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Cocaine Exposure During the Brain Growth Spurt Failed to Produce Cerebellar Purkinje Cell Loss in Rat Pups

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ABSTRACT Previous studies in our laboratory indicated that cocaine exposure during the brain growth spurt period, a developmental stage vulnerable to various teratogens, did not produce microencephaly (gross brain weight measures). However, neonatal cocaine exposure has been shown to affect motor coordination and balance, which are both sensitive to cerebellar damage. The purpose of this study was to investigate whether cocaine exposure during the brain growth spurt period could result in the loss of cerebellar Purkinje cells, a neuronal population known to be vulnerable to other teratogenic insults. Sprague-Dawley rat pups were randomly assigned to either cocaine-treated groups (40, 80 mg/kg s.c.) or a gastrostomy control group, and were reared using an artificial-rearing method from postnatal days (PDs) 4 through 9. On PD 10, these animals were perfused and the cerebella were extracted and processed for cell counts. Estimates of Purkinje cell numbers were obtained using a 3-dimensional optical disector method. The results using this stereological method demonstrated no significant Purkinje cell loss in response to cocaine treatment, even at a dose which has been shown to result in high mortality. The failure of cocaine to produce significant Purkinje cell loss (present finding) or microencephaly (previous finding) adds to the evidence indicating that cocaine is not a potent neuroteratogen.

Whether cocaine is a potent teratogen remains a controversial issue. According to a recent commentary discussion [published in Neurotoxicology and Teratology (vol. 15, no. 5)], several leading investigators within the field of prenatal cocaine research indicated there are still important unanswered questions related to understanding the effects of maternal cocaine exposure on the offspring, both from clinical and experimental perspectives. Currently, there is no consensus regarding the teratogenic effects of cocaine resulting from experimental findings, possibly due to differences in drug dosage, methodology or animal species used, and it is important to reconcile these differences in the literature. Clinically, it is unclear whether the teratogenic effects of cocaine are uniquely and solely associated with prenatal cocaine exposure or the result of the interactive action of many other risk factors, such as maternal polydrug abuse, conditions of maternal health, or timing of the exposure during pregnancy.

Much of the published clinical data support the general notion that prenatal cocaine exposure produces various obstetric complications (Handler et al., '91; Neuspiel et al., '94; Ryan et al., '87; Singer et al., '94), as well as a general somatic growth retardation (Chouteau et al., '88; Hadeed and Siegel, '89; Kliegman et al., '94; Petitti and Coleman, '90) and various neurobehavioral alterations in the offspring of maternal cocaine abusers (Chasnoff et al., '85; '89b; Griffith et al., '88; Legido et al., '92). However, the interpretation of the clinical results consistently has been plagued by many of the potential confounding variables, such as sampling bias, socioeconomic status and the accuracy of self-report data (Jacobson and Jacobson, '90; Weinberg et al., '92). Therefore, in order to have a thorough understanding of how prenatal cocaine exposure, per se, is responsible for documented medical complications, animal model systems incorporating stringent methodological controls are valuable tools in addressing this controversial issue.

The postnatal rat model system has been widely employed in assessing the effects of teratogens on brain development, particularly in the field of fetal alcohol syndrome (Diaz, 1991; Marcussen et al., '94; Phillips and Krueger, '90; Riley and Meyer, '84; Samson and Grant, '84; Tavares and Silva, '93). The postnatal rat model is unique insofar as the timing of the brain growth spurt occurs after birth (Dobbing and Sands, '73, '79). Brain development in humans begins at the time

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of conception and continues into postnatal life with a rapid period of development occurring during the third trimester; this rapid brain growth has been termed the brain growth spurt (Dobbing and Sands, '79). In the rat, the brain growth spurt occurs entirely postnatally. Therefore, in order to study the effects of cocaine on brain development during the brain growth spurt requires exposing rats to cocaine during the neonatal period to extrapolate the findings from rat to human.

