

A Mechanism for the Inhibition of Neural Progenitor Cell Proliferation by Cocaine

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Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; CNS, central nervous system; ER, endoplasmic reticulum; IP, intraperitoneally; LDH, lactate dehydrogenase; ROS, reactive oxygen species; RT-PCR, reverse transcription PCR; SEM, standard error of the mean; SVZ, subventricular zone; VZ, ventricular zone

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ABSTRACT

Background

Prenatal exposure of the developing brain to cocaine causes morphological and behavioral abnormalities. Recent studies indicate that cocaine-induced proliferation inhibition and/or apoptosis in neural progenitor cells may play a pivotal role in causing these abnormalities. To understand the molecular mechanism through which cocaine inhibits cell proliferation in neural progenitors, we sought to identify the molecules that are responsible for mediating the effect of cocaine on cell cycle regulation.

Methods and Findings

Microarray analysis followed by quantitative real-time reverse transcription PCR was used to screen cocaine-responsive and cell cycle-related genes in a neural progenitor cell line where cocaine exposure caused a robust anti-proliferative effect by interfering with the G1-to-S transition. *Cyclin A2*, among genes related to the G1-to-S cell cycle transition, was most strongly down-regulated by cocaine. Down-regulation of cyclin A was also found in cocaine-treated human primary neural and A2B5+ progenitor cells, as well as in rat fetal brains exposed to cocaine in utero. Reversing cyclin A down-regulation by gene transfer counteracted the proliferation inhibition caused by cocaine. Further, we found that cocaine-induced accumulation of reactive oxygen species, which involves N-oxidation of cocaine via cytochrome P450, promotes cyclin A down-regulation by causing an endoplasmic reticulum (ER) stress response, as indicated by increased phosphorylation of eIF2 α and expression of ATF4. In the developing rat brain, the P450 inhibitor cimetidine counteracted cocaine-induced inhibition of neural progenitor cell proliferation as well as down-regulation of cyclin A.

Conclusions

Our results demonstrate that down-regulation of cyclin A underlies cocaine-induced proliferation inhibition in neural progenitors. The down-regulation of cyclin A is initiated by N-oxidative metabolism of cocaine and consequent ER stress. Inhibition of cocaine N-oxidative metabolism by P450 inhibitors may provide a preventive strategy for counteracting the adverse effects of cocaine on fetal brain development.

The Editors' Summary of this article follows the references.

Introduction

Abuse of cocaine during pregnancy exposes several hundred thousand infants per year to cocaine in the United States alone [1]. A variety of disorders of central nervous system (CNS) development, e.g., intrauterine growth retardation [2], interference with neuronal migration and differentiation [3], and neurobehavioral deficits [4,5], have been associated with prenatal exposure to cocaine. Adverse effects of cocaine on brain development have also been demonstrated in nonhuman primates. Prenatal cocaine exposure results in neurobehavioral deficits in subhuman primate infants or adolescents, including deficits in attention and motor maturity [6]. At the cellular level, cocaine exposure induces neocortical cytoarchitectural abnormalities including a decrease in the number of cortical neurons and abnormal positioning of cortical neurons in the primate embryonic cerebral wall [7,8]. Notably, these abnormalities are found only when cocaine is administered during the second trimester (E40–E102), the period when proliferation of neural progenitors is most active [9]. The specific actions of cocaine in the second trimester and the decrease of neuron numbers in the cortex suggest that cocaine may affect important cellular functions of neural progenitor cells.

In vitro, cocaine has been shown to influence several cell biological functions such as cell survival and mitogenesis independent of its action on monoaminergic systems. One in vitro study showed that a single 30-min exposure to 1 μM cocaine results in late-onset (>72 h) cell death in differentiated human neuronal progenitor cells [10]. On the other hand, accumulating evidence highlights an inhibitory effect of cocaine on neural progenitor cell proliferation. Cocaine (1–100 μM , 7 d) was shown in an in vitro study to inhibit the proliferation of human neural precursor cells without producing a cytotoxic effect [11]. Cocaine has also been shown to cause genetic toxicity and disturbances in chromosome segregation during meiosis [12,13]. These findings suggest that cocaine may influence cell cycle control. Because the proliferation of neural progenitors is an important factor that eventually contributes to determining numbers of neurons and brain cytoarchitecture, clarifying the action of cocaine on cell cycle control might provide an avenue for understanding the mechanisms underlying cocaine-induced retardation of brain development.

The aim of the present study is to clarify the effect of cocaine on proliferation of neural progenitors and elucidate the underlying molecular mechanisms. Both human and animal studies have demonstrated that cocaine can cross the placental barrier and enter the fetal brain rapidly after maternal cocaine use [14,15]. Plasma cocaine concentrations after intranasal application of 1.5 mg/kg cocaine in human volunteers were between 0.4 and 1.6 μM [16], while plasma cocaine concentrations are often considerably higher in tolerant abusers, reaching ~ 13 μM [17]. A previous study found that concentrations of cocaine in maternal rat brain are higher (3- to 4-fold) than in plasma [15], and cocaine concentrations in fetal brain are 50%–90% of those found in the maternal brain [15], indicating that the high range of cocaine concentrations in the fetal brain may reach ~ 20 –47 μM . Moreover, cocaine concentrations up to 100 μM and higher have been reported in postmortem brains of chronic human cocaine users after acute intoxication [18]. Based on

these reports, we considered the cocaine dose range from 1 to 100 μM to be comparable to the range of exposure of the fetal brain to cocaine. Therefore, we employed cocaine in this concentration range to investigate its effects on neural progenitor cell proliferation.

Materials and Methods

Drugs

Cocaine hydrochloride was provided by the National Institute on Drug Abuse. SKF-525A, cimetidine, α -tocopherol, 3(2)-*tert*-butyl-4-hydroxyanisole (BHA), and deferoxamine (DFO) were obtained from Sigma-Aldrich.

Cell Culture

The AF5 neural progenitor cell line was maintained as previously described [19]. The AF5 cell line is homogenous and over long-term culture maintains growth stability [19], differentiation capacity [19,20], and an intact p53 function [19,21]. Notably, the total length of the cell cycle of AF5 cells (24 h) is close to that in the rodent ventricular zone (VZ) (18 h at E15 or later) [22]. 5×10^3 cells/well were plated in 96-well plates for cell proliferation and cytotoxicity assays, and 4×10^4 cells/well in 12-well plates for immunostaining and reactive oxygen species (ROS) measurement 24 h prior to use. Primary human fetal CNS cells (ScienCell Research Laboratories) were from ~ 20 -wk human fetal cerebral cortexes, obtained in accordance with principles embodied in the Declaration of Helsinki (Code of Ethics of the World Medical Association) and were cultured at 37 $^{\circ}\text{C}$, 5% CO_2 using the recommended human cell media obtained from ScienCell Research Laboratories, except that human neural progenitor cells were maintained in DMEM/F12 (1:1, Invitrogen) supplemented with N2 supplement (R & D Systems), 20 ng/ml EGF (R & D Systems), 20 ng/ml bFGF (R & D Systems), 5 $\mu\text{g/ml}$ heparin (Sigma-Aldrich), 100 U/ml penicillin G, 100 $\mu\text{g/ml}$ streptomycin, and 50 $\mu\text{g/ml}$ gentamicin (Sigma-Aldrich). Purity of respective types of CNS cells was evaluated by immunocytochemistry using antibodies against specific cell markers. For evaluation of neural progenitors, neurospheres were first dissociated into single cells using papain (Worthington Biochemical Corporation) for 10 min at 37 $^{\circ}\text{C}$, and plated onto laminin/poly-L-ornithine-coated slides. Once attached, cells were fixed with 4% paraformaldehyde and evaluated by immunocytochemistry. A2B5 progenitor cells differ from neural progenitor cells in that they are committed to a glial cell lineage, eventually differentiating to type-2 astrocytes or oligodendrocytes, whereas neural progenitor cells can become both neurons and glial cells. The human neurons used in this study were MAP-2 positive, and immunoreactive for glutamate (44% \pm 3%) and GABA (69% \pm 5%), but not for tyrosine hydroxylase (TH), choline acetyltransferase (ChAT), or 5-hydroxytryptamine (5-HT) (unpublished data).

Cell Proliferation and Cytotoxicity Assays

AF5 cells were treated with cocaine at various concentrations, and cell proliferation was measured using CyQUANT cell proliferation assay (Invitrogen). Cocaine-induced cytotoxicity was evaluated by lactate dehydrogenase (LDH) release from the cytosol into the medium after exposure of AF5 cells to various concentrations of cocaine for 24 h, according to the manufacturer's protocol (Roche Applied Science). For single-

stranded DNA immunostaining, cells were fixed with methanol/PBS (6:1) for 24 h at -20°C followed by incubation in formamide at 70°C for 5 min. Fixed cells were immunostained with mouse anti-single-stranded DNA monoclonal antibodies (1:10, Chemicon) and fluorescein-conjugated anti-mouse IgM (1:200, Jackson ImmunoResearch). Data were represented as: (number of single-stranded DNA-positive nuclei/number of DAPI-positive total nuclei) $\times 100\%$.

FACS Analysis

Cultures were synchronized by maintaining in serum-free medium for 24 h, followed by exposure to 0, 10, or 100 μM cocaine in serum-containing medium for 24 h. AF5 cells were analyzed by flow cytometry on a FACS Calibur flow cytometer (Becton Dickinson). Proportions of cells in the G1, S, and G2/M phases of the cell cycle were determined by using ModFit LT software (Verity Software House).

5-Bromo-2'-Deoxyuridine Incorporation and Mitosis Assay

AF5 cultures were treated with 20 μM 5-bromo-2'-deoxyuridine (BrdU) (BD Biosciences) in the presence/absence of cocaine for 24 h, fixed with 95% ethanol, and permeabilized with 2 N HCl. Nonspecific staining was blocked with 5% normal goat serum and 0.1% Nonidet P-40 in PBS for 20 min at room temperature. Cells were double-labeled with monoclonal mouse anti-BrdU (1:100, BD Biosciences) and polyclonal rabbit anti-phospho-histone H3 (1:200, Upstate Biotechnology) overnight at 4°C . After washing, secondary antibodies Alexa Flour 594 goat anti-mouse and Alexa Flour 488 goat anti-rabbit (1:500, Invitrogen) were applied, and nuclei were labeled with DAPI. Data were obtained by dividing numbers of nuclei positive for BrdU or phospho-histone H3 by total numbers of nuclei.

Microarray Analysis

Total RNA was extracted from AF5 cultures using RNA STAT-60 (TEL-TEST). cDNA microarray analysis was performed using a mouse developmental cDNA microarray containing 15k clones derived from early Kargul libraries using procedures for processing as previously described [23]. z-Score transformation was employed to compare array data between different treatments [24]. z-Score transformation allows analysis of array data independent of the original pixel intensities and can be used in calculation of *p*-values for significance estimates. To calculate gene expression changes after cocaine treatment, z scores were converted to z ratios, which represent fold-like changes for each gene.

Immunocytochemistry

Primary human fetal CNS cells were fixed in 4% paraformaldehyde in PBS for 10 min and processed for immunostaining [20]. The following primary antibodies were used: rabbit anti-nestin (1:200, Chemicon); mouse anti-A2B5 (supernatant, clone 105, 1:3, ATCC); mouse anti-MAP2 (1:500, BD Biosciences); mouse anti-OX42 (1:200, BioLegend); and mouse anti-GFAP (1:200, Sigma-Aldrich). Cells were developed using Alexa Fluor 488 goat anti-rabbit or mouse secondary antibody (1:500, Invitrogen), and nuclei were labeled with DAPI.

Quantitative Real-Time Reverse Transcription-PCR

Reverse transcription was performed as described previously [20]. To quantify the *cyclin A2* transcript, quantitative

real-time reverse transcription (RT)-PCR using the DNA Engine Opticon Fluorescence Detection System (MJ Research) was performed using SYBR green according to the manufacturer's protocol. The primer sequences and sizes of the PCR products for rat *cyclin A2* were ATATGAAGAGG-CAGCCAGACA (sense), AGGCAGCTCCAGCAATAAGCG (antisense), 483 bp; and for human *cyclin A2* were GCAAA-CAGTAAACAGCCTGCG (sense), TCAACTAACCCAGTCCAC-GAGG (antisense), 386 bp. The results were analyzed using Opticon software. Relative expression was determined by normalizing to 18S ribosomal RNA (Ambion) using 1.0 for the control.

Animals and Experimental Design

Pregnant Sprague-Dawley rats (Charles River Laboratories) received cocaine at early (E13 and E14), middle (E15 and E16), or late periods (E17 and E18) of neocortical neurogenesis. Rats received 20 mg/kg cocaine (intraperitoneally [IP]) twice at an interval of 12 h followed by 50 mg/kg BrdU (Sigma-Aldrich), IP, 24 h after the last injection of cocaine. Rats were euthanized by CO_2 inhalation 2 h after BrdU. Control animals received physiological saline. All animal procedures were performed according to the "Guide for the Care and Use of Laboratory Animals," according to an animal protocol approved by the Institutional Animal Care and Use Committee of the NIDA Intramural Research Program.

Quantitation of Cocaine in the Fetal Rat Brain

For measurements of cocaine concentrations, prefrontal cortex and peri-ventricular region were dissected from fetal rat brains at the early period of neurogenesis (E15). Tissues from all fetuses of each pregnant dam were pooled to become one individual sample. Detection and quantification of cocaine in fetal rat brain was accomplished utilizing a modification of a previously published method [25]. The method employed ultrasonic homogenization of brain tissue in pH 4.0 sodium acetate buffer followed by solid phase extraction. Extracts were derivatized with *N,O*-bis-[trimethylsilyl]trifluoroacetamide (BSTFA). Cocaine was separated by capillary gas chromatography and simultaneously quantified by electron impact mass spectrometry in selected ion mode. The calibration curve for cocaine was linear to 15,000 ng/g of brain, and the limit of quantification was 50 ng/g. This method provided sufficient analytical sensitivity to allow quantification of cocaine in small amounts of tissue.

