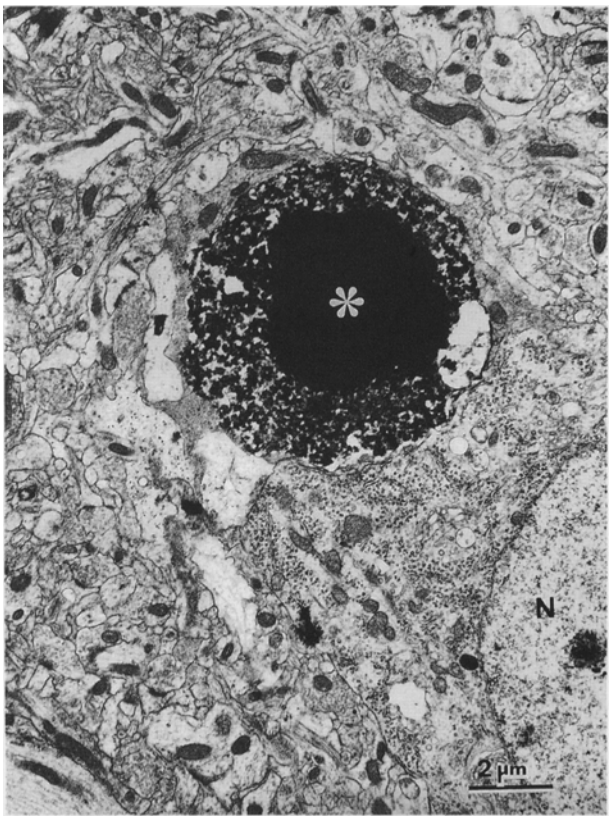

Fig. 4. Electron micrograph of a degenerated neuron in a PND30 cocaine-treated rat. Note the dense condensation of the nuclear chromatin (*); N—nuclear profile of a cortical neuron.

Numerical Density of Prelimbic Neurons

No differences were detected in the numerical density of prelimbic neurons in layers II–III and V–VI on PND14; on PND30, no differences were detected as well; C and CO groups presented an age-dependent decrease in the number of neurons per unit volume on layers II–III ($F = 2.40$, $p < 0.05$) (Fig. 9).

Discussion

It is now well documented that infants born to mothers who abuse cocaine during pregnancy exhibit diverse behavioral and neurologic abnormalities (Chasnoff et al., 1985; Neuspiel and Hamel, 1991; Gingras et al., 1992). Neurotransmitter systems of the central nervous system are highly affected by prenatal exposure to cocaine (Dow-Edwards, 1989; Karoum et al., 1990; Akbari and Azmitia, 1992; Akbari et al., 1992), and, among them, the dopaminergic systems seem to be one of the most vulnerable (Galloway, 1988). In fact, it has been pointed out that functional effects of prenatal exposure to cocaine have been mediated, at least in part, by changes in the development of the catecholaminergic systems (Karoum et al., 1990; Maisonneuve et al., 1990; Akbari and Azmitia, 1992). There are several works reporting that gestational exposure to cocaine alters the functioning of the dopaminergic system (DA) by inhibiting reuptake of dopamine and stimulating the dopamine release (Galloway, 1988). In addition to enhancing the brain tyrosine hydroxylase activity (Akbari and Azmitia, 1992; Meyer and Dupont, 1993), cocaine also interacts with other neurotransmitters, such as norepinephrine and serotonin (Karoum et al., 1990; Akbari et al., 1992).

In this work, we studied the quantitative organization of the rat medial prefrontal cortex, which extends from the frontal pole to the level of the anterior commissure (Van Eden and Uylings, 1985a,b) and is situated at the medial area of the hemispheres; its prelimbic subarea contains the highest density and the more extensive distribution of DA fibers (Van Eden et al., 1987). Within this area and for methodological purposes, we considered together layers II and III and layers V and VI in order to achieve a larger reference area of the cortex; moreover, there are recognizable differences in the topography of the afferents for each of its cortical layers (Van Eden et al., 1987).