Previous work from our laboratory indicated that cocaine exposure during the brain growth spurt period did not produce gross brain growth deficits in a rat model system, even at a dose which significantly increased the mortality rate (Chen et al., '93, '94a). This was a surprising finding, given that microcephaly is often reported as the result of cocaine exposure during gestation (Chasnoff et al., '89a; Little and Snell, '91; Ryan et al., '87). Recent efforts in elucidating interactive effects of polydrug use (i.e., cocaine and alcohol) in rodent model systems have failed to reveal any significant effects of cocaine alone or an interaction with alcohol treatment on various dependent measures including gross brain weight. On the other hand, alcohol administration alone effectively produces a high incidence of gross malformations and brain growth restrictions in some studies (Chen et al., '94b; Randall et al., '94).

Although we found no obvious deficits in gross brain growth parameters as assessed in our previous experimental settings, microscopic alterations in specific neuronal populations may occur in response to cocaine treatment. In this study, we investigated the effects of cocaine exposure during the brain growth spurt period on the viability of cerebellar Purkinje cells. The rationale for counting cerebellar Purkinje cells is threefold. First, it is known that cerebellar Purkinje cells are vulnerable to a variety of neuroteratogenic insults, including alcohol (Goodlett et al., '90; Hamre and West, '93) and hypoxia (Cervous-Navarro and Diemer, '91; Cervous-Navarro et al., '91) during the brain growth spurt. Second, the topographic organization of cerebellar Purkinje cells is well defined, which facilitates obtaining good estimates of neuronal numbers. Third and most importantly, cocaine exposure in neonatal rats has been shown to affect motor coordination and balance, measures which reflect cerebellar damage (Barron and Irvine, '94). State-of-the-art stereological methods were used in this study to estimate the total number of cerebellar Purkinje cells.

METHODS

Subjects

The subjects were 26 male and female Sprague-Dawley rat pups derived from 9 time-mated litters which were reared as part of the study published previously reporting the effects of cocaine on mortality, somatic growth and gross brain weight (Chen et al., '94a). The animals were born and reared in a vivarium at the University of Iowa. The day that sperm was detected in the vaginal smear was designated as gestational day 0, thus, gestational day 22 was defined as postnatal day 0 (postnatal day [PD], rather than gestational day, is used throughout the manuscript). Pups within each litter were culled to 10 on PD 1, using cross-fostering procedures as necessary. On PD 4, the rat pups were randomly assigned to one of the three treatment groups (40, 80 mg/kg/day cocaine or gastrostomy control groups) and no more than two pups from a litter were assigned to the same treatment condition. Pups were reared in an artificial rearing apparatus from PDs 4 through 10, and sacrificed on PD 10.

Artificial rearing procedure

The details regarding the artificial rearing are described in West et al. ('84). Briefly, on PD 4, pups were anesthetized with ether and underwent gastrostomy surgery to implant the gastrostomy tubes. From PDs 4 to 10, pups were maintained in the artificial rearing apparatus and received their daily nutritional requirements through formula (diet)-filled syringes, which were operated by a timer-activated infusion pump (Harvard Apparatus, model 935). We have found that the artificial rearing method is critical for maintaining appropriate somatic growth following early postnatal cocaine administration (Chen et al., '94a).

Drug administration

A 20-mg/ml cocaine HCl (Sigma) stock solution (in sterile dH_2O) was prepared twice weekly and stored in brown bottles at 4°C. Two cocaine-treated groups (40 and 80 mg/kg) received cocaine injection (s.c.) daily from PDs 4 through 9 and one gastrostomy control received an equivolume injection of sterile dH_2O using a Hamilton syringe and a 30-gauge needle. The injection sites varied from day to day but were always on the dorsal aspect of the thoracic region. Daily injections were performed 24 hr apart from PDs 4–9.

