Chronic Cocaine Exposure in the Fetal Rhesus Monkey: Consequences for Early Development of Dopamine Neurons

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The effects of cocaine on the development of neurons containing tyrosine hydroxylase (TH) were investigated in fetal rhesus macaques. Pregnant monkeys were treated with cocaine 3 mg/kg or saline i.m., four times a day from day 18 of pregnancy until days 40 or 60. Cocaine concentration in plasma from females thus treated was approximately 800 ng/ml 10–20 min following injection. At the time of C-section, plasma levels of cocaine in fetal blood were 231 ± 70 ng/ml. The brains from 40 and 60 d old fetuses were examined using immunocytochemistry and *in situ* hybridization. The appearance of neurons containing TH by day 40 of gestation was not different between control and cocaine-treated fetal monkeys. In both groups ($N = 3$ each) TH-IR neurons and TH mRNA were located in the mesencephalon and dorsal hypothalamus by day 40, and fiber projections extended to the developing striatum. Also in the day 60 fetuses, the TH-IR neurons were distributed similarly in both groups ($N = 5$ each), but the TH mRNA content, measured by quantitative *in situ* hybridization, was reduced in the substantia nigra (SN) and ventral tegmental area (VTA) after cocaine treatment. These data suggest that exposure to cocaine in fetal life does not affect the development of TH or the expression of its mRNA on day 40 of gestation. By day 60, however, the expression of TH mRNA was significantly reduced. This latter effect can be explained by reduced dopamine synthesis in the cocaine-treated fetuses.

[Key words: mesencephalon, dopamine neurons, tyrosine hydroxylase, cocaine, fetal monkey, immunocytochemistry, *in situ* hybridization]

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Maternal drug use during pregnancy has increased during the last several years, and estimates of infants exposed to cocaine *in utero*, as high as 8% have been made (Neerhof 1989). Infants, exposed to cocaine *in utero*, are often smaller, less coordinated, and more irritable than normal infants (MacGregor et al., 1987; Hume et al., 1989; Schneider et al., 1989; Fries et al., 1993). In humans, the most serious consequences of *in utero* cocaine exposure, are behavior and learning difficulties observed in childhood which are presumed to be the result of abnormal organization of the nervous system (Chasnoff et al., 1989a,b; Schneider et al., 1989; Struthers, Hansen, 1992; Azuma, Chasnoff, 1993). However, the neurological effects of cocaine in infants and children are difficult to determine due to the inherent limitations that accompany research with human subjects.

A number of animal studies support the conclusion that subjects exposed to cocaine prenatally have altered brain functions (Dow-Edwards et al., 1990; Heyser et al., 1990; Clow et al., 1991; Gressens et al., 1992; Heyser et al., 1992; Minabe et al., 1992; Peris et al., 1992; Leslie et al., 1994; Wang and Pitts, 1994). Offspring thus exposed show deficits in cognitive function prepubertally which persists into adulthood in rats (Heyser et al., 1990, 1992) and rabbits (Romano and Harvey, 1994). The brains of adult animals exposed to cocaine *in utero*, are sensitized to subsequent cocaine exposure, and basal locomotion patterns of females are elevated (Peris et al., 1992). Others found widespread reductions in metabolic rates in various brain regions of adult male rats which had received cocaine prenatally (Dow-Edwards et al., 1990). Collectively, these data suggest that prenatal cocaine exposure has lasting effects on brain functions.

Cocaine is a powerfully reinforcing drug, an effect due to its capacity to bind to the transporter of dopaminergic nerve terminals (Ritz et al., 1987; Kuhar et al., 1991). Cocaine increases synaptic dopamine concentrations by blocking reuptake of spontaneously released dopamine, and it is the increased concentrations of dopamine that is believed to underlie the neurobehavioral effects of cocaine (Ritz et al., 1987; Kuhar et al., 1991; Koob, 1992; Robinson and Berridge, 1993).

The effects of cocaine on the developing dopamine neurons in primates are not known. In the rhesus monkey which has a gestation period of approximately 165 d, all mesencephalic dopamine neurons arise in a short time span between embryonic days 36 and 43 (Levitt, Rakic, 1982). The peak of cell genesis in the ventral tegmental area (VTA) and the substantia nigra occurs between day 38 and 40 of gestation (Levitt, Rakic, 1982). In both monkey and human fetuses, TH-immunoreactive (TH-IR) neurons first appear during the 5th embryonic week (Verney

et al., 1991; Ronnekleiv, 1992), indicating that the early development of dopamine neurons is similar in human and monkey.

The purpose of the present study was to elucidate the effects of cocaine on the developing dopamine neurons of the rhesus monkey. In particular, we wish to determine the earliest time in fetal development when the actions of cocaine are manifested. The rhesus monkey was chosen as the experimental subject because its placenta and the development of its fetus are both very similar to the human (Gribnau and Geijsberts, 1981; Faber and Thornburg, 1983).

Materials and Methods

Animals. Animal care and use were in accordance with our Institutional guidelines based on NIH standards. Adult female rhesus monkeys (*Macaca mulatta*) were paired with fertile males for 3 d beginning on day 9–18 of their menstrual cycle, based on an analysis of their previous menstrual cycle lengths. Pregnancy was determined by RIA analysis of estrogen (>100 pg/ml) and progesterone (>2.5 ng/ml) in blood samples obtained at days 13–17 after pairing (Hess et al., 1981). The second day of pairing was chosen as the day of conception; gestation times were calculated from that point.

Determination of dose and route of cocaine administration. Synthesized cocaine HCl was obtained from the Research Triangle Institute (Research Triangle Park, NC) through the National Institute of Drug Abuse (Bethesda, MD). First, we investigated whether cocaine could be administered orally dissolved in juice or mixed with palatable fruit-snacks. However, we were not successful in getting the monkeys to consistently consume the food or drinks that contained cocaine. Thus, we decided that an intramuscular (i.m.) injection was the best route of cocaine administration. Initially, the proper dose of cocaine was determined in two pregnant animals. On day 18 of pregnancy, these females, body weight 8.2 and 8.3 kg, repeatedly were given cocaine injections i.m. (3.0 mg/kg body weight) four times daily at 0800, 1200, 1600, and 2000 hr. In these animals, the plasma cocaine levels were monitored at 10 d intervals until day 60 of pregnancy. On the selected days, a 2.0 ml blood sample was taken at 1130, one-half hour prior to the 1200 noon cocaine dose, and afterwards at 1210, 1220, 1300, and 1400 hr. This sampling protocol was repeated four times during the course of gestation on days 28, 38, 48, and 58, in order to determine whether cocaine clearance changes during pregnancy. To prevent degradation of the cocaine, sodium fluoride was added to the collected samples at a final concentration of 50 mM (Sandberg, Olsen, 1992). From these samples, the half-life ($t_{1/2}$) of cocaine in plasma was calculated using SIGMAPLOT. The measured values were plotted on a semilogarithmic scale and fitted by linear regression. The slope of the fitted lines were used to calculate the half-life.

Cocaine treatment. Based on initial observation of cocaine concentrations in maternal and fetal plasma, consultation with Dr. William L. Woolverton that 4 mg/kg may kill an adult monkey, and the findings that 3.2–6.5 mg/kg cocaine i.v. cause convulsions in rhesus monkey (Matzuzaki, 1978), a dose of 3 mg/kg was used in subsequent studies. Cocaine (3 mg dissolved in 50 μ l 0.9 % saline/kg) was injected i.m. four times daily at 0800, 1200, 1600, and 2000 hr. Animals receiving saline were used as controls. In both groups the injection site was rotated between hips and shoulders. Treatments were begun on days 18–20 of pregnancy in females weighing between 5–8 kg.

The fetuses were delivered by caesarean (C)-section. On the day of C-section, each animal received the final injection at 0840. Shortly thereafter the animal was sedated with ketamine, transported to the surgical area and anesthetized with halothane. At the time of surgery maternal and fetal blood samples, as well as samples of amniotic and chorionic fluids were obtained. Two maternal blood samples were obtained from experimental animals: the first within 20–30 min of the final cocaine/saline injection (0900–0910), and the second concurrent with drawing fetal blood, and amniotic and chorionic fluid samples (0920–0930). Blood sera, amniotic and chorionic fluid samples were stored frozen at -20°C until assayed for cocaine and its major metabolites using HPLC according to Sandberg and Olsen (1990). Briefly, samples (75–100 μ l) were prepared by a solid phase extraction procedure on a 50 mg C-18 Bond Elut column (Varian, Harbor City, CA) with lidocaine as the internal standard and immediately assayed by HPLC. The sensitivity of the assay was 35 ng for cocaine and norcocaine, and 18 ng for benzoylecgonine (BE) and benzoynorecgonine

(BN). Coefficient of variation within an assay was less than 7%. Samples were assayed in duplicates and values averaged.

Tissue preparation. Prior to dissection, three parameters were recorded: body weight, crown-rump length, and head circumference. Then, the brain was dissected under a microscope. Brains were fixed in freshly prepared, ice-cold, 4% paraformaldehyde in Sorensen's Buffer, pH 7.2 ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, 67 mM/67 mM, 20/80, v/v). Fixation times varied from 4 to 6 hr depending on the size of the brain. Following fixation the brains were soaked in 20% sucrose buffer (0.1 M sodium phosphate, pH 7.2) overnight at 4°C . All solutions were prepared using diethyl pyrocarbonate (DEPC)-treated water and RNase-free reagents. Brains were then infiltrated with O.C.T. embedding media (Miles Laboratories), placed in Tissue-Tek cryomolds, frozen in liquid isopentane (2-methylbutane) chilled to -55°C on dry ice, and were stored in liquid nitrogen. The brains were sectioned coronally ($N = 4$, each group) or horizontally ($N = 1$, each group) at 15 μ m on a Leica cryostat, mounted on poly-L-lysine coated microscope slides, and stored at -85°C .

Immunocytochemistry. Tissue sections were processed immunocytochemically by a three-step avidin/biotin method using as markers horseradish peroxidase or Cy3 fluorochrome (Jackson Immuno Research) as follows: tissue sections were reacted with affinity purified rabbit anti-tyrosine hydroxylase (TH, Pei-Freez) at a final dilution of 1:1000 for 48 hr at 4°C . A 30 min post-primary wash in phosphate buffer was followed by a 60 min room temperature incubation with biotinylated goat anti-rabbit IgG at 1:300 (Vector Laboratories), another 30 min wash and a 60 min room temperature incubation with either the streptavidin/HRP (1.5 μ g/ml) or the streptavidin/Cy3 (1:500) marker. The HRP slides were washed for 30 min in phosphate buffer, and then reacted with 3,3'-diaminobenzidine tetrahydrochloride dihydrate (Aldrich Chemical Co.) by the glucose oxidase/ β -D-glucose method (Itoh et al., 1979) to generate hydrogen peroxide. The HRP slides were either directly coverslipped with Glycerin-Jelly (Sigma Chemical Co.), or taken through alcohol and xylene, and coverslipped with Entellan (Merck). The Cy3 fluorescent slides were washed for 60 min in phosphate buffer, and coverslipped with buffered glycerol.

