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Cocaine selectively increases proliferation in the adult murine hippocampus

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Abstract

Cocaine abuse continues to be a significant problem in the USA and elsewhere. Cocaine is an indirect agonist for dopamine, norepinephrine and serotonin with numerous potential downstream effects, including processes and signals associated with adult neurogenesis. Since drug addiction is associated with brain plasticity, we hypothesized that cocaine exposure would alter cellular proliferation in two adult neurogenic regions (the subventricular and subgranular zones). We used bromodeoxyuridine (BrdU) to track newly generated cells in the brains of adult mice after chronic cocaine or saline exposures. No differences were found in the number or migration patterns of BrdU-labeled cells in the forebrain neurogenic areas. However, cocaine produced a significant increase in the number of hippocampal BrdU-labeled cells.

Keywords

Cocaine; subventricular zone; subgranular zone; adult neurogenesis

It is well documented that both short-term and chronic exposures to recreational drugs can induce structural changes in the brain [25,34], alterations in learning and memory [29], as well as changes in mood and cognition [2,39]. Although the mechanism underlying these changes is complex, alterations in neurogenesis may be a contributing factor [13]. The process of neurogenesis is well described in the adult nervous system [28]. It predominantly occurs in the dentate gyrus of the hippocampal formation and the subventricular zone (aSVZ) of the lateral ventricles, structures that are thought to contribute to memory formation, mood regulation and olfaction [44].

A number of factors influence adult neurogenesis, including aging, stress, exercise, genes, the environment, and learning as well as circulating factors such as hormones, growth factors and neurotransmitters [44]. Neurogenesis in the subgranular zone of the adult hippocampus (SGZ) is decreased by corticosteroids [17] whereas antidepressant treatment results in increased proliferation and survival of newborn hippocampal cells, possibly through serotonin (5-HT) or norepinephrine-dependent mechanisms [43]. The monoamine

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dopamine (DA) has also been shown to regulate adult neurogenesis. DA receptors and DA afferent fibers are localized within neurogenic regions and dopamine agonists, antagonists, lesions and denervation affect proliferation [20,41].

Cocaine is a powerful and addictive psychostimulant drug. Based on the SAMSHA National Survey on Drug Use and Health Report, 5.3 million people in the US reported abusing cocaine in 2008 [36]. Cocaine is an indirect agonist for dopamine, norepinephrine and serotonin, resulting in elevated synaptic levels of these neurotransmitters. Given the potential role of neurotransmitters in the regulation of adult neurogenesis, we hypothesized that chronic cocaine exposure would disrupt aSVZ and SGZ proliferation.

A decrease in cellular proliferation in the subgranular zone of the adult hippocampus (SGZ) is reported for rodents after exposure to methamphetamine, ethanol, opiates, nicotine, and antidepressants [1,12,26,30,37]. The role of cocaine in this process is more controversial. Several studies show alterations in hippocampal neurogenesis following chronic cocaine exposure [3,10,31,43], while others have not found any changes [11]. In the aSVZ, ethanol decreases proliferation, while opiates have no effect [9,12]. In addition, serotonin depletion and selective dopamine agonists stimulate aSVZ proliferation while dopamine depletion inhibits it [8,20]. A recent study examining the role of short-term self-administered cocaine on aSVZ proliferation [31] found that there is a 20% reduction in cell proliferation without any alterations in aSVZ volume. In this study, we assessed the effects of longer-term, chronic cocaine exposure and short-term abstinence on cell proliferation in the SGZ and aSVZ of mice. We also examined the migratory pattern of aSVZ cells as they travel through the rostral migratory stream (RMS) toward the olfactory bulb and the pattern of BrdU labeling in the SGZ along the dorso-ventral axis of the hippocampus. As in previous studies, we found that chronic cocaine exposure alters neurogenesis and add that this effect is specific to the dorsal, but not ventral hippocampal subgranular zone. However, we did not find any significant changes in the number, nor the migratory pattern of BrdU-labeled cells in the aSVZ/RMS.

Methods

Cocaine Administration

Adult (3-4 month) male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were given subcutaneous injections of 20mg/kg/day of cocaine HCl (Sigma, St. Louis, MO) or saline administered as two 10mg/kg injections at 0900 and 1700 for a total of 28 days [24]. On day 29, each animal received a single intraperitoneal (ip) injection of 50µg/g 5-bromo-2'-deoxyuridine (BrdU; Boehringer Mannheim, Mannheim, Germany) in a 5mg/ml solution of 0.007N NaOH. Three additional ip BrdU injections were given at 2 hour intervals. Analysis was performed at one, three, and five days after the initial BrdU injection (n =5 per group).

All procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals.

Histology

Mice were anesthetized with Avertin and perfused transcardially with PBS followed by 4% paraformaldehyde. The brains were extracted, postfixed overnight, processed for paraffin embedding, and blocked in the sagittal plane. Serial sections at 5µm were cut and mounted onto polyionic slides. One half of each brain was randomly assigned for analysis of BrdU labeling in the aSVZ/RMS while the other half was used for a similar analysis in the SGZ.

Immunohistochemistry for Bromodeoxyuridine—Every other slide was baked at 65°C in a 50% formamide solution in SSC for two hours, rinsed in SSC and incubated in 2N HCl at 37°C for 30 minutes. The slides were submersed in 0.1M boric acid (pH 8.5) for 10 minutes, 3% H₂O₂ for 10 minutes, and 1% BSA in PBS with 0.1% triton X-100 for 30 minutes. The slides were incubated overnight at 4°C in a humidified chamber with mouse monoclonal anti-BrdU (1:100; Dako, Carpinteria, CA), a biotinylated secondary antibody for 30 minutes and horseradish peroxidase conjugated avidin-biotin complex for 30 minutes (Vector, Burlingame, CA), and visualized using DAB (KPL, Gaithersburg, MD). The slides were counterstained with cresyl violet, dehydrated in graded ethanols, cleared in xylenes and coverslipped with Permount.

Immunohistochemistry for Tenascin—Alternating sections were processed for tenascin immunohistochemistry as described above using a rabbit polyclonal tenascin antibody (1:500; Chemicon, Temecula, CA). Antigen retrieval was performed in a 0.01M sodium citrate solution for 10 minutes at 95°C.

Analysis of BrdU Labeling

Analysis of the Migratory Patterns of BrdU-labeled cells in the aSVZ—The center of the aSVZ/RMS was located using anatomical landmarks, BrdU+ cell quantification and tenascin immunohistochemistry [11,12,22]. Tenascin staining confirmed that seventeen slides contained the aSVZ/RMS (two slides medial to the center (-100µm to -50µm), the central slide (0µm) and fourteen slides lateral to the center (50µm to 700µm)), which were analyzed using the Bioquant NOVA Image Analysis System (R & M Biometrics, Nashville, TN). For each section, the x,y coordinates of a line representing the center of the aSVZ/RMS were recorded using Bioquant NOVA. The number and x,y coordinates of each of the BrdU+ cells in the forebrain were also recorded. A least squares formula was used to determine the distance of each BrdU+ cell from the line representing the center of the aSVZ/RMS. The measurements represent each cell's distance from the center of the aSVZ/RMS (i.e., 0µm-50µm, 51µm-100µm, 101µm-150µm, 151µm-200µm, 201µm-250µm, or >250µm). These data were used to assess the number and migratory pattern of BrdU+ cells in the aSVZ/RMS across two different treatment conditions and three different post-labeling time points using a factorial design in SPSS (v16.0).

Quantification of BrdU-labeled cells in the SGZ—Slides containing the entire hippocampus were identified using hematoxylin staining and anatomical landmarks (0.36mm to 3.72mm lateral and -0.82mm to -4.24mm posterior to Bregma) [32]. Ten slides spaced evenly through the SGZ were chosen for analysis. The SGZ was defined as the area bordering the granule cell layer and hilus. The area one to two cell widths from the granule cell layer and hilus was excluded from analysis [13]. The number of BrdU+ cells in the entire SGZ [18] or its dorsal or ventral aspects [16] was calculated for each slide to provide a total estimated number of BrdU+ cells in each partition of the SGZ for each animal. These data were compared across two different treatment conditions and three different post-labeling time points using a mixed model factorial design in SPSS (v16.0).

Results

Effects of Chronic Cocaine Exposure on BrdU Labeling in the aSVZ

In each of the brain sections analyzed (-100µm to 700µm), regardless of time point of measurement (1, 3, or 5 days post-labeling) or experimental treatment (saline or chronic cocaine), the 0µm-50µm bin contained the vast majority of BrdU+ cells (Figure 1B-D). There were no significant differences in the distribution of cells within bins across treatment conditions ($p>0.05$). Thus, cell migration out of the confined regions of the aSVZ/RMS did

not appear altered by chronic cocaine administration. For a few brains, the individual sections were reconstructed in Bioquant NOVA using 3-dimensional rendering. As expected, the overall patterns revealed a progression of labeled cells from the aSVZ towards the OB over the 5 days of post-labeling we examined. However, we did not find any qualitative differences in the migratory pattern of aSVZ neurons following cocaine exposure.

The data within sections were collapsed to assess the effects of chronic cocaine on the total number of BrdU+ cells (Figure 1A). A $2 \times 3 \times 17$ factorial design was constructed to assess the contributions of each independent variable on the total number of BrdU+ cells. The independent variables include: 1) chronic cocaine or saline treatment; 2) 1, 3 or 5 days post-labeling; and 3) section of analysis (from -100 μ m to 700 μ m relative to the aSVZ/RMS at 50 μ m increments). The overall model is significant, $F(97, 194) = 8.20, p < .001$, with an interaction of days post-labeling by experimental group, $F(2, 194) = 8.95, p < .001$. The interaction justifies individual comparisons at 1, 3, and 5 days in a 2 (group) $\times$ 3 (days) factorial design, but no significant effects were found with these permutations ($p > .05$).

The data were further collapsed to assess the total number of BrdU+ cells across all sections for each time point of analysis. No significant differences were found when comparing either the total or average number of BrdU+ cells ($p > .05$).

Effects of Chronic Cocaine Exposure on BrdU Labeling in the SGZ

An analysis of BrdU+ cells in the adult hippocampal SGZ as a function of the treatment condition and number of days post-labeling revealed that chronic cocaine treatment results in a significantly greater number of BrdU+ cells in the SGZ ($\bar{X} = 1206.22$, SEM = 113.58) when compared to saline treated animals ($\bar{X} = 872.97$, SEM = 225.47), $F(1, 9) = 5.165, p < .05$ (Figure 2A). Furthermore, the total number of labeled cells observed in the hippocampus significantly decreased as the post-labeling time increased, $F(2, 9) = 41.54, p < .001$. A Bonferroni post hoc comparison revealed that there were fewer BrdU+ cells at 3 days post labeling ($\bar{X} = 699.92$, SEM = 138.72) and 5 days post labeling ($\bar{X} = 407.07$, SEM = 17.52) compared to the 1 day post labeling time point ($\bar{X} = 1911.81$, SEM = 209.27) ($p < .001$). There were no differences in the number of BrdU+ cells between saline and cocaine-treated animals at 5 days post labeling.

An analysis of BrdU-labeling along the dorso-ventral axis of the SGZ showed more labeling in the dorsal ($\bar{X} = 921.88$, SEM = 98.51) compared to the ventral hippocampus ($\bar{X} = 117.71$, SEM = 29.71), $F(1, 9) = 40.931, p < .001$ (Figure 2B). The observed spatial labeling differences were not consistent across the post-labeling time frame when also considering drug treatment. Post hoc analysis of this significant three-way interaction effect, $F(2, 9) = 4.28, p < .05$, revealed increases in dorsal SGZ BrdU labeling at 1 and 3 days post labeling in cocaine-treated animals ($\bar{X} = 1995.28$, SEM = 127.57 and $\bar{X} = 892.29$, SEM = 11.66, respectively) compared to saline-treated animals ($\bar{X} = 1463.68$, SEM = 146.80 and $\bar{X} = 495.07$, SEM = 67.95, respectively) ($p < .05$). No significant differences were noted in the ventral SGZ or at the 5 days post labeling time point.

Discussion

Chronic cocaine exposure resulted in an initial non-significant decrease in aSVZ cell proliferation that normalized after 5 days of cocaine withdrawal, but did not affect the overall number or pattern of migration of proliferating cells in the aSVZ (Figure 1). Chronic cocaine exposure also affected BrdU labeling in the adult SGZ of the hippocampus, which is in keeping with previous reports. However, our finding that SGZ BrdU labeling increased is

in contrast with previous reports showing either a reduction [3,10,31,43] or no effect [11] in neurogenesis in the hippocampus after chronic cocaine exposure.

We found no significant differences in the number or distribution of cells out of the aSVZ/RMS after chronic cocaine treatment ($\bar{X}$ =9780; SEM=549.72) compared to control animals ($\bar{X}$ =9347.33; SEM=406.26). However, the total number of BrdU labeled cells after cