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Cocaine Alters Proliferation, Migration, and Differentiation of Human Fetal Brain-Derived Neural Precursor Cells

Shuxian Hu, Maxim C.-J. Cheeran, Wen S. Sheng, Hsiao T. Ni, James R. Lokensgard, and Phillip K. Peterson

Neuroimmunology Laboratory, Center for Infectious Diseases and Microbiology Translational Research, University of Minnesota Medical School, Minneapolis, Minnesota (S.H., M.C.-J.C., W.S.S., J.R.L., P.K.P.); and Stem Cell Department, R&D Systems, Inc., Minneapolis, Minnesota (H.T.N.)

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Corresponding author: Dr. Phillip K. Peterson, Center for Infectious Diseases and Microbiology Translational Research, 3-216 LRB/MTRF, 2001 6th Street SE., University of Minnesota Medical School, Minneapolis, MN 55455

Tel: 612-626-9923; fax: 612-626-9924; email: peter137@umn.edu

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CXCR, CXC chemokine receptor

CXCL, CXC chemokine ligand

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Abstract

Maternal use of cocaine during pregnancy is associated with sustained morphological brain abnormalities and sustained cognitive deficits in the offspring. Here, we use a cell culture model of highly enriched human fetal brain-derived neural precursor cells (NPCs) to assess the effects of cocaine treatment on their proliferation, migration, and differentiation. Our data show that cocaine treatment markedly inhibited the proliferation of NPCs, a phenomenon which was associated with cell cycle arrest, possibly due to increased expression of the cyclin-dependent kinase inhibitor p21. In addition, treatment of NPCs with cocaine inhibited their migratory response to CXCL12 (stromal cell-derived factor-1, SDF-1 α), a finding that correlated with cocaine-induced downregulation of CXCR4 on NPCs. Finally, these data demonstrated that NPCs exposed to cocaine underwent differentiation into cells expressing neuronal markers, which was associated with an inhibition of SOX2, a transcription factor that inhibits NPC differentiation. Taken together, these results point to several cellular mechanisms whereby exposure of human NSCs to cocaine in utero could contribute to subsequent neurodevelopmental and neurocognitive deficits.

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Introduction

The impact of in utero exposure to psychostimulants on neural development has been the subject of growing concern and research interest. In the case of cocaine, recent evidence indicates that maternal use of cocaine during pregnancy is associated with significant impairment of cognitive development (Lester et al., 2002; Singer et al., 2002). It appears that cocaine-induced cognitive abnormalities which are detectable during the first 2 years of life continue to contribute to learning difficulties at school age (Singer et al., 2002). Although the mechanisms responsible for cocaine's effect on the developing human brain are unknown, administration of cocaine to pregnant animals is associated with permanent morphological abnormalities of the brain and sustained cognitive deficits in the offspring (Lidow and Song, 2001; Harvey, 2004).

During the past several years, increased attention has been focused on the biological role of neural stem cells (NSCs) in brain development (McKay, 1997; Piper et al., 2000; Uchida et al., 2000; Sommer and Rao, 2002) as well as during neurogenesis in the adult brain (Gage et al., 1998; Kempermann, 2002; Nunes et al., 2003). Based on their functional properties of self-renewal (i.e., sustained proliferative capacity), motility (i.e., migration throughout the brain), and multipotency (i.e., ability to differentiate into glial cells or neurons), NSCs have been considered as a potential therapeutic modality for brain repair (Cao et al., 2002; Peterson, 2002; Hallbergson et al., 2003). Also, NSC cultures have been increasingly used as models of neurodegenerative disorders (Jakel et al., 2004) and as pharmacological tools for evaluating the neurotoxic and therapeutic potential of drugs (Conti et al., 2003). Thus far, however, relatively few psychoactive agents have been evaluated in these models and little or nothing is known about the

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effects of cocaine on NSCs. In the present study, a cell culture model of highly enriched human fetal brain-derived neural precursor cells (NPCs) (Ni et al, 2004) which share the functional properties of NSCs, was used to assess the effects of cocaine on these neural progenitors.

Materials and Methods

Reagents. All reagents were obtained from the sources indicated: glucose, glutamine, poly-D-lysine, cocaine hydrochloride, penicillin/streptomycin, paraformaldehyde and trypsin (Sigma, St. Louis, MO); (-)-cocaine-base, (+)-cocaine-base (National Institute on Drug Abuse, Bethesda, MD); DMEM/F-12 medium and gentamicin (Invitrogen, Carlsbad, CA); human fibroblast growth factor-basic (hFGFb), human epidermal growth factor (hEGF), N2 plus supplement, CXCL12/SDF-1 α , anti-human nestin, anti-human Tuj1, anti-human CXCR4 and anti-human SOX2 antibodies (R&D Systems, Minneapolis, MN); fetal bovine serum (FBS, Hyclone, Logan, UT); ³H-thymidine (GE Healthcare, Piscataway, NJ); 5-bromo-2'-deoxyuridine (BrdU) and anti-BrdU antibody (Roche, Indianapolis, IN); anti-human glial fibrillary acidic protein (GFAP) antibody (Sternberger Monoclonals, Lutherville, MD); anti-PCNA antibody (BD Biosciences, San Diego, CA); 4'-6-diamidino-2-phenylindole (DAPI) (Intergen, Purchase, NY) and gp120 (Protein Sciences, Meriden, CT).

NPC cultures. NPC cultures were prepared from 7- to-9-week-old human fetal brain as previously described (Ni et al, 2004). Human fetal brain tissues obtained under a protocol approved by our Human Subjects Research Committee were mechanically dissociated,

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resuspended in DMEM/F12 media (containing 8 mM glucose and glutamine, N2 plus supplement, penicillin and streptomycin, and 20 ng/ml human hFGFb/20 ng/ml hEGF) and plated onto poly-D-lysine coated 10-cm tissue culture dishes or 24-well plates when indicated. This stage is considered as passage 0. When cell cultures reached 50-60% confluence, they were subcultured using trypsin removal (0.0125%) at a density of 2×10^5 cells per 10-cm culture dish and considered as passage 1. Medium was replaced every other day. NPC cultures at passages 1-3 were used throughout the study. These NPCs express the neural stem cell markers nestin (>90% positive) and CD133 (>80% positive) and are glial fibrillary acidic protein (GFAP, a marker for astrocytes)- and microtubule-associated protein 2 (a neuronal cell marker)-negative (Ni. et al, 2004).

NPC proliferation assay. Cocaine (10^{-10} to 10^{-4} M) was added one day after plating NPCs onto 24-well culture plates or 4-well chamber slides (1×10^2 cells per well) and was re-added every other day during culture media replacement for a total of 7 days. Either ^3H -thymidine was added to the 24-well plates (1 μCi per well) and incubated overnight before being harvested and measured for ^3H -thymidine incorporation in a beta-counter, or BrdU (10 $\mu\text{g}/\text{ml}$) was added to NPC chamber slides overnight before being fixed and stained with anti-BrdU antibody.

NPC differentiation assay. Two experimental paradigms were used: **A)** One day after plating NPCs onto 4-chamber slides (1×10^2 cells per well), NPC cultures were treated with cocaine (10^{-8} , 10^{-6} and 10^{-4} M) for 7 days with media and cocaine replacement every other day. NPC culture slides were fixed with 4% paraformaldehyde and stained for

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nestin, Tuj1 (a neuronal marker) and GFAP. **B)** Seven days after plating NPCs onto 10-cm culture dishes (2×10^5 cells per dish) with media replacement every other day, NPC (50-60% confluence) containing growth factors (FGFb and EGF), were subjected to cocaine treatment (10^{-8} , 10^{-6} and 10^{-4} M) for 7 days. At the designated time point, NPCs were collected for flow cytometric analysis for differentiation into neuronal and astrocytic cell populations.

NPC migration assay. NPCs were added to upper chambers of a 96-well chemotaxis device (Neuro Probe Inc., Gaithersburg, MD) (10^6 cells/well) separated from the lower chambers with an 8 μ m-pore size of polyvinylpyrrolidone-free polycarbonate filter. The lower chambers were filled with chemoattractants. After 6 h of incubation, NPCs that had migrated from upper chambers into lower chambers were quantified by Diff-Quik staining (Dade Diagnostics, Aguada, PR). To determine the effect of blockade of CXCR4, NPCs were treated with anti-CXCR4 antibody (10 μ g/mL) or human immunodeficiency virus type 1 (HIV-1) gp120 (10^{-9} and 10^{-8} M) for 30 min before being used in chemotaxis assay towards CXCL12/SDF-1 α .

Immunocytochemical staining. Anti-nestin, anti-Tuj1 and anti-BrdU antibodies were used at the concentration of 10 μ g/ml on fixed cells. Anti-GFAP antibody was used at 1:200 dilution. Cells were fixed with 4% paraformaldehyde and 0.15% picric acid in PBS at room temperature for 20 min and were then permeated and blocked with 0.1% triton X-100, 1% BSA and 10% normal donkey serum in PBS at room temperature for 45 min. After blocking, cells were incubated with diluted primary antibody overnight at 4 $^{\circ}$ C and

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sequentially with fluorescence-coupled anti-mouse IgG Ab (Jackson Laboratory, Bar Harbor, Maine) at room temperature in the dark for 1 h. Cells were washed between each step with 0.1% BSA in PBS.

Flow cytometry. Staining of cells with mouse anti-human SOX2-phycoerythrin and anti-human CXCR4 receptor-phycoerythrin antibodies was performed according to the manufacturer's suggested procedures. For nestin staining, cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min and washed twice with PBS. After washing, cells were resuspended in SAP buffer (2% FCS, 0.5% saponin, and 0.1% sodium azide in PBS), and mouse anti-human nestin or mouse IgG₁ isotype control antibody (R&D Systems) was added at the final concentration of 10 µg/ml to 2.5 x 10⁵ cells in a total reaction volume of 200 µl. Following incubation for 20 min at room temperature, excess nestin or isotype control antibody was removed by washing with SAP buffer, and cells were resuspended in 200 µl of SAP buffer with 1 µg of goat anti-mouse IgG-FITC (Caltag, San Francisco, CA). The samples were incubated for 20 min in the dark at room temperature, washed once each with SAP buffer and PBS, resuspended in 400 µl of PBS and analyzed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). Five thousand events were collected and analyzed using CELL Quest software.

Cell death detection ELISA. To measure apoptosis, cell lysates from culture medium- or cocaine-treated NPC cultures in 24-well plates were collected for histone-associated DNA fragmentation measurement according to the manufacturer's protocol. Briefly, cell

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lysates were added to the streptavidin-coated 96-well ELISA plates together with anti-histone biotinylated and anti-DNA-peroxidase antibodies. After incubation and washing, DNA fragments were captured and detected by a chromogenic enzyme-substrate reaction.

Statistical analysis. Data are expressed as mean \pm SEM. For comparison of means of multiple groups, ANOVA was used, followed by Scheffe's test.

Results

Effect of Cocaine on NPC Proliferation. Like NSCs, one of the salient characteristics of the NPCs used in this study is their capacity for self-renewal. To study the effect of cocaine on this functional property, NPCs were treated with varying concentrations of the cocaine hydrochloride, and cell proliferation was assessed after 7 days of culture. In these experiments, cocaine was found to inhibit the proliferation of NPCs in a dose-dependent manner, and significant inhibitory effects were observed at concentrations $\geq 10^{-6}$ M, as assessed by ^3H -thymidine uptake (Fig. 1A). As the pharmacological action of the naturally occurring enantiomer, (-)-cocaine, is thought to involve inhibition of biogenic amine transporters, a process that is not observed with (+)-cocaine, we compared the effects of the two enantiomers on NPC proliferation. As is shown in Fig. 1B, the unnatural enantiomer had no effect on the proliferative activity of NPCs. Next, the ^3H -thymidine uptake assay was used to evaluate a time-course of cocaine-induced inhibition of NPC proliferation. Within 3 days of exposure to cocaine (10^{-6} M), NPC proliferative activity was found to be significantly impaired, and this inhibitory effect was even more pronounced at the end of the 7 day assay period (Fig. 1C). When examined using

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immunochemistry, a marked decrease in the number of proliferative cells was observed following cocaine treatment (10^{-6} M), as judged by their ability to incorporate bromodeoxyuridine (BrdU) (Fig. 1D).

To determine whether the inhibition of NPC proliferation by cocaine was related to induction of cell death, apoptosis of the treated NPCs was quantified after 7 days of culture in the absence (control) or presence of cocaine at doses ranging from 10^{-12} to 10^{-4} M. As human NPCs are known to express the chemokine coreceptor CXCR4 (Ni et al., 2004), which is a binding site for HIV-1 gp120, we included gp120 in this experiment as a positive control. These experiments showed that in marked contrast to gp120 (10 nM), which induced significant apoptosis, cocaine treatment itself did not affect NPC survival, as assessed using an ELISA for histone-associated DNA fragments (Fig. 2A). Even though cocaine by itself had no effect on cell survival, when it was added along with varying concentrations of gp120, it potentiated gp120-induced NPC apoptosis (Fig. 2B).

To investigate whether cocaine arrested progression of NPCs through the cell cycle, the cultures were assessed for PCNA (proliferating cell nuclear antigen), a cell cycle gene that plays a key role in entry of cells into S phase. Treatment of NPCs with cocaine (10^{-6} M) demonstrated marked downregulation of the expression of this gene product, as determined by immunocytochemical staining. This cocaine-induced downregulation was quantified by counting PCNA-positive cells microscopically (Fig. 3A). These data suggest that cocaine-mediated inhibition of NPC proliferation is associated with retarded entry from the G1 into the S phase of the cell cycle. We went on to examine the effect of cocaine treatment of NPCs on expression of the cyclin-dependent kinase inhibitor p21, as the induction of p21 is known to lead to cell cycle arrest.

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Treatment of NPCs with cocaine (10^{-10} M or 10^{-4} M) robustly stimulated the expression of p21 (Fig. 3B). Thus, these results show that cocaine's effect on the proliferation of NPCs is correlated with increased expression of p21.

Effect of Cocaine on NPC Migratory Activity. Another defining feature of NSCs is their ability to migrate to areas distal to the ventricular and subventricular zones where they originate. Although the processes that govern NSC migration within the nervous system are incompletely understood, CXCL12 recently has been shown to regulate migration of sensory neural progenitors in the mouse embryo (Belmadani et al., 2005), and human NPCs are known to migrate towards this CXCR4 ligand in vitro (Ni et al., 2004). Thus, we next studied the effect of treatment of NPCs with cocaine on their migratory response to CXCL12. As is shown in Fig. 4A, cocaine inhibited the migratory activity of NPCs in a concentration-dependent manner. Because this migratory response involves CXCR4, which is expressed on a large majority of NPCs (Ni et al., 2004), we used flow cytometry to test the hypothesis that cocaine treatment would be associated with downregulation of CXCR4 expression, as a potential explanation of this inhibitory effect of cocaine. In support of this hypothesis, cocaine (10^{-6} M) downregulated the expression of CXCR4 on treated NPCs (Fig. 4B). While 96% of untreated (control) NPCs expressed CXCR4, only 68% of cocaine-treated cells expressed this chemokine receptor. Also, the intensity of fluorescence was diminished by cocaine treatment indicating that, in addition to fewer cells displaying CXCR4, the total number of receptors was reduced following exposure to cocaine.

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Effect of Cocaine on NPC Differentiation. Human NPCs that are maintained in culture medium containing the growth factors hFGFb and hEGF do not express markers indicative of differentiation into neurons or glia (Ni et al., 2004). To determine whether exposure of NPCs to cocaine affects the expression of cellular differentiation markers, cocaine was added to the growth factor-containing medium, and the cells were examined by immunocytochemistry for the presence of Tuj1 and GFAP. After 7 days of culture in medium containing cocaine (10^{-6} M), the morphology of NPCs differed from that of untreated cells, and the cocaine-treated NPC cultures harbored a considerable number of cells that expressed Tuj1 (Fig. 5A), but few which expressed GFAP (Fig. 5B). These data demonstrate that cocaine treatment induced cellular differentiation of the NPCs into cells possessing a neuronal, but not an astrocytic phenotype.

Based upon the key role of the transcription factor SOX2 in inhibiting NPC differentiation (Graham et al., 2003), we hypothesized that exposure of these cells to cocaine would result in downregulation of the constitutive expression of SOX2 as a potential mechanism whereby cocaine induces the differentiation of NPCs into neurons. To test this hypothesis, NPCs were both left untreated or treated with cocaine and SOX2 expression was assessed using FACS analysis. While untreated (control) NPCs expressed SOX2 (Figure 6A), treatment with cocaine (10^{-6} M) for 3 days completely suppressed SOX2 expression (Fig. 6B). These results demonstrate that cocaine treatment of NPCs is able to downregulate the expression of transcription factors which are required for stem cell maintenance.

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Discussion

The fate of NSCs in the developing, as well as in the adult, brain is regulated by complex interactions between a large number of growth factors, neurotransmitters, and neuropeptides (Cameron et al., 1998; Sommer and Rao, 2002), and cell culture models, such as the one used in this study, can provide potentially valuable insight regarding the mechanisms whereby extrinsic factors impact the fate of neuroprogenitors. The results of the present study of human fetal brain-derived NPC cultures, indicate that cocaine is another extrinsic factor that can significantly affect three critical functional properties of NSCs. First, cocaine treatment was shown to inhibit the proliferation of NPCs, a phenomenon which was associated with cell cycle arrest, which appeared to be due to increased expression of the cyclin-dependent kinase inhibitor p21. Second, treatment of NPCs with cocaine inhibited their migratory response to CXCL12, a finding that was associated with cocaine-induced downregulation of CXCR4 on NPCs. And third, NPCs exposed to cocaine underwent changes indicative of differentiation into neurons, which was associated with an inhibition of SOX2, a transcriptional factor that inhibits NSC differentiation. Although additional work is required for a comprehensive understanding of how cocaine alters each of these cellular processes, as well as to determine whether the effects we observed were mediated by cocaine itself or by a metabolite generated by NPCs, these findings provide initial information regarding several mechanisms that could underlie the effects of cocaine on neurogenesis in the human brain.

Previous *in vitro* studies investigating the effects of cocaine on neuroglioblastoma cells (Johnson and Weissman, 1988), PC-12 cells (Zachor et al., 1994), and cortical neurons of fetal mice (Nassogne et al., 1997), as well as *in utero* exposure to cocaine

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administered to pregnant animals (Mayes, 1999; Lidow and Song, 2001; Harvey, 2004) support the notion that cocaine has a variety of adverse effects on neurodevelopment. Because of potential differences among animal species, the use of human neuroprogenitors to model neurological disease has been recommended (Jakel et al., 2004). Thus, although the biological significance of the findings in the present study must be interpreted with caution, as they were derived using an *in vitro* NPC culture model, nonetheless, they point to cellular mechanisms whereby exposure of human NSCs to cocaine *in utero* could contribute to subsequent neurocognitive deficits.

Of the different classes of psychoactive drugs that have potential for abuse, to date opiates have been the best studied in terms of their effects on NSCs. These studies, which have used various *in vitro* and experimental rodent models, have shown that exposure of embryonic and adult neuroprogenitor cells to opiates inhibits neurogenesis (Eisch et al., 2000; Hauser et al., 2000; Persson et al., 2003; Mandyam et al., 2004). In one study of mixed glial cell cultures isolated from day old mouse striatum, morphine was found to synergistically increase HIV-1 Tat protein-mediated cytotoxicity of glial cell precursors (Khurdayan et al., 2004). This observation is interesting in light of the finding in the present study that while cocaine by itself had no effect on cell survival, it synergistically increased HIV-1 gp120-induced apoptosis of human NPCs. This synergistic activity of cocaine is consistent with the report that subchronic administration of cocaine in rats had no effect on viability of cortical neurons but that cocaine significantly enhanced neuronal apoptosis induced by gp120 (Bagetta et al., 2004). Also, the finding in the present study that gp120 induces apoptosis of human NPCs adds to

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recent evidence that interactions of gp120 with NSCs may contribute to HIV-1 neuropathogenesis (Krathwohl and Kaiser, 2004; Tran et al., 2005).

Because neurogenesis in the adult brain is associated with memory formation and learning (Gould et al., 1999; Shors et al., 2001), the findings in the present study using human NPCs may also have relevance to the development of neurocognitive deficits of adults who are using cocaine. Mounting evidence from studies of other substances of abuse including alcohol suggests that alterations in neurogenesis may be involved in their neurobehavioral effects (Duman et al., 2001; Powrozek et al., 2004; He et al., 2005), and it also appears that NSC proliferation may underlie the therapeutic effect of certain antipsychotic agents (Kippin et al., 2005). Although *in vitro* studies have major limitations and great caution is needed in extrapolating these findings to the clinical arena, it is possible that the human NPC cultures used in the present study could be applicable not only as a model to study cocaine-induced neurotoxicity but also to investigate the potential involvement of NSCs in the process of cocaine dependency.

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Footnotes

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Legends for Figures

Fig. 1. Effect of cocaine on proliferation of human NPCs. Cultures of human NPCs were (A) treated with medium alone (control) or cocaine (10^{-10} to 10^{-4} M) beginning at day 1. ^3H -thymidine was added at day 6 for 16 h followed by sample collection on day 7. Data presented are mean \pm SEM of 5 experiments using NPCs derived from different brain specimens (**, $P < 0.01$ versus control). (B) Differential, dose-responsive effects of (+) and (-) -cocaine enantiomers on NPC proliferation. Data presented are mean \pm SEM and are representative of experiments using NPCs derived from two different brain specimens (* P , <0.05 and **, $P <0.01$ vs. untreated). (C) NPCs were incubated in medium alone (untreated) or treated with cocaine (10^{-6} M) for 0, 24, 72, and 120 h before adding ^3H -thymidine and collecting samples to measure incorporation (cpm). Data presented are mean \pm SEM of 5 experiments using NPCs derived from different brain specimens (**, $P <0.01$ vs. untreated). (D) NPCs grown on culture slides were treated with cocaine (10^{-6} M) for 6 days followed by adding BrdU for 16h. The cells were then fixed with paraformaldehyde and stained for incorporation of BrdU using an anti-BrdU antibody (green cells) contrasted with DAPI nuclear staining (blue).

Fig. 2. Effect of cocaine treatment on apoptosis of NPCs. NPC cultures were (A) incubated in either medium alone (control), or treated with cocaine (10^{-12} to 10^{-4} M) or gp120 (10 nM, positive control) for 7 days before collecting cell lysates for assessment of cell death using ELISA of histone-associated DNA fragments. Data presented are mean \pm SEM using NPCs derived from three different brain specimens (**, $P < 0.01$ versus control). (B) NPCs were incubated in medium alone (control), or treated with cocaine

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(10^{-6} M), gp120 (0.1, 1, and 10 nM), or a combination of cocaine and gp120. Data presented are mean \pm SEM using data obtained from three different brain specimens (*, $P < 0.05$; ** $P < 0.01$ versus gp120 alone).

Fig. 3. Effect of cocaine treatment on the NPC cell cycle. Cultures were treated with medium or cocaine, fixed, and stained with anti-PCNA (proliferating cell nuclear antigen) antibody. (A) NPCs were incubated in medium alone (0) or treated with cocaine (10^{-8} to 10^{-4} M) for 7 days and cell counts from five high power fields of anti-PCNA antibody-stained cells were obtained using fluorescence microscopy. Data presented are mean \pm SEM counts of five fields from three experiments using NPCs from different brain specimens (*, $P < 0.05$; ** $P < 0.01$ versus control). (B) Nuclear extracts collected from NPC cultures incubated in medium alone (0) or treated with cocaine (10^{-10} to 10^{-4} M) for 7 days were assessed by ELISA for the cyclin-dependent kinase inhibitor p21. Data are presented as mean \pm SEM of six experiments using NPCs obtained from three different brain specimens (*, $P < 0.05$; ** $P < 0.01$).

Fig. 4. Effects of cocaine on NPC migration and CXCR4 expression. (A) NPCs were incubated in medium alone (0) or treated with cocaine (10^{-9} to 10^{-4} M) for 60 min prior to assessing their migration towards CXCL12/SDF-1 α (10 ng/ml) using a chemotaxis chamber. Migrated NPCs retained on the membrane were stained with Diff-Quik and quantified by cell count of five high power fields (HPF). Data presented are mean \pm SEM of six experiments using NPCs from two different brain specimens (** $P < 0.01$ versus control). (B) NPC cultures incubated in medium alone (unshaded curve) or cocaine

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(shaded curve, 10^{-6} M) for 7 days were collected and stained with anti-human CXCR4 antibody for flow cytometric analysis. Isotype-matched control antibody was used as a negative control (black curve). Data shown are representative of two experiments using NPCs derived from different brain specimens.

Fig. 5. Effect of cocaine on the differentiation of NPCs. NPCs were cultured in proliferation media containing the growth factors hFGFb/hEGF until 50-60% confluence. The cells were then treated with medium containing cocaine (10^{-8} to 10^{-4} M) for 7 days and counts of (A) anti-Tuj1 antibody-stained or (B) anti-GFAP antibody-stained cells were obtained using fluorescence microscopy. Data presented are expressed as mean \pm SEM percent positive cells from 3 experiments for Tuj-1 and 2 experiments for GFAP using different brain specimens (**, $P < 0.01$ versus control).

Fig. 6. Effect of cocaine treatment on SOX2 expression in NPCs. NPC cultures were (A) left untreated or (B) treated with cocaine (10^{-6} M) for 3 days and then collected for analysis of SOX2 (a transcription factor required for stem-cell maintenance) expression by flow cytometry (shaded curves). Isotype IgG1 was used as a negative control (black line).

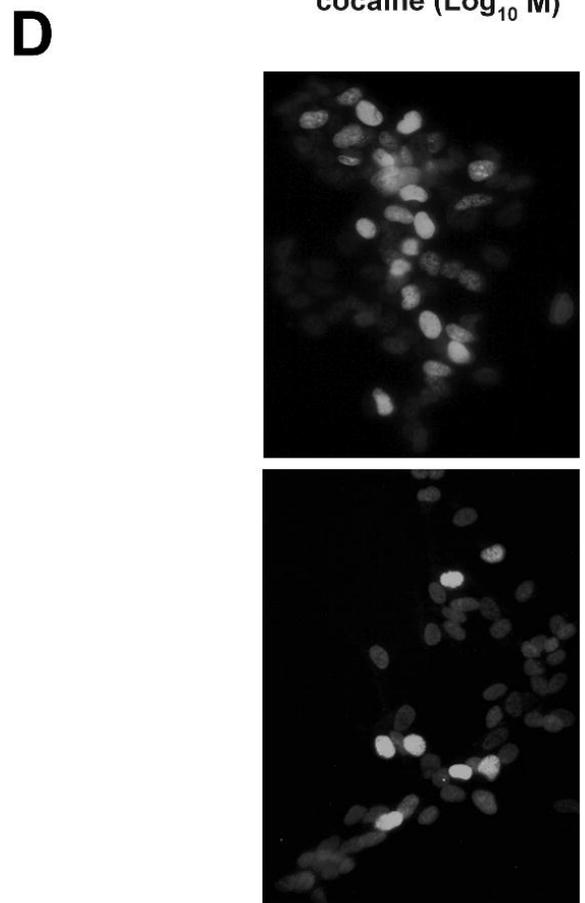
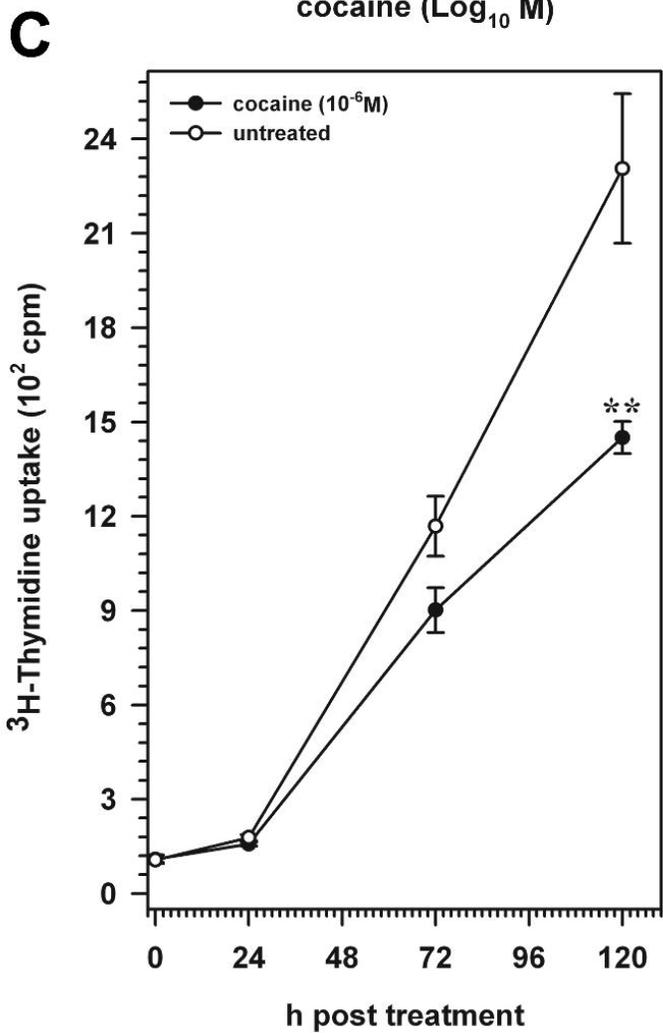
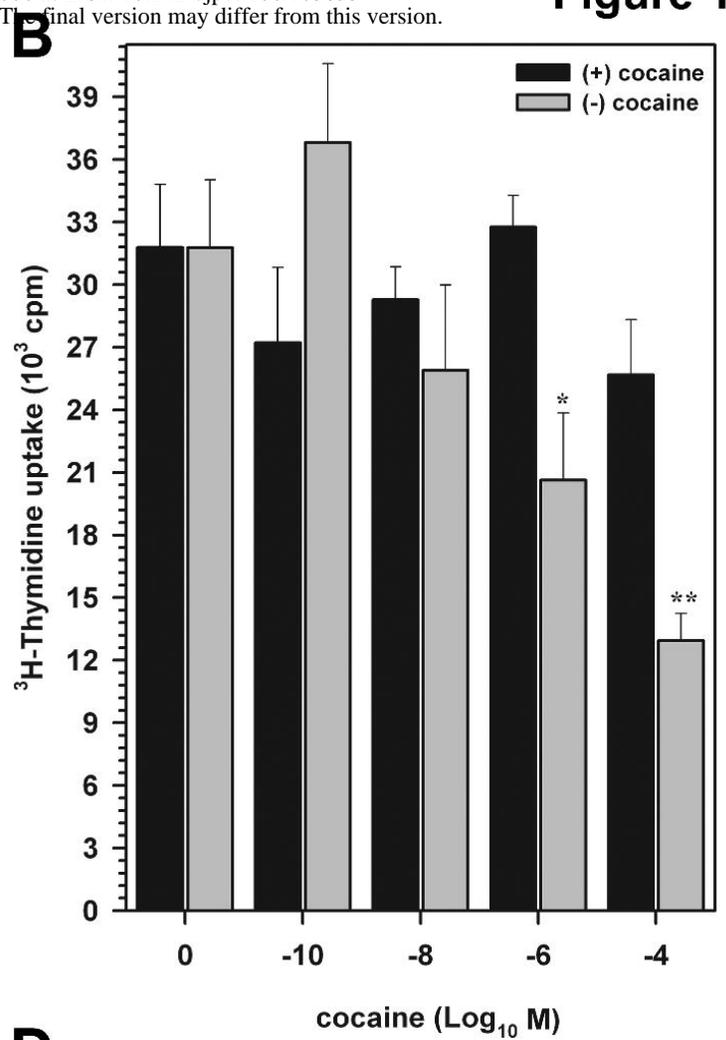
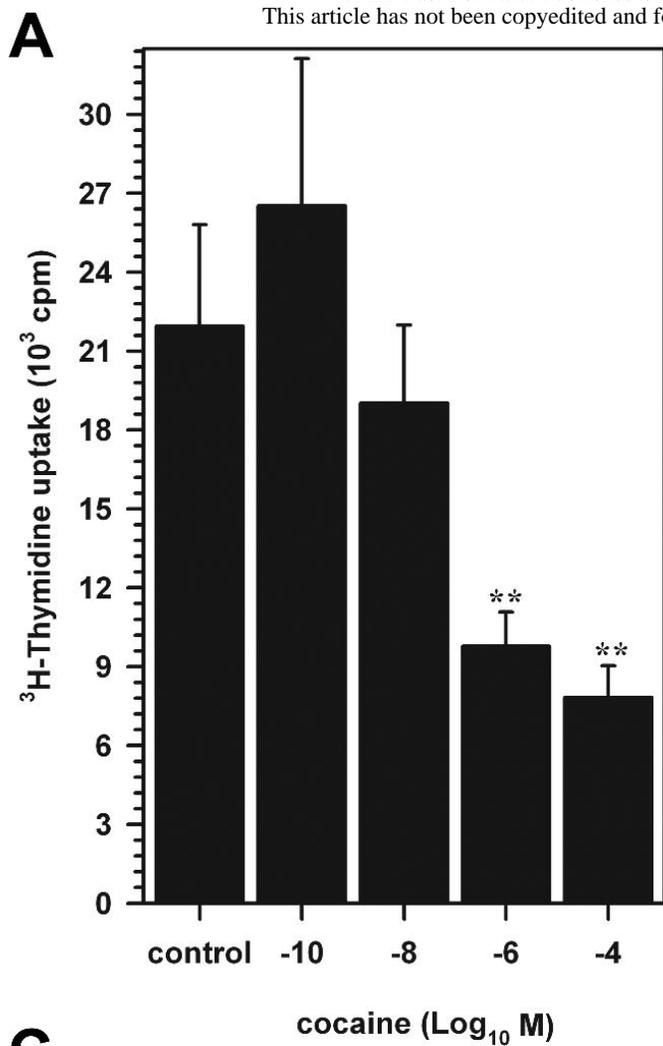


