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Effects of Prenatal Cocaine Exposure in the Photoreceptor Cells of the Rat Retina

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Abstract

Despite the increasing evidence of eye abnormalities, the effects of prenatal exposure to cocaine on the visual system are still poorly understood. This study was aimed at analyzing the qualitative and quantitative organization of the retinal photoreceptor cells (PR) and outer nuclear layer (ONL) after prenatal exposure to cocaine in the rat. Pregnant Wistar rats were given sc injections of cocaine hydrochloride (60 mg/kg body wt/d) or saline or were not manipulated; analyses were performed in the 14- and 30-d-old male offspring. Radial semithin and ultrathin sections of epon-embedded flat mounts of the retina showed displaced PR-like cells in the inner nuclear layer (INL), picnotic PR nuclei in INL, and ONL, and retinal PR rosettes and outer-segment debris in the subretinal space. The quantitative study showed an increased density of PR-like nuclei in the INL in PND14 cocaine-treated rats that were within normal values at PND30; no changes were detected in the PR mean nuclear diameter and in the packing density of PR nuclei in the ONL. These data constitute the first morphological demonstration of photoreceptor damage after prenatal cocaine-exposure probably owing to a direct action of the drug and/or to the cocaine-induced ischemia/hypoxia.

Index Entries: Retina; photoreceptors; cocaine; prenatal exposure; rat; morphometry.

Introduction

Clinical studies on the use of drugs during periods of fetal development provide increasing evidence that prenatal exposure to cocaine is associated with a number of teratogenic amd neurobehavioral abnormalities (Chasnoff et al., 1986; Gingras et al., 1992; Volpe, 1992). Despite the compelling evidence of specific ocular abnormalities in infants exposed prenatally to drugs of abuse, particularly to cocaine, e.g., optic nerve hypoplasia, microphthalmia with retinal dysgenesis, and retinal colobomas (Dixon et al., 1987; Isenberg et al., 1987; Ferriero et al., 1989; Dominguez et al., 1991; Good et al., 1992), little work has been done on the morphological effects of prenatal cocaine-exposure on the developing visual system. Previous

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studies in rodents demonstrated gross morphological changes in the visual system (Mahalik et al., 1980; Church et al., 1990; Webster et al., 1991). More detailed studies revealed that pre- and postnatal exposure to psychostimulants altered the qualitative and quantitative organization of the optic nerve and ganglion cell layer of the retina in rats exposed both prenatally (Salgado-Borges et al., 1992; Silva-Araújo et al., 1992) and postnatally to cocaine (Silva-Araújo et al., 1991, 1993). Recently, we have also shown that neonatal exposure to cocaine induced epiretinal membrane formation allied to changes in the organization of the photoreceptor layer, namely the appearance of photoreceptor rosettes (Silva-Araújo et al., 1994). The changes of the photoreceptor layer were thought to be related to the vulnerability of the retina to variation in the concentration of oxygen, e.g., hypoxia/ischemia (Hughes, 1991), which is also associated with exposure to cocaine (Webster and Brown-Woodman, 1990; Fantel et al., 1992; Gingras et al., 1992; Wallace et al., 1992).

To evaluate the role of gestational exposure to cocaine as a cause of altered developmental maturation of the visual pathways, it was necessary to study the effects on photoreceptors of the retina. We report the morphological organization of the photoreceptors of 14- and 30-d-old rats exposed prenatally to cocaine, in an attempt to determine whether some of the quantitative parameters were altered by this experimental circumstance. Part of this work has been presented in abstract form (Tavares et al., 1993).

Methods

Animals, Treatments, and Experimental Design

Rats used were the male offspring born from nulliparous Wistar female 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 adult females (60-d-old) from 8:00 PM to 8:00 AM the next morning. Identification of a vaginal plug or a sperm-positive vaginal cytology determined the gestational day (GD) 1. The females were housed individually in plastic breeding cages and weighed on alternate days.

From GD8–GD22, females assigned to the cocaine group were given injections of cocaine hydrochloride (Sigma, St. Louis, MO) dosed in 60 mg/kg body wt/d, administered sc in 0.9% saline (3 mL/kg body wt/d) until GD22. The daily dose was given in two equal parts, the first being injected between 8:30 and 9:00 AM and the second between 6:00 and 8:00 PM. The site of injection was moved around the rat dorsal surface in order to minimize skin necrosis. During the same experimental period (GD8–GD22), a control group of dams was given an equivalent volume of saline vehicle and designated as saline group; another group that was not manipulated constituted the noninjected controls. Food and water were given ad libitum to all experimental groups. Overall 36 litters were used, 12 litters from 12 different females assigned to each of the three experimental groups (Silva et al., 1994).

After delivery, the total number of offspring was recorded and each animal sexed and weighed. The litters were then culled to eight pups (four males and four females). From each litter pups were weaned on postnatal day (PND) 21.

For the quantitative evaluations, two males were taken at random from each of six females, totaling 12 rats/treatment (control, saline, and cocaine). Half of the subjects in each treatment were randomized to be sacrificed on PND14 and the other half on PND30.

Tissue Preparation

At PND14 and PND30, the rats were deeply anesthetized with ether and perfused transcardially with a fixative solution of 1% glutaraldehyde and 1% paraformaldehyde in 0.12*M*

phosphate buffer at pH 7.4 (Palay and Chan-Palay, 1974). The right eye was selected for the quantitative analysis. Before enucleation, the eyes were oriented by an India ink mark at 12 h. 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 smaller sections were made at 3, 6, and 9 h and the temporal/dorsal quadrant was identified by a short peripheral cut. The retinas were immersed overnight in the same fixative and then rinsed for 2 h in cacodylate buffer; postfixation was made in cacodylated buffered 1% osmium tetroxide followed by dehydration in ascending concentrations of ethanol, immersion in mixtures of resin/propylene oxide, and embedded flat in epoxic resin (TAAB). Each quadrant was cut, re-embedded in resin, and vertical semithin sections (2 μm) were obtained with an ultramicrotome and stained with toluidine blue; ultrathin sections were double-stained with uranyl acetate and lead citrate.

Quantitative Analysis

In vertical semithin sections of each retinal quadrant (temporal/dorsal, temporal/ventral, nasal/dorsal, and nasal/ventral) the following parameters were determined.

Surface Area of the ONL

Per animal and in vertical sections of two quadrants, the boundaries of the ONL of a retinal meridian were drawn using a camera lucida and a final magnification of ×200. The surface area of the ONL was measured using a MOP-Videoplan.

Mean Nuclear Diameter of PR

Per animal and per quadrant, random areas of the central (up to 1 mm from the optic disk), peripheral (up to 1 mm from the ora serrata), and intermediate (up to 500 μm from the midpart of a whole retinal section) parts of the ONL were photographed with a ×100 objective, and prints were obtained with a ×2000 final magnification. The nuclear profiles of the PR located within an area of the reference test grid

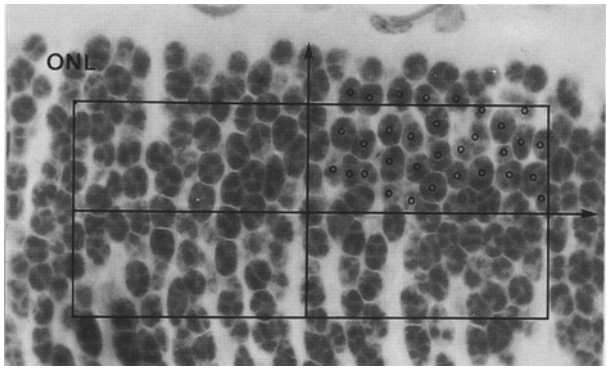

Fig. 1. Test grid reference area used for determination of the quantitative parameters. The "forbidden" line of a reference area is shown by arrows. Open circles represent the profiles counted in a reference area. ONL—outer nuclear layer.

(Fig. 1) were drawn, and the diameters measured with the aid of a MOP-Videoplan. The mean diameter was then calculated (Weibel, 1979). On average, 200 PR nuclei were measured/animal. Assuming that these nuclei could be considered spheres, the values were corrected for the overestimation of large profiles resulting from section thickness effect (Weibel, 1979); for this correction, the section thickness was estimated as 2 μm.

Packing Density (Number per Unit Surface Area) of PR in the ONL

The packing density of PR nuclei located in the ONL was determined using the same prints and the test grid, and applying the rule of the "forbidden" line (Gundersen, 1977).

Packing Density (Number per Unit Surface Area) of PR in the INL

Using a reference test grid of 80 × 50 mm and a final magnification of ×640, the number of photoreceptor-like nuclei located in the INL was counted. The rule of the "forbidden" line was applied for these counts as well. The counts were performed in 8 reference areas/quadrant, in each animal. The density of these cells (number per unit area surface of INL) was then calculated. The criteria for identification of photoreceptors was based on the descrip-

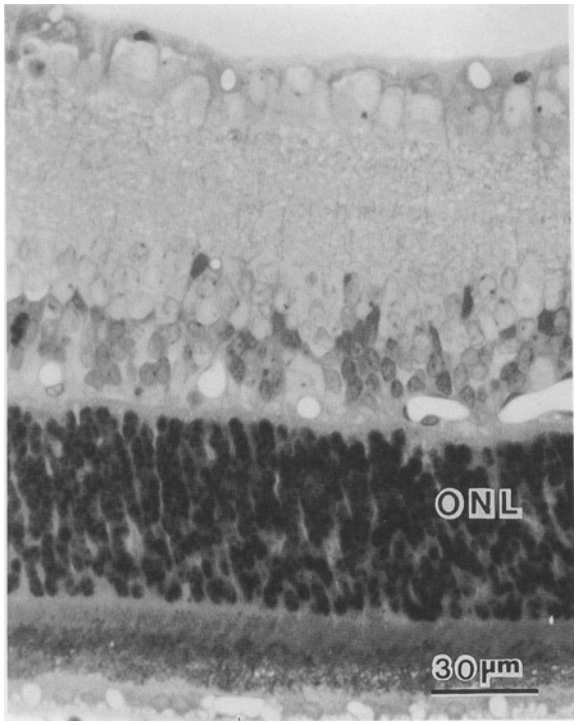

Fig. 2. Light micrograph from a vertical semithin section of a PND14 saline rat. ONL—outer nuclear layer.

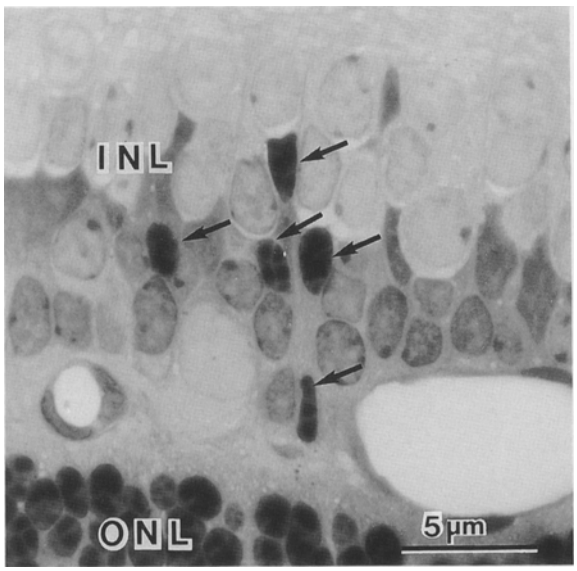

Fig. 3. Light micrograph from a vertical semithin section of the INL of a PND14 cocaine-treated rat. Note the profiles of the ectopic photoreceptor nuclei (arrows). ONL—outer nuclear layer.

tions given in previous papers on displaced photoreceptor-like cells in developing rodent retina (Spira et al., 1984; Akari et al., 1988).

Statistical Analysis

The data were analyzed by Kruskal-Wallis test, followed by pairwise comparisons with the nonparametric Mann-Whitney U-test (Conover, 1980).

Results

Animals and Treatments

There were no significant differences in the maternal body weight gain from GD1 to GD22 among the three experimental groups, in the gestational length, or in the litter size. At PND14, the mean body, brain, and cerebellar weights were not significantly different among the three groups. At PND30, the saline group had higher brain weights than the other experimental groups (Silva et al., 1995).

Qualitative Observations

Figures 2 and 3 are light micrographs of semithin vertical sections obtained through the retinas of a PND14 saline rat (Fig. 2) and a PND14 cocaine-treated rat (Fig. 3). At the light microscopic level, the main changes observed in PND14 cocaine-treated rats consisted of an increase in PR-like nuclei scattered in the INL (Fig. 3); these cells, located in the INL, showed the same nuclear features as rod cells, and were frequently observed in the middle and inner portions of the INL. However, they were not found in the outer plexiform layer; these profiles were also found in PND14 controls, although they were less abundant.

At PND30, few PR-like cells were found in the INL of the cocaine-exposed animals and control groups. The PR-like cells were identified as PRs by electron microscopic study of the cocaine-exposed retinas (Fig. 4); the nuclear chromatin patterns and staining densities of their somas were identical to rods located in the ONL. They were smaller than the

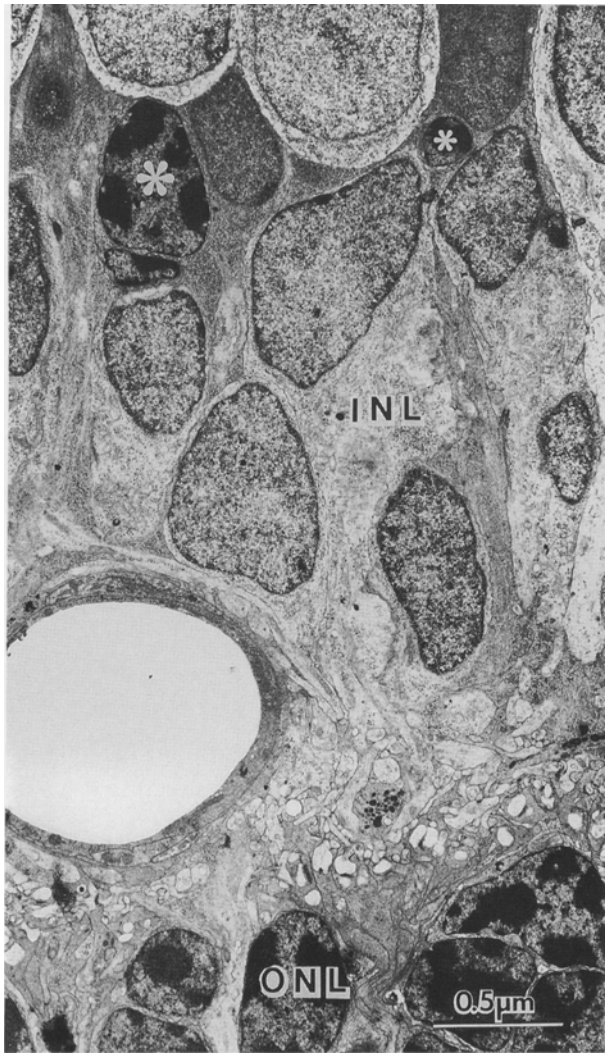

Fig. 4. Electron micrograph from the INL and ONL of a PND14 cocaine-treated rat. Note the ectopic photoreceptor nuclear profiles (*) amidst the other neuronal profiles.

surrounding cells in the INL and had a scanty cytoplasm; the nuclei were elongated or round in sections (Fig. 3). Cells with similar characteristics, also located in the INL, have been identified as PRs by their morphological characteristics (Spira et al., 1984) and by their immunoreactivity for opsin and 5'-nucleotidase (Akari et al., 1988).

