Effect of Cocaine on Cell Proliferation in the Cerebral Wall of Monkey Fetuses

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This study examined the effect of cocaine on cell proliferation in the fetal monkey cerebral wall. Pregnant monkeys received cocaine daily (10 mg/kg, orally, in fruit treats, at 07.00 h and 19.00 h) beginning on the 40th day of pregnancy (E40). The control animals received fruit treats only. One set of monkeys was used to examine the state of cell proliferation in the fetal cerebral wall at peak cocaine levels. These animals were injected with [3H]thymidine intravenously on E73. 1.5 h after the morning drug or placebo administration. Another set of monkeys was used to determine the state of cell proliferation after cocaine concentration declined to ineffective levels. These animals were injected with [3H]thymidine on the same day of pregnancy 10 h after the treatment. Cesarean sections were performed 40 min after the radioisotope injection. The right hemispheres were processed for autoradiography. The left hemispheres were used for biochemical analysis of the radioisotope incorporation into DNA. The third set of monkeys was used to determine whether chronic cocaine treatment extends the timing of neocortical neuronogenesis. These monkeys received their final cocaine treatment on E102 (the last day of normal neocortical neuronogenesis) and were injected with [3H]thymidine 24 h later. On E113, the fetal brains were processed for emulsion autoradiography. We found a significant decrease in the density of [3H]thymidine-labeled cells and in the levels of this radioisotope incorporation into DNA in the fetal cerebral wall 1.5 h after cocaine administration. In contrast, 10 h after cocaine administration we detected a significantly elevated density of radiolabeled cells, and abnormally high levels of [3H]thymidine incorporation into DNA. This suggests that chronic intermittent administration of cocaine results in significant periodic fluctuations in cell production within the fetal corticoproliferative zones. We detected no cocaine-induced extension in neocortical neuronogenesis.

Introduction
Cocaine abuse is a significant problem not only in the general population but also among pregnant women (Keller and Snyder-Keller, 2000). This raises the question of the effects of this drug on fetal development. In pursuit of an answer to this question, we developed a primate model of prenatal cocaine exposure. In our model, cocaine is administered to pregnant rhesus monkeys orally twice daily during the second trimester, which is the time of neocortical neuronogenesis in primates (Rakic, 1982, 1994). This developmental period has been previously identified as one of the most teratogen-sensitive phases of brain development (Kameyama, 1991). The oral administration of cocaine to monkeys models the snorting of cocaine by human addicts because the pharmacokinetics of cocaine administered orally closely resembles that achieved through intranasal application (Wilkinson et al., 1980; Jatlow, 1988; Jufer et al., 1998; Fattinger et al., 2000).

We observed that monkeys born from the mothers receiving cocaine in accordance with our model displayed abnormal neocortical cytoarchitecture, with many neurons being unable to assume their proper position within the cortical plate (Lidow, 1995, 1998; Lidow and Song, 2001). These findings were quite similar to those reported for mice exposed to cocaine in utero (Gressens et al., 1992; Kosofsky et al., 1994). Furthermore, our investigations went on to demonstrate that prenatally cocaine-exposed monkeys had a significantly reduced density and number of neocortical neurons (Lidow and Song, 2001). In search of the mechanisms of the latter effect of prenatal cocaine administration, we examined its ability to induce cell death in the fetal brain. This examination showed that the presence of cocaine increased the occurrence of cell death in the fetal monkey cerebral wall during corticogenesis (He et al., 1999). However, the rise in cell death may not be the sole reason for the cocaine-induced decrease in density and number of neocortical neurons. It is conceivable that the aforementioned decrease may also reflect the apparent ability of this drug to interfere with cell proliferation. Such ability of cocaine has been demonstrated in cultures of fibroblasts (Di Francesco et al., 1990), pheochromocytoma PC12 cells (Zachor et al., 1994; Tosk et al., 1996), C6 glioma cells (Grag et al., 1993) and splenic T lymphocytes (Klein et al., 1988; Berkeley et al., 1994; Piccotti et al., 1997). In addition, cocaine-induced suppression of cell proliferation has been reported in the cerebellum and cerebral wall of neonatal rats (Anderson-Brown et al., 1990). Therefore, the present study was designed to determine whether the cocaine treatment used in our nonhuman primate model of prenatal cocaine exposure indeed affects cell proliferation in the fetal cerebral wall. Specifically, we addressed the following questions: (i) whether there is a reduction in cell proliferation during peak levels of cocaine in the organism; (ii) whether an initial reduction in cell proliferation is followed by a burst in proliferative activity when the concentration of cocaine declines to ineffective levels; and (iii) whether the chronic cocaine treatment used in our model extends the timing of neocortical neuronogenesis, as has been seen during development of the cerebral cortex in ethanol-exposed animals (Miller, 1986, 1987).

Materials and Methods

Animals
For this study, healthy time-pregnant rhesus monkeys (Macaca mulatta), 5–7 years of age, were purchased from the Oregon Regional Primate Research Center (Beaverton, OR, USA). The monkeys arrived at the University of Maryland Animal Facilities between pregnancy days 35 and 37 (E35–E37). Eleven of these animals were administered cocaine hydrochloride (Research Technology Branch, National Institute of Drug Abuse, Rockville, MD, USA) daily beginning on E40, the day when neocortical neurons are first generated in the transient fetal proliferative zones (Rakic, 1982, 1994). This is also the day of pregnancy on which we began cocaine treatment in our previous studies (Lidow, 1995; He et al., 1999; Lidow and Song, 2001). Cocaine (10 mg/kg) was administered orally (in fruit treats) twice daily: at 07.00 h and 19.00 h. Previously, we demonstrated that cocaine presented in this way to pregnant monkeys...
achieves its peak plasma concentration in the fetus within 1.5 h (Zhou et al., 2001). The fetal plasma concentration of the drug then declines to the levels undetectable by chromatographic–mass spectrometric technique within the next 8 h (Zhou et al., 2001). While the pharmacokinetics of cocaine in the fetal brain was not examined, the available literature indicates that it should closely follow the one observed in the fetal circulation (Wiggins, 1992). Eleven control pregnant monkeys received fruit treats only. During the study, all monkeys received High Protein Monkey Chow (Ralston Purina Co., St. Louis, MO, USA) and were given fresh fruits twice a day. Water was available ad libitum. As cocaine is a known appetite suppressant (Lidow et al., 1999), the daily food consumption was monitored for all pregnant monkeys involved in the present study from the time of their arrival to the University of Maryland until the time when their fetuses were removed for examination. The body weight of these animals was also recorded on a weekly basis. We did not observe any decline either in food consumption or in body weight among cocaine-treated pregnant monkeys. Furthermore, food consumption and body weight of the latter monkeys were well within the range of those seen in drug-naïve pregnant animals. The failure of cocaine to suppress appetite in pregnant monkeys was also observed in all of our previous studies (Lidow, 1995; He et al., 1999; Lidow and Song, 2001) and in studies of Morris et al. (Morris et al., 1996, 1997).

**[3H]Thymidine Injections**

On E73, eight cocaine-treated and eight drug-naïve pregnant animals received intravenous injections of 10 mCi/kg [3H]thymidine in 5 ml of sterile distilled water (New England Nuclear Co., Boston, MA, USA). This embryonic age was chosen because on this day both ventricular and subventricular transient proliferative areas are active in most developing neocortical regions (Rakic, 1982, 1994). It has been demonstrated that alcohol (Miller and Nowakowski, 1991) and such neurotransmitters as GABA and glutamate (Haydar et al., 2000) can differentially regulate the rate of cell proliferation in these two zones. Therefore, it was reasonable for us to wish to evaluate the effect of cocaine exposure on proliferative activity in each of these zones. In half of the animals, [3H]thymidine injection was administered 1.5 h after the morning cocaine or placebo treatment. As was mentioned earlier, this is the time at which we detected maximal concentration of cocaine in the fetal circulation (Zhong et al., 2001). The other half of the animals received [3H]thymidine injection 10 h after the morning treatment. By that time, cocaine could no longer be detected in the plasma of fetuses (Zhong et al., 2001). This allowed us to evaluate [3H]thymidine incorporation into the DNA of proliferating cells under the peak levels of cocaine, achieved in our model of prenatal cocaine exposure and after the drug had been eliminated from the fetal circulation. Forty minutes after the [3H]thymidine injection, all fetuses were removed by Cesarean section, and their brains dissected out. The right cerebral hemispheres were blocked and fixed by immersion into 4% paraformaldehyde for 6 h at 4°C. This was followed by immersion overnight in phosphate buffer containing 10% sucrose and, then, for 24 h in solution containing 20% sucrose. Finally, the tissue was frozen to −70°C prior to processing for emulsion autoradiography. The cerebral wall of the left hemispheres was dissected out and homogenized in 10 volumes of ice-cold water to access the incorporation of the radioisotope into DNA.

Analysis of the Radioisotope Incorporation into DNA of the Proliferating Cells

The analysis of [3H]thymidine incorporation into DNA was performed as described by Anderson-Brown et al. (Anderson-Brown et al., 1990) with modifications. After the homogenates of the brain tissue were generated, 0.5 ml aliquots (three per sample) of the these homogenates were used to assess the total uptake of the radiolabel with a Beckman LS5811 Scintillation Counter (Fullerton, CA, USA). Six additional 0.5 ml aliquots per sample were precipitated with 10% trichloroacetic acid, sedimented at 1000 × g for 15 min, and the resultant pellets washed twice by resuspension and centrifugation. Three of these aliquots were used to measure radioisotope incorporation into DNA. The pellets from these aliquots were digested with hyamine hydroxide, and counted on a Beckman LS5811 Scintillation Counter (Fullerton, CA, USA). The remaining three aliquots were used for the determination of the amount of DNA with a Hitachi U-1100 Spectrophotometer (Tokyo, Japan). For the comparative analysis, the [3H]thymidine incorporation into DNA from the tissue of each animal was expressed as: DPMt/DPMs, where DPMt is the sample DNA (mean of three aliquots)/DPMs, the tissue DNA (mean of three aliquots) × 10^-3 (Kornblum et al., 1987), where DPM is disintegrations of the radioisotope per minute in a given sample. This expression takes into consideration the fact that the incorporation of radiolabel into macromolecules is dependent upon the amount of the radiolabel taken up by the tissue (Kornblum et al., 1987; Anderson-Brown et al., 1990).

Statistical Analysis

For all experimental paradigms used in this study, the comparison between values generated by the cocaine-exposed and drug-naïve fetuses was performed with two-tailed Student’s t-test. The differences were considered significant when P < 0.05.
Results

Analysis of $[^{3}H]$Thymidine Labeling in Tissue Sections from 73-day-old Fetuses

The examination of the $[^{3}H]$thymidine labeling in sections of the developing cerebral wall of 73-day-old fetuses revealed no differences in the laminar position of the radiolabeled cell nuclei between drug-naive and cocaine-exposed tissue. On all sections, the radiolabeled nuclei were situated predominantly in the ventricular and subventricular proliferative zones. In the ventricular zone, these nuclei were largely positioned near its border with the subventricular zone (Fig. 1), while the labeled nuclei were observed throughout the entire thickness of the subventricular zone (Fig. 1).

The qualitative examination of tissue sections from the fetuses of the placebo- and cocaine-treated mothers receiving $[^{3}H]$thymidine injection 1.5 h after the treatment showed that the latter fetuses displayed a clearly detectable decrease in the density of radiolabeled nuclei in both the ventricular and subventricular proliferative zones across the entire cerebral wall. This observation was further supported by the counting of radiolabeled nuclei. Counting demonstrated that the number of labeled nuclei under 1 mm of pial surface on the sections through these two regions of the cerebral wall of the cocaine-exposed fetuses contained less than half of the labeled nuclei seen in comparable sections from the drug-naive fetuses (Fig. 2). This decrease was equally strong and statistically significant in the ventricular and subventricular zones of both VCW and PCW.

A comparison of the sections of cocaine-exposed fetuses from the mothers receiving $[^{3}H]$thymidine injections 1.5 and 10 h after drug treatment showed that the latter fetuses contained a much higher density of radiolabeled nuclei (Fig. 1). Moreover, the cerebral wall of these fetuses contained a much higher...
The present study demonstrates that cocaine can interfere with cell proliferation in the ventricular and subventricular transient zones of the fetal primate cerebral wall. This is shown by both the autoradiographic visualization of [3H]thymidine-labeled nuclei and by biochemical examinations of the incorporation of this radionucleotide into DNA. These findings are in agreement with the earlier reports of cocaine-induced inhibition of DNA synthesis in cultures of several cell types (Klein et al., 1988; Di Francesco et al., 1990; Zachor et al., 1994; Tosk et al., 1996; Piccotti et al., 1997) and in the brains of neonatal rats (Anderson-Brown et al., 1990).

Most importantly, we have found that the initial suppression of cell proliferation produced by an individual cocaine administration is followed by a significant compensatory burst of proliferative activity when the levels of the drug decline to ineffective levels. The data obtained in the present study do not allow us to discern exactly how cocaine administration leads to this fluctuation in cell proliferation. However, earlier investigations in fibroblast cultures demonstrated that addition of cocaine causes an accumulation of dividing cells at the G1/S border of the cell cycle (Di Francesco et al., 1990). We speculate that a similar accumulation of cells poised to enter the S phase also takes places in the cerebral wall of monkey fetuses during the several hours after cocaine administration. In such case, the eventual decline in cocaine levels would release the accumulated cells to enter the S phase of the cell cycle in numbers exceeding those in control brains.

While the chronic intermittent cocaine treatment employed in our model induces fluctuations in the proliferation of cortical cells, the ability of this treatment to produce changes in the overall number of cells generated by the cortical proliferative zones is much less certain. Indeed, each cocaine-induced reduction in cell proliferation during the course of the treatment is likely followed by a compensatory increase in cell division. Furthermore, on average, the magnitude of a decrease in the density of [3H]thymidine-labeled cells seen during peak cocaine levels is matched in magnitude by an increase in the density of such cells observed 10 h later. This suggests that a reduction in actual cell production may not be among the main causes of...
decrease in cell number in the neocortex of animals born from cocaine-treated mothers (Lidow, 1995; Lidow and Song, 2001). In such case, this reduction may largely reflect the unusually high levels of postmitotic death of cortical cells that were previously detected in cocaine-exposed monkey fetuses (He et al., 1999). At this time, we do not know whether and how the cocaine-induced fluctuations in cell proliferation may affect corticogenesis. Presently, we are conducting studies to address this question.

In addition to cocaine, several other widely abused substances, including marijuana, amphetamine, alcohol, morphine and nicotine, are known to be capable of suppressing the

**Figure 4.** Micrographs of the coronal sections through the cerebral wall containing the primary visual cortex of 113-day-old fetuses from either a drug-naive mother (A) or a mother receiving cocaine between E40 and E102 (B). The right images show the cresyl violet staining of the sections. The left images show the same sections in dark field, which allows a visualization of nuclei labeled with [3H]thymidine administered on E103. VENT – lateral ventricle; WM – white matter; I–VI – cortical layers. Scale bar = 200µm. Note the absence of clearly detectable differences in the distribution and density of the radiolabeled nuclei between cocaine-exposed and drug-naive fetuses. The insert shows a typical cresyl violet image of a radiolabeled nucleus (pointed to by the arrow). The nucleus is small with a dispersed chromatin pattern indicating that this nucleus belongs to a glial cell (Williams and Rakic, 1988; Selemon et al., 1999). For the insert, scale bar = 10µm.