Cyclin A Expression and BrdU Labeling In Vivo

For cyclin A expression studies, tissues (prefrontal cortex and peri-ventricular region) from fetal rat brain were dissected. Tissues from three fetal rats were pooled for each individual assay to obtain sufficient material. RNA and proteins were extracted with RNA STAT-60 (TEL-TEST) and lysis buffer, respectively. For BrdU labeling, coronal brain sections were labeled with monoclonal mouse anti-BrdU (1:200, BD Biosciences) and polyclonal rabbit anti-Ki67 (1:500, Novocastra Laboratories) overnight at 4°C and visualized using Alexa Flour 594 goat anti-mouse and Alexa Flour 488 goat anti-rabbit antibodies (Invitrogen). BrdU labeling index [(number of BrdU-positive nuclei/number of Ki67-positive nuclei) $\times 100\%$] was calculated in the regions with 160 μm width (red rectangles in Figure S1) that locate on one-third and two-thirds of the cerebral cortex defined by

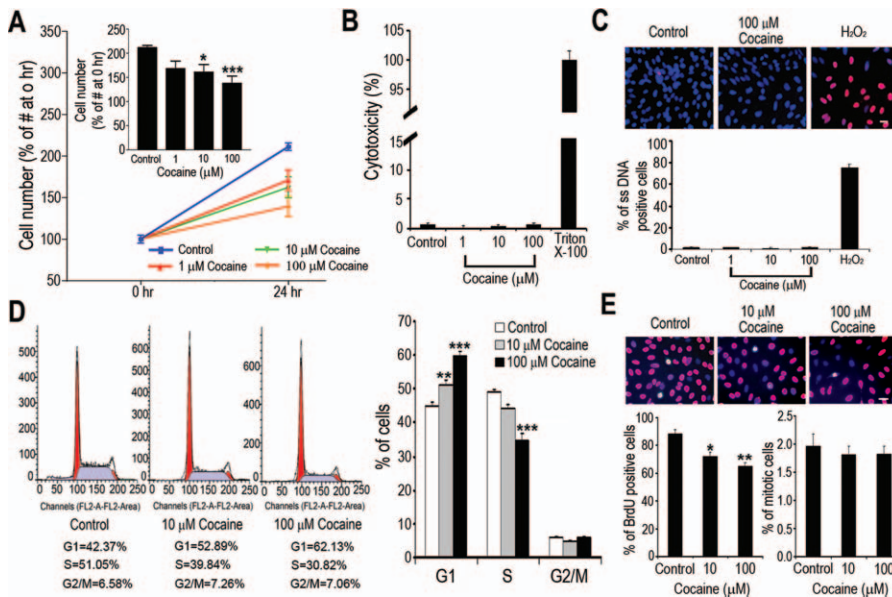


Figure 1. Cocaine-Induced Proliferation Inhibition in AF5 Cells

(A) AF5 cells were treated with cocaine at concentrations from 1–100 μM for 24 h. Inset: dose-dependent inhibition of cell proliferation by cocaine (24 h). Data are presented as means ± SEM of four replicates from ten separate experiments. *, $p = 0.010$; ***, $p < 0.001$ compared to control. (B) LDH activity in the medium from cocaine-treated AF5 cells (24 h). Cytotoxicity was expressed as a percentage of the maximum LDH activity (LDH released by 2% Triton X-100). Data represent six separate experiments (means ± SEM of triplicate observations). (C) Effect of cocaine (24 h) on single-stranded DNA (ssDNA) immunostaining (red). DAPI in blue. Data are presented as percentage of ssDNA positive cells from 15 fields of three wells in each group (>450 cells/group). Scale bar = 10 μm. (D) Cell cycle distribution measured by FACS in AF5 cells treated with cocaine (24 h). Note changes in the scales in y-axis. A graph shows means ± SEM from six independent experiments. **, $p = 0.004$; ***, $p < 0.001$ as compared to each control. (E) BrdU-positive (red) and mitotic (green staining, phospho-histone H3-positive) cells in cocaine-treated AF5 cultures (24 h with BrdU). Cells that coexpress BrdU and phospho-histone H3 appear yellow, DAPI in blue. Scale bar = 10 μm. Sixteen fields from four wells were examined for quantification (>1,000 cells/group). *, $p = 0.013$; **, $p = 0.004$ compared to control. doi:10.1371/journal.pmed.0050117.g001

arrowheads at the superior sagittal sinus (SSS) and the causal pole of the internal capsule in Figure S1. Six brain sections from both brain hemispheres were examined in each animal. The outer half of the VZ (the half farthest from the ventricle) contains a layer of closely packed cells positive for Ki67 and BrdU S-phase incorporation. The outer edge of this epithelium-like layer was used to define the boundary between VZ and subventricular zone (SVZ).

Western Blot Analysis

Western blotting was performed as previously described [20] using antibodies to CDK2 and pRb (BD Biosciences), α-tubulin (Sigma-Aldrich), phospho-CDK2 (Thr-160), phospho-eIF2α (Ser-51), and eIF2α (Cell Signaling), cyclin A, ATF1, ATF2, ATF3, ATF4, phospho-CREB (Ser-133), CREB, JunB, JunD, c-Jun, c-Fos, p21, and p27 (Santa Cruz Biotechnology). Immunoreactive bands were densitometrically quantitated using Kodak Image Station 440 CF.

Transfection

AF5 cells were transfected with a plasmid encoding *cyclin A* (pRc/CMV-CycA; gift of P. Hinds, Harvard Medical School, Boston, Massachusetts, United States of America [26]) or a control plasmid (pcDNA3.1; Invitrogen). Briefly, one million AF5 cells in suspension (100 μl) were mixed with 2 μg of either plasmid, and electroporation was performed using nucleofection protocol T-20 (Amaxa Biosystems). Immediately after nucleofection, cells were plated in six-well plates for the

Western blot analysis and 96-well plates for the CyQUANT cell proliferation assay.

Analysis of Endogenous ROS Formation

Endogenous ROS were measured by incubating AF5 cells with 100 μM 2', 7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) during the last 20 min of indicated treatments. The treated AF5 cells were washed, dissolved with 1% Triton X-100 in PBS, and fluorescence was measured at an excitation wavelength of 485 nm, and an emission wavelength of 530 nm using a fluorescence microplate reader.

Statistical Analysis

All values were expressed as means ± standard error of the mean (SEM). Mean values were compared using the Student's *t*-test for two-sample comparisons, or analysis of variance (ANOVA) followed by Newman-Keuls tests for multiple comparisons as indicated in the legends for Figures 1–7, S2, and S6. The criterion for statistical significance was $p < 0.05$. It should be noted that this study includes a large number of statistical tests; thus, it is possible that some differences, even though statistically significant, are due to chance. For all differences found to be statistically significant by Student's *t*-tests, the differences were additionally assessed by non-parametric Mann-Whitney U tests, the results of which are listed in Table S1. *p*-Values obtained by both tests were similar. Also, as compared to the experiments performed in vitro, the variability between animals was relatively large;

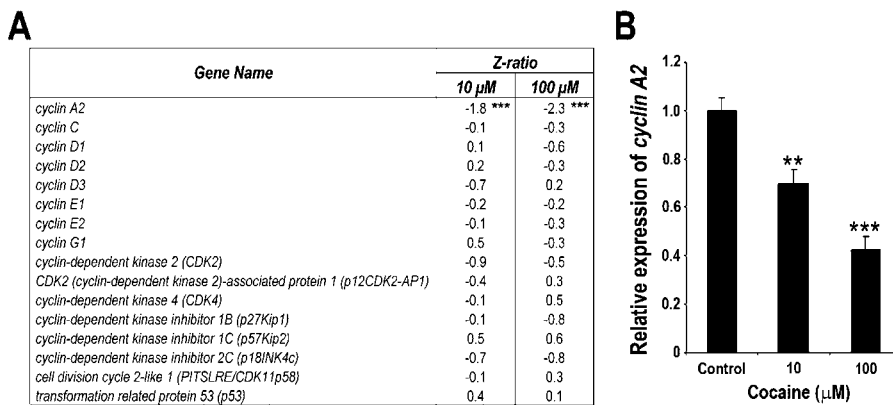


Figure 2. Microarray and Quantitative Real-Time RT-PCR Analyses of Cocaine-Treated AF5 Cells

(A) Microarray analysis of G1/S phase transition regulators after cocaine treatment (10 and 100 μ M, 24 h). ***, $p < 0.001$ compared to control, $n = 4$. (B) Confirmation of changes in *cyclin A2* expression (10 and 100 μ M cocaine, 24 h) by quantitative real-time RT-PCR. Data are expressed as fold changes in relationship to the control condition. **, $p = 0.004$; ***, $p < 0.001$ compared to control, $n = 3-7$. doi:10.1371/journal.pmed.0050117.g002

therefore, scattergram plots showing the data for individual animals are included as Figures S4 and S7.

Results

Cocaine Inhibits Cell Proliferation without Affecting Cell Survival

Log-phase cultures of AF5 cells were treated with varying concentrations of cocaine (1–100 μ M) for 24 h. The number of cells in each condition was then measured and compared to the number at the initiation of the treatment. As shown in Figure 1A, the number of AF5 cells in untreated cultures doubled in 24 h, while numbers of cells in cocaine-treated cultures were significantly reduced in a dose-dependent manner as compared to controls. When exposure of AF5 cells to 100 μ M cocaine was extended to 6 d, the inhibitory effect was even more pronounced (Figure S2). Because cocaine substantially inhibited proliferation of AF5 cells after only 24 h exposure, we employed this exposure duration for subsequent experiments.

The effect of cocaine was not due to an increase in cell death, as we did not observe a change in either extracellular LDH activity (Figure 1B) or numbers of apoptotic nuclei immunoreactive for single-strand DNA (Figure 1C). We also did not observe any morphological changes indicative of necrosis or apoptosis in cocaine-treated AF5 cells. Therefore, as has been reported for several other cell types [11,27,28], cocaine inhibits proliferation of AF5 neural progenitor cells.

Cocaine Interferes with the G1/S Transition

We next examined the cell cycle distribution of cocaine-treated cells by FACS (Figure 1D). Cocaine resulted in a dose-dependent increase in cells in G1 phase and a reduction in the number of cells in S phase, indicating that cocaine suppresses the G1-to-S phase transition (Figure 1D). To further clarify the transition suppression, cell populations going through S phase were monitored by BrdU incorporation over 24 h. Both 10 μ M and 100 μ M cocaine significantly decreased the percentage of BrdU-positive cells that had entered S phase, supporting the notion that cocaine interferes with the G1/S cell cycle transition (Figure 1E). The percentage of mitotic cells was low (<3%) as measured

by phospho-histone H3 immunocytochemistry, and was not affected by cocaine (Figure 1E).

Microarray Screening Identifies Cyclin A2 as a G1/S Phase Transition Controller Affected by Cocaine

To identify molecules that could mediate the cocaine-induced G1/S transition impairment, we used a microarray that contains 93 cell cycle-related genes including 16 G1/S phase transition controllers (*cyclin A2*, *C*, *D1*, *D2*, *D3*, *E1*, *E2*, *G1*, *CDK2*, *p12CDK2-AP1*, *CDK4*, *p27Kip1*, *p57Kip2*, *p18INK4c*, *PITSLRE/CDK11p58*, and *p53*). Of these, cocaine (10 and 100 μ M, 24 h) significantly down-regulated only *cyclin A2* (Figure 2A). The decrease in *cyclin A2* expression by 10 or 100 μ M cocaine was confirmed by quantitative real-time RT-PCR (Figure 2B).

To characterize the time course of cocaine-induced down-regulation of cyclin A, we treated AF5 cells with 100 μ M cocaine for 6 d and found that the cyclin A protein level, as measured by Western blotting, had started to decrease by day 1, continued to decline at day 3, and finally resulted in an undetectable amount of cyclin A protein by day 6 (Figure S3). These data show that cocaine-induced inhibition of AF5 cell proliferation is correlated with down-regulation of cyclin A.

Cocaine Down-Regulates Cyclin A2 Expression in Primary Human Fetal CNS Progenitor Cells in Culture

To determine whether our findings in AF5 cells are relevant to primary cells, we measured *cyclin A2* mRNA in several types of primary human fetal CNS cells obtained from ~20-wk (second trimester) human fetal cerebral cortexes. As measured by quantitative real-time RT-PCR, in vitro cocaine exposure (100 μ M, 24 h) significantly decreased *cyclin A2* mRNA level in both human neural progenitor cells (>95% of cells are Nestin-positive and A2B5-negative, unpublished data) and A2B5+ progenitor cells (>90% of cells are A2B5-positive and Nestin-positive, unpublished data), whereas *cyclin A2* mRNA was not altered in neurons or microglia (Figure 3A). In contrast, cocaine increased the *cyclin A2* transcript in human astrocytes (Figure 3A).