Figure 2

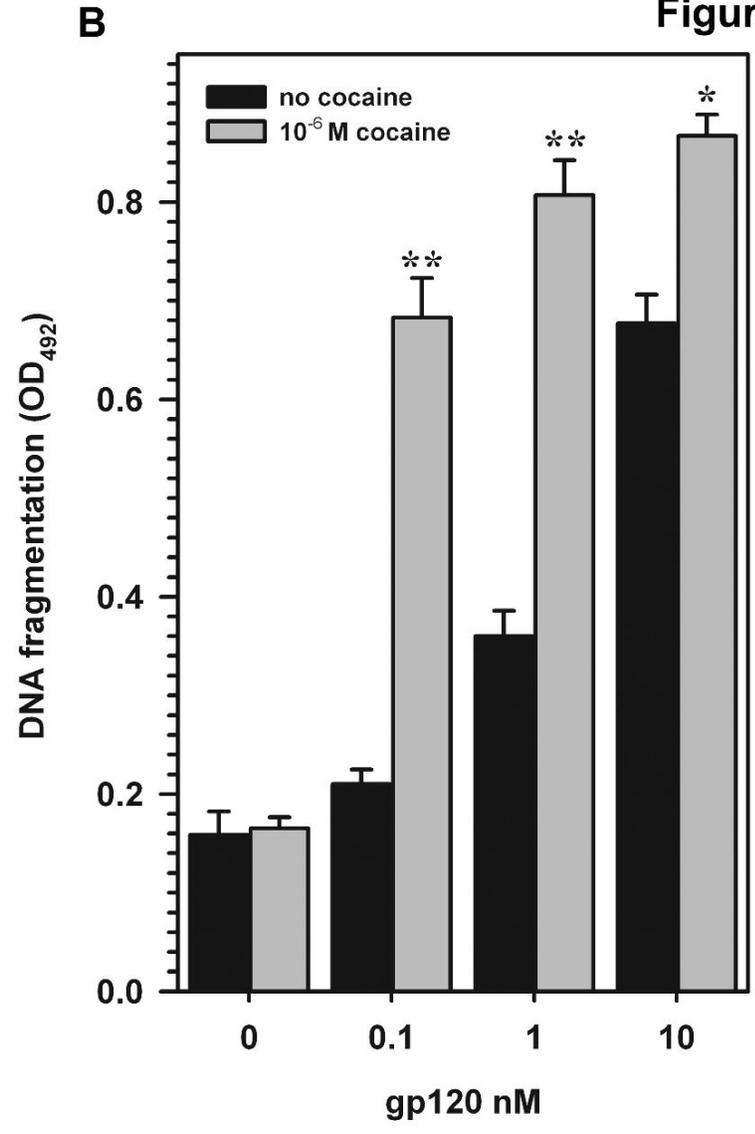
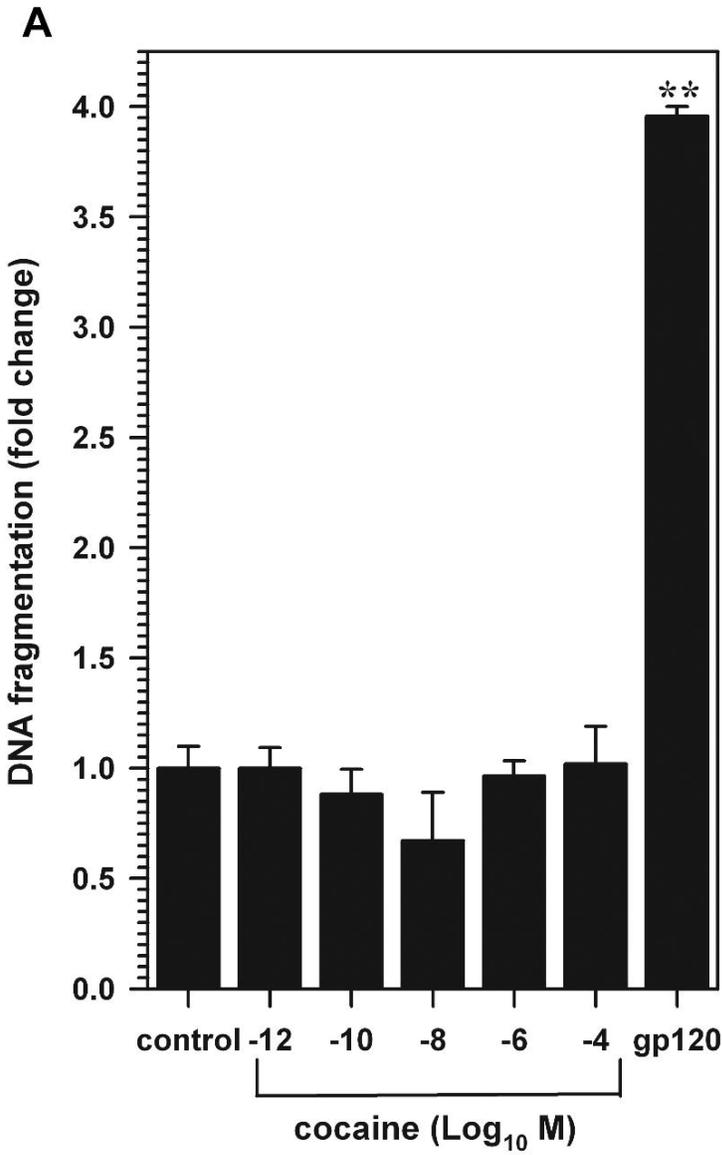