The retinas of PND30 cocaine-exposed rats contained photoreceptor rosettes, which were

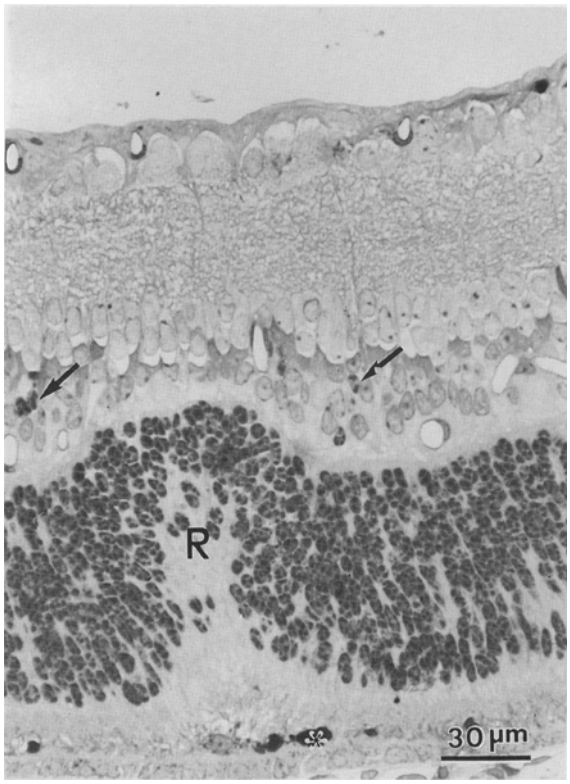

Fig. 5. Light micrograph from a vertical semithin section of a PND30 cocaine-treated rat. Photoreceptor rosette (R) and cellular debris in the subretinal space (*) can be identified. Ectopic photoreceptors can be seen (arrows).

less frequent in controls (Figs. 5 and 6). The rosettes were very similar to those found in rats exposed postnatally to cocaine (Silva-Araújo et al., 1994). Electron microscopy revealed that these PR rosettes had shortened, degenerated outer segments (Fig. 7).

Quantitative Results

The mean surface area of the ONL (Fig. 8) was not significantly different in the cocaine-treated group (PND14 and PND30) as compared with the age-matched control groups. The mean nuclear diameters of PRs (Fig. 9) and the packing density of PRs in the ONL (Fig. 10) were not significantly different in the three age-matched groups; PR-like cells in the INL were significantly increased in all hemi-retinas in

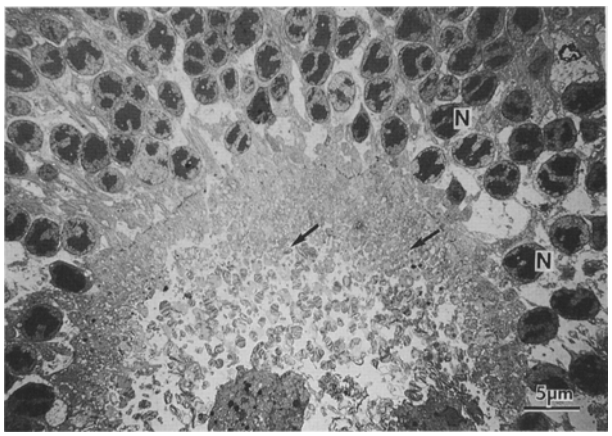

Fig. 6. Electron micrograph of a rosette formation in a PND30 cocaine-treated rat. Photoreceptor nuclei (N) and disorganization of the PR segments (arrows) can be seen.

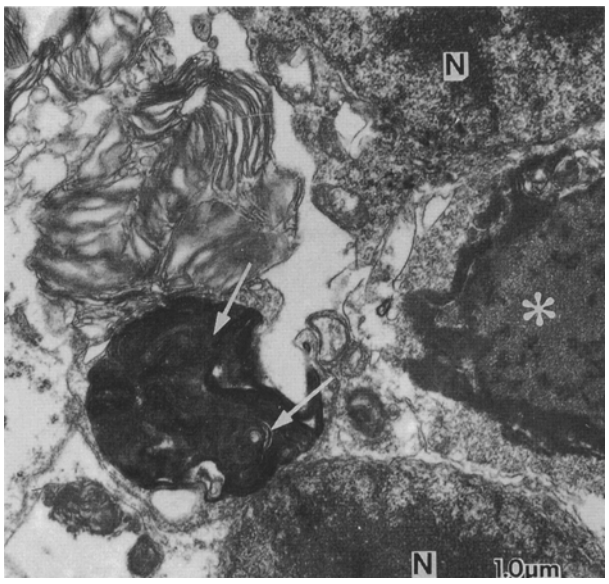

Fig. 7. Electron micrograph of the ONL of a PND30 cocaine-treated rat. A degenerated PR outer segment (arrows) can be identified. N—photoreceptor nuclear profiles; (*) degenerated profile of a photoreceptor nucleus.