The cyclin A protein level was also significantly decreased by cocaine (10 and 100 μ M, 24 h) in both human neural and A2B5+ progenitor cells (Figure 3B and 3C). Cocaine caused a

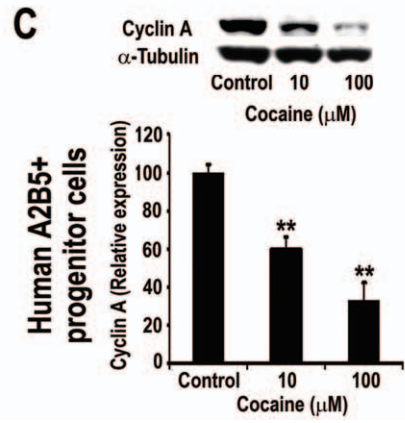
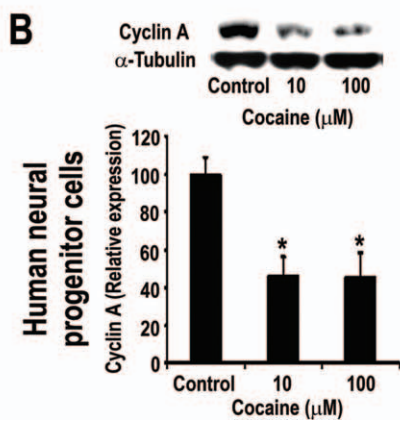
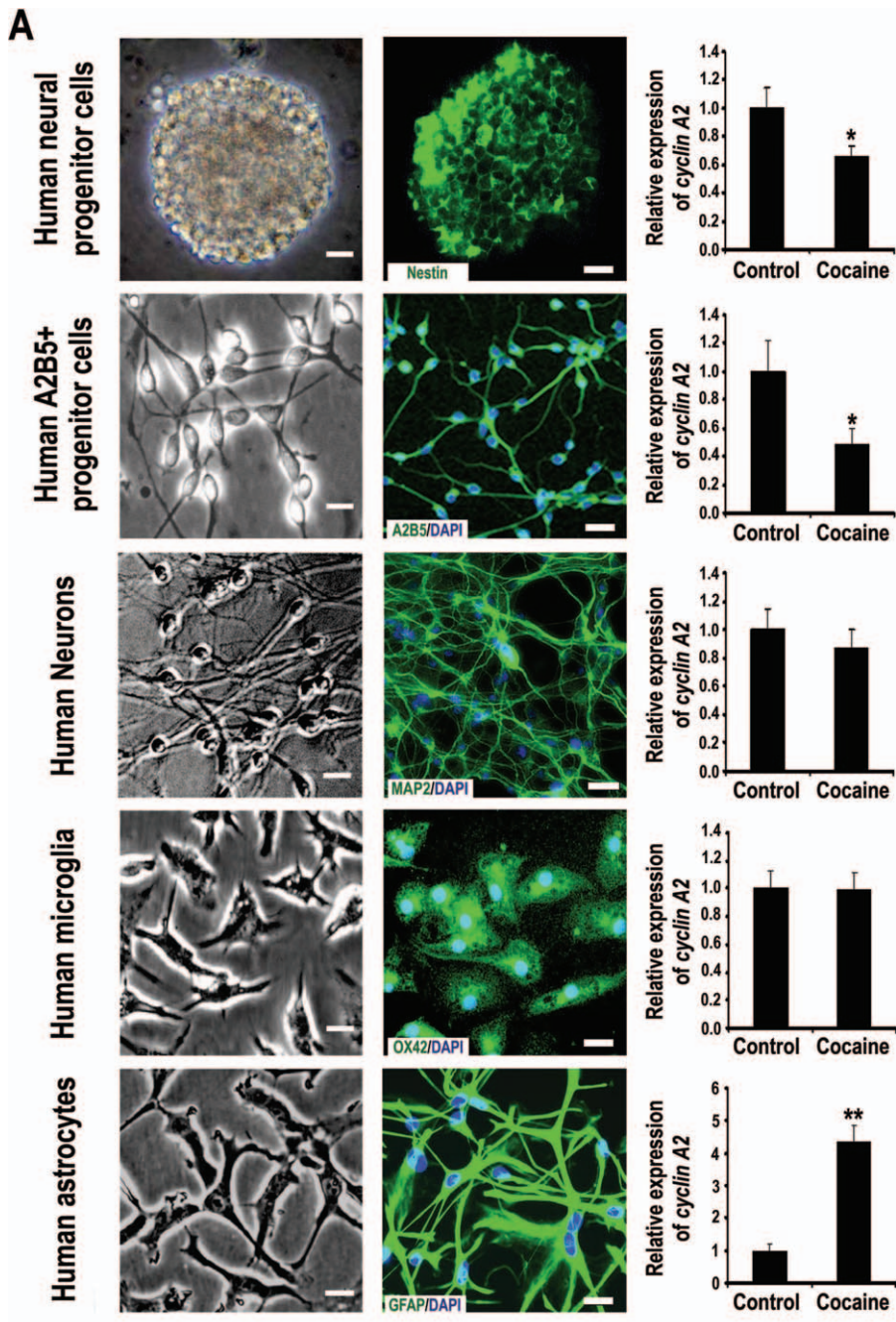


Figure 3. Expression of Cyclin A in Primary Human Fetal CNS Cells Treated with Cocaine

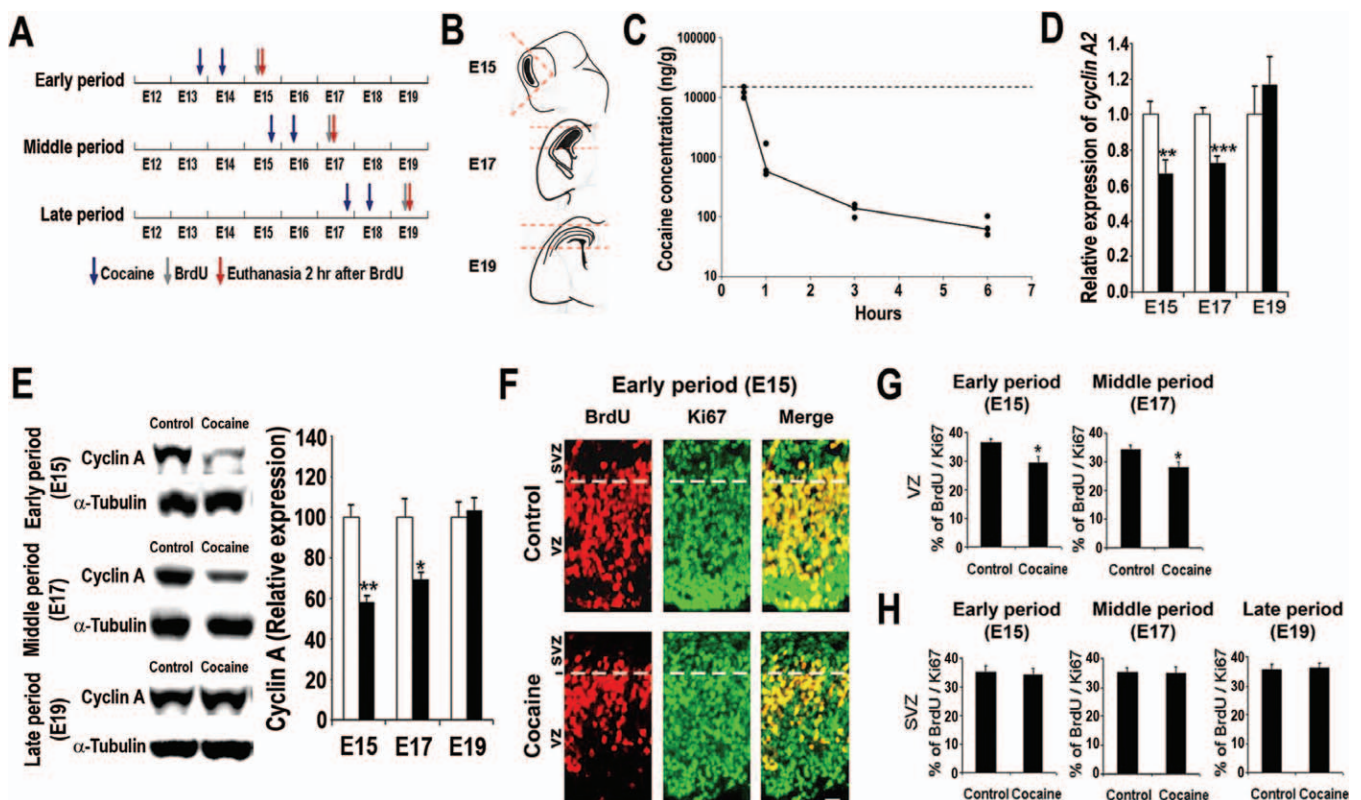
(A) Five different types of human primary cells from cerebral cortex (8–21 d in vitro), characterized by the CNS cell type-specific markers including nestin (green) for neural progenitor cells, A2B5 (green) for A2B5+ progenitor cells, MAP2 (green) for neurons, OX42 (green) for microglia, and GFAP (green) for astrocytes, were treated with 100 μ M cocaine for 24 h, and *cyclin A2* was measured by quantitative real-time RT-PCR analysis. DAPI nuclear staining is shown in blue. Human neural progenitor cells, *, $p = 0.039$ compared to control; human A2B5+ progenitor cells, *, $p = 0.040$ compared to control; human astrocytes, **, $p = 0.001$ compared to control. $n = 3-5$, from three to five individual brains. All scale bars are 25 μ m, except for morphology and marker expression of A2B5+ progenitor cells scale bars are 15 and 35 μ m, respectively.

(B–C) Western blot analysis of cyclin A in cocaine (24 h)-treated human neural and A2B5+ progenitor cells. The expression of cyclin A was normalized to α -tubulin and expressed as ratios to the control values. For human neural progenitor cells, 10 μ M cocaine, *, $p = 0.013$ compared to control; 100 μ M cocaine, *, $p = 0.027$ compared to control. For human A2B5+ progenitor cells, 10 μ M cocaine, **, $p = 0.006$ compared to control; 100 μ M cocaine, **, $p = 0.001$ compared to control. $n = 3$ from three individual brains.
doi:10.1371/journal.pmed.0050117.g003

maximum down-regulation of cyclin A protein level at 10 μ M in human neural progenitor cells (Figure 3B), but decreased expression of cyclin A in a dose-dependent manner in A2B5+ progenitor cells (Figure 3C). These data verify that cocaine down-regulates cyclin A in both types of progenitor cells derived from human fetal cerebral cortexes.

Cocaine Down-Regulates Cyclin A2 Expression and Inhibits Cell Cycle Progression in Fetal Rat Brains

We next examined cyclin A expression in fetal rat brains exposed to cocaine in utero. Neocortical neurogenesis occurs within two proliferative strata of the embryonic cerebral wall, which is adjacent to the ventricle. Neocortical neurogenesis

**Figure 4.** Effects of Cocaine on Cyclin A Expression in the Developing Rat Neocortex

(A) Experimental paradigm for cocaine and BrdU injections. Twenty mg/kg of cocaine was injected twice (IP) to pregnant rats at an interval of 12 h. BrdU was injected (IP) 24 h after cocaine.

(B) Diagrams of E15 (sagittal), E17 (coronal), and E19 (coronal) fetal brains showing the areas used for fetal brain cocaine concentration measurements and cyclin A assays (inside red dotted lines). Further anatomical detail can be obtained from Paxinos et al. [76].

(C) Time course of cocaine concentrations in developing rat neocortex at early period of neurogenesis determined by gas chromatography-mass spectrometry (GC-MS). The maximum concentration shown is 15,000 ng/g, indicating that higher concentrations are over the limit (15,000 ng/g) of quantification. $n = 3$ from three individual pregnant dams.

(D) *Cyclin A2* mRNA expression in cocaine-treated (closed bars) fetal cortex measured by quantitative real-time RT-PCR. Data are expressed as fold changes in relationship to the control condition (open bars). E15, **, $p = 0.00995$ compared to control; E17, ***, $p < 0.001$ compared to control. $n = 4-7$ from three individual pregnant dams.

(E) Western blot analysis of cyclin A in developing neocortex. The expression of cyclin A was normalized to α -tubulin and expressed as ratios to the control values. E15, **, $p = 0.001$ compared to control; E17, *, $p = 0.010$ compared to control. $n = 4-7$ from three individual pregnant dams.

(F) BrdU incorporation in peri-ventricular region of cocaine-exposed fetuses during the early period of neurogenesis (E15). Images showing immunoreactivity of BrdU (red) and Ki67 (green). Scale bar is 20 μ m.

(G) Percentage of cortical progenitor cells (Ki67-positive cells) in VZ that entered S phase (BrdU-positive). E15, *, $p = 0.014$ compared to control; E17, *, $p = 0.025$ compared to control. $n = 6$ from three to four individual pregnant dams.

(H) Percentage of cortical progenitor cells (Ki67-positive cells) in SVZ that entered S phase (BrdU-positive). $n = 6$ from three to four individual pregnant dams. These differences were not statistically significant.

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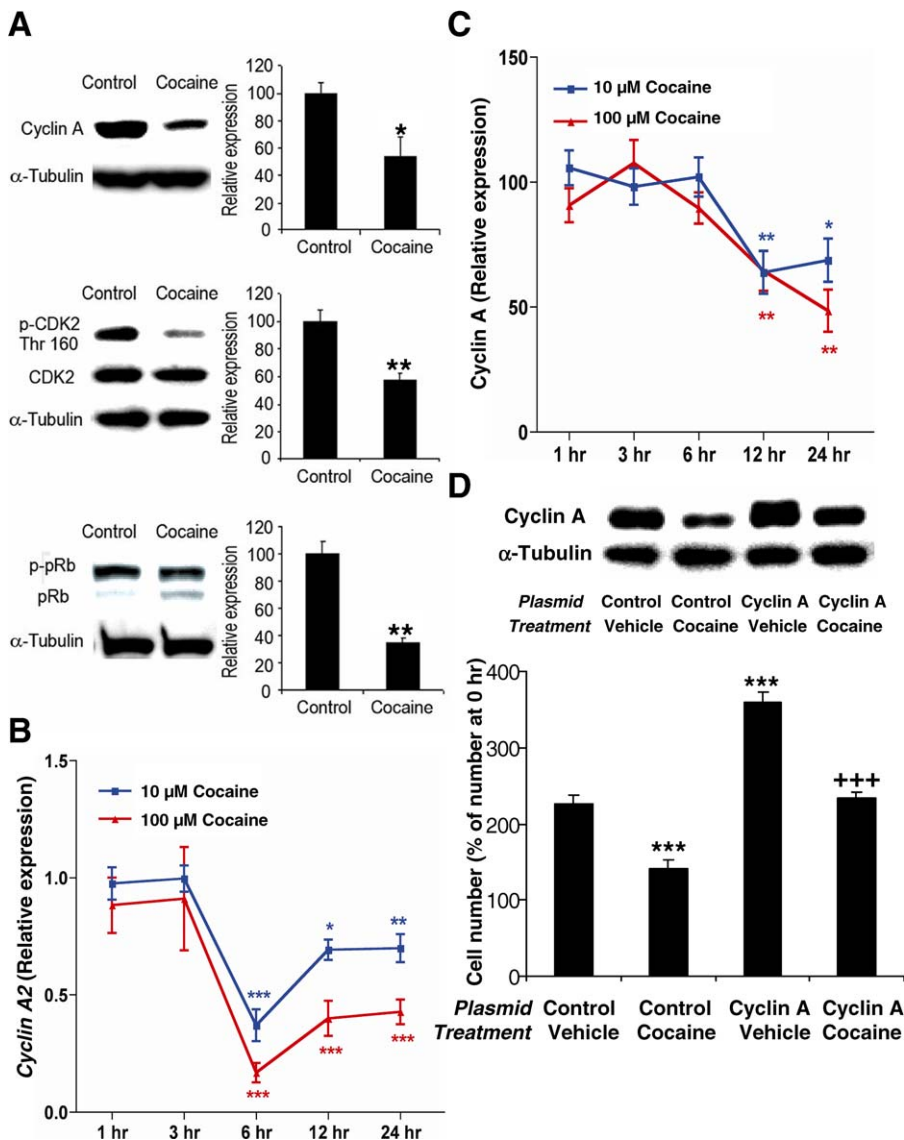


Figure 5. Role of Cyclin A Expression in Cocaine-Induced Proliferation Inhibition

(A) Western blot analysis of cyclin A and its downstream proteins in cocaine-treated AF5 cells. AF5 cells were treated with vehicle or 100 μ M cocaine for 24 h. Signals of cyclin A were normalized to α -tubulin. Phosphorylation status of CDK2 and pRb were determined by normalizing phosphorylated forms to total CDK2 proteins and unphosphorylated forms of pRb, respectively. Intensities of bands were densitometrically analyzed. Data represent means of three to five independent experiments. Cyclin A, *, $p = 0.031$ compared to control; p-CDK2, **, $p = 0.002$ compared to control; p-pRb, **, $p = 0.002$ compared to control.

(B) Time course of *cyclin A2* mRNA levels in AF5 cells treated with 10 and 100 μ M cocaine, as measured by quantitative real-time RT-PCR. The expression of *cyclin A2* was expressed as fold changes in relationship to the control values. For 10 μ M cocaine, 6 h, ***, $p < 0.001$ compared to control; 12 h, *, $p = 0.012$ compared to control; 24 h, **, $p = 0.004$ compared to control. For 100 μ M cocaine, ***, $p < 0.001$ compared to each control. $n = 3-7$.

(C) Time course of cyclin A protein levels in AF5 cells treated with 10 and 100 μ M cocaine. The expression of cyclin A was normalized to α -tubulin and expressed as ratios to the control values. For 10 μ M cocaine, 12 h, **, $p = 0.007$ compared to control; 24 h, *, $p = 0.012$ compared to control. For 100 μ M cocaine, 12 h, **, $p = 0.003$ compared to control; 24 h, **, $p = 0.002$ compared to control. $n = 3-6$.

(D) Effect of cyclin A overexpression on cocaine-induced proliferation inhibition. Cyclin A protein levels and cell proliferation were measured 24 h after electroporation of the CMV-Cyc A vector and 100 μ M cocaine treatment. Cyclin A: cells transfected with pRc/CMV-CycA. Control: cells transfected with the empty vector. Data are presented as percentage of control cell numbers at 0 h. ***, $p < 0.001$ compared to control; +++, $p < 0.001$ compared to treatment with cocaine only. $n = 8$.

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starts at E12 and ends at E19 in the rat [29]. During this process, the VZ appears first and is followed by the SVZ. To investigate the time-frame within which neocortical development is susceptible to prenatal cocaine exposure, we examined three different time periods: the early neurogenesis period from E13 to E15, the middle period of neurogenesis from E15 to E17, and the late neurogenesis period from E17 to E19. Pregnant animals received cocaine according to the

injection schedule shown in Figure 4A, and the frontal cortex of developing fetuses was dissected as shown in Figure 4B.

To examine cocaine concentrations in the fetal neocortex under our injection schedule and for comparison to our in vitro studies, tissue concentrations of cocaine were analyzed at the early period of neurogenesis after the second cocaine administration. Cocaine concentrations in fetal neocortex reached at least 30 μ M (9,812 ng/g) 0.5 h after injection,

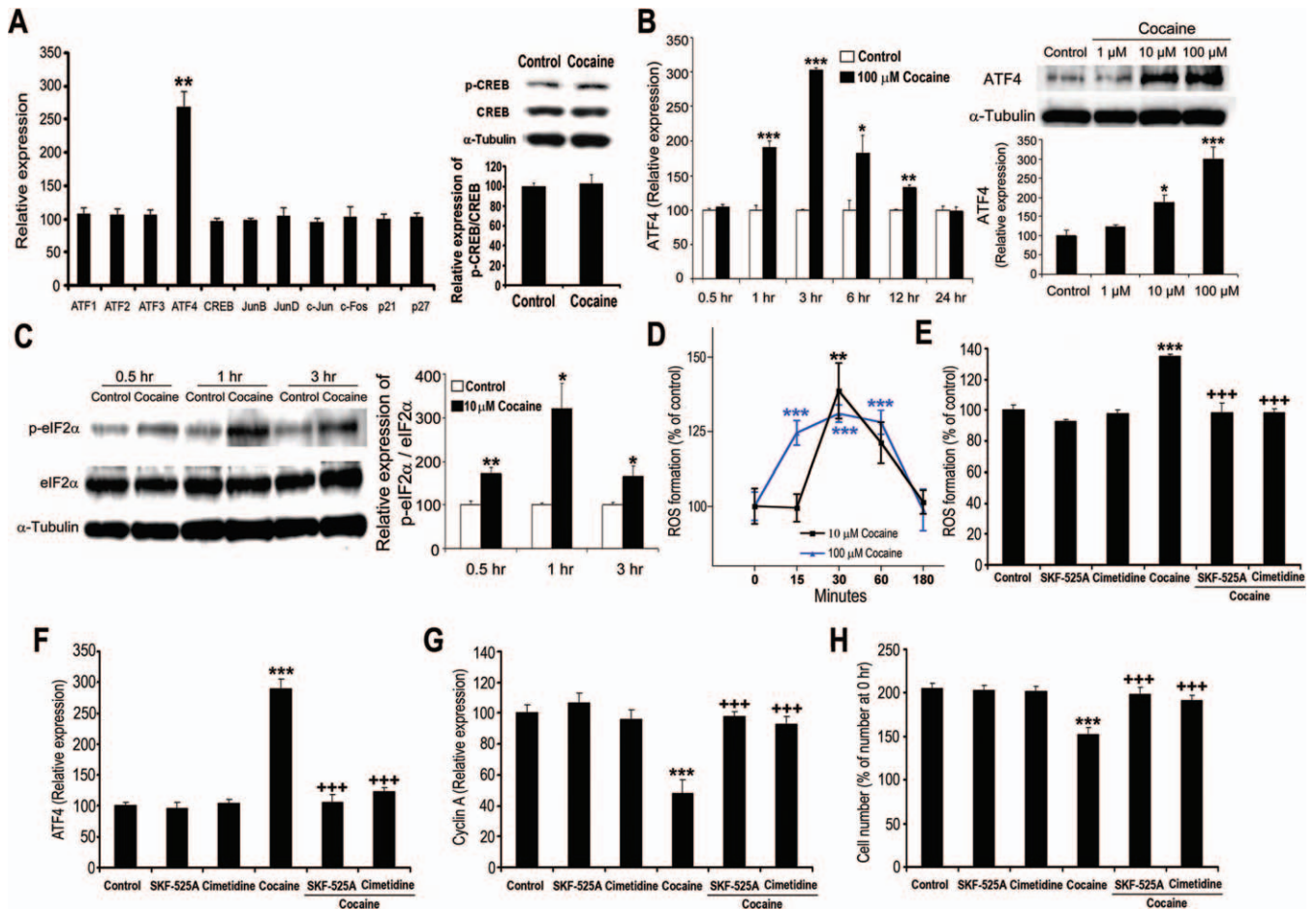


Figure 6. Cocaine-Induced Cyclin A Down-Regulation and Proliferation Inhibition Involves Oxidative ER Stress Signaling

(A) Expression of transcription factors, CDK inhibitors, and the activity of CREB following cocaine treatment. AF5 cells were exposed to cocaine for 3 h, and transcription factors and CDK inhibitors that regulate the transcription of *cyclin A2* were measured by immunoblotting. Signals were normalized to α -tubulin and expressed as percent of control. Phosphorylated CREB was normalized to total CREB proteins. **, $p = 0.001$ compared to control. $n = 4-6$. (B) Time course of ATF4 up-regulation in AF5 cells treated with cocaine. For dose-response experiments, AF5 cells were exposed to cocaine for 3 h. The expression of ATF4 was normalized to α -tubulin and expressed as percentage of the control values. For time course experiments, 1 h and 3 h, ***, $p < 0.001$ compared to the respective controls; 6 h, *, $p = 0.039$ compared to control; 12 h, **, $p = 0.008$ compared to control. For dose-response experiments, 10 μ M cocaine, *, $p = 0.031$ compared to control; 100 μ M cocaine, ***, $p < 0.001$ compared to control. $n = 3$.

(C) Time course of phosphorylation of eIF2 α in AF5 cells treated with 10 μ M cocaine. The phosphorylation status of eIF2 α was determined by normalizing phosphorylated forms to total eIF2 α proteins and expressed as percentage of the control values. 0.5 h, **, $p = 0.005$ compared to control; 1 h, *, $p = 0.031$ compared to control; 3 h, *, $p = 0.045$ compared to control. $n = 3$.

(D) ROS formation in AF5 cells treated with cocaine determined by DCFH-DA. Data are presented as percentage of control. **, $p = 0.003$; ***, $p < 0.001$ compared to each control. $n = 4-9$.

(E-H) Effects of P450 inhibitors on ROS formation (E), expression of ATF4 (F) and cyclin A (G), and proliferation of AF5 cells (H). SKF-525A (100 μ M) or cimetidine (100 μ M) were applied 30 min before 100 μ M cocaine. ROS and ATF4 protein levels were measured 30 min and 3 h after cocaine, respectively, whereas cyclin A protein levels and cell proliferation were measured 24 h after cocaine. For Western blot analysis, the expression of ATF4 and cyclin A was normalized to α -tubulin. For the cell proliferation assay, data are shown as percentages of control cell numbers at 0 h. ***, $p < 0.001$ compared to each control; +++, $p < 0.001$ compared to each cocaine treatment. $n = 6-8$ for ROS measurement; $n = 3-6$ for ATF4 and cyclin A measurements; and $n = 6$ for the cell proliferation assay.

doi:10.1371/journal.pmed.0050117.g006

dropped to ~ 2 μ M (median value of 594 ng/g) at 1 h, and gradually declined to ~ 0.2 μ M (median value of 63 ng/g) at 6 h (Figure 4C). Thus, the injection schedule used produced exposure of fetal brains to maximum concentrations of cocaine within the range of doses that we found to inhibit proliferation of progenitor cells in vitro.

Down-regulation of *cyclin A2* mRNA was seen in prefrontal cortex of the developing fetuses when cocaine was injected at either early or middle periods of neurogenesis (Figures 4D and S4A). *Cyclin A2* mRNA was not, however, changed by injections during the late period of neurogenesis (Figures 4D and S4A). Similarly, cyclin A protein was also significantly

decreased by cocaine in both early and middle neurogenesis periods, but not during the late period of neurogenesis (Figures 4E and S4B). This result suggests that cocaine down-regulates cyclin A expression only during earlier periods of neocortical neurogenesis that involve active proliferation of neural progenitor cells in the VZ.

Since cocaine causes down-regulation of cyclin A in fetal brains, we also examined cell cycle progression of neural progenitors in the VZ and SVZ in fetal brains in utero exposed to cocaine. Pulse labeling with BrdU was used to quantify cortical progenitor cells that had entered S phase during a period of 2 h, whereas Ki67 immunocytochemistry

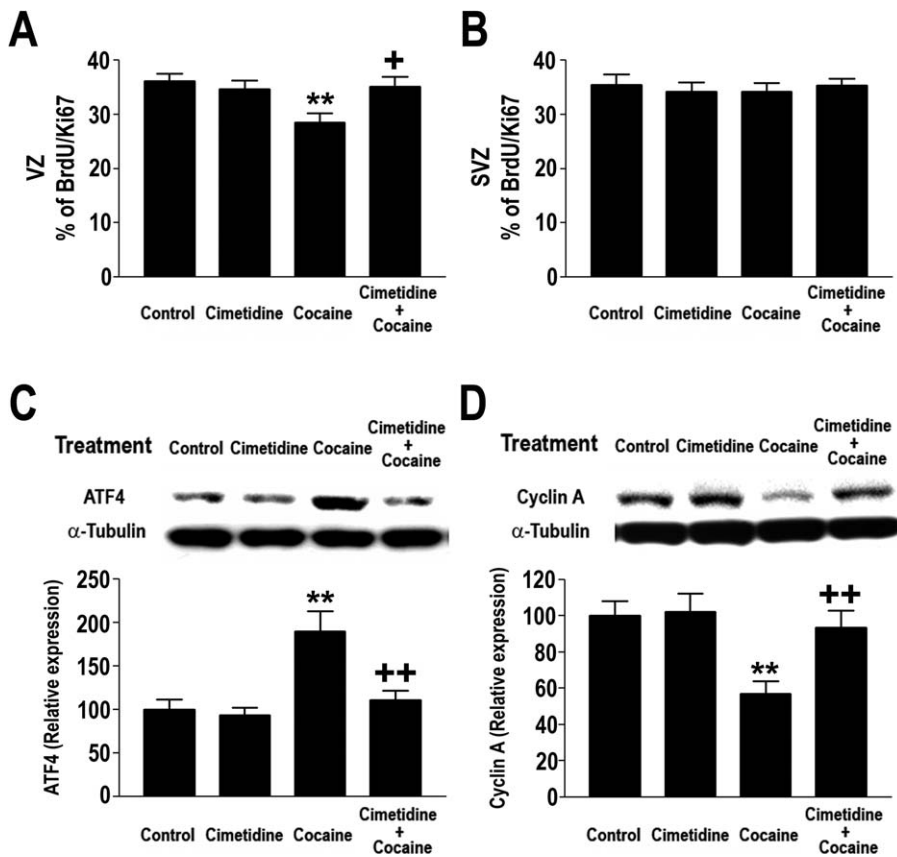


Figure 7. Recovery of Cocaine-Induced Changes in the Developing Neocortex by the P450 Inhibitor Cimetidine

Pregnant rats at early period of neurogenesis (E13–E15) were pretreated with 100 mg/kg of cimetidine (IP) 1 h before receiving 20 mg/kg of cocaine using the regimen described in Figure 4A. (A and B) Effects of cimetidine on the BrdU incorporation in the developing rat neocortical VZ and SVZ of cocaine-exposed fetuses. (A) Percentage of cortical progenitor cells (Ki67-positive cells) in VZ that entered S phase (BrdU-positive). **, $p = 0.009$ compared to control; +, $p = 0.018$ compared to cocaine treatment. $n =$ six to nine samples from three pregnant dams. (B) Percentage of cortical progenitor cells (Ki67-positive cells) in SVZ that entered S phase (BrdU-positive). $n = 6–9$ from three individual pregnant dams. These differences were not statistically significant. (C and D) Effects of cimetidine on expression of ATF4 and cyclin A in prefrontal cortex of cocaine-exposed fetuses. ATF4 protein levels were measured 3 h after the last injection of cocaine. Cyclin A protein was measured 24 h after the last injection of cocaine. For Western blot analysis, the expression of ATF4 and cyclin A was normalized to α -tubulin. For ATF4, **, $p = 0.001$ compared to control; ++, $p = 0.002$ compared to cocaine treatment. For cyclin A, **, $p = 0.007$ compared to control; ++, $p = 0.008$ compared to cocaine treatment. $n =$ five to six samples from three pregnant dams for ATF4 measurement, and $n = 6$ samples from three pregnant dams for cyclin A measurement. doi:10.1371/journal.pmed.0050117.g007

was used to monitor the total fraction of progenitors that are in any phase of the cell cycle except for G_0 [30]. Cocaine decreased the number of BrdU-labeled progenitor cells in the VZ during both the early and middle neurogenesis periods (Figures 4F, 4G, S4C, and S5), suggesting that cocaine inhibits the proliferation of progenitor cells in the VZ.