Preparation of TH oligonucleotide probes. Three TH oligonucleotide probes were synthesized based on published cDNA sequences: a 45-mer, 5'-TCG CCC AAC GAA CCG CGG GGA CTG CCC TCT TAC CAT GAT GGC CTC from monkey (*Macaca fuscata*) that recognizes TH1 and TH2 (Ichikawa et al., 1990); a 39-mer, 5'-CGC CGC CTC CCG CTC CTT GCG GCG GGC GTC CTC GAT GAG GCT and 48-mer, 5'-CCT GCG GAA GCC CTT GCC CTT GGC CTG TGG CGT GGT GGC GTC GGG GGT GGG CAT from human TH N-terminal sequence that recognizes TH 1 (Grima et al., 1987). In addition, some sections were tested with a rhesus monkey specific cRNA probe (186 bases) that recognizes TH 1 (Pau et al., unpublished). Since all three oligonucleotide probes and the riboprobe gave similar results, the study was performed for the most part, using the monkey specific TH 45-mer.

Probe labeling. The TH oligonucleotide probes were 3'-end labeled with ^{35}S -dATP using terminal deoxynucleotidyltransferase according to previously described procedures (Ronnekleiv et al., 1991).

In situ hybridization. *In situ* hybridization was performed according to the method of Lewis et al. (1985) with minor modifications as described previously (Ronnekleiv et al., 1991). Tissues were brought to room temperature, pretreated in 0.1 M triethanolamine (TEA) buffer for 3 min and for 10 min in 0.25% acetic anhydride in 0.1 M TEA to reduce background. Sections were covered with 25–50 μ l of hybridization buffer (50% formamide, 10% dextran sulfate, $1 \times$ Denhardt's, $2 \times$ SSC, 100 μ g/ml yeast transfer RNA, 125 μ g/ml sonicated salmon sperm DNA and 100 mM dithiothreitol) and allowed to prehybridize for 60 min at 45°C . After prehybridization, the sections were quickly rinsed in $2.0 \times$ SSC buffer. The labeled probe was heat denatured in a 90°C water bath, chilled on ice, diluted with hybridization buffer and used at final concentrations of $1\text{--}3 \times 10^4$ dpm/ μ l. Depending upon the tissue surface area, either 25 μ l or 50 μ l of hybridization solution was applied to each section. Then, the sections were covered with glass coverslips, sealed with DPX (Gallard and Schleisenger) and hybridized for at least 18 hr at $45\text{--}50^{\circ}\text{C}$. Following hybridization, the slides were washed in $2.0 \times$, $1.0 \times$, and $0.5 \times$ SSC for 45 min each at $45\text{--}50^{\circ}\text{C}$, to a final stringency of $0.1 \times$ SSC at 55°C . The sections were dehydrated in 50, 70, 80, 90, and 100% ethanol for 2 min each. The 50–90% ethanol contained 300 mM ammonium acetate.

Autoradiography. Slides were dipped in Kodak NTB3 liquid emulsion diluted 1:1 with distilled water, air-dried in a humid atmosphere for 3–5 hr, sealed with Drierite, and allowed to incubate at 4°C for 14

d. Slides were developed in D19 developer, fixed in Kodak Fixer, and counterstained with Gills' 3× Hematoxylin (diluted 1:12 with water). Slides were then dehydrated in ethanol, cleared in xylene, and coverslipped with Entellan.

Control experiments. Control experiments performed included RNase pretreatment of sections prior to probe hybridization; washing of hybridized sections in increasing temperatures up to 65°C, and immunocytochemistry for TH and *in situ* hybridization for TH mRNA on adjacent tissue sections. In addition, *in situ* hybridization with the TH probes followed by TH immunocytochemistry on the same tissue sections revealed colocalization of TH mRNA and TH protein.

Data analysis (immunocytochemistry). Sections through the midbrain and caudal diencephalon were evaluated and photographed under bright-field and dark-field illumination using a Leitz Laborlux microscope or a Zeiss microscope equipped with a Darklight Attachment (Fostec, Inc.). Camera lucida drawings of immunoreactive cells were prepared from some of the sections. Fluorescent images were analyzed and photographed with a Leitz microscope outfitted with incident-light epifluorescence generated by a 100 W mercury vapor lamp through a Leitz M₂ filter.

Data analysis (in situ hybridization). The densities of autoradiographic grains were counted under dark-field illumination using a Macintosh computer, and software obtained from Dr. Donald Clifton (Chowen et al., 1991). Because the cells within the substantia nigra area were small and densely packed together, it was not possible to accurately count grains over individual cells. Therefore, grains were counted within a set area, approximately 50 μm^2 , from various regions of the midbrain from control and cocaine treated fetal monkeys, and a mean value was calculated for each fetus. The mean values were analyzed by Student's *t* test, and *P* < 0.05 was considered statistically significant.

To further evaluate the difference in TH mRNA levels between control and cocaine treated fetal monkeys, the total area of autoradiographic grains was measured in different coronal sections through the midbrain as follows: Four sections from rostral to caudal SN/VTA area, 12–15 sections apart (180–225 μm), were photographed with Tri-X-Pan film (ASA 400; Eastman Kodak, Rochester, NY) on a Zeiss microscope through a 2.5× objective using dark-field illumination. The resulting negatives were scanned at the exact same magnification by a Macintosh Quadra 960 computer using a Nikon LS-3510 AF 35 mm film scanner. Using the PHOTOSHOP software, the SN/VTA area covered with grains was traced and subsequently measured. Statistical differences between the two groups and the four areas were evaluated using a two-way repeated measure ANOVA. Values were expressed as group means and SEM.

