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Effects of Prenatal Cocaine Exposure in the Retinal Ganglion Cell Layer of the Rat

A Morphometric Analysis

A. Silva-Araújo,[1] M. C. Silva,[2] P. Abreu-Dias,[1] and M. A. Tavares*,[3]

[1]Department of Ophthalmology and [2]Institute for Biomedical Sciences, Largo Abel Salazar, 4000; and [3]Institute of Anatomy, Medical School of Porto, Al. Hernai Montero, 4200 Porto, Portugal

Abstract

To study the effects of prenatal cocaine-exposure on the developing retinal ganglion cell layer of the rat, female Wistar rats were administered subcutaneously (sc) cocaine hydrochloride (60 mg/kg body wt/d) or saline, or were not manipulated from gestational d 8–22. Male offspring were sacrificed at postnatal day 14 and 30. Radial semithin sections of epon-embedded flat mounts of the retinal quadrants were used to evaluate the following parameters along the centro-peripheral axis:

1. Thickness of ganglion cell plus nerve fiber layer;
2. Nuclear size of ganglion cell layer neurons; and
3. Linear density (number per unit length) of ganglion cell layer neurons.

To study the effects of cocaine and age on the retinal areas (temporal/nasal, dorsal/ventral), a repeated measures analysis of variance was used for each of the parameters mentioned above. All parameters were affected by prenatal exposure to cocaine. The thickness of the ganglion cell plus nerve fiber layer was reduced in cocaine-exposed rats in comparison with the saline group. Nuclear diameters were smaller in the cocaine than in the saline and control groups. The linear density was higher in the cocaine-exposed group than in the control and saline groups. The age-dependent decrease in the linear density from postnatal day 14–30 was higher in the cocaine-exposed rats than in the saline group; the decrease in the linear density along the centroperipheral axis found in both the control and saline groups was not significant in the cocaine-treated group. These morphometric findings strongly support the view that prenatal cocaine-exposure induces marked changes in the organization of the developing retina.

Index Entries: Retina; ganglion cell layer; cocaine; prenatal exposure; rat; morphometry.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

A substantial number of pregnant women use cocaine (Chasnoff et al., 1986; Volpe, 1992). This is a major concern since the lipophilic properties of cocaine permit it to cross the placenta rapidly and to accumulate in fetal tissues, including the developing CNS (Shah et al., 1980).

Among the increasing number of studies describing the adverse effects associated with exposure to cocaine during the developmental periods (for review, *see* Volpe, 1992), some focus on ophthalmic abnormalities following gestational exposure to this drug (Dixon et al., 1987; Isenberg et al., 1987; Teske and Trese, 1987; Ferriero et al., 1989; Dominguez et al., 1991; Good et al., 1992). The most recent survey reported optic nerve abnormalities, delayed visual maturation, and prolonged eyelid edema in 13 cocaine-exposed infants (Good et al., 1992). Previous reports of ophthalmic findings included dilated iris vessels (Isenberg et al., 1987), retinopathy of prematurity and persistent primary vitreous (Teske and Trease, 1987; Dominguez et al., 1991), and retinal dysgenesis (Ferriero et al., 1989).

From the escalating number of experimental investigations in the field of prenatal cocaine exposure, only a few report gross morphological abnormalities in components of the visual system. Webster et al. (1991) documented that late gestational exposure to cocaine in the rat leads to brain damage and bilateral involvement of the eye. Cases of anophthalmia were also observed in mice (Mahalik et al., 1980) and rats (Church et al., 1990). We recently demonstrated (Silva-Araújo et al., 1993), that early and chronic postnatal exposure of rats to cocaine affected the postnatal development of both the retinal ganglion cell layer and the optic nerve.

The retina of the rat is a well-defined layered structure whose regular morphological pattern allows an easy identification of any structural disruption induced by experimental situations, pathological conditions, or developmental exposure to neurotoxins (Borges et al., 1990; Hughes, 1991; Silva-Araújo et al., 1993). Moreover, the assessment of changes occurring in a specific retinal layer, for example, the ganglion cell layer, may serve as an index of visual function, since the optic nerve axons originate from this layer.

We have measured standardized reference areas in the retinal ganglion cell layer of 14- and 30-d-old rats exposed prenatally to cocaine in order to assess possible effects of this drug on the morphological organization of this retinal region.

Methods

Subjects and Breeding

Subjects were the male offspring generated from nulliparous Wistar rats bred in our laboratory and purchased from the Colony of the Gulbenkian Institute of Science, Oeiras, Portugal. At the onset of breeding, males were placed with 60-d-old females from 8:00 PM until 8:00 AM the following morning. The presence of a vaginal plug or a sperm-positive vaginal cytology determined pregnancy (gestational day [GD] 1 = day found sperm positive). The females were weighed, placed in individual cages, and on GD8, assigned to the experimental groups.

Drug Administration

Females were given sc injections of cocaine hydrochloride (Sigma, St. Louis, MO), 60 mg/kg body wt/d administered in 0.9% saline (3 mL/kg body wt/d) from GD8–GD22. Each dose was split, with the first portion being injected between 8:30 and 9:00 AM and the second portion between 6:00 and 8:00 PM. The cocaine was diluted in a solution of saline, and the site of injection varied daily on the dorsal surface in order to minimize skin necrosis.

A control group was given isovolumetric doses of saline during the same experimental period (GD8–GD22), and another group was not manipulated. Food and water were available ad libitum.

Following delivery, the total number of offspring was recorded, and each animal was weighed, sexed, and examined. The litters were then culled to eight pups with preference given to four males and four females. Pups were weaned on postnatal day (PND) 21, housed in pairs with like-sexed age-mates, and sacrificed on PND14 and PND30. Brains were weighed after perfusion.

Experimental Design

Two male rats were obtained at random from each of six females, totaling 12 rats/treatment (control, saline, cocaine); half of the subjects in each treatment were randomized to be sacrificed on PND14, and the other half were sacrificed on PND30. Overall, 36 litters were used, 12 litters from 12 different females assigned to each of the 3 experimental groups (Silva et al., 1995).

Since the main objective of this work was to evaluate the effects of prenatal cocaine exposure in two different periods of the postnatal development and screen the whole extent of the retinal ganglion cell layer, we used animals aged 14 and 30 d and considered different retinal reference areas—nasal/temporal, dorsal/ventral—analyzing them along the centroperipheral axis.

Tissue Preparation

At PND14 and PND30, the rats were anesthetized and perfused transcardially with a solution of 1% glutaraldehyde/1% paraformaldehyde in 0.12*M* phosphate buffer at pH 7.4 (Palay and Chan-Palay, 1974). Before enucleation, the eyeballs were oriented by an India ink mark at 12 h. The right eye was selected for the quantitative analysis; the anterior segment and the lens were removed, and a deep cut was made along the India ink mark toward the optic disk. Three other cuts were made at 3, 6, and 9 h; a short peripheral cut identified the temporal/dorsal quadrant.

The retinas were immersed in the same fixative overnight, rinsed for 2 h in cacodylate buffer and postfixed in cacodylated buffered 1% osmium tetroxide, dehydrated in ascending concentrations of ethanol followed by mixtures of resin/propylene oxide, and embedded flat in epoxic resin (TAAB). Each quadrant was cut and subsequently re-embedded in resin; vertical semithin sections were cut from the centrum to the periphery of each quadrant and stained with toluidine blue.

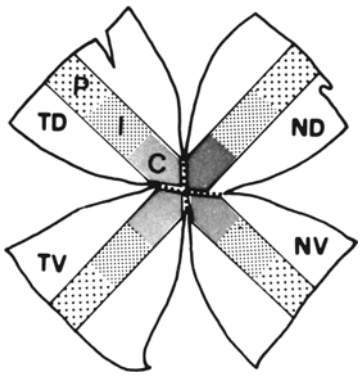

Fig. 1. Schematic representation of retinal reference areas; four quadrants were cut radially: temporal dorsal (TD), temporal ventral (TV), nasal dorsal (ND), and nasal ventral (NV); central (C), intermediate (I), and peripheral (P) parts of each quadrant were used for evaluation of selected quantitative parameters.

Quantitative Analysis

Per animal, all four quadrants were used for the quantitative studies (Fig. 1). In each vertical section, a 150-μm extent of the central (up to 1 mm from the optic disk), intermediate (up to 500 μm from the midpart of a whole retinal section), and peripheral (up to 1 mm from the ora serrata) parts of the retina reference areas was evaluated. The following parameters were determined.

Thickness of the Ganglion Cell Layer (GCL) Plus Nerve Fiber Layer

Per animal, and per quadrant, two semithin sections (2 μm) representative of the whole extent of the retina, spaced by at least 60 μm, were drawn with the aid of a camera lucida at

a magnification of 640×. The thickness of the GCL/nerve fiber layer was determined as previously described (Silva-Araújo et al., 1993). Briefly, in tracings perpendicular to the inner retinal layers, the extent of the layer was measured in the central, intermediate, and peripheral portions of the retina.

Mean Nuclear Diameter of GCL Neurons (GCLN)

Discrimination between neuronal vs nonneuronal profiles was made according to the indications of Perry (1981), as applied in a previous report (Silva-Araújo et al., 1993). In each selected quadrant, the nuclear profiles of the GCLN were drawn using a tracing technique at a magnification of 640× (Fig. 2). An average of 240 cells/animal were drawn and their diameters measured with the aid of a MOP-Videoplan; the mean nuclear diameter was calculated taking the mean value between its maximum diameter and the diameter perpendicular to its mean point. A correction for the section thickness was introduced (Weibel, 1979).

Linear Density of GCLN

In each section, the extent of the GCL inner limit was drawn and measured. The number of GCLN nuclear profiles within its limits was counted along the centroperipheral axis, and the linear density of GCLN (number per unit length) was then calculated based on the extent of the layer and number of nuclear profiles.

Data Analysis

The analysis of each quantitative parameter followed a 3 × 2 × 2 × 2 × 3 factorial experiment with repeated measures on the last three factors (retinal reference areas): nasal/temporal, dorsal/ventral, and central/intermediate/peripheral) (Winer, 1991).

The data were analyzed by ANOVA using the BMDP statistical software/1990 release. Contrasts were employed to assess the different patterns of variation found in the data, both for main effects and interactions.

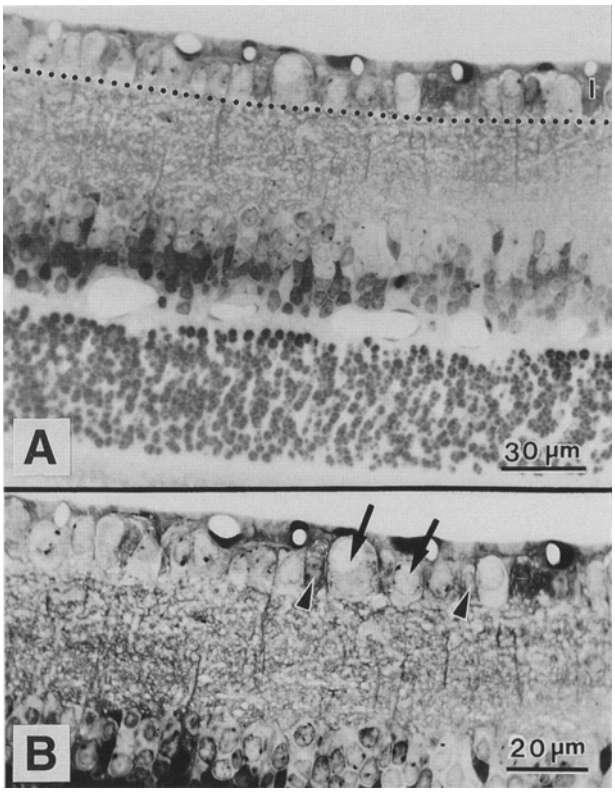

Fig. 2. **(A)** Vertical semithin section from a 14-d-old cocaine-exposed rat. I—Ganglion cell layer plus nerve fiber layer (dots). **(B)** Higher magnification of layer I. Note neuronal profiles (arrows) and nonneuronal profiles (arrowheads).

Results

Animals and Treatments

No differences were detected in the number of pups per litter or in the ratio of male to female per litter. On PND14, the mean body, brain, and cerebellar weights of rats in the three experimental groups were not significantly different; on PND30, the saline group presented, on average, higher body and brain weights than cocaine and control groups (Silva et al., 1995).

Qualitative Observations

Figure 2 A and B is a light micrograph of a semithin section through a retina of a PND14 cocaine-treated rat, as used for qualitative and

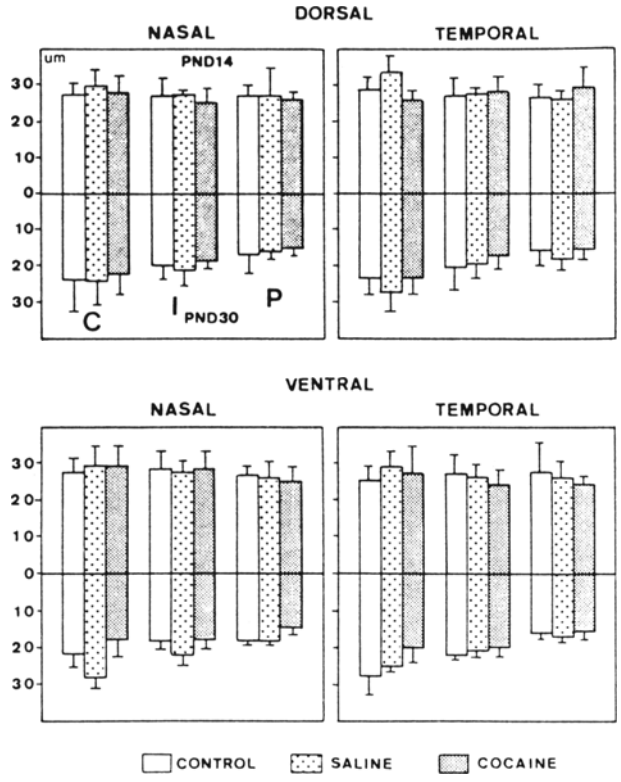

Fig. 3. Thickness of the ganglion cell layer plus nerve fiber layer. C—central; I—intermediate; P—peripheral.

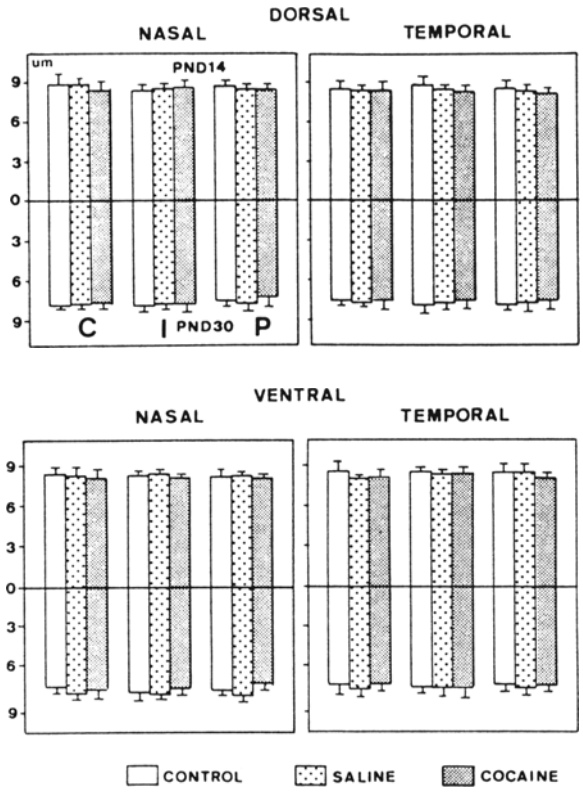

Fig. 4. Nuclear diameters of ganglion cell layer neurons. C—central; I—intermediate; P—peripheral.

quantitative analysis. At the light microscopic level, no marked changes were detected in the organization of the ganglion cell layer in the three groups on PND14 and PND30. The GCLN were easily identified in the semithin sections (Fig. 2A and B).

Quantitative Results

The summary of the quantitative results (means and standard deviations) of the retinal GCL morphological parameters is shown in Figs. 3–5. The selected design enabled the testing of several factors that contribute to the variability of the experimental data: those related to treatment effects (main effect of treatment, treatment by age interaction, treatment by location interactions, and treatment by age by location interactions) and those not involving treatment effects (main effect of age, main effect of location, and age by location interactions).

Effects of Age and Location

The quantitative parameters of the GCL showed on average a significant reduction from PND14 to PND30 (Tables 1–3). A significant reduction of the thickness of GCL/nerve fiber layer and of the linear density of GCLN was also found along the centroperipheral axis. The age-related reduction in the thickness of the retinal GCL/nerve fiber layer and linear density of GCLN (CIP × age interaction)—Tables 1 and 3—increased along the centroperipheral axis.

Effects of Treatment

All the quantitative parameters were affected by treatment. The thickness of the retinal GCL/nerve fiber layer was reduced in

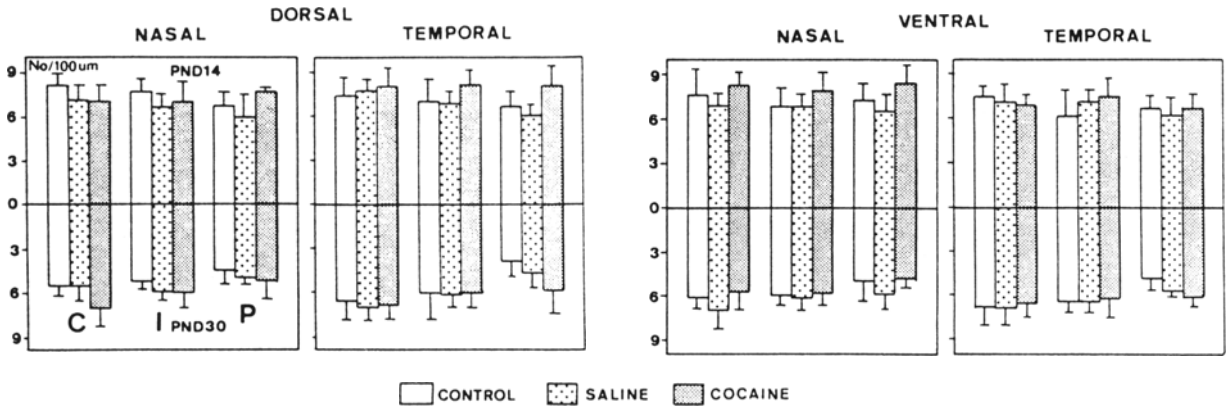

Fig. 5. Linear density (no. per unit length) of ganglion cell layer neurons. C—central; I—intermediate; P—peripheral.

Table 1
Repeated Measures ANOVA for the Thickness of Retinal Ganglion Cell Layer Plus Nerve Fiber Layer

Source of variation	df	Sum of squares	Mean square	*F*
Treatment	2	381.70	190.85	4.53[a]
Age	1	6123.09	6123.09	145.34[b]
Treatment × age	2	91.45	45.72	1.09
Across animals	30	1263.89	42.12	—
NT[c]	1	0.65	0.65	0.03
NT × treatment	2	3.90	1.95	0.08
NT × age	1	23.15	23.15	0.98
NT × treatment × age	2	24.91	12.46	0.53
Within animals	30	706.85	23.56	—
DV[d]	1	7.95	7.95	0.47
DV × treatment	2	19.12	9.56	0.56
DV × age	1	11.02	11.02	0.65
DV × treatment × age	2	29.40	14.70	0.86
Within animals	30	512.20	17.07	—
CIP[e]	2	1525.98	762.99	33.44[b]
CIP × treatment	4	154.25	38.56	1.69
CIP × age	2	557.24	278.62	12.21[b]
CIP × treatment × age	4	36.03	9.01	0.39
Within animals	60	1368.97	22.82	—

[a] $p < 0.05$.
[b] $p < 0.001$, otherwise not significant.
[c] Nasal/temporal.
[d] Dorsal/ventral.
[e] Central/intermediate/peripheral.

the cocaine group when compared with the saline group (F [1,30] = 8.98, $p < 0.01$) (Fig. 6A).

The nuclear diameters were, on average, smaller in the cocaine group than in the control group (F [1,30] = 4.72, $p < 0.05$) and in the saline controls (F [1,30] = 5.45, $p < 0.05$) (Fig. 6B).

The linear density was higher in the cocaine-exposed group than in the control group (F

Table 2
Repeated Measures ANOVA for the Nuclear Diameters of Ganglion Cell Layer Neurons

Source of variation	df	Sum of squares	Mean square	*F*
Treatment	2	4.05	2.03	3.40[a]
Age	1	46.01	46.01	77.09[b]
Treatment × age	2	0.99	0.50	0.83
Across animals	30	17.91	0.60	—
NT[c]	1	1.12	1.12	3.81
NT × treatment	2	0.42	0.21	0.72
NT × age	1	0.31	0.32	1.08
NT × treatment × age	2	0.33	0.17	0.58
Within animals	30	8.82	0.29	—
DV[d]	1	0.39	0.39	1.37
DV × treatment	2	0.48	0.24	0.84
DV × age	1	0.01	0.01	0.05
DV × treatment × age	2	0.37	0.18	0.64
Within animals	30	8.66	0.29	—
CIP[e]	2	1.08	0.54	3.25[a]
CIP × treatment	4	0.54	0.14	0.82
CIP × age	2	0.15	0.08	0.46
CIP × treatment × age	4	0.67	0.17	1.00
Within animals	60	10.00	0.17	—

[a] $p < 0.05$.
[b] $p < 0.001$, otherwise not significant.
[c] Nasal/temporal.
[d] Dorsal/ventral.
[e] Central/intermediate/peripheral.

[1,30] = 6.42, $p < 0.05$) and saline group (F [1,30] = 5.42, $p < 0.05$). Moreover, there was a greater decrease with age in the linear density of the GCLN of the cocaine-exposed rats in comparison with rats in the control group (F [1,30] = 5.37, $p < 0.05$) (Fig. 6C).

An effect of treatment was also found along the centroperipheral axis. Although in control and saline rats there was a significant decrease in the linear density of GCLN along this axis, this decrease was not significant in the cocaine-exposed rats (Fig. 6D).

Discussion

The effects of cocaine use on the fetus have been extensively reviewed in a recent paper (Volpe, 1992). Experimental models provided additional evidence that cocaine affects the development of different sensory systems (Church and Overbeck, 1991; Webster et al., 1991; Salamy et al., 1992). In an earlier study, we demonstrated that early and chronic exposure to cocaine and amphetamine affected the structural organization of the optic nerve (Silva-Araújo et al., 1991), that the thickness of the retinal GCL was reduced, and that a higher packing density of GCLN was found along with the presence of degenerated profiles (Silva-Araújo et al., 1993). These data led to the suggestion that neonatal cocaine exposure in the rat induced morphological changes in the developing visual pathways. However, it remained to be assessed whether early prenatal exposure to this drug might also induce long-term changes, since recent reports documented gross visual abnormalities in humans (Dixon et al., 1987; Isenberg et al., 1987; Teske and Trese, 1987; Ferriero et al., 1989; Dominguez

Table 3
Repeated Measures ANOVA for the Linear Density of Ganglion Cell Layer Neurons

Source of variation	df	Sum of squares	Mean square	F
Treatment	2	20.28	10.14	3.96[a]
Age	1	170.49	170.49	66.57[c]
Treatment × age	2	17.14	8.56	3.35
Across animals	30	76.84	2.56	—
NT[d]	1	1.58	1.58	1.58
NT × treatment	2	1.47	0.74	0.74
NT × age	1	6.84	6.84	6.84[a]
NT × treatment × age	2	3.39	1.69	1.70
Within animals	30	30.00	1.00	—
DV[e]	1	1.35	1.35	0.90
DV × treatment	2	4.94	2.47	1.65
DV × age	1	1.83	1.83	1.22
DV × treatment × age	2	5.30	2.65	1.77
Within animals	30	44.92	1.49	—
CIP[f]	2	66.58	33.29	35.30[c]
CIP × treatment	4	10.02	2.51	2.66[a]
CIP × age	2	12.78	6.39	6.77[b]
CIP × treatment × age	4	6.10	1.52	1.62
Within animals	60	56.58	0.94	—

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$, otherwise not significant.
[d] Nasal/temporal.
[e] Dorsal/ventral.
[f] Central/intermediate/peripheral.

et al., 1991; Good et al., 1992) and animals (Mahalik et al., 1980; Church et al., 1990) exposed prenatally to cocaine and/or other illicit drugs.

As shown previously (Spear et al., 1989; Church et al., 1990), gestational exposure to cocaine did not interfere with the duration of pregnancy and number of live male and female pups per litter. Since used previously, the sc route was chosen for administration of the drug for its consistency with the available laboratory reports on behavioral teratogenicity of cocaine (Spear et al., 1989). The period of exposure corresponds to the period of neural tube closure until the day before birth, covering the major period of neural differentiation.

The results of the present study provide evidence that administration of 60 mg/kg/d of cocaine from GD8–22 induces a number of quantitative alterations in the organization of the retinal GCLN, despite the lack of observable light microscopic qualitative changes. The alterations could be ascribed to the developmental pattern (age effects), to the treatment imposed (treatment effects), and to various interactive factors. The purpose of the quantitative and statistical evaluation was to discriminate among the factors that might explain the variability found in the different retinal GCL parameters.

It is known that the neuronal population of the rat retinal ganglion cell layer decreases substantially during early postnatal development (Potts et al., 1982; Sengelaub and Finlay, 1982; Horsburgh and Sefton, 1987; Reese and Colello, 1992). The higher density of neurons on PND14 compared with PND30 may reflect the last phase of the ongoing process of natural cell

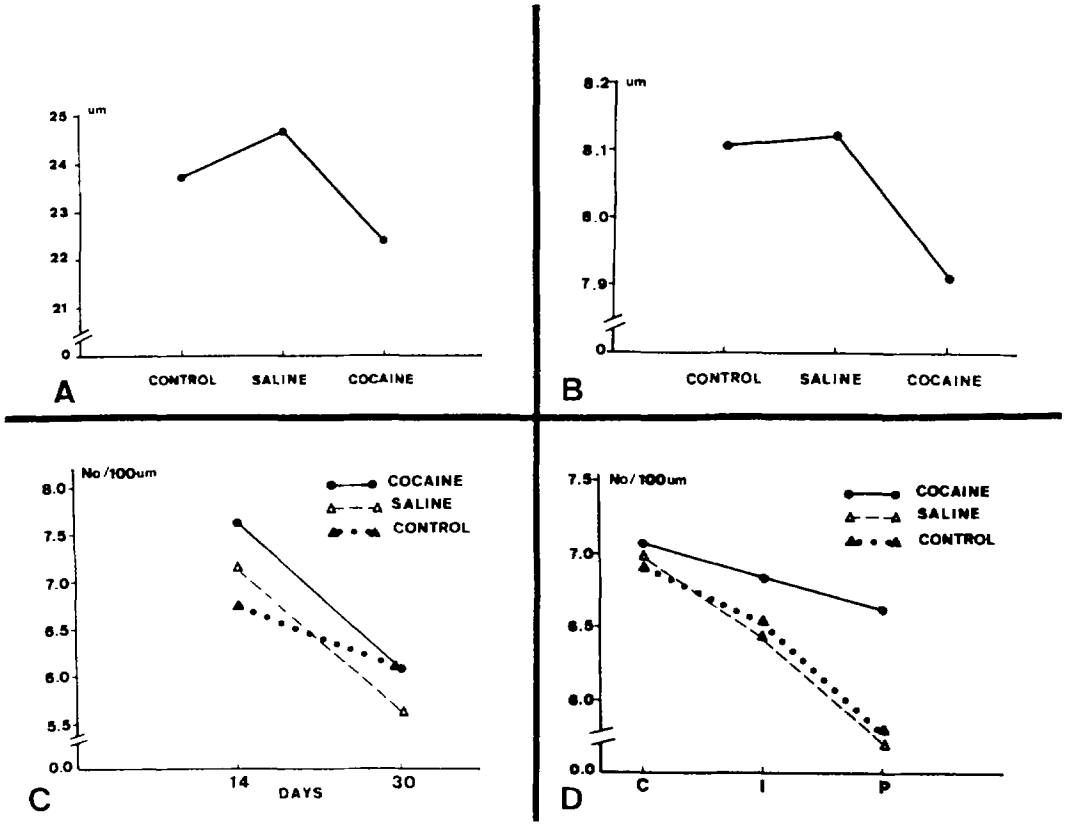

Fig. 6. Graphic representation of treatment effects. **(A)** layer thickness; **(B)** nuclear diameters; **(C)** linear density and age; **(D)** linear density along the centroperipheral axis.

death in the postnatal period after the overproduction of neurons. In cocaine-treated animals, there is a significant delay as compared with age-matched controls, reflecting an alteration of the normal maturational postnatal changes in the retina, which could also affect the retinal growth known to continue after PND14 and/or specific development of the GCLN.

In this study, we evaluated all the neurons in the GCL. The GCL of the vertebrate retina contains different types of neuronal and nonneuronal elements, which were identified according to the criteria of Perry (1981). However, discrimination between ganglion cells and amacrine cells, which constitute a substantial number of GCL neurons in the retina of the rat, would require tracing techniques (Perry, 1981; Reese and Colello, 1992), which were not used in the present study. Although vertical semithin sections of the retina provide a clear view of the neuronal morphology, the number of cells analyzed is far smaller than the number analyzed using whole-mounted retinas (Horsburgh and Sefton, 1987).

The delay observed in the establishment of normal cell numbers may cause marked changes in the final adjustment of the various neuronal populations to the afferent and efferent patterns of each cell. This fact was emphasized by Linden and Perry (1982), who emphasized the role of cell death in the production of regular spacing between ganglion cells. Moreover, there is evidence that the activity of retinal ganglion cells influences normal synaptic development in the retina circuitry (Dubin et al., 1986; Kalil et al., 1986). In fact, the development of a complex structure like the retina requires the coordination of a

number of specific processes, including cytogenesis, cellular migration, dendritic differentiation, axonal growth, synaptogenesis, and cell death. This set of requisites makes the retina highly sensitive to experimental and/or pathological situations occurring in different periods of pre- and/or postnatal development.

It is known that cocaine abuse causes reduced uterine blood flow, impaired oxygen transfer, and fetal hypoxemia (Woods et al., 1987). It is also recognized that cocaine interferes directly with a number of neurochemical systems, e.g., the dopaminergic system (Akbari and Azmitia, 1992).

It has been recently suggested that prenatal exposure to cocaine has deleterious effects on biomarkers of cell development in both fetal and neonatal rats (Seidler and Slotkin, 1993). The functional deficits found after fetal cocaine-exposure probably reflect the effects on specific cell populations (Seidler and Slotkin, 1993). Whether any cellular population in the retina is specifically vulnerable to this drug remains to be clarified. We suggest that our data from rat retinas have important implications for the development of humans exposed gestationally to cocaine.

Acknowledgments

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