In contrast to the findings in the VZ, BrdU positive progenitor cells in the SVZ were not changed by cocaine (Figures 4F, 4H, S4D, and S5). Also, since cortical germinal zones in the late period of neurogenesis comprise SVZ only, cocaine did not change BrdU labeling during this period (Figures 4H, S4D, and S5). Taken together, these data suggest that cocaine promotes cyclin A down-regulation and cell proliferation inhibition both in vitro and in vivo.

Cocaine Inhibits Cyclin A2 Downstream Cell Cycle Effectors: Decreased Phosphorylated CDK2 and pRb

Western blotting confirmed that cocaine (100 μ M, 24 h) decreases cyclin A2 protein in AF5 cells (Figure 5A). Cyclin A induces a conformational change in CDK2, and thereby

permits activation of CDK2 through phosphorylation of Thr-160 catalyzed by CDK-activating kinase (CAK) [31]. Activated CDK2 phosphorylates the downstream molecule pRb, which in turn promotes the expression of genes required for progression from G1 to S. Western blotting showed that cocaine significantly decreased the phosphorylated forms of CDK2 and pRb (Figure 5A), indicating that cocaine-induced down-regulation of cyclin A results in a hypoactive state of the downstream cell cycle signaling pathway.

Reversal of Cocaine-Induced Proliferation Inhibition by Cyclin A Transfection

To demonstrate a causal relationship between cyclin A down-regulation and cocaine-induced inhibition of AF5 cell proliferation, we attempted to compensate for cyclin A down-regulation by gene transfer using an expression vector encoding cyclin A (pRc/CMV-CycA).

To examine the appropriate timing of transfection, we first examined the time course of cyclin A expression after either cocaine treatment or vector transfection. Quantitative real-

time RT-PCR showed that *cyclin A2* mRNA was decreased as early as 6 h after 10 or 100 μM cocaine, and the down-regulation lasted up to 24 h (Figure 5B). Cyclin A protein was significantly decreased 12 h after 10 or 100 μM cocaine with nearly maximum effects (64%) at 12 h for 10 μM cocaine, and a 48% decrease at 24 h for 100 μM cocaine (Figure 5C). Since pRc/CMV-CycA transfection (with $\sim 80\%$ transfection efficiency) also showed the maximum level of overexpression at 24 h post-transfection (unpublished data), we exposed cells to 100 μM cocaine immediately after electroporation of the CMV-Cyc A vector, and measured cyclin A protein and cell proliferation 24 h later. As shown in Figure 5D, cyclin A transfection counteracted the cocaine-induced down-regulation of cyclin A, as well as the inhibition of proliferation caused by cocaine. These data suggest that cocaine-induced down-regulation of cyclin A contributes to the proliferation inhibition seen in cocaine-exposed AF5 cells.

Identification of the eIF2 α -ATF4 Pathway in Transcriptional Down-Regulation of Cyclin A by Cocaine

We next examined the levels of several transcription factors involved in regulation of the *cyclin A2* promoter activity. Western blot analysis showed that cocaine (100 μM , 3 h) significantly up-regulated only ATF4 among a total of nine candidate transcription factors tested, including ATF1–4, CREB, JunB, JunD, c-Jun, and c-Fos (Figure 6A). The phosphorylation status of CREB, which is involved in activation of *cyclin A* transcription at G1/S [32], was not affected by 100 μM cocaine at 3 h (Figure 6A). In addition, cocaine did not change levels of p21 and p27, CDK inhibitors that decrease transcription of *cyclin A2* (Figure 6A) [33,34].

An increase in ATF4 protein occurred as early as 1 h after 100 μM cocaine exposure, and reached the maximal level at 3 h (Figure 6B). Cocaine at concentrations higher than 1 μM resulted in dose-dependent induction of ATF4 (Figure 6B). The phosphorylated form of the alpha subunit of translation initiation factor 2 (eIF2 α) is known to promote ATF4 induction [35]. Indeed, we found that 10 μM cocaine significantly increased the phosphorylated form of eIF2 α 0.5 h after exposure to cocaine, preceding induction of ATF4 (Figure 6C).

Role of N-Oxidative Metabolism of Cocaine in Generation of ROS, Endoplasmic Reticulum Stress, Induction of ATF4, Inhibition of Cyclin A, and Cell Proliferation

The eIF2 α -ATF4 pathway is activated by PERK, an endoplasmic reticulum (ER) stress sensor protein [35,36]. The ER stress pathway has been shown to be activated by a variety of chemicals or pathological stress including oxidative ER stress [35]. Notably it has been demonstrated that the cytochrome P450 dependent N-oxidative pathway is responsible for generation of ROS and glutathione (GSH) depletion during cocaine biotransformation in the liver [37]. We therefore hypothesized that ROS generation caused by the N-oxidative metabolism of cocaine may trigger activation of the eIF2 α -ATF4 pathway.

Accordingly, we first examined whether cocaine induces ROS production in progenitor cells. As shown in Figure 6D, 10 μM cocaine caused a significant increase in ROS 30 min after treatment. Cocaine at 100 μM caused ROS generation even earlier, with a significant increase 15 min after the exposure.

Because the rise of cocaine concentrations over 10 μM lasts for less than 1 h in fetal brains after cocaine injection (Figure 4C), AF5 cells were next treated with 30 μM cocaine for 30 min followed by incubation in cocaine-free medium for 2.5 h to examine ATF4 protein levels. We found that ATF4 was significantly up-regulated by this transient 30 min exposure to cocaine (Figure S6). Thus, exposure to cocaine for a period of only 30 min can generate ROS sufficient to initiate ER stress, and result in up-regulation of ATF4 2.5 h later.

Pretreatment with the cytochrome P450 inhibitors SKF-525A or cimetidine, drugs that have been shown to potently block N-oxidative metabolism of cocaine, completely blocked cocaine-induced ROS formation (Figure 6E), confirming that N-oxidative metabolism of cocaine is involved in ROS formation in AF5 cells. Further, ROS generation appears to be the source of cocaine-induced ER stress, as both SKF-525A and cimetidine also completely inhibited cocaine-induced ATF4 up-regulation and cyclin A down-regulation (Figure 6F and 6G).

Finally, we tested effects of SKF-525A and cimetidine on the inhibition of cell proliferation by cocaine. Both drugs significantly diminished cocaine-induced proliferation inhibition (100 μM for 24 h) (Figure 6H). On the other hand, the lipophilic free radical scavengers α -tocopherol and BHA and the iron chelator DFO (each 50 μM) did not prevent cocaine-induced proliferation inhibition (unpublished data). These data indicate that cytochrome P450-dependent ROS formation occurring during cocaine metabolism is responsible for both cocaine-induced proliferation inhibition and cyclin A down-regulation.

Cimetidine Reverses Effects of Cocaine In Utero in a Rat Model

To determine whether P450 inhibitors can block cocaine-induced proliferation inhibition in neural progenitor cells in the developing neocortex, pregnant rats at the early period of neurogenesis (E13–E15) were pretreated with 100 mg/kg cimetidine IP 1 h before each cocaine administration. Cimetidine is known to cross the placenta [38–40]. Cimetidine itself did not affect neural progenitor cell proliferation, survival, density, or fetal mortality (Figures 7A, 7B, S7A, S7B, and S8A–S8E). As shown in Figures 7A and S7A, pretreatment of pregnant rats with cimetidine resulted in recovery of the cocaine-induced decrease in BrdU-positive progenitor cells in the VZ. As expected, no differences in BrdU-positive progenitor cells were observed in the SVZ (Figures 7B and S7B).

To determine whether the protection afforded by cimetidine was due to the recovery of cocaine-induced down-regulation of cyclin A and mediation by ER stress, the effects of cimetidine on expression of ATF4 and cyclin A were also measured in prefrontal cortex of cocaine-treated fetuses. Pretreatment of pregnant rats with cimetidine significantly inhibited the cocaine-induced up-regulation of ATF4 and the down-regulation of cyclin A (Figures 7C, 7D, S7C, S7D). These results suggest that blockade of cocaine N-oxidative metabolism by the P450 inhibitor cimetidine reverses cocaine-induced proliferation inhibition of neural progenitor cells in the VZ through normalizing cocaine-induced oxidative ER stress and consequent cyclin A down-regulation.

Discussion

In the present study, we found that (1) cocaine causes proliferation inhibition and cyclin A down-regulation in

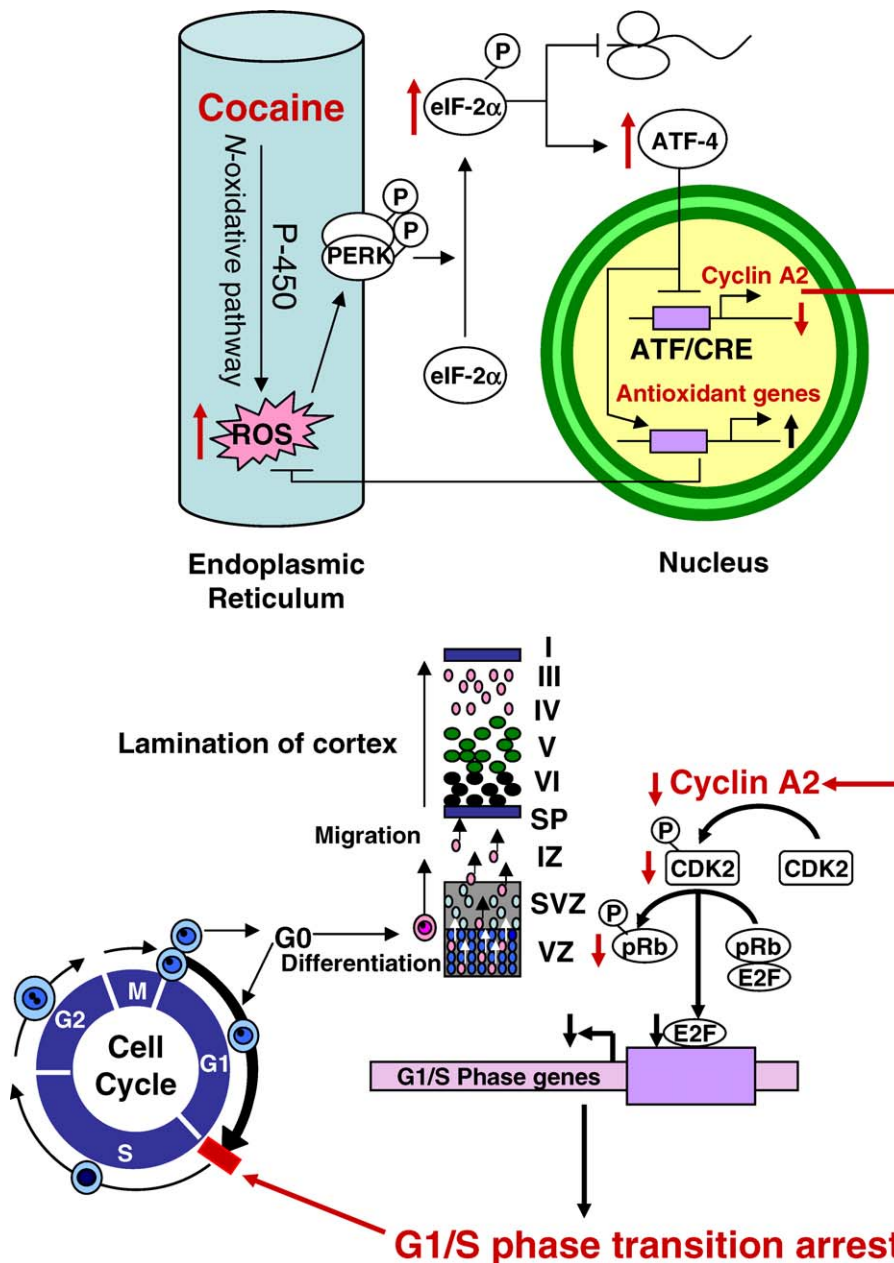


Figure 8. Schematic Illustrating the Mechanism of Cocaine's Effect on Brain Development Described in this Study

During cortical neurogenesis, ventricular progenitors (blue) withdraw from the cell cycle (pink) and start to differentiate and migrate to their specific destinations to form cortical layers. Exposure to cocaine during neocortical development causes rapid ROS accumulation in ER via cytochrome P450 dependent N-oxidative metabolism. ROS-induced oxidative ER stress leads to activation of the PERK/eIF2 α /ATF4 pathway. ATF4 represses the transcription of *cyclin A2* in neural progenitor cells. Blocking cell cycle progression by cyclin A down-regulation may result in fewer immature neurons migrating into the cortex to form the cortical layers. I, III, IV, V, and VI, cortical layers I, III, IV, V, and VI; IZ, intermediate zone; SP, superficial portion of the intermediate zone; SVZ, subventricular zones; VZ, ventricular zone. Up-regulation or activation of transcripts or proteins is denoted by \uparrow ; down-regulation or inactivation of transcripts or proteins is denoted by \downarrow . Changes indicated by red arrows were confirmed by quantitative real-time RT-PCR or Western blot analysis in this study.