In this experimental model, pair-feeding and injection of saline were used to control both the effects of undernutrition (Heyser and Spear, 1993) and handling stress (Deutch and Roth, 1990; Horner et al., 1991). There were some recent studies reporting alterations in the neurotransmitter systems, namely, the catecholaminergic system, depending on the quality of diet provided to the animals (Thibault, 1992; Venero et al., 1992), which may justify the

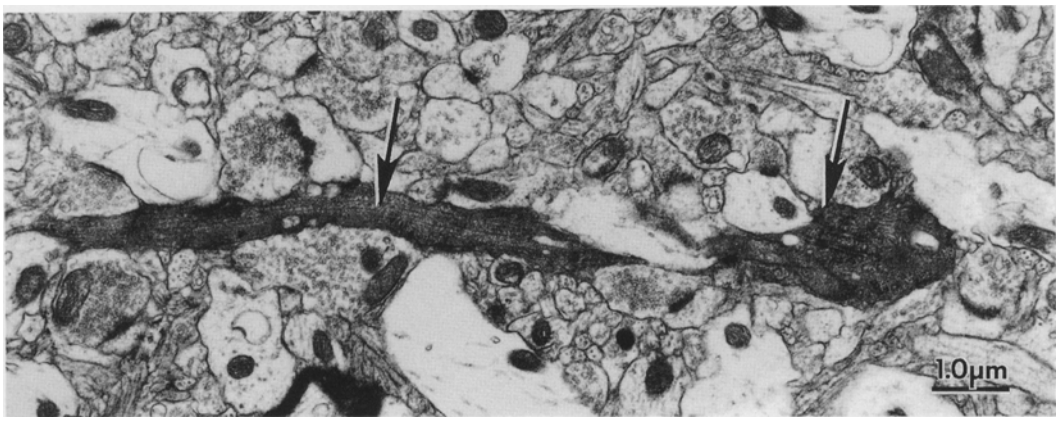

Fig. 5. Electron micrograph of a dark degenerated dendrite (arrows) in the neuropil of the prelimbic cortex of a PND30 cocaine-treated rat.

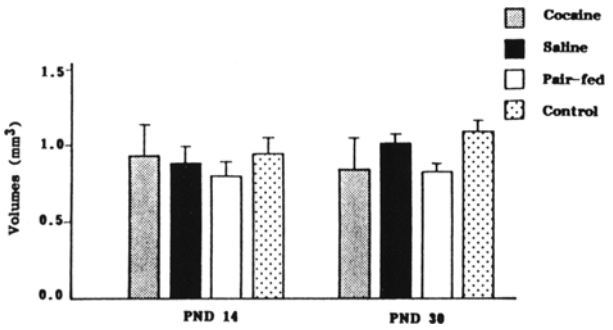

Fig. 6. Graphic representation of the mean volumes of the prelimbic area of the rat after gestational exposure to cocaine and respective controls. Columns represent mean and vertical bars 1 SD.

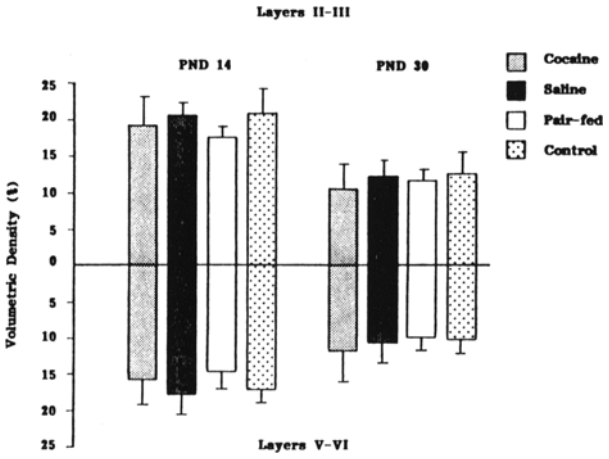

Fig. 7. Graphic representation of the mean neuronal volumetric density (V_V%) of the prelimbic area of the rat after gestational exposure to cocaine and respective controls. Columns represent means and vertical bars 1 SD.

reported behavioral alterations. In spite of this, no alterations were detected in the morphometric parameters of the prefrontal cortex of the pair-fed groups when compared to the other controls.

With an incidence of approx 5%, the cocaine-treated animals presented degenerative changes and signs of vascular disruption in the cerebral cortex. However, in accordance with other work (Spear et al., 1989; Church et al., 1990), our results show that administering cocaine to pregnant rats from GD8 to GD22 produces no significant effects on gestational length, litter size, and male to female offspring ratio; also, no significant differences were detected in maternal weight gain among the experimental groups. In these experimental groups, there were also no significant differences in body weight gain at PND14 or at PND30.