Brain measures and tissue preparation

On PD 10, pups were weighed, given an overdose of Nembutal (Abbott Labs, N. Chicago) and perfused intracardically with saline followed by 4% (w/v) paraformaldehyde (in 0.1M phosphate buffer, pH 7.4). The cerebellum was removed, weighed, and postfixed in additional fixative for 9–12 months until tissue preparation for stereological cell counts. Each cerebellum was embedded in 4% agar and cut sagittally into 2-mm slabs. These 2-mm slabs were then dehydrated through increasing concentrations of alcohol (70%, 95%, and 100%), processed through a series of graded concentrations of infiltration solution (25%, 50%, 75%, and 100%; 100% infiltration solution is prepared by mixing 50 ml hydroxyethlymethacrylate and 0.5 g benzol peroxide; Historesin Embedding kit, Leica) and embedded in Historesin (15 ml of 100% infiltration solution and 1 ml dimethyl sulfoxide). Serial sectioning of each slab was done at 30 μm in the sagittal plane with a Reichert-

Jung rotary microtome (model 2055, Leica) using glass knives. Every fifth section was saved, mounted onto glass slides, dried overnight at 60°C, stained in cresyl violet and coverslipped.

Stereological equipment

The optical disector was utilized to estimate the density of cerebellar Purkinje cells and the reference volume of cerebellum. The Nikon Optiphot microscope used in this study had a motor-driven stage [X, Y] and the adjacent attached microcator measured the Z-axis. Slides were viewed with an oil-immersion ×60 objective lens with a 1.4 numerical aperture. The image from the microscope was transferred to Amiga 2000 computer using a SONY CCD-IRIS color video camera. The software GRID (GRID software package, Medicosoft Corp., Copenhagen, Denmark) provide the point counting and counting frame templates, as well as their respective areas.

Stereological methods

The estimated total number of cerebellar Purkinje cells (N) was estimated from measuring of reference volume (Vref; the volume that contains the population of the cells) and numerical density (Nv) of the cells within this Vref.

$$N = Vref \times Nv$$

A systematic random sampling method was employed to determine the first counted section (flocculus tissue was excluded prior to the sampling) and every fifth slide was counted thereafter. Details regarding the stereological methods used in the present study have been described elsewhere (Bonthius et al., '92).

Estimation of reference volume. The reference volume was determined using Cavalieri's Principle (Gundersen et al., '88), and it was calculated by the equation listed below (Gundersen and Jensen, '87). The GRID software provided templates of points in various arrays that were used in the point counting for reference volume estimation. The template used in the present study appeared on the screen as lines of crosses (+) of a known area. When the cerebellar tissue transected the upper right quadrant of a cross (+), it was counted. Pia and residual matter (e.g., remnants of inferior cerebellar peduncle) were not included in this or other calculations.

$$Vref = \sum pi \times A(pi) \times t$$

where $\sum pi$ is the sum of the number of points (pi) counted, A(pi) is the known area associated with each point, and t is the known distance between two serial sections counted (Fig. 1).

Estimation of cell density. Purkinje cell density was determined following the optical disector method (Gundersen et al., '88; West and Gundersen, '90), which was calculated as shown below. The placement of the disector frame for the estimation of cell density was determined by a random starting point outside the region of interest.

$$Nv = \frac{\sum Q}{\sum \text{disector} \times A(fr) \times h}$$

where $\sum Q$ is the sum of the Purkinje cells counted (nucleoli as counting units) from each disector frame, $\sum$ disector is the sum of the number of disector frames counted, A(fr) is the known area associated with each disector frame, and h is the known distance between two disector planes.

The two-dimensional frame generated by the software appeared as a rectangle with two red and two green lines. White ("green" from video monitor) lines served as "inclusion" lines (upper and right side of rectangle) and the dark ("red" from video monitor) lines represented "exclusion" lines (lower and left side of rectangle). Completing the third dimension (the z-axis) were two planes separated by 10 μm (measured by the microcator); the top plane was an "exclusion" plane and the bottom one was an "inclusion" plane. Nucleoli that touched an exclusion plane were not counted; those that touched the inclusion plane were counted as were all nucleoli that fall within the counting frame (Fig. 2).