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neural progenitor cells both in vitro and in vivo; (2) restoring cyclin A reverses proliferation inhibition induced by cocaine; and (3) ROS-induced ER stress, activating the eIF2 α -ATF4 pathway, is involved in cyclin A down-regulation induced by cocaine. Thus, this study identifies ES stress-induced cyclin A down-regulation as an important molecular event involved in cocaine-induced proliferation inhibition in neural progenitor cells. A diagram, illustrating this pathway is shown in Figure 8.

The Role of Cyclin A in Neural Progenitor Cells Exposed to Cocaine

Using the AF5 neural progenitor cell line, we determined that cocaine treatment for 24 h causes proliferation inhibition at concentrations higher than 1 μ M. Similar results were reported by Hu et al. [11] showing that cocaine ranging 1–100 μ M (7 d) suppresses growth of human neural precursor cells, as measured by thymidine incorporation. Nevertheless,

this group also did not detect proliferation inhibition by 1 μM cocaine for 24 h, although a significant effect was produced by treatment with 1 μM cocaine for 5 d [11]. The findings of Hu et al. [11] are therefore consistent with our data.

Poon et al. [10] found that 1 μM cocaine treatment for 30 min induced late-onset cell death (72 h later) in differentiated human neuronal progenitor cells. We did not observe cell death in cocaine (1–100 μM)-treated AF5 cells (Figure 1B and 1C) or in primary human fetal neural and A2B5+ progenitor cells (unpublished data) 24 h after treatment, although we did not examine cell death at later time points such as 72 h. In agreement with our findings, cocaine treatment (1 pM–100 μM) for 7 d did not induce cell death in undifferentiated human neural precursor cells [11]. Under certain conditions, cocaine can selectively induce cell death in neurons without affecting viability of other types of CNS cells [41]. The differentiated neuronal progenitor cells employed by Poon et al. [10] may therefore be relatively more sensitive to an effect of cocaine in promoting cell death as compared to undifferentiated neural progenitor cells.

Although cocaine has been shown to inhibit DNA synthesis as measured by thymidine incorporation in human neural precursor cells [11], the mode of action of cocaine on cell cycle progression has not previously been determined. We found that cocaine causes cell proliferation inhibition by interfering with the G1-to-S transition. Although one report suggested a disturbance in chromosome segregation induced by 2,500 μM cocaine treatment (14 or 24 h) of mouse oocytes [13], our flow cytometric and immunocytochemical analyses show that cocaine (10–100 μM) does not have any effect on the mitotic phase at least in our system (Figure 1D and 1E). A cDNA microarray identified *cyclin A2* as a candidate molecule related to cocaine-mediated G1/S phase arrest. Cocaine causes specific down-regulation of cyclin A2 in AF5 cells, primary neural and A2B5+ progenitor cells, and fetal brains exposed to cocaine in utero. Further, compensating for cocaine-induced cyclin A down-regulation by gene transfer counteracted the growth-suppressing action of cocaine in AF5 cells (Figure 5D).

Reversal of cocaine-induced proliferation inhibition by cyclin A transfection is not, in itself, proof that the effect of cocaine on neural progenitor cell proliferation is caused by down-regulation of cyclin A. Nevertheless, among known modulators of the G1-to-S transition, microarray analysis identified only *cyclin A2* as being down-regulated by cocaine. The cyclin A transfection results are consistent with the hypothesis that cocaine decreases proliferation via down-regulation of cyclin A. Moreover, the fact that the ROS-induced ER stress consequently promoted transcriptional down-regulation of cyclin A is further evidence that reductions in cyclin A signaling are responsible for decreased proliferation. These findings, taken together, suggest that cyclin A down-regulation constitutes at least one molecular mechanism by which cocaine causes dysfunction of neural progenitor cells.

Cocaine-induced *cyclin A2* down-regulation was observed in human neural progenitor and A2B5+ progenitor cells, but not in human neurons and microglia. In contrast to progenitor cells, the *cyclin A2* transcript was increased by cocaine in human astrocytes. Although we do not know the mechanism underlying the proliferative effect of cocaine on

astrocytes, it is worth noting that cellular stress inhibits proliferation in CNS progenitors, whereas it activates proliferation in astrocytes, leading to gliosis in the brain [42]. Cocaine thus has at least two pharmacological actions on cellular proliferation in a cell type-specific manner. Moreover, in utero cocaine exposure down-regulated cyclin A expression in progenitor cells in the VZ, but not in the progenitor cells in the SVZ. Although the reason for this specific mode of action is unknown, variations in expression and metabolic capacities of cytochrome P450s, plasma membrane permeability of cocaine, and variations in cellular stress response mechanisms could be involved. Future studies exploring the activity of cytochrome p450s and oxidative stress in different types of CNS cells will address this issue.

Oxidative ER Stress in Cocaine-Induced Cyclin A Down-Regulation

Screening of molecules that can regulate the promoter activity of *cyclin A2* demonstrated that ATF4 is specifically up-regulated by cocaine exposure; this effect of cocaine occurs 3 h prior to *cyclin A2* down-regulation. Upon ER stress such as ER oxidation, the ER sensor protein PERK phosphorylates eIF2 α , subsequently promoting translational activation of ATF4 [35,36]. ATF4 promotes not only up-regulation of genes involved in redox control (e.g., glutathione biosynthesis) [35], but also promotes down-regulation of cyclin A during G1-to-S progression, which can lead to cell cycle arrest [43]. Thus, ER oxidation is capable of causing cell cycle arrest via the eIF2 α -ATF4 pathway.

In addition to promoting dopamine auto-oxidation, cocaine can itself get through the cell membrane in its nonprotonated form, where it produces ROS via N-oxidative metabolism catalyzed by cytochrome P450 at the ER [37]. Cocaine is first N-demethylated to norcocaine [44,45], followed by oxidation to N-hydroxynorcocaine [44–47] by cytochrome P450 and flavin-containing monooxygenases. N-hydroxynorcocaine is further converted to norcocaine nitroxide by one-electron oxidation, also through cytochrome P450 activity [44,48]. Norcocaine nitroxide can be rapidly reduced to N-hydroxynorcocaine again by flavoproteins such as cytochrome P450 reductase and FAD-monoxygenase [44,48]. Redox cycling between norcocaine nitroxide and N-hydroxynorcocaine is responsible for generation of ROS such as superoxide anion and hydrogen peroxide [37,44]. In addition, depletion of NADPH, an essential cofactor for maintenance of reduced glutathione, during the futile redox cycling of N-hydroxynorcocaine/norcocaine nitroxide is able to disrupt the homeostatic effect of cellular glutathione [48], possibly also contributing to cellular oxidative stress.

Indeed, we found that cocaine induces endogenous ROS accumulation as early as 15 min after treatment of AF5 cells (Figure 6D). Further, the P450 inhibitors SKF-525A and cimetidine, which have been shown to inhibit N-oxidative metabolism of cocaine, blocked cocaine-induced endogenous ROS generation, translational activation of ATF4, down-regulation of cyclin A, and proliferation inhibition in AF5 cells (Figure 6E–6H). In contrast, lipophilic free radical scavengers and the iron chelator DFO did not block cocaine-induced inhibition of proliferation, suggesting that lipid peroxidation and iron-mediated ROS production (Fenton reaction) in mitochondria are not primarily involved in cocaine-induced ROS production in AF5 cells. We there-

fore conclude that cocaine biotransformation by microsomal cytochrome P450 is the source of cocaine-induced ROS generation, which promotes cell cycle arrest via ER stress-induced cyclin A down-regulation (Figure 6E–6H).

In cultured human neural precursor cells, cocaine-induced (1–100 μ M for 7 d) cell proliferation inhibition has been suggested to be related to increased expression of p21, a major transcriptional target of p53, as well as down-regulation of nuclear antigen (PCNA), an essential DNA replication factor [11]. Activation of the p53-p21 pathway has been implicated in hyperoxia-induced inhibition of G1/S progression, through suppression of CDK activity and subsequent repression of *cyclin A2* transcription and inactivation of PCNA [34,49]. Increased expression of p21 was not observed 24 h after exposure to cocaine in our study; however, we did detect an increase of p21 48 h after exposure to cocaine (unpublished data). These data suggest that the p53-p21 pathway may be involved in longer-term effects of cocaine, but not in the short-term action of cocaine that promotes down-regulation of cyclin A2 within 24 h.

Cocaine has been shown to inhibit protein synthesis in rat fibroblasts, associated with cocaine-induced inhibition of cell proliferation [28]. One of the cellular adaptive responses to oxidative ER stress is inhibition of translation, which may explain the inhibition of protein synthesis observed in cocaine-treated fibroblasts. Also, cocaine acts as a local anesthetic by inhibiting sodium influx into cells. An in vitro study, however, has shown that cocaine-induced proliferation inhibition in lymphocytes was not related to sodium channel blockade [50].

Effects of Cocaine in Developing Cerebral Cortex

Neural progenitor cells in ventricular proliferating zones are multipotent, having the potential to differentiate to neurons destined for different layers of the cortex. Alterations in cell cycle dynamics of neural progenitor cells have been linked to the production of various sizes of cortical areas and to the thickness of cortical layers [51,52]. In addition, disruption of neural progenitor proliferation can result in microcephaly [53], which has been reported in infants born to cocaine-abusing mothers [3]. We demonstrated here that oxidative ER stress-mediated down-regulation of cyclin A contributes to cocaine-induced inhibition of neural progenitor cell proliferation in the developing neocortex (Figure 8). Because cell cycle duration is regulated mainly via G1 phase and/or the G1-to-S transition during embryonic neurogenesis [54,55], cocaine-induced G1/S cell cycle arrest mediated by down-regulation of cyclin A may have a substantial impact on proliferation of progenitor cells, as well as on ensuing migration and differentiation (Figure 8).

Cocaine inhibited proliferation of AF5 cells in concentrations as low as 10 μ M for 24 h of exposure. As shown in Figure 4C, the concentrations of cocaine in the developing neocortex following the second IP administrations of 20 mg/kg cocaine peaked higher than 30 μ M within 30 min. A short duration of exposure of the fetal brain to cocaine may in fact be deleterious since, as shown in Figure S6, exposure of AF5 cells to 30 μ M cocaine for 30 min significantly up-regulated the expression of ATF4 at 3 h. Thus, exposure to 30 μ M cocaine for 30 min is sufficient to increase endogenous ROS to a degree that activates the ATF4 signaling pathway. Therefore, it is conceivable that exposure to cocaine for

fairly short durations could initiate the molecular mechanisms underlying cocaine-induced inhibition of neural progenitor cell proliferation.

Maternal cocaine exposure has been associated with uterine vasoconstriction [56], which could cause low oxygenation of the developing fetal brain. A number of studies indicate, however, that cocaine-induced vasoconstriction is unlikely to mediate impairments in brain development [57–59] as measured by brain structure and DNA synthesis. Also, Lidow and Rakic [60] have shown that neurotransmitter receptors expressed in neural progenitor cells, including dopaminergic, serotonergic, and adrenergic receptors, are related to cell proliferation stimulation or inhibition in the developing primate occipital lobe. Therefore, it is possible that cocaine-induced accumulation of monoamine neurotransmitters and activation of corresponding receptors also contribute to the inhibition of cell cycle progression by cocaine, especially in vivo. Moreover, in addition to ROS induced by N-oxidation of cocaine, auto-oxidation of monoamines could contribute to ATF4-mediated cyclin A down-regulation in fetal brains. Nevertheless, our data demonstrated that cytochrome P450-dependent N-oxidation of cocaine promotes cyclin A2 down-regulation by causing ER stress, which constitutes at least one mechanism underlying cocaine-induced inhibition of ventricular progenitor cell proliferation in the developing neocortex.

Possible Clinical Use of Cimetidine

We have shown that pretreatment with the P450 inhibitor cimetidine effectively abolished both the cocaine-induced inhibition of neural progenitor cell proliferation in the VZ of the developing rat brain, and cyclin A down-regulation (Figures 7A, 7D, S7A, and S7D). This effect of cimetidine appeared to be mediated by prevention of N-oxidative metabolism of cocaine and consequent ER stress, as cimetidine also prevented the cocaine-induced increase in ATF4 (Figures 7C and S7C). These effects of cimetidine in the developing rat brain were consistent with the efficacy of cimetidine in blocking effects of cocaine on the AF5 cell line in vitro.

Cimetidine belongs to a class of drugs called histamine H₂ receptor antagonists, and is often prescribed for the treatment of gastroesophageal reflux diseases and peptic ulcer diseases. Cimetidine crosses the placenta by passive diffusion [38] and has been allocated to pregnancy category B by the FDA, which means that cimetidine is not expected to be harmful to the fetus. There are conflicting reports on anti-androgenic effects of cimetidine in animals exposed in utero [61–64]; however, no anti-androgenic effects of cimetidine have so far been reported in human pregnancy.

We demonstrated that giving cimetidine (100 mg/kg IP twice at an interval of 12 h) to pregnant rats at an early period of neurogenesis did not affect proliferation (Figures 7A, 7B, S7A, and S7B), survival (Figure S8A and S8B), or density (Figure S8C and S8D) of neural progenitor cells in the VZ or SVZ. We also did not observe an increase in perinatal mortality in cimetidine-treated animals (Figure S8E). The expression of histamine H₂ receptors in the cerebral cortex is very low [65,66]; therefore, H₂ receptor activity is not likely to be involved in the ability of cimetidine to decrease cocaine-induced changes in the fetal brain.

The question naturally arises as to whether cimetidine or a

similar drug could be employed to prevent the adverse effects of cocaine on brain development. Presumably, the manner in which this would be accomplished would be that women of child-bearing age with a history of cocaine abuse, and who are at risk for subsequent cocaine abuse, would be asked to take cimetidine as a prophylactic measure. Several issues would have to be addressed before this could be done. Most importantly, the possibility that cimetidine increases the systemic toxicity of cocaine, perhaps by interfering with cocaine metabolism and lengthening the half-life of cocaine, would have to be considered. Another question to be addressed is the half-life of the preventive drug used that would be necessary to achieve a satisfactory level of patient compliance; it is possible that a drug with a very long half-life, e.g., a depot preparation, would be needed. Also, although the mechanism that is described here appears to at least contribute to the developmental toxicity of cocaine, it is not necessarily the entire cause of cocaine's adverse effects on development. Thus, the long-term efficacy of cimetidine (or alternative drugs) on brain development and function, and efficacy in a second larger species, will also need to be examined.