PND14 cocaine-treated rats when compared with any of the control groups (Fig. 11). No significant differences were detected between the noninjected control and saline groups (Fig. 11).

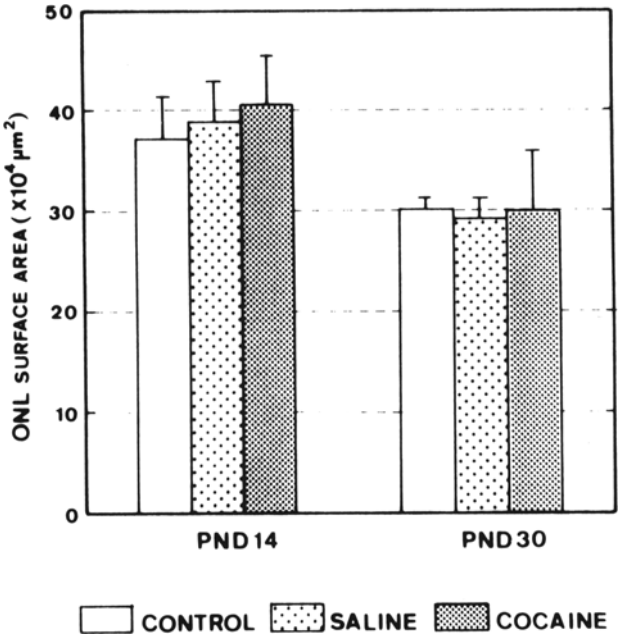

Fig. 8. Graphic representation of the surface area of the retinal ONL.

The differences found in PND14 cocaine-treated rats were no longer present in PND30 cocaine-treated rats (Fig. 11).

Discussion

The present study demonstrated that prenatal exposure to cocaine from GD8–22 causes striking alterations in the retinal photoreceptor cells of 14- and 30-d-old exposed male offspring. Changes in the structural organization of photoreceptors were observed in both ages (PND14, PND30) when cocaine-treated rats were compared with controls. Allied to the alteration of the structural organization of the photoreceptors, e.g., increased number of displaced rods in the INL and the appearance of photoreceptor rosettes, the morphometric parameters of this retinal cellular population were also affected, including an increase in the number of ectopic PR-like cells in the INL in PND14 cocaine-treated rats. Although morphological changes could still be detected in PND30 cocaine-treated rats, the other quanti-

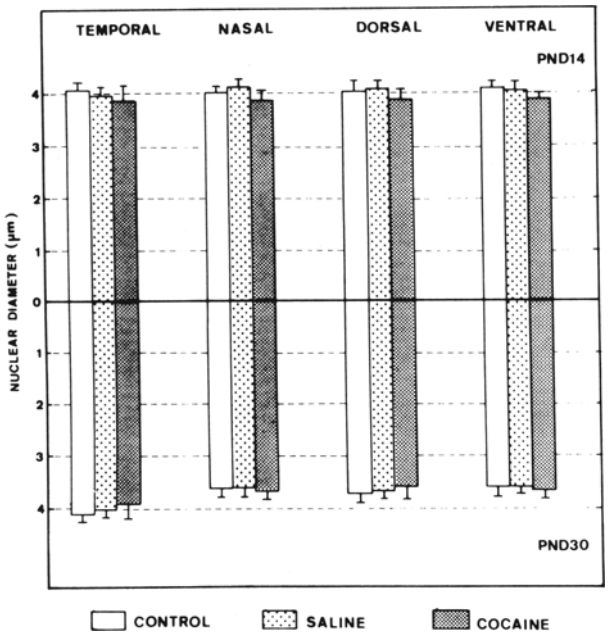

Fig. 9. Graphic representation of the mean nuclear diameters of the photoreceptor nuclei in the ONL.

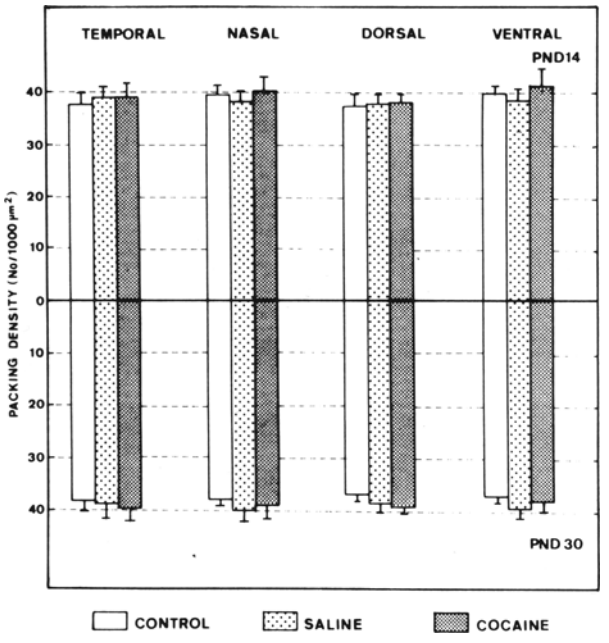

Fig. 10. Graphic representation of the packing density (number/unit surface area) of the photoreceptor nuclei in the retinal ONL.

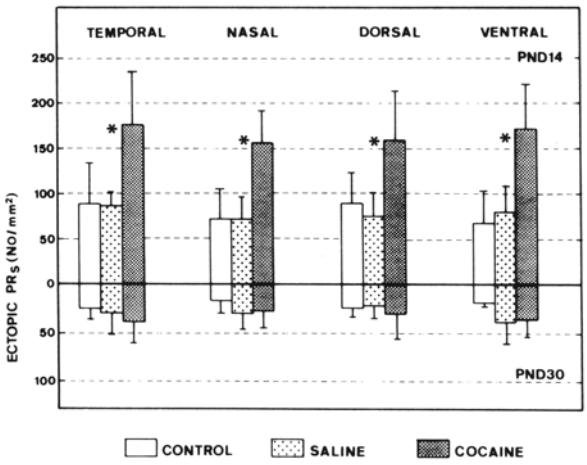

Fig. 11. Graphic representation of the packing density (number/unit surface area) of the ectopic photoreceptor nuclei located in the INL. $*p < 0.001$ cocaine vs control vs saline (PND14).

tative parameters were within normal values. The morphological aspects of PR maturation have been widely described in the developing rat retina by light and electron microscopy (Weidman and Kuwabara, 1968, 1969; Galbavy and Olson, 1979). Ectopic photoreceptors have been described as a normal finding in the developing rat retina (Young, 1984), their number gradually decreasing with age and no longer being detected by the end of the first postnatal month. Cells resembling those of PR, but with the soma located in the INL have been also reported in the early postnatal mice (Saynal and Bal, 1973; Blanks and Bok, 1977) and rats (Raedler and Sievers, 1975; Spira et al., 1984; Akari et al., 1988).

Although immunocytochemical studies were not undertaken in the present work, there are a few electron microscopic and histochemical studies (Spira et al., 1984; Akari et al., 1988) using specific antibodies against opsin and histochemistry for 5'-nucleotidase, which is found specifically on the plasma membrane of rod cells (Kreutzberg and Hussein, 1984), demonstrating that the PR-like cells located during development in the INL are, in fact, photoreceptors. What has not yet been clarified is their final destination: migration into their usual

position in the ONL, pycnosis, and death, or differentiation into bipolar cells remaining in the INL (Spira et al., 1984).

We could not detect the PR-like cells in the outer plexiform layer by PND14, an age at which this layer is already well differentiated, suggesting that these cells are not migrating back to the ONL. Since no differences were detected in the number of ectopic PRs between uninjected control and saline groups, the effects of manipulation were not a confounding factor for this finding. However, whether the ectopic PRs undergo degeneration or phenotypic conversion between PND14 and PND30 remains to be clarified. We could not identify outer segments or synaptic terminals in the PR-like cells located in the INL, as also reported by Akari et al. (1988); however, although these cells were found to have outer segments and presynaptic terminal profiles (Spira et al., 1984), they do not appear to establish normal synaptic junctions, unlike other neuronal ectopias (Landis, 1973). If this did occur, long-term alterations might be expected in the synaptic organization in the retina of prenatally cocaine-exposed rats.

In some of the 30-d-old cocaine-exposed rats, the retinas contained photoreceptor rosettes, which, as found for the displaced cells in the INL, had no preferential location among the retinal quadrants. Some models of retinal degeneration show differential vulnerability of the retinal regions, e.g., in light-induced retinal degeneration, the dorsal part of the retina is more affected than the other parts (Rapp et al., 1985; Edward et al., 1993). In our study, regional differences in PR damage were not evident morphologically as opposed to the findings of the ganglion cell layer neurons whose parameters were diferentially affected in the retinal quadrants (Silva-Araújo et al., 1993). Moreover, the quantitative increase of the PR-like cells in the INL at PND14 was not confined to a certain retinal location, but was found throughout the whole retinal surface area.

As found previously (Church et al., 1990), prenatal exposure to cocaine did not affect the gestational weight gain and length, the litter size, or the ratio of male to female per litter. Also, the growth of the animals until PND14 and PND30 was in good accord with other studies (Church et al., 1990).

The dose of cocaine used in this experiment was based in previous studies (Spear et al., 1989) on prenatal exposure to cocaine, which demonstrated that lower doses did not induce significant behavioral effects. It is very questionable to draw comparisons on doses of consumption with human data since the human situation always has many confounding variables and most of the subjects are polydrug abusers. In this context and owing to the limitations imposed by the human data, the animal model can contribute to the assessment of the ocular abnormalities described in cocaine-exposed infants, providing a valuable insight into the temporal development and vulnerability of the visual system.

Development of both the central nervous system structure and the retina requires the coordination of several processes, including cytogenesis, cellular migration, dendritic differentiation, axonal growth, synaptogenesis, and cell death. These requisites make the retina highly vulnerable to experimental and/or pathological conditions occurring during pre- and/or postnatal development. It is known that PR cells are a retinal neuronal population whose ordered specialization is attained during different perods of retinal development (Young, 1984). The delay observed in the acquisition of the normal pattern of photoreceptors in cocaine-treated rats may cause alterations in the final organization of the retinal circuitry. Several mechanisms may underlie the observed effects of prenatal cocaine exposure in the rat. There is evidence that cocaine interferes with several neurochemical systems and has deleterious effects on biomarkers of cell development in both fetal and neonatal rats (Seidler and Slotkin, 1993). In the retina, its effects are not confined to a specific cellular population, since alterations were also found in the ganglion cell layer (Silva-Araújo et al.,1992). The widespread effects of cocaine-exposure on the fetus may be the result of vascular effects of cocaine,

since cocaine use during pregnancy causes reduction of the uterine blood flow, impaired oxygen transfer, and fetal hypoxemia (for review, *see* Volpe, 1992). The transient vasoconstriction induced by cocaine also results in ischemia/reperfusion damage to the fetus (Fantel et al., 1992). The deleterious effects of cocaine are probably exacerbated by the fetal hypoxia/ischemia and/or hypertension caused by the drug (Gingras et al., 1992; Horn, 1992; Volpe, 1992).

Moreover, it is known that ischemia/hypoxia induced by pre- or neonatal exposure to cocaine has marked effects in the retina, causing hemorrhagic lesions and formation of epiretinal membranes (Silva-Araújo et al., 1994). The formation of photoreceptor rosettes has been documented after ischemia of the retina (Hughes, 1991), and this mechanism may also underlie the rosettes found in PND30 rats exposed prenatally to cocaine.

Although some abnormal parameters are only transitory, as seems to be the case of the displaced photoreceptors, other alterations appear to be permanent, such as the retinal photorecetor rosettes. The effects of such changes on development of connectivity patterns in the retina remain to be established.

Acknowledgments

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