Statistical analysis

All statistical analyses were conducted using one-way ANOVAs with Experimental Treatment as the independent factor ($\alpha = 0.05$) (Keppel, '91). There were no significant main effects of gender or interactions of gender with the experimental treatment on any of the dependent measures, therefore, the results have been combined across gender for data analyses. Furthermore, the data obtained from the two pups from the same litter assigned to the same treatment group were treated as independent representations in the data analysis.

RESULTS

Body weight, cerebellum weight, and cerebellum/body weight ratio

Three separate one-way ANOVAs conducted on the data of body weight, cerebellum weight and cerebellum to body weight ratio failed to indicate significant differences among groups (all $P > 0.1$) indicating that cocaine treatment, regardless of dose, did not affect body or cerebellum growth measured at PD 10 using the artificial rearing method (Table 1).

Estimated neuronal number, reference volume, and density

Statistical analyses revealed that cocaine treatments did not produce any significant changes in the number of cerebellar Purkinje cells, the reference volume of the cerebellum, or Purkinje cell density in the cerebellum (all $P > 0.5$) compared with gastrostomy control, even at a dose (80 mg/kg/day) which is found to result in a high mortality rate (Chen et al., '94a) (Table 2).

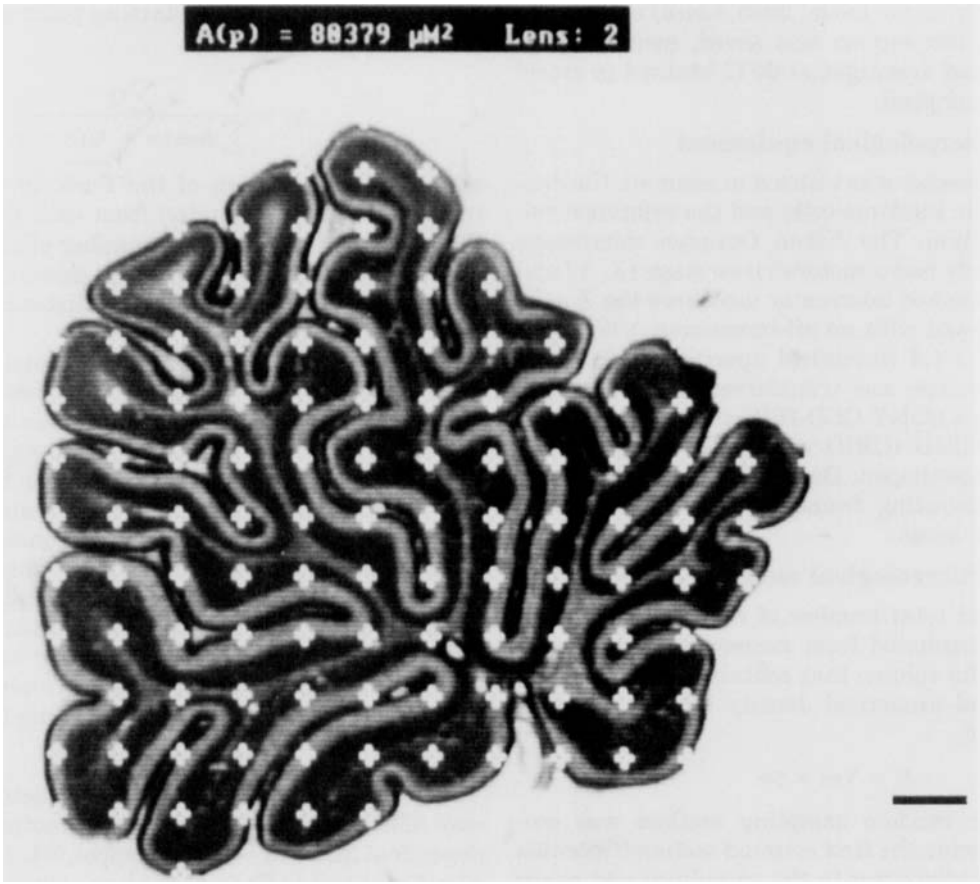

Fig. 1. Photograph taken from the computer monitor demonstrating the cresyl violet stained sagittal section of the cerebellum from a gastrostomy control rat pup at PD 10. The area associated with each point [A(pi)] is shown at the top of the photo. The reference volume [Vref] is determined using Cavalieri's principle. Bar = 340 μm.

Verification of section thickness

The thickness of the sections was verified by measuring each slab both before and after sectioning, and the difference in length was then divided by the total number of sections cut from that slab. Twenty-two slabs were randomly selected for the verification of thickness and the average section thickness was 29.08 μm (±0.83 μm). These results indicate that the sections closely approached 30 μm.

Precision of the estimates

The precision of the estimates was calculated and expressed as the coefficient of error (CE). The average CEs for the total number of points counted over sectional profiles ($\sum$P), the number of the frames examined ($\sum$F), the number of Purkinje cell nucleoli sampled ($\sum$Q) were 0.087, 0.089, and 0.108 respectively. These CEs reflect a reliable estimate of total Purkinje cell number.

DISCUSSION

The present findings indicate that cocaine exposure during the period of the brain growth spurt, the most dynamic period of central nervous system (CNS) development, did not reduce the total number of cerebellar Purkinje cells on PD 10 as assessed with stereological methods. In addition to the lack of effect on the estimated number of Purkinje cells, neither the reference volume of the cerebellum nor the density of the Purkinje cell per unit (mm^3) were altered in response to neonatal cocaine treatment. These results, taken together with our previous data showing no effect of neonatal cocaine on gross brain weight parameters (Chen et al., '94a, b), demonstrate a clear lack of injurious effects of cocaine on these neuroanatomical measurements.

The total number of Purkinje cells estimated from neonatal rat pups in this study was found to be approximately 0.5 million ($5.10 \pm 0.38 \times 10^5$) in the gastrostomy control group, which is higher than Purkinje cell estimates from other studies in adult rats using the

Fig. 2. Photograph taken from the computer monitor demonstrating the Purkinje cells of the cerebellum from a gastrostomy control rat pup at PD 10. The disector frame [fr] and the area associated with each disector frame [A(fr)] are shown in the center and the top of the photo. Any Purkinje cell nucleoli falling on the dark lines (lower and left side of the disector frame) of the disector frame are excluded and nucleoli falling on the white lines (upper and right side of the disector frame) of the frame are included. In terms of the Z axis (not shown in this photo), the distance between two planes (h) was 10 µm, any nucleoli seen on the top plane are excluded and the ones seen on the bottom plane are included. Bar = 12 µm.

TABLE 1. Effects of neonatal cocaine administration on body weight, cerebellum weight, and cerebellum to body weight ratio measures on postnatal day 10

Dose of cocaine (mg/kg)	N	Body weight (g)	Cerebellum weight (mg)	Cerebellar weight/ body weight ratio (%)
0 (GC)	9	18.2 ± 1.2	56.5 ± 3.4	0.31 ± 0.013
40	9	16.7 ± 0.6	51.7 ± 2.0	0.31 ± 0.011
80	8	18.6 ± 0.6	56.8 ± 3.0	0.31 ± 0.017

GC, gastrostomy control group.

TABLE 2. Effects of neonatal cocaine administration on reference volume, Purkinje cell number of the cerebellum, and the density of the Purkinje cell in the cerebellum

Dose of cocaine (mg/kg)	N	Reference volume (mm^3)	Purkinje cell number ($\times 10^5$)	Density ($\times 10^4$) (cells per mm^3)
0 (GC)	9	25.0 ± 1.3	5.10 ± 0.38	2.05 ± 0.13
40	9	24.9 ± 1.5	5.10 ± 0.36	2.10 ± 0.18
80	8	25.0 ± 0.7	5.22 ± 0.38	2.11 ± 0.18

GC, gastrostomy control group.

fractionator methods (Bedi et al., '92; Campbell et al., '88; Warren et al., '88). It is unclear at present whether the higher number of total Purkinje cells in neonates simply reflects the developmental profile or the programmed cell death as it occurs during the development into adulthood. However, further experimental evidence is required to examine the difference between different aged animals and different stereology methods.