To determine the number of cells containing TH mRNA, cells within 150 μm^2 were counted under bright-field illumination using an eyepiece square grid reticle on a Leitz Laborlux microscope. The total number of cells and the number of cells that contained autoradiographic grains at least five times background levels were counted using a 25× objective. Three to five sections from the midbrain area of each animal were counted without knowledge of treatment groups. Within each section, cells were counted in the medial and lateral SN, and in the VTA. The mean number of cells from each area was calculated and used for further analysis. Statistical differences between the two groups and the three areas were evaluated using a two-way analysis of variance (ANOVA). Values were expressed as group mean and SEM.

Results

Cocaine levels in pregnant monkeys. Two pregnant rhesus monkeys were treated with cocaine daily from days 18–60 of pregnancy. Blood samples for cocaine measurements were obtained on days 28, 38, 48, and 58 of pregnancy for cocaine measurements. Both animals showed similar plasma cocaine profiles (Fig. 1). Following a cocaine injection at 0800 hr on day 28 of pregnancy, cocaine was not detectable in plasma by 1130 hr (Fig. 1), the metabolites benzoylecgonine (BE) and benzoynorecgonine (BN), however, were present in low quantities (175 ng/ml and 180 ng/ml, respectively). After the 1200 hr cocaine injection, plasma levels of cocaine 10 and 20 min later were highly elevated (Fig. 1). The concentrations, however, decreased to ≈ 300 ng/ml after 1 hr, and were reduced further 2 hr after treatment (Fig. 1). Over the same time period there was a grad-

Figure 1. Plasma cocaine concentrations in two female monkeys on days 28, 38, 48, and 58 of pregnancy. The animals were given four injections of cocaine (3 mg/kg, i.m.) daily from day 18 of pregnancy at 0800, 1200, 1600, and 2000 hr. On the days indicated in the upper right-hand corner, blood samples were obtained at 1130, 1210, 1220, 1300, and 1400 hr.

ual elevation of the metabolites of cocaine, BE and BN, to a plateau of 330 and 350 ng/ml, respectively, by 1 hr, but declined to 280 and 285 ng/ml, 2 hr after treatment. Plasma levels of cocaine (Fig. 1) as well as its metabolites (data not shown) remained consistent following a cocaine challenge at the various stages of pregnancy. The average half-life for cocaine in plasma was 41, 34, 55, and 39 min on gestational days 28, 38, 48, and 58, respectively.

Maternal effects of cocaine. Pregnant females treated with cocaine did not show any long-term overt signs of cocaine intolerance. They maintained body weight as well as control subjects, but, immediately after a cocaine injection some animals showed signs of agitation and hyperactivity.

Cocaine levels in body fluids at the time of C-section. Mean concentrations of cocaine, BE and BN in maternal plasma, amniotic and chorionic fluids and in fetal plasma at the time of C-section (day 60 animals), are illustrated in Figure 2. Twenty to 30 min after the last cocaine injection at approximately 0840 hr, maternal plasma cocaine levels were 408 ± 72 ng/ml. The concentrations of cocaine in amniotic and chorionic fluids obtained 15–20 min later, were 124 ± 40 and 132 ± 45 ng/ml,

Figure 2. Levels of cocaine (COC), benzoylcegonine (BE), and benzoynorecgonine (BN) in maternal and fetal plasma, and amniotic and chorionic fluids at the time of C-section on day 60 of pregnancy. Data are presented as means (bars) $\pm$ SEM (vertical lines). Number of animals/group is indicated in parentheses at the base of each bar. The final cocaine injection was given at 0840 designated by ($\uparrow$), and the samples obtained at the time-interval indicated along the abscissa. The lowercase letters *a-c* indicate significantly different values as compared to COC, BE, and BN, respectively, at other time points; $P < 0.05$.

Figure 3. Camera lucida drawings of sections through the diencephalon (A, B) and midbrain (C, D) of control (A, C) and cocaine-treated (B, D) fetuses on day 40 of gestation. The large dots represent TH-IR cells, the small dots represent fibers. Scale bar, 500 μ m.

respectively. Shortly thereafter, the fetal plasma sample and a second maternal blood sample contained approximately the same cocaine content, 231 ± 70 and 220 ± 47 ng/ml, respectively (Fig. 2). The metabolites BE and BN were found to be nearly equal in the first and second maternal blood sample and in fetal plasma (Fig. 2), with values ranging from 125 to 141 ng/ml for BE and 125 to 149 ng/ml for BN. The highest quantities of metabolites were found in amniotic and chorionic fluids (Fig. 2), which contained 309 ± 43 and 237 ± 17 ng/ml versus 190 ± 13 and 268 ± 35 ng/ml for BE and BN, respectively. The metabolite norcocaine was also measured. However, with the exception of a few samples that contained small amounts, norcocaine was not detected in the mother or fetus.

Fetal growth. Maternal cocaine treatment did not significantly affect body weight or crown rump length on days 40 or 60 of gestation, or head circumference on day 60. Body weight of control and cocaine-treated fetuses on day 40 was 0.52 ± 0.02 gm and 0.59 ± 0.07 gm, respectively, in the day 40 group ($N = 3$, each), and 9.42 ± 0.30 gm versus 10.33 ± 0.72 gm in the day 60 group ($N = 5$, each). The crown-rump length on day 40 of control and cocaine-treated fetuses was 1.81 ± 0.06 and 1.73 ± 0.05 cm, respectively. On day 60, control and cocaine-treated fetuses had crown-rump lengths of 5.09 ± 0.09 and 4.80 ± 0.20 cm, respectively. The head circumference measured only in the day 60 fetal monkeys, was 5.80 ± 0.09 cm for controls and 5.61 ± 0.15 cm for the cocaine group.

Tyrosine hydroxylase (TH) in day 40 fetal macaques. TH immunoreactive (TH-IR) cells and fibers were present in the mesencephalic area and the dorsal hypothalamus of day 40 fetuses (Figs. 3, 4). In the coronal plane, small fusiform and pyramidal-shaped cells (5–10 μ m in diameter) were often clumped together in the ventral mesencephalon. Caudally, these cells were arranged in two layers in the ventral and intermediate zone, more rostrally the cells were evenly distributed within the ventromedial mesencephalon. In the rostral part of the midbrain the TH-IR cells were located ventrolateral, and TH-IR fiber bundles extended dorsolaterally. TH-IR cells were not found in the germinal zone throughout the mesencephalon. However, TH-IR fibers extended into the germinal zone near the ventricle (Fig. 3). Within the diencephalon, TH positive cells were present in the dorsal hypothalamus (Fig. 3). TH mRNA was detected in the

Figure 4. Dark-field photomicrographs of adjacent horizontal sections through the diencephalon (upper part of each panel) and mesencephalon (lower part of each panel) of control (*A, B*) and cocaine-treated (*C, D*) fetuses on day 40 of gestation. *A* and *C* illustrate the distribution of TH-IR, and *B* and *D* the distribution of TH mRNA. Clusters of TH-IR cells were present in the diencephalon (arrowhead) and the mesencephalon. Autoradiographic grains indicative of TH mRNA were primarily observed in the mesencephalon. V3, Third ventricle; V4, fourth ventricle. Scale bar, 400 μ m.

Day 60

Figure 5. Camera lucida drawings of coronal sections through caudal diencephalon (*A,B; F,G*) and rostral to caudal mesencephalon (*C-E*, and *H-J*) of a representative control (*left panel*) and cocaine-treated (*right panel*) fetus on day 60 of gestation. The dots represent TH-IR cells. The number of immunoreactive cells are not accurately displayed, that is, each dot may represent more than a single cell since it was often difficult to distinguish individual neurons that were clustered together. In *A,B* and *F,G*, distribution of small, faintly TH-IR cells in the dorsal hypothalamic area, is illustrated by small dots (*arrows*), and the intermediate type cells are indicated (*arrowheads*). Abbreviations: ARH, arcuate nucleus hypothalamus; cp, cerebral peduncle; fr, fasciculus retroflexus; IPN, interpeduncular nucleus; LI, linear nucleus of the raphe; SA, sylvian aqueduct; SN, substantia nigra; V3, third ventricle; Scale bar, 1 mm.

mesencephalic area and showed a distribution similar to that of TH-IR cells (Fig. 4). A faint hybridization signal was also detected in the dorsal hypothalamus in the same regions that contained TH-IR cells (data not shown). At this stage of development, no difference in location or content of immunoreactive TH and TH mRNA could be determined when comparing control and cocaine-treated animals ($N = 3$ each; Figs. 3, 4).

TH in day 60 fetal macaques. By day 60 of gestation the fetal monkey brain was greatly increased in size as compared to the day 40 fetus, consequently, the area occupied by TH-IR cell-groups within the mesencephalon and the diencephalon were

greatly increased in size. Morphologically, the TH-IR neurons of the SN/VTA area in day 60 fetuses were larger (5–20 μm in diameter), exhibited a greater number of processes, and stained more intensely for TH. The relative distribution of TH-IR cells within the caudal diencephalon and the midbrain in a representative control fetus is shown in Figure 5*A–E*. The cells of the substantia nigra extended from the caudal part of the midbrain and rostrally into the hypothalamus along the dorsal and medial borders of the cerebral peduncle (Fig. 5*A–E*). Clusters of densely packed cells were found in the developing linear nucleus of the raphe (Fig. 5*C–E*). Within the substantia nigra, no clear distinc-

tion was apparent between the distribution of TH-IR cells in the dorsal (pars compacta) versus the ventral (pars reticulata) region (Figs. 5C–E). The TH positive cells in day 60 fetuses exhibited distinct morphological differences in that larger (10×12 to $15 \times 30 \mu\text{m}$ in diameter), more intensely stained cells were found in the substantia nigra area throughout the midbrain and caudal diencephalon. Further rostral in the posterior hypothalamus, scattered, faintly stained, small round to fusiform cells (6×6 to $8 \times 12 \mu\text{m}$ in diameters) with thin processes were observed (Fig. 5A,B). Dorsal and rostral to these cells in the periventricular area, an intermediate type of cell was present (5×10 to $12 \times 20 \mu\text{m}$ in diameters) (Figs. 5A,B). The majority of these cells were larger than the cells observed in the posterior hypothalamus and exhibited more densely stained processes, but they were not as large as the cells of the substantia nigra area.