**Figure 5.** Histogram representing the number of [3H]thymidine-labeled nuclei under 1mm of the pial surface on sections of the cerebral wall of the right hemisphere containing the developing primary visual cortex from 113-day-old fetuses of drug-naive mothers and mothers treated with cocaine between E40 and E102. [3H]Thymidine in both animal groups was injected on E103. Each column represents a mean value for three control or three experimental animals ± SD. Note a lack of significant differences between experimental and control fetuses (P > 0.05; two-tailed Student’s t-test).
proliferation of neural cells (Huot, 1974; Bendek and Hahn, 1981; Miller and Nowakowski, 1991; Stiene-Martin and Haeuser, 1993; Levin and Slotkin, 1998). However, the present study, to our knowledge, is the first that demonstrates the ability of the developing brain to support a significant compensatory burst of proliferative activity following suppression of cell division by one such drug. We believe that this is because very few previous studies were designed to test this possibility. We expect future investigations to show that such compensatory aftereffect can also accompany administration of at least some of the other drugs of abuse listed above.

At this time, however, the only other well-documented substantial compensatory response to suppression of cell proliferation in the developing brain is an extension of the timing of neocortical neuronogenesis in the fetuses of rats chronically receiving an alcohol-containing liquid diet (Miller, 1986, 1987). Interestingly, we found that cocaine exposure employed in our model did not result in such protraction of the period of generation of neocortical neurons. Specifically, we detected no differences between cocaine-exposed and drug-naïve animals in the number of cells in the cerebral wall labeled with [3H]thymidine administered one day after the end of the normal neocortical neuronogenesis. Furthermore, the labeled cells in all animals represented glia positioned in the white matter and layer I of the cortex. This may indicate that, as suggested above, the intermittent cocaine treatment employed in the present study does not result in a large enough overall reduction in the number of generated neurons to trigger an extension of the period of neocortical neuronogenesis. This is in contrast to a paradigm of essentially continuous suppression of cell proliferation in studies employing alcohol-containing liquid diet (Miller, 1986, 1987), which does not allow for a periodic compensation of a drug-induced reduction in cortical cell production. Alternatively, cocaine may affect proliferative activity in the fetal cerebral wall by mechanisms that do not allow for compensation by extending the period of neuronogenesis.

The mechanisms involved in the ability of cocaine to influence cell division in the fetal cerebral wall are now open for discussion. Several such mechanisms can be identified. For example, the fact that cocaine can block proliferation in cell cultures (Klein et al., 1988; Di Francesco et al., 1990; Grag et al., 1993; Zachor et al., 1994; Tosk et al., 1996; Piccotti et al., 1997) points to its direct effect on dividing cells. This effect is probably related to the ability of cocaine to induce elevation in the levels of intracellular free Ca2+, which has been shown to be responsible for cocaine-instigated suppression of proliferation in cultures of human T lymphocytes (Matsui et al., 1993). In addition, cocaine may affect cell proliferation in the fetal cerebral wall by modulating the extracellular levels of monoaminergic neurotransmitters (Lidow, 1998; Lidow et al., 1999), which are likely involved in regulating the rate of cell proliferation in the fetal brain (Lidow and Wang, 1995; Wang and Lidow, 1997; Wang et al., 1997). The inhibition of cell proliferation in the fetal brain following cocaine administration may also be related to hypoxia, which has been shown to accompany the cocaine-induced constriction of the uterine artery and fetal brain vessels (Moore et al., 1986; Woods et al., 1987; Morgan et al., 1991). The ability of hypoxia to affect the progression of cells throughout the cell cycle has been well documented (Spiro et al., 1984; Amellem and Pettersen, 1991). We hope that future studies will address the contribution of each of these mechanisms to the ability of cocaine to interfere with cell proliferation in the fetal primate cerebral wall.

Notes
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References