The coefficients of error (CEs) in this study are somewhat higher than other studies (~10%) indicating that the sampling schemes may need to be modified to increase the precision of estimation. The CEs could be decreased by increasing the counting frames within each section or increasing the total number of sections counted within each cerebellum or changing the size of

the disector frame. Although these procedures may optimize the CEs, it is important to note that early postnatal cocaine exposure did not reduce the number of Purkinje cells, since the cocaine-treated groups appear to be higher in total Purkinje cell number and density.

In the rat, Purkinje cells are formed prenatally and undergo differentiation and maturation postnatally (Altman, '69; '82). Several histological landmarks during the early postnatal period have been commonly used to indicate the sequence of normal cerebellar development. Some of those landmarks include the thickness of the external granular layer, the shape and size of the Purkinje cells, the orientation of the apical cytoplasm of the Purkinje cells and the arrangement of the Purkinje cell monolayer between the molecular and granular layers. Although these parameters of development were not a direct focus in this study, we observed no qualitative differences between cocaine treated and gastrostomy control animals in comparisons of, for example, the thickness of the external granular layer in corresponding lobules. This suggests that there were no apparent morphological changes in the development of cerebellar cortex on PD 10 due to cocaine treatment. Given the circuitry of the cerebellum and particularly the fact that the Purkinje cell provides the sole output from the cerebellar cortex, the use of Purkinje cell number as a dependent measure seems parsimonious, as well as functionally interesting.

The present findings using anatomical measures are inconsistent with the neurobehavioral assessments of early postnatal cocaine exposure indicating behavioral deficits associated with cerebellar function in a rat model system (Barron and Irvine, '94). One possible explanation is that the neuroanatomical effects of neonatal cocaine exposure are latent on PD 10 and in fact are expressed later in development at the age when behavioral measures have been performed. Another possibility is that the neurochemical interactions involved in normal function of cerebellar circuitry are more vulnerable to neonatal cocaine treatment than is neuronal survival. For example, the number of climbing fibers from the inferior olive or the availability of their postsynaptic sites in synapsing with Purkinje cells may be altered following neonatal cocaine treatment. Therefore, these possibilities cannot be disregarded in terms of reconciling the neuroanatomical and behavioral outcomes.

Despite the negative findings of this study, we would like to emphasize that there is a substantial body of evidence indicating that prenatal cocaine treatment produces a variety of behavioral, neurochemical and neuroanatomical changes (for review, see Spear, '95). With regard to the cerebellar functions, prenatal cocaine exposure has been shown to affect various motor activities (Church and Overbeck, '90; Johns et al., '92). Most importantly, prenatal cocaine exposure results in deficits in the performance of some conditioning tasks (Heyser et al., '90; '92). These prenatal cocaine-induced deficits in cognitive function may be mediated through cerebellar activities, since recent reports have suggested that cerebellum is involved in some of the cognitive processes (Akshoomoff and Courchesne, '92; Canavan et al., '94; Grafman et al., '92; Leiner et al., '93; Middleton and Strick, '94). Thus, the critical difference between the results of prenatal cocaine studies and the present study, notwithstanding the difference in dependent variable, may involve the timing of cocaine exposure during cerebellar development.

In conclusion, the present study failed to demonstrate Purkinje cell loss in animals that were artificially reared and given cocaine treatment during a vulnerable portion of the brain growth spurt. These results suggest that cocaine is not a potent neuroteratogen compared with other neuroteratogens, such as alcohol. It is possible that some of the neurological dysfunctions observed clinically in cocaine exposed offspring may be associated with other risk factors, such as polydrug use or the timing of cocaine exposure. Therefore, investigating the interactive effects of polydrug use, either synergistic, additive, or antagonistic, and the temporal windows of vulnerability may provide critical information with respect to identifying potential teratogens and developing intervention strategies.

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