The distribution and staining intensity of the different TH cell types were similar in control and cocaine-treated fetal macaques (Fig. 5A–E vs Fig. 5F–J). However, in cocaine-treated fetuses the number of TH-IR cells and the area occupied by TH-IR cells appeared to be less than in control subjects. Due to difficulties in quantifying the immunocytochemical data in fetal tissues, all quantifications were performed on the *in situ* hybridization materials. In control and cocaine-treated fetal brains, adjacent sections were either stained immunocytochemically for TH or subjected to *in situ* hybridization for TH mRNA: TH-IR neurons and TH mRNA were present in the same areas of both groups of animals (Figs. 6, 7). In the controls, TH mRNA and TH-IR were closely matched and all midbrain areas showed an equivalent distribution of TH mRNA and TH-IR (Figs. 6A,B; E,F; and 7A,B; E,F). Also in the cocaine-treated fetuses there was a close match between the distribution of TH mRNA and TH-IR with the exception of the lateral substantia nigra (Figs. 6C,D; G,H; and 7C,D; G,H). Thus, although TH-IR neurons were present in the lateral substantia nigra area of cocaine-treated animals, the TH mRNA levels were often below detectability (Figs. 6C,G; 7C,G vs Figs. 6D,H; 7D,H). Overall, the density of TH mRNA was significantly reduced in the cocaine-treated group as compared to controls (Fig. 8; $P < 0.05$).

Drawings of the area covered by grains, indicative of TH mRNA, from representative matched midbrain sections in controls and cocaine-treated animals, are illustrated in Figure 9. The control group consisted of two males and two females, and the cocaine-treated group consisted of three males and one female (Fig. 9). For each individual control and cocaine-treated fetus, the distribution of TH mRNA was relatively consistent throughout the midbrain. However, all of the cocaine-treated fetuses had midbrain areas covered by silver grains that were smaller than that of controls. The area covered by grains in four matched sections from rostral (A) to caudal (D) regions of the midbrain were calculated. Statistical analysis revealed that the area covered by grains (indicative of TH mRNA) in the midbrain was significantly reduced in cocaine-treated fetal brains as compared to saline-treated controls (Table 1; $P < 0.01$).

For further analysis, the total number of cells and the number of cells containing TH mRNA per $150 \mu\text{m}^2$ in the medial and lateral substantia nigra and the VTA were quantified. This anal-

ysis revealed that the overall cell density was not different between control and cocaine-treated fetuses, however, the number of cells containing TH mRNA was significantly reduced in the cocaine-treated animals (Figs. 10, 11; $P < 0.02$).

Discussion

This study describes the effects of cocaine in the developing rhesus macaque fetus at days 40 and 60 of gestation, a critical period of development. The primary findings are the following. (1) Cocaine and its major metabolites were detected in amniotic and chorionic fluids, and were present in significant concentrations in fetal plasma. (2) Body weight, crown-rump length, or head circumference were not different between control and cocaine-treated fetuses. (3) TH-IR and TH mRNA were observed in the day 40 fetal brains, and no apparent effects of cocaine on TH-IR or TH mRNA could be detected at this stage of gestation. (4) By day 60, fetal cocaine exposure significantly reduced the density of TH mRNA, the area occupied by TH mRNA, and the number of neurons in the SN/VTA that expressed TH mRNA.

In this study pregnant female monkeys were treated with cocaine from day 18 to day 40 or day 60 of gestation. Following a cocaine injection, peak plasma levels were reached within 10–20 min and cleared from plasma by 3.5 hr. We found no significant difference in maternal plasma cocaine levels, metabolites, or the half-life of cocaine in plasma, when measured at four different times during pregnancy. This would indicate that the clearance of cocaine from plasma remained the same at the different stages of gestation. A similar conclusion was reached by Duhart et al. (1993) who studied cocaine pharmacokinetics in pregnant monkeys during the last trimester as compared to non-pregnant monkeys. They found that chronically exposed pregnant monkeys metabolize and eliminate cocaine similar to non-pregnant monkeys (Duhart et al., 1993). Moreover, the half-life for cocaine in plasma was 114 and 66 min after a 0.3 and 1 mg/kg dose, respectively. These values of cocaine elimination times are higher than that found in the present study after a 3 mg/kg dose (34–55 min), which may be related to the different doses used (Barnett et al., 1981; Sandberg and Olsen, 1991). By day 150 of pregnancy in the monkey, cocaine reaches peak levels in maternal blood within 10–20 min after an i.m. injection, whereas in the fetus cocaine reaches peak levels within 30–120 min (Binienda et al., 1993). The peak cocaine levels in the fetus are only approximately one-third of that found in maternal blood (Binienda et al., 1993). In our study we measured cocaine levels in fetal plasma within 45–50 min after an i.m. injection of the mother and found it to be approximately one-third of the maximum levels obtained in maternal plasma. Thus, we conclude that the fetal cocaine values obtained (150–460 ng/ml) probably represent peak values. In the present study we did not measure the concentration of cocaine in the fetal brains. However, based on studies in other species, the levels of cocaine in the fetal monkey brains would be equal to or greater than that found in plasma (Sandberg and Olsen, 1992). In adult humans plasma cocaine concentrations of 150–200 ng/ml are sufficient to elicit self-reports of euphoria (Van Dyke et al., 1978). Therefore, the cocaine exposure of the monkey fetuses in our study should be

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Figure 6. Low power dark-field photomicrographs of adjacent (A, B; C, D; E, F; G, H) coronal sections through matched areas of the midbrain of two controls (A, B; E, F) and two cocaine-treated (C, D; G, H) fetuses on day 60 of gestation. Sections were subjected to immunocytochemistry (A, C, E, G) and the respective adjacent sections (B, D, F, H) to *in situ* hybridization. Abbreviations: LI, linear nucleus of the raphe; cp, cerebral peduncle; fr, fasciculus retroflexus; SN, substantia nigra; VTA, ventral tegmental area; SA, sylvian aqueduct. Scale bar, 400 μm .