Limitations of This Study

During neocortical neurogenesis, the two germinal compartments VZ and SVZ are composed of heterogeneous populations of neural progenitor cells. It is difficult to isolate, define, and examine specific sub-types of neural progenitor cells derived from primary fetal cortex. It is also problematic to examine regional cellular and molecular parameters *in vivo* in tissue sections derived from embryonic rat brains at different ages following intrauterine cocaine administration. The AF5 neural progenitor cell line used in this study may not have properties identical to those of cortical VZ progenitors; however, the AF5 cell line was able to reveal the molecular mechanisms (e.g., free radical-induced ER stress), which are involved in cocaine-induced proliferation inhibition of neural progenitor cells. The AF5 cell model not only allowed us to identify these molecular mechanisms, but also was remarkably predictive of the *in vivo* findings. Nevertheless, the expression and metabolic capacities of cytochrome P450 in neural progenitor cells of developing rodent and human brains are not identical, which to some extent limits extrapolation of our findings to cocaine-exposed human fetal brains.

A second issue involves the dose of cimetidine that is required to prevent the effects of cocaine. The dose of cimetidine used to block the histamine H₂ receptor in rodents is ~2.5–10 mg/kg [67]; however, doses of cimetidine up to 100 mg/kg are used to inhibit the activity of cytochrome P450 in rodents [68,69]. We therefore used 100 mg/kg cimetidine for the present study. On a mg/kg basis, this dose is about ten times higher than the human therapeutic dose. Thus, it is not clear whether cimetidine, or any similar drug, would be effective in preventing the adverse effects of cocaine on neural progenitor cells in human cocaine abuse. It will therefore be essential to determine whether clinically relevant doses of cimetidine have the capacity to inhibit the adverse effects of exposure of the human fetus to cocaine, as well as whether there are other P450 inhibitors that are more effective.

Conclusion

Understanding the molecular mechanisms by which *in utero* cocaine exposure causes proliferation inhibition of neural progenitor cells is important for developing prevention and therapeutic strategies against long-lasting neurological and behavioral dysfunction caused by exposure of the developing fetus to cocaine. Future research might be focused on exploring the molecular and biochemical mechanisms involved in cellular functions such as differentiation and migration changed by cocaine in neural progenitor cells, determining the expression and metabolic capacity of cytochrome P450s in various subtypes of cortical and subcortical progenitor cells, and investigating whether oxidative ER stress is involved in other disorders that have been shown to be related to P450-dependent oxidative metabolism of cocaine, such as immunosuppression [70–72], hepatocyte injury [37,69,73,74], and cardiotoxicity [75].

Supporting Information

Figure S1. Cortical Regions Used for Measurements of BrdU Labeling

Coronal brain sections of E15, E17, and E19 rat fetal brains stained with cresyl violet illustrate cortical regions used for BrdU labeling measurements (one-third and two-thirds of the cerebral cortex from the superior sagittal sinus [SSS] to the caudal pole of the internal capsule, red rectangles) for cocaine treatment at the early period of neocortical neurogenesis (E13–E15), the middle period (E15–E17), and the late period of neocortical neurogenesis (E17–E19). The scale bar is 0.5 mm.

Found at doi:10.1371/journal.pmed.0050117.sg001 (4.9 MB TIF).

Figure S2. Time Course of Cocaine-Induced Proliferation Inhibition in AF5 Cells

AF5 cells were treated with 100 μ M cocaine every day during a medium change for a total of 6 d. Data are presented as percentage of control cell numbers at 0 h. Day 1: **, $p = 0.001$ compared to control; days 3 and 6: ***, $p < 0.001$ compared to each control; $n = 4$.

Found at doi:10.1371/journal.pmed.0050117.sg002 (698 KB TIF).

Figure S3. Time Course of Cocaine-Induced Down-Regulation of Cyclin A in AF5 Cells

AF5 cells were treated with 100 μ M for 6 d, and cyclin A protein levels were assessed by immunoblotting at day 1, 3, and 6.

Found at doi:10.1371/journal.pmed.0050117.sg003 (532 KB PPT).

Figure S4. Data from Figure 4 Shown in Scattergram Form

- (A) Scattergram of Figure 4D.
- (B) Scattergram of Figure 4E.
- (C) Scattergram of Figure 4G.
- (D) Scattergram of Figure 4H.

Found at doi:10.1371/journal.pmed.0050117.sg004 (78 KB PPT).

Figure S5. BrdU Incorporation in the Developing Rat Neocortex for the Middle and Late Neurogenesis Periods

The VZ and SVZ of cocaine-exposed fetuses during middle (E15–E17) and late (E17–E19) periods of neocortical neurogenesis are shown. Immunoreactivity for BrdU (red) and Ki67 (green) are shown for E17 and E19 fetal brains. Cocaine decreased the number of BrdU-labeled progenitor cells in VZ at the middle of the neurogenesis period. Images for the early period of neurogenesis are shown in Figure 4F. The scale bar is 20 μ m.

Found at doi:10.1371/journal.pmed.0050117.sg005 (530 KB PDF).

Figure S6. ATF4 Expression for AF5 Cells Treated with 30 μ M Cocaine for 30 Min

AF5 cells were treated with cocaine on the basis of the concentrations curve measured in fetal brain following cocaine injections (Figure 4C). AF5 cells were treated with 30 μ M cocaine for 30 min, and ATF4 protein level was measured by immunoblotting. The expression of ATF4 was normalized to α -tubulin. **, $p = 0.006$ compared to control, $n = 4$.

Found at doi:10.1371/journal.pmed.0050117.sg006 (1.3 MB PDF).

Figure S7. Data from Figure 7 Shown in Scattergram Form

- (A) Scattergram of Figure 7A.
 (B) Scattergram of Figure 7B.
 (C) Scattergram of Figure 7C.
 (D) Scattergram of Figure 7D.

Found at doi:10.1371/journal.pmed.0050117.sg007 (58 KB PPT).

Figure S8. Developmental Indicators Following Prenatal Administration of Cocaine and Cimetidine

Pregnant rats at the early period of neurogenesis (E13–E15) were pretreated with 100 mg/kg of cimetidine IP 1 h before receiving 20 mg/kg of cocaine using the regimen described in Figure 4A.

(A and B) Effects of cimetidine on cell death in the developing rat neocortical VZ and SVZ of cocaine-exposed fetuses. Apoptotic index [(number of condensed nuclei/number of total nuclei) × 100%] was calculated in the regions used for quantitative evaluation of the BrdU labeling described in the methods. *n* = six samples from three pregnant dams.

(C and D) Effects of cimetidine on cell density in the developing rat neocortical VZ and SVZ of cocaine-exposed fetuses. Cell density [(number of total nuclei/rectangle area with 160 μm-width) × 100%] was calculated in the regions used for quantitative evaluation of the BrdU labeling described in the methods. *n* = six samples from three pregnant dams.

(E) Effects of cimetidine on the mortality of cocaine-exposed fetuses. Perinatal mortality was calculated by (number of dead fetuses/number of total fetuses) × 100%. *n* = four to five samples from four to five pregnant dams. None of the differences were statistically significant.

Found at doi:10.1371/journal.pmed.0050117.sg008 (937 KB TIF).

Table S1. Comparison of *p*-Values Obtained by *t*-Tests and Mann-Whitney U Tests

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Author contributions. CTL, TH, and WJF conceived and designed the study. CTL, JC, SYT, and JFS performed the experiments involving cell proliferation and cytotoxicity assays, transfection, immunocytochemistry, and in vivo experiments. CTL and JC performed the quantitative real-time RT-PCR. SLE and RA performed the Western blot analysis and helped with in vivo experiments. JS prepared the human primary CNS cells. KGB performed the microarray analysis. CTL, TH, and WJF interpreted the data. RHL and MAH performed the measurements of cocaine concentrations in fetal brain. CTL and WJF drafted the manuscript. CTL, TH, TPS, HMG, and WJF performed critical revision of the manuscript.

References

- Substance Abuse and Mental Health Services Administration (1997) National Household Survey on Drug Abuse: Main Findings 1995. Washington (D.C.): US Government Printing Office. Department of Health and Human Services Publication Number SMS: 95–3127.
- Chiriboga CA, Brust JC, Bateman D, Hauser WA (1999) Dose-response effect of fetal cocaine exposure on newborn neurologic function. *Pediatrics* 103: 79–85.
- Bellini C, Massocco D, Serra G (2000) Prenatal cocaine exposure and the expanding spectrum of brain malformations. *Arch Intern Med* 160: 2393.
- Lewis BA, Singer LT, Short EJ, Minnes S, Arendt R, et al. (2004) Four-year language outcomes of children exposed to cocaine in utero. *Neurotoxicol Teratol* 26: 617–627.
- Linares TJ, Singer LT, Kirchner HL, Short EJ, Min MO, et al. (2006) Mental health outcomes of cocaine-exposed children at 6 years of age. *J Pediatr Psychol* 31: 85–97.
- He N, Bai J, Champoux M, Suomi SJ, Lidow MS (2004) Neurobehavioral deficits in neonatal rhesus monkeys exposed to cocaine in utero. *Neurotoxicol Teratol* 26: 13–21.
- Lidow MS (1995) Prenatal cocaine exposure adversely affects development of the primate cerebral cortex. *Synapse* 21: 332–341.

- Lidow MS, Song ZM (2001) Primates exposed to cocaine in utero display reduced density and number of cerebral cortical neurons. *J Comp Neurol* 435: 263–275.
- Lidow MS, Bozian D, Song ZM (2001) Cocaine affects cerebral neocortical cytoarchitecture in primates only if administered during neocortical neurogenesis. *Brain Res Dev Brain Res* 128: 45–52.
- Poon HF, Abdullah L, Mullan MA, Mullan MJ, Crawford FC (2007) Cocaine-induced oxidative stress precedes cell death in human neuronal progenitor cells. *Neurochem Int* 50: 69–73.
- Hu S, Cheeran MC, Sheng WS, Ni HT, Lokensgard JR, et al. (2006) Cocaine alters proliferation, migration, and differentiation of human fetal brain-derived neural precursor cells. *J Pharmacol Exp Ther* 318: 1280–1286.
- Yu RC, Lee TC, Wang TC, Li JH (1999) Genetic toxicity of cocaine. *Carcinogenesis* 20: 1193–1199.
- Combelles CM, Carabatsos MJ, London SN, Mailhes JB, Albertini DF (2000) Centrosome-specific perturbations during in vitro maturation of mouse oocytes exposed to cocaine. *Exp Cell Res* 260: 116–126.
- Schenker S, Yang Y, Johnson RF, Downing JW, Schenken RS, et al. (1993) The transfer of cocaine and its metabolites across the term human placenta. *Clin Pharmacol Ther* 53: 329–339.
- Wiggins RC, Rolsten C, Ruiz B, Davis CM (1989) Pharmacokinetics of cocaine: basic studies of route, dosage, pregnancy and lactation. *Neurotoxicology* 10: 367–381.
- Van Dyke C, Barash PG, Jatlow P, Byck R (1976) Cocaine: plasma concentrations after intranasal application in man. *Science* 191: 859–861.
- Stephens BG, Jentzen JM, Karch S, Mash DC, Wetli CV (2004) Criteria for the interpretation of cocaine levels in human biological samples and their relation to the cause of death. *Am J Forensic Med Pathol* 25: 1–10.
- Kalasinsky KS, Bosy TZ, Schmunk GA, Ang L, Adams V, et al. (2000) Regional distribution of cocaine in postmortem brain of chronic human cocaine users. *J Forensic Sci* 45: 1041–1048.
- Truckenmiller ME, Vawter MP, Zhang P, Conejero-Goldberg C, Dillon-Carter O, et al. (2002) AF5, a CNS cell line immortalized with an n-terminal fragment of SV40 large T: growth, differentiation, genetic stability and gene expression. *Exp Neurol* 175: 318–337.
- Sanchez JF, Crooks DR, Lee CT, Schoen CJ, Amable R, et al. (2006) GABAergic lineage differentiation of AF5 neural progenitor cells in vitro. *Cell Tissue Res* 324: 1–8.
- McNeill-Blue C, Wetmore BA, Sanchez JF, Freed WJ, Merrick BA (2006) Apoptosis mediated by p53 in rat neural AF5 cells following treatment with hydrogen peroxide and staurosporine. *Brain Res* 1112: 1–15.
- Takahashi T, Nowakowski RS, Caviness VS Jr. (1995) Early ontogeny of the secondary proliferative population of the embryonic murine cerebral wall. *J Neurosci* 15: 6058–6068.
- Tanaka TS, Jaradat SA, Lim MK, Kargul GJ, Wang X, et al. (2000) Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. *Proc Natl Acad Sci USA* 97: 9127–9132.
- Cheadle C, Vawter MP, Freed WJ, Becker KG (2003) Analysis of microarray data using Z score transformation. *J Mol Diagn* 5: 73–81.
- Lowe RH, Barnes AJ, Lehrmann E, Freed WJ, Kleinman JE, et al. (2006) A validated positive chemical ionization GC/MS method for the identification and quantification of amphetamine, opiates, cocaine, and metabolites in human postmortem brain. *J Mass Spectrom* 41: 175–184.
- Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SL, et al. (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* 70: 993–1006.
- Zachor D, Cherkas JK, Fay CT, Ocran I (1994) Cocaine differentially inhibits neuronal differentiation and proliferation in vitro. *J Clin Invest* 93: 1179–1185.
- Francesco P, Pica F, Favalli C, Tubaro E, Garaci E (1990) Inhibition of rat fibroblast cell proliferation at specific cell cycle stages by cocaine. *Cell Biol Int Rep* 14: 549–558.
- von Waechter R, Jaensch B. (1972) Generation times of the matrix cells during embryonic brain development: an autoradiographic study in rats. *Brain Res* 46: 235–250.
- Chenn A, Walsh CA (2002) Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* 297: 365–369.
- Jeffrey PD, Russo AA, Polyak K, Gibbs E, Hurwitz J, et al. (1995) Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature* 376: 313–320.
- Desdouets C, Matesic G, Molina CA, Foulkes NS, Sassone-Corsi P, et al. (1995) Cell cycle regulation of cyclin A gene expression by the cyclic AMP-responsive transcription factors CREB and CREM. *Mol Cell Biol* 15: 3301–3309.
- Chen D, Krasinski K, Sylvester A, Chen J, Nisen PD, et al. (1997) Downregulation of cyclin-dependent kinase 2 activity and cyclin A promoter activity in vascular smooth muscle cells by p27(KIP1), an inhibitor of neointima formation in the rat carotid artery. *J Clin Invest* 99: 2334–2341.
- Spitkovsky D, Schulze A, Boye B, Jansen-Durr P (1997) Down-regulation of cyclin A gene expression upon genotoxic stress correlates with reduced binding of free E2F to the promoter. *Cell Growth Differ* 8: 699–710.
- Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, et al. (2003) An integrated

- stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* 11: 619–633.
36. Rutkowski DT, Kaufman RJ (2004) A trip to the ER: coping with stress. *Trends Cell Biol* 14: 20–28.
 37. Boelsterli UA, Goldlin C (1991) Biomechanisms of cocaine-induced hepatocyte injury mediated by the formation of reactive metabolites. *Arch Toxicol* 65: 351–360.
 38. Schenker S, Dicke J, Johnson RF, Mor LL, Henderson GI (1987) Human placental transport of cimetidine. *J Clin Invest* 80: 1428–1434.
 39. Howe JP, McGowan WA, Moore J, McCaughey W, Dundee JW (1981) The placental transfer of cimetidine. *Anaesthesia* 36: 371–375.
 40. Ching MS, Mihaly GW, Morgan DJ, Date NM, Hardy KJ, et al. (1987) Low clearance of cimetidine across the human placenta. *J Pharmacol Exp Ther* 241: 1006–1009.
 41. Nassogne MC, Evrard P, Courtoy PJ (1995) Selective neuronal toxicity of cocaine in embryonic mouse brain cocultures. *Proc Natl Acad Sci USA* 92: 11029–11033.
 42. Fattore L, Puddu MC, Picciau S, Cappai A, Fratta W, et al. (2002) Astroglial in vivo response to cocaine in mouse dentate gyrus: a quantitative and qualitative analysis by confocal microscopy. *Neuroscience* 110: 1–6.
 43. Shimizu M, Nomura Y, Suzuki H, Ichikawa E, Takeuchi A, et al. (1998) Activation of the rat cyclin A promoter by ATF2 and Jun family members and its suppression by ATF4. *Exp Cell Res* 239: 93–103.
 44. Kloss MW, Rosen GM, Rauckman EJ (1984) Biotransformation of norcocaine to norcocaine nitroxide by rat brain microsomes. *Psychopharmacology (Berl)* 84: 221–224.
 45. Toennes SW, Thiel M, Walther M, Kauert GF (2003) Studies on metabolic pathways of cocaine and its metabolites using microsome preparations from rat organs. *Chem Res Toxicol* 16: 375–381.
 46. Shuster L, Casey E, Welankiar SS (1983) Metabolism of cocaine and norcocaine to N-hydroxynorcocaine. *Biochem Pharmacol* 32: 3045–3051.
 47. Benuck M, Reith ME, Lajtha A (1988) Presence of the toxic metabolite N-hydroxy-norcocaine in brain and liver of the mouse. *Biochem Pharmacol* 37: 1169–1172.
 48. Rauckman EJ, Rosen GM, Cavagnaro J (1982) Norcocaine nitroxide. A potential hepatotoxic metabolite of cocaine. *Mol Pharmacol* 21: 458–463.
 49. Helt CE, Stavensky RJ, Lee YJ, Bambara RA, Keng PC, et al. (2004) The Cdk and PCNA domains on p21Cip1 both function to inhibit G1/S progression during hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 286: L506–L513.
 50. Berkeley MB, Daussin S, Hernandez MC, Bayer BM (1994) In vitro effects of cocaine, lidocaine and monoamine uptake inhibitors on lymphocyte proliferative responses. *Immunopharmacol Immunotoxicol* 16: 165–178.
 51. Dehay C, Giroud P, Berland M, Smart I, Kennedy H (1993) Modulation of the cell cycle contributes to the parcellation of the primate visual cortex. *Nature* 366: 464–466.
 52. Polleux F, Dehay C, Moraillon B, Kennedy H (1997) Regulation of neuroblast cell-cycle kinetics plays a crucial role in the generation of unique features of neocortical areas. *J Neurosci* 17: 7763–7783.
 53. Sheen VL, Ganesh VS, Topcu M, Sebire G, Bodell A, et al. (2004) Mutations in ARFGEF2 implicate vesicle trafficking in neural progenitor proliferation and migration in the human cerebral cortex. *Nat Genet* 36: 69–76.
 54. Miyama S, Takahashi T, Nowakowski RS, Caviness VS Jr. (1997) A gradient in the duration of the G1 phase in the murine neocortical proliferative epithelium. *Cereb Cortex* 7: 678–689.
 55. Dehay C, Savatier P, Cortay V, Kennedy H (2001) Cell-cycle kinetics of neocortical precursors are influenced by embryonic thalamic axons. *J Neurosci* 21: 201–214.
 56. Fantel AG, Person RE, Burroughs-Gleim CJ, Mackler B (1990) Direct embryotoxicity of cocaine in rats: effects on mitochondrial activity, cardiac function, and growth and development in vitro. *Teratology* 42: 35–43.
 57. Wilkins AS, Marota JJ, Tabit E, Kosofsky BE (1998) Transplacental cocaine exposure. 3: Mechanisms underlying altered brain development. *Neurotoxicol Teratol* 20: 239–249.
 58. Anderson-Brown T, Slotkin TA, Seidler FJ (1990) Cocaine acutely inhibits DNA synthesis in developing rat brain regions: Evidence for direct actions. *Brain Res* 537: 197–202.
 59. He N, Lidow MS (2004) Cerebral cortical abnormalities seen in a non-human primate model of prenatal cocaine exposure are not related to vasoconstriction. *Neurotoxicology* 25: 419–432.
 60. Lidow MS, Rakic P (1995) Neurotransmitter receptors in the proliferative zones of the developing primate occipital lobe. *J Comp Neurol* 360: 393–402.
 61. Hoie EB, Swigart SA, Nelson RM, Leuschen MP (1994) Development of secondary sex characteristics in male rats after fetal and perinatal cimetidine exposure. *J Pharm Sci* 83: 107–109.
 62. Walker TF, Bott JH, Bond BC (1987) Cimetidine does not demasculinize male rat offspring exposed in utero. *Fundam Appl Toxicol* 8: 188–197.
 63. Parker S, Udani M, Gavalier JS, Van Thiel DH (1984) Pre- and neonatal exposure to cimetidine but not ranitidine adversely affects adult sexual functioning of male rats. *Neurobehav Toxicol Teratol* 6: 313–318.
 64. Takeshi S, Kai H, Suita S (2002) Effects of the prenatal administration of cimetidine on testicular descent and genital differentiation in rats. *Surgery* 131: S301–S305.
 65. Ruat M, Traiffort E, Arrang JM, Leurs R, Schwartz JC (1991) Cloning and tissue expression of a rat histamine H2-receptor gene. *Biochem Biophys Res Commun* 179: 1470–1478.
 66. Honrubia MA, Vilaró MT, Palacios JM, Mengod G (2000) Distribution of the histamine H(2) receptor in monkey brain and its mRNA localization in monkey and human brain. *Synapse* 38: 343–354.
 67. Wong CL (1995) Effects of a histamine H2 receptor agonist and antagonist on restraint-induced antinociception in female mice. *Eur J Pharmacol* 279: 109–113.
 68. Ndikum-Moffor FM, Schoeb TR, Roberts SM (1998) Liver toxicity from norcocaine nitroxide, an N-oxidative metabolite of cocaine. *J Pharmacol Exp Ther* 284: 413–419.
 69. Peterson FJ, Knodell RG, Lindemann NJ, Steele NM (1983) Prevention of acetaminophen and cocaine hepatotoxicity in mice by cimetidine treatment. *Gastroenterology* 85: 122–129.
 70. Pacifici R, Fiaschi AI, Micheli L, Centini F, Giorgi G, et al. (2003) Immunosuppression and oxidative stress induced by acute and chronic exposure to cocaine in rat. *Int Immunopharmacol* 3: 581–592.
 71. Jeong TC, Jordan SD, Matulka RA, Stanulis ED, Kaminski EJ, et al. (1995) Role of metabolism by esterase and cytochrome P-450 in cocaine-induced suppression of the antibody response. *J Pharmacol Exp Ther* 272: 407–416.
 72. Jeong TC, Jordan SD, Matulka RA, Stanulis ED, Park SS, et al. (1996) Immunosuppression induced by acute exposure to cocaine is dependent on metabolism by cytochrome P-450. *J Pharmacol Exp Ther* 276: 1257–1265.
 73. Bouis P, Boelsterli UA (1990) Modulation of cocaine metabolism in primary rat hepatocyte cultures: effects on irreversible binding and protein biosynthesis. *Toxicol Appl Pharmacol* 104: 429–439.
 74. LeDuc BW, Sinclair PR, Walton HS, Sinclair JF, Greenblatt DJ, et al. (1994) Cocaine toxicity in cultured chicken hepatocytes: role of cytochrome P450. *Toxicol Appl Pharmacol* 125: 322–332.
 75. Wang JF, Yang Y, Sullivan MF, Min J, Cai J, et al. (2002) Induction of cardiac cytochrome p450 in cocaine-treated mice. *Exp Biol Med* 227: 182–188.
 76. Paxinos G, Ashwell K, Tork I (1994) Atlas of the developing rat nervous system. San Diego: Academic Press.

Editors' Summary

Background. Every year, cocaine abuse by mothers during pregnancy exposes thousands of unborn infants (fetuses) to this powerful and addictive stimulant. Maternal cocaine abuse during early pregnancy increases the risk of miscarriage; its use during late pregnancy slows the baby's growth and can trigger premature labor. Babies exposed to cocaine shortly before birth are often irritable and have disturbed sleep patterns. They can also be very sensitive to sound and touch and consequently hard to comfort. These problems usually resolve spontaneously within the first few weeks of life but some permanent birth defects are also associated with frequent cocaine abuse during pregnancy. In particular, babies exposed to cocaine before birth sometimes have small heads—an abnormality that generally indicates a small brain—and, although they usually have normal intelligence, the development of their thinking skills and language is often delayed, and they can have behavioral problems.

Why Was This Study Done? Exposure to cocaine before birth clearly interferes with some aspects of brain development. More specifically, it reduces the number and position of neurons (the cells that transmit information in the form of electrical impulses around the body) within the brain. All neurons develop from neural progenitor cells, and previous research suggests that cocaine exposure before birth inhibits the proliferation of these cells in the developing brain. It would be useful to understand exactly how cocaine affects neural progenitor cells, because it might then be possible to prevent the drug's adverse effects on brain development. In this study, therefore, the researchers investigate the molecular mechanism that underlies cocaine's effect on neural progenitor cells.

What Did the Researchers Do and Find? When the researchers investigated the effects of cocaine on AF5 cells (rat neural progenitor cells that grow indefinitely in the laboratory), they found that concentrations of cocaine similar to those measured in fetal brains after maternal drug exposure inhibited the proliferation of AF5 cells by blocking the "G1-to-S transition." This is a stage that cells have to pass through between each round of cell division (the production of two daughter cells from one parent cell). Next, the researchers showed that cocaine-treated AF5 cells made much less cyclin A2, a protein that controls the G1-to-S transition, than untreated cells. Cocaine also decreased cyclin A2 levels in neural progenitor cells freshly isolated from human fetal brains and in fetal rat brains exposed to the drug while in their mother's womb. Treatment of AF5 cells with a cyclin A2 expression vector (a piece of DNA that directs the production of cyclin A2) counteracted the down-regulation of cyclin A2 and restored AF5 proliferation in the presence of cocaine. Other experiments indicate that

the reduction of cyclin A2 by cocaine in AF5 cells involves the accumulation of "reactive oxygen species," by-products of the breakdown of cocaine by a protein that is a member of a family of proteins called cytochrome P450. Finally, treatment of pregnant rats with cimetidine (which inhibits the action of cytochrome P450) counteracted both the inhibition of neural progenitor cell proliferation and the cyclin A2 down-regulation that cocaine exposure induced in the brains of their unborn pups.

What Do These Findings Mean? These findings show that the cocaine-induced inhibition of neural progenitor cell proliferation involves, at least in part, interfering with the production (that is, causing down-regulation) of cyclin A2. They also show that this down-regulation is induced by the breakdown of cocaine by cytochrome P450, and that in both a rat cell line and in fetal rats, the cytochrome P450 inhibitor cimetidine (a drug that is already used clinically for stomach problems) can block the adverse effects of cocaine on the proliferation of neural progenitor cells. These findings need to be confirmed in animals more closely related to people than rats, and the long-term effects of cimetidine need to be investigated, in particular its effects on cocaine toxicity. Nevertheless these results raise the possibility that giving cimetidine or other drugs with similar effects to pregnant women who are addicted to cocaine might prevent some of the harm that their drug habit does to their unborn children, although it is not clear whether there is a dosage of cimetidine that might be both safe and adequate for this purpose.

Additional Information. Please access these Web sites via the online version of this summary at <http://dx.doi.org/10.1371/journal.pmed.0050117>.

- A *PLoS Medicine* Perspective article by Steven Hyman further discusses this study
- The US National Institute on Drug Abuse provides a fact sheet on cocaine (in English and Spanish)
- The UK charity Release provides information and advice to the public and professionals about the law and drugs, including information about cocaine
- MedlinePlus also provides a list of links to information about cocaine (in English and Spanish)
- The March of Dimes Foundation, a US nonprofit organization for the improvement of child health, provides information about illicit drug use during pregnancy (in English and Spanish)
- The Organization of Teratology Information Specialists also provides a fact sheet on cocaine and pregnancy (in English, Spanish, and French)