For the analysis of the gross morphological parameters of the prelimbic area, determination of the volumes of the prelimbic area was undertaken since it is preferred to the measure of the cortical thickness (Van Eden and Uylings, 1985a,b). Moreover, determination of

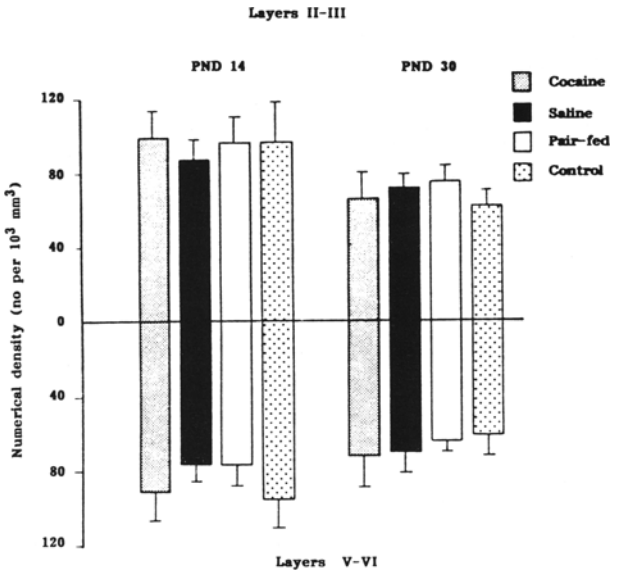

Fig. 8. Graphic representation of the packing density of neurons (N_A—number per unit area) of the prelimbic area of the rat after gestational exposure to cocaine and respective controls. Columns represent means and vertical bars 1 SD.

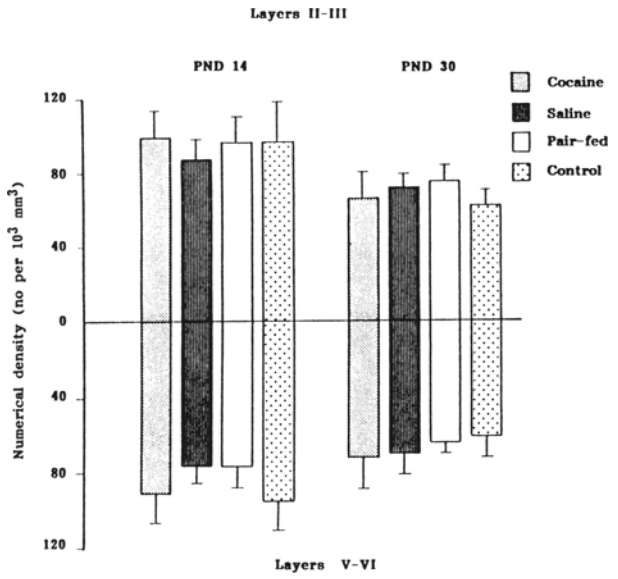

Fig. 9. Graphic representation of the numerical density of neurons (N_V—number per unit volume of reference) of the prelimbic area of the rat after gestational exposure to cocaine and respective controls. Columns represent means and vertical bars 1 SD.

volumes of anatomically defined brain regions has been shown to be a sensitive method for quantification and localization of brain damage owing to experimental conditions, as is the case in ischemic brain damage (Beck et al., 1993). Our results of the volumetric study of the prelimbic area, corrected for shrinkage in both ages, agree with previous work in the normal development of the prefrontal cortex of the rat (Van Eden and Uylings, 1985b) and allowed the detection of differences that, although not significant, are suggestive of a decrease by PND30. Moreover, a great interanimal variation was found in the PND14 cocaine-treated group.

The present results showed no differences in either the fraction of the prelimbic cortex occupied by neurons, in the number of neurons per unit surface area, or in their number per unit volume of reference area in the groups of rats gestationally exposed to cocaine. The determination, with the dissector, of the number of cells per unit volume overcame some disadvantages of the classical morphometric techniques (Weibel, 1979), namely, the questions raised concerning the meaning of the number per unit area. This technique allows one to obtain unbiased values of NVS, independent of cell orientation and distribution of the cell nuclei and not biased by the thickness of the section and/or lost caps (Braendgaard and Gundersen, 1986).

The morphometric data do not appear to be sufficient to account for the vast array of the neurobehavioral and biochemical alterations described as associated with prenatal exposure to cocaine. It has been shown that, during the brain growth spurt, the rat brain exhibited a greater resistance to cocaine exposure than the somatic growth (Chen et al., 1993). Despite the need for further complementary evaluations, namely, the immunocytochemical evaluation and the biochemical determination of DA and its metabolites, the spare found in the gross morphological parameters point to a relatively structural resistance of this CNS area to the cocaine insults that may constitute a good foundation for a long-term recovery in the

behavioral functions described after gestational exposure to this abused substance.

Acknowledgments

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