Figure 8. TH mRNA levels (number of autoradiographic grains per 2500 μm^2) in the SN/VTA of control and cocaine-treated fetuses. *, $P < 0.05$ cocaine-treated versus controls.

of sufficient quantities to affect the developing neurons. The cocaine metabolites BE and BN were found in highest concentrations in the amniotic and chorionic fluids, whereas the cocaine levels in these fluids were low in comparison to that found in maternal or fetal plasma. This would suggest that rapid metabolism of cocaine occurred in the placenta and might help explain why fetal cocaine levels only reached one-third of maximum levels observed in the maternal circulation (this study, and Binienda et al., 1993). The human placenta has been shown *in vitro*

to metabolize cocaine, which may serve to protect the fetus (Roe et al., 1990).

In infants and children exposed to cocaine *in utero*, the most consistent observation has been decreased body weight and head circumference (MacGregor et al., 1987; Chasnoff et al., 1989b; Neerhof, 1989; Zuckerman, 1989; Coles et al., 1992; Griffith et al., 1994). In the present study the body weight, crown-rump length and head circumference of the cocaine-treated fetal monkeys were similar to that of controls up to gestational day 60. It is possible that if these monkeys had gone to term, we would have found differences in one or more of these growth indexes. However, the possibility also exists that maintenance of proper maternal nutrition assured normal growth of the offspring in spite of exposure to cocaine. A series of studies using the rat as experimental animal support the latter hypothesis (Clow et al., 1991; Heyser et al., 1992; Peris et al., 1992; Keller et al., 1994).

In the rhesus monkey the dopamine neurons of the midbrain first appear between days 36 and 43, with peak neurogenesis around days 38–40 of gestation (Levitt, Rakic, 1982). The rate-limiting enzyme of dopamine synthesis, TH, can be detected in the mesencephalon and the diencephalon of the rhesus monkey at approximately day 38 of gestation (Ronnekleiv, 1992). By days 40–41, TH-IR cells show more widespread distribution in the fetal brain and the TH-IR fibers are present in the developing striatum (Ronnekleiv, 1992, and present study). In addition, TH mRNA was found to be highly expressed in the mesencephalon in day 40 fetuses but was barely detectable in dorsal hypothalamic areas, which contain clusters of TH-IR cells. The difference in TH mRNA expression could be due to higher concentration of TH-containing cells in the ventral mesencephalon, or that these cells are metabolically more active as compared to TH-containing cells in the dorsal hypothalamus.

Figure 9. Computer tracings of the areas covered by autoradiographic grains in sections from the caudal midbrain areas (C-D in Table 1) of control and cocaine-treated fetuses on day 60 of gestation. Each animal number and sex are indicated. Scale bar, 1.5 mm.

Figure 7. High power dark-field photomicrographs of coronal sections through the rostral (A–D) and caudal (E–H) midbrain of control (A, B, E, F) and cocaine-treated (C, D, G, H) day 60 fetuses illustrating the distribution of TH-IR (A, C, E, G) and TH mRNA (B, D, F, H) in representative matched sections. Abbreviations: LI, linear nucleus of the raphe; cp, cerebral peduncle; fr, fasciculus retroflexus; IPN, interpeduncular nucleus; SN, substantia nigra; VTA, ventral tegmental area; SA, sylvian aqueduct. Scale bar, 200 μm .

Table 1. Midbrain areas covered by autoradiographic grains

Group	Number of animals	Areas from rostral to caudal in mm ²			
		A	B	C	D
Control	4	2.96 ± 0.20	3.42 ± 0.23	3.70 ± 0.40	3.75 ± 0.43
Cocaine	4	2.25 ± 0.08*	2.56 ± 0.33*	2.76 ± 0.35*	2.82 ± 0.36*

The area covered by silver grains in four matched sections from rostral (A) to caudal (D) regions of the midbrain in control and cocaine-treated day 60 fetal monkeys were calculated and analyzed. This analysis revealed that the area covered by grains, indicative of TH mRNA, was significantly reduced in cocaine-treated subjects. A, B, C, D: Areas through the midbrain from rostral (A) to caudal (D) approximately 10–15 sections apart (150–225 μ m). The numbers are expressed as mean $\pm$ SEM.

* $p < 0.01$ ANOVA for repeated measures.

By day 60 of gestation, the areas occupied by TH-IR neurons in the caudal diencephalon and the midbrain had increased greatly in size, and the neurons had differentiated into at least three groups based on neuronal size. In the diencephalon TH-IR cells

were present in the dorsal periventricular, ventral, and lateral areas of the hypothalamus. However, TH-IR cells were not detected in the arcuate (infundibular) nucleus. Also in other species such as the rat and the sheep, the arcuate (A 12) TH-IR neurons

Figure 10. Bright-field photomicrographs of coronal sections through the midbrain of control (A) and cocaine-treated (B) fetuses illustrating the cellular distribution of autoradiographic grains in the SN area. The sections were counterstained with Hematoxylin. Scale bar, 30 μ m.

Figure 11. The average number of cells containing TH mRNA (A) and the average number of combined (labeled + unlabeled) cells (B). The unlabeled, Nissl-stained cells are most likely a mixture of dopamine and other (GABA?) cells. SN_{med}, SN_{lat}, and VTA, medial and lateral parts of the substantia nigra and the ventral tegmental area, respectively. *, $P < 0.05$ cocaine versus control.

are last to develop (Daikoku et al., 1986; Tillet, Thibault, 1987). In the present study, the TH-IR neurons in the day 60 fetuses were evenly distributed throughout the substantia nigra. Thus, the pars compacta and the pars reticulata could not be distinguished from each other at this stage of gestation. This observation is in agreement with the findings of Levitt and Rakic (1982), who noted that embryonic day 65 was the first time that the substantia nigra could be separated into its two subdivisions based on toluidine blue stained sections. In this respect, the neurons of the rhesus monkey substantia nigra continue to develop throughout gestation (Levitt and Rakic, 1982).

In the present study, *in utero* cocaine exposure had no apparent effect on the distribution of TH-IR neurons or TH mRNA in the day 40 fetuses. This observation indicates that the timing of TH-IR cell origin and the initial cell migration are not affected by fetal cocaine treatment. Similarly, the timing of axonal projections migrating into the developing striatum appear not to be affected by cocaine. Cocaine exerts its action through binding at the dopamine transporter (DAT) and thus inhibiting dopamine reuptake (Ritz et al., 1987). Since DAT mRNA is very faintly expressed in the midbrain of day 45 fetal monkeys and significantly increased by day 60 (Choi et al., 1994, and unpublished observations), we hypothesize that in the day 40 fetus the dopamine transporter has not yet developed, and therefore, cocaine is not effective in influencing the initial development of TH-IR or TH mRNA levels.

The major finding of the present study was that TH mRNA levels were significantly reduced in the SN/VTA area as a consequence of cocaine exposure up to day 60 of gestation. TH-IR was not quantified in the present study; however, the staining intensity of individual neurons appeared not to differ in cells from control and cocaine treated subjects, whereas, the number of neurons immunoreactive to TH appeared to be less in the cocaine exposed group. Chronic treatment with cocaine in adult Fischer 344 rats results in increased TH activity and mRNA levels in the substantia nigra and the VTA (Vrana et al., 1993). In adult male Sprague Dawley rats daily cocaine injections has no effect on TH-IR or mRNA at 2 hr, but causes a significant

elevation of TH-IR at 24 hr after the last cocaine injection (Sorg et al., 1992). In a similar study, Beitner-Johnson and Nestler (1991) found that chronic cocaine treatment increases TH-IR in the VTA, but not in the substantia nigra, measured at 30 min and 24 hr after the last cocaine injection. Thus, it appears that the effects of cocaine on mesencephalic TH differ between fetal monkeys and adult rats.

The reduced number of neurons expressing TH mRNA in the day 60 cocaine-treated fetal monkeys could be the result of reduced rate of dopamine synthesis, which has been described in cocaine-treated rat pups at postnatal days 1 and 7 (Seidler et al., 1995). That is, some of the neurons are undetectable due to low levels of TH mRNA. This was most apparent in adjacent tissue sections subjected to TH immunocytochemistry and *in situ* hybridization. TH-IR cells and fibers were detected in lateral substantia nigra areas; whereas, TH mRNA was barely detectable in the same regions. Thus, it is possible that the feedback circuitry that regulates dopamine synthesis and TH mRNA production or degradation had been altered in the cocaine-treated fetal monkeys. It is also likely that the overall number of dopamine neurons in the substantia nigra/VTA area were reduced as a result of prenatal cocaine exposure. Since cocaine appears not to be neurotoxic (Bennett et al., 1993), a potential reduction in mesencephalic dopamine neurons in the fetus could be the result of reduced trophic activity (Weese-Mayer et al., 1993).

In the present study we demonstrated that monkey fetuses exposed chronically to cocaine exhibit alterations in TH mRNA levels in the midbrain during the beginning of the second trimester of gestation. In the same animal model at least two of the dopamine receptor-subtypes mRNA levels are upregulated in the rostral forebrain regions, and enkephalin and dynorphin mRNA levels are also altered as a result of prenatal cocaine (Chai et al., 1994; Choi et al., 1994). We do not know if these cocaine-induced changes in the developing monkey brain are temporary or permanent. However, based on the observation that cocaine exposure during the first trimester produce neurobehavioral and other problems in human infants (Chasnoff and Griffith, 1989; Chasnoff et al., 1989a), a critical period for cocaine actions prenatally may exist. Therefore, we believe that the changes in the developing dopamine neurons of the cocaine-treated fetal monkey may be protracted. In support of this conclusion, recent studies in the rat demonstrated that prenatal treatment with cocaine results in a significant reduction in the number of spontaneously active dopamine neurons in the SN and VTA of 2 week old, and adult offspring (Minabe et al., 1992; Wang and Pitts, 1994). These findings illustrate that prenatal cocaine exposure may permanently alter normal function of mesencephalic dopamine neurons. Numerous studies using the rat as an experimental animal, have attempted to elucidate specific long-term effects of *in utero* cocaine exposure on dopamine neurons, their receptors and target neurons (Scalzo et al., 1990; Clow et al., 1991; de Bartolomeis et al., 1994; Keller et al., 1994; Leslie et al., 1994). Some of these studies have been inconclusive in that no effects could be documented (de Bartolomeis et al., 1994), whereas others found changes in dopamine receptors, the dopamine transporter and some of the opioid target neurons of dopamine (Scalzo et al., 1990; Clow et al., 1991; Leslie et al., 1994).

In summary, we have demonstrated that intermittent cocaine treatment of pregnant monkeys resulted in cocaine levels in the fetus that were approximately one-third of maximum levels in the maternal circulation. The cocaine treatment had no apparent

effects on TH-IR or TH mRNA in the day 40 fetuses. However, by day 60 TH mRNA in fetal macaques was significantly reduced. We hypothesize that cocaine reduces the synthetic activity of the developing dopamine neurons perhaps through alterations in their integrated feedback circuitry.

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