Figure 3

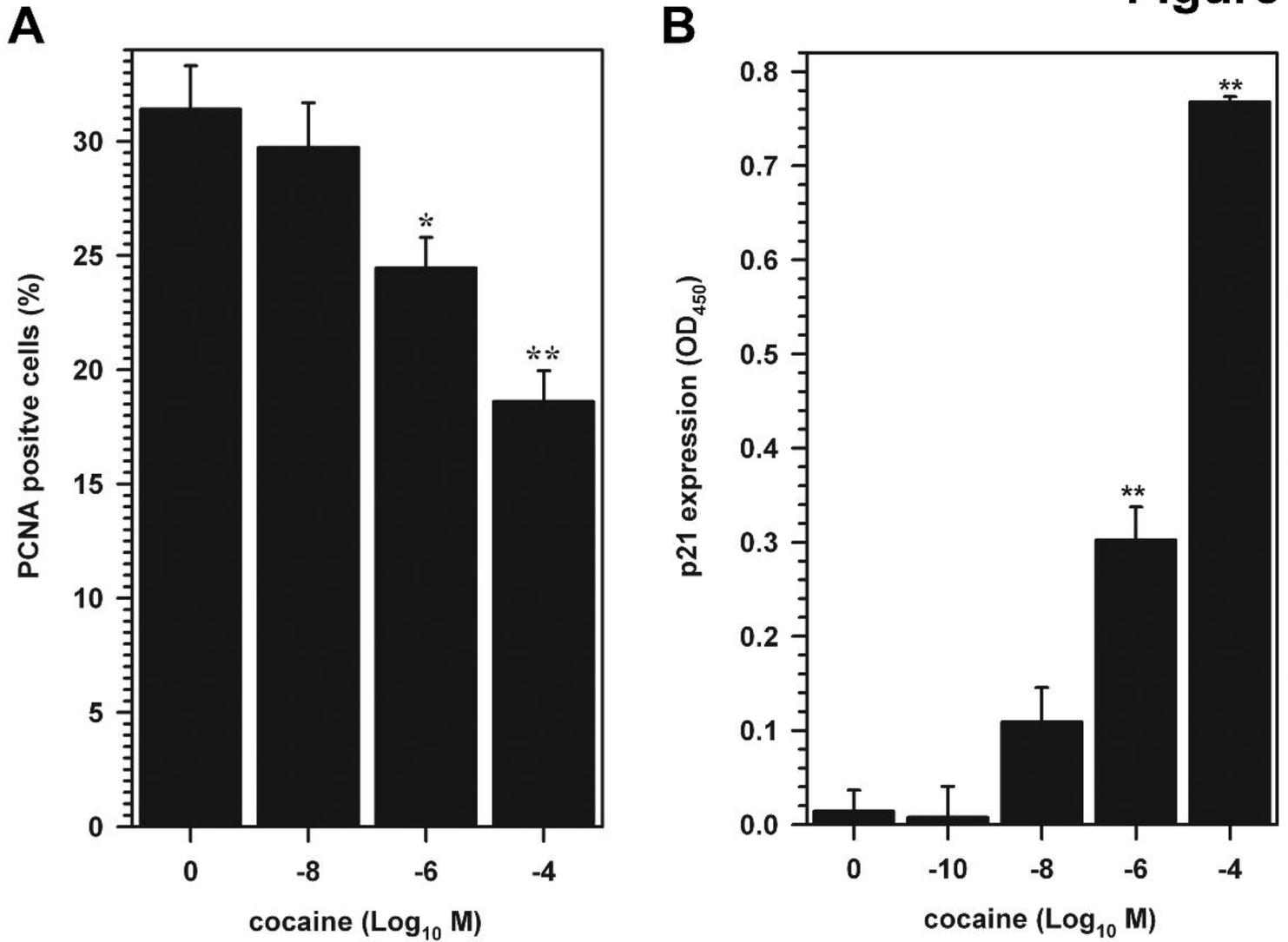


Figure 4

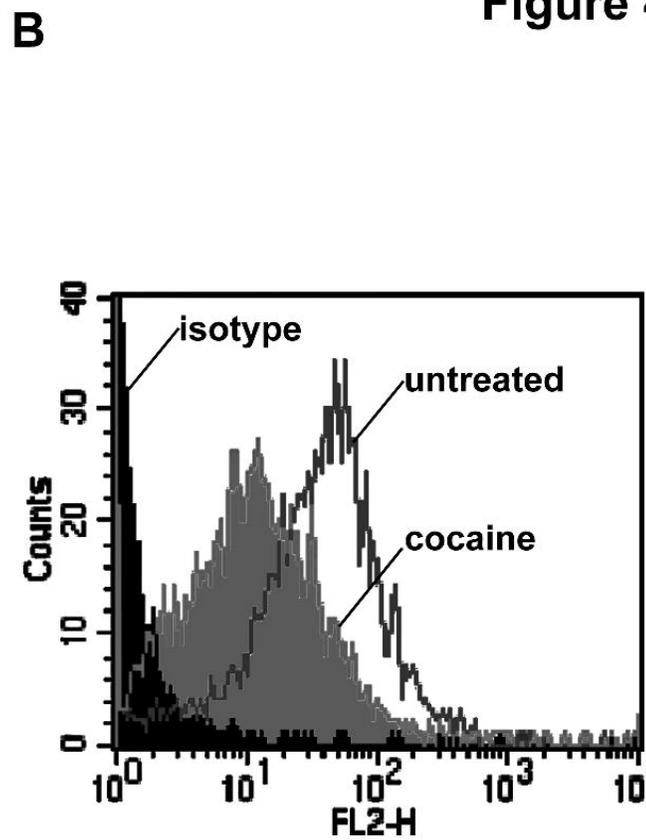
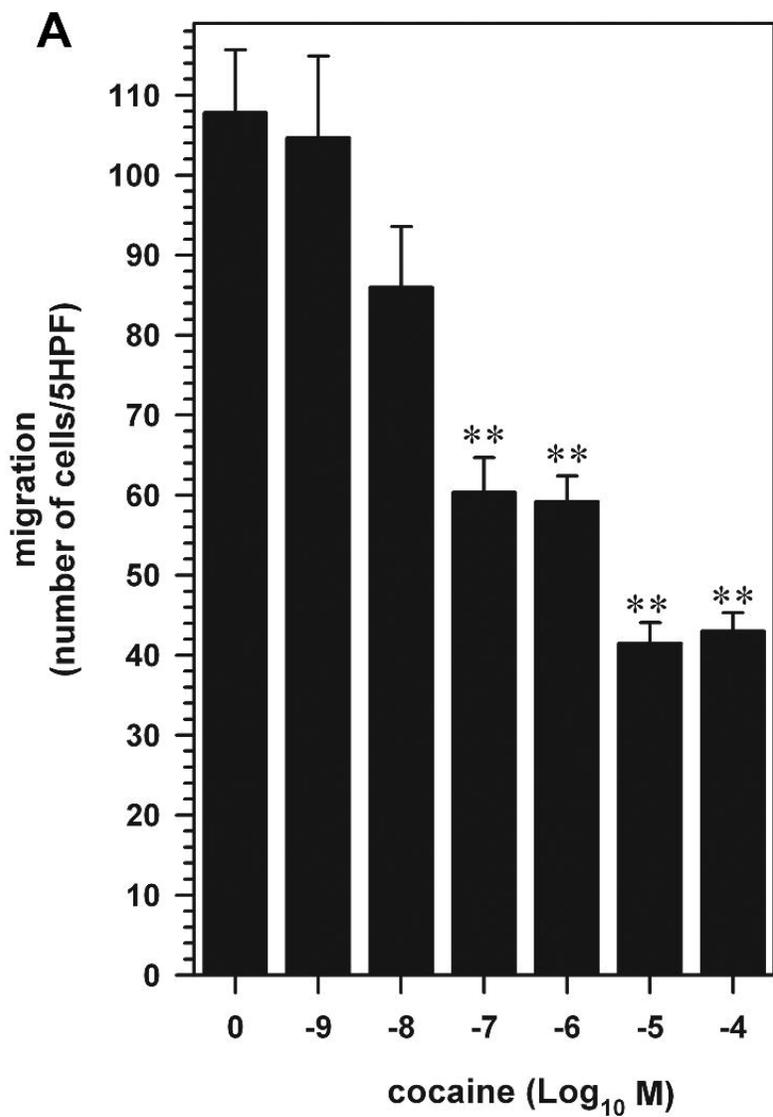


Figure 5

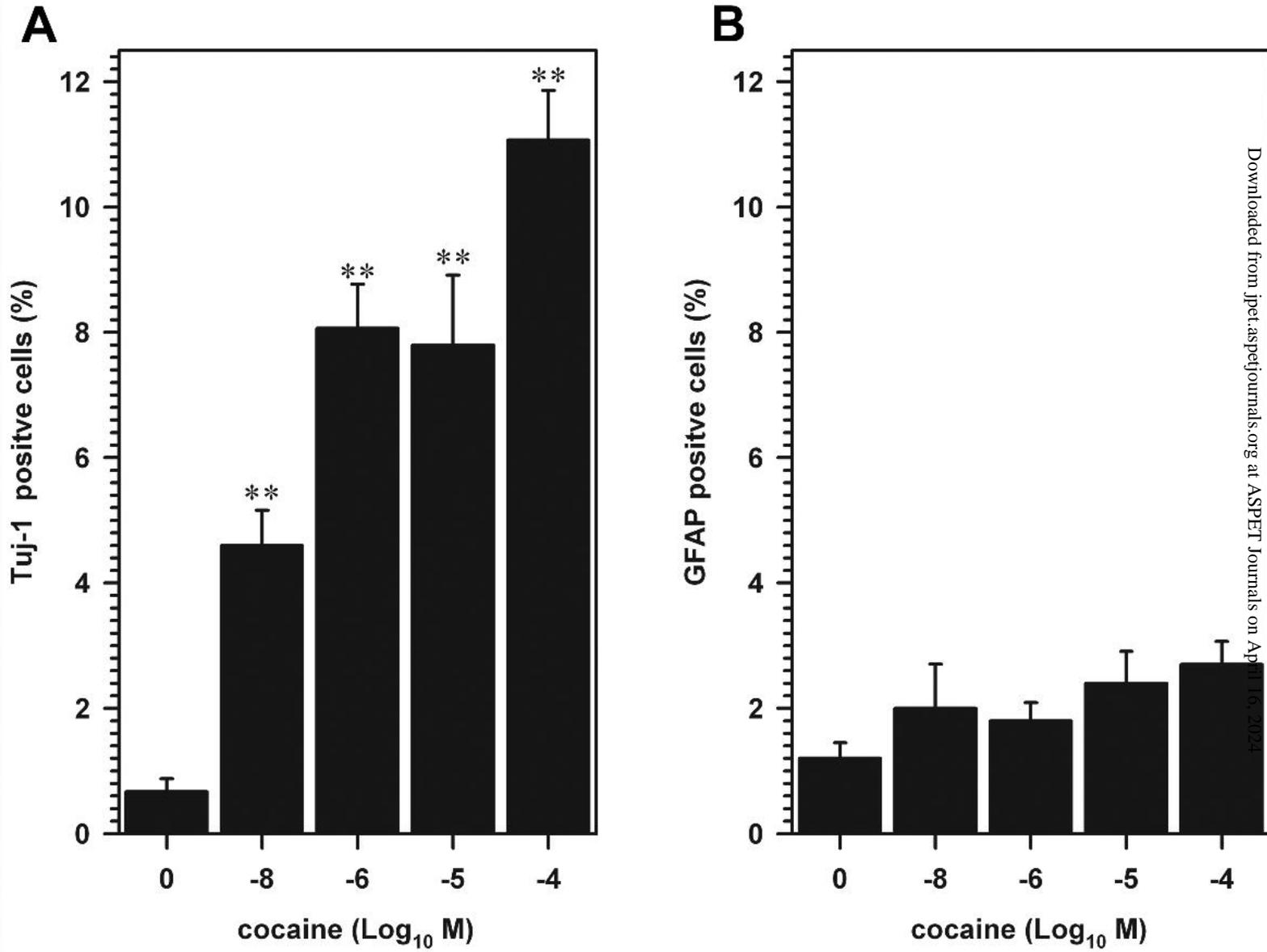


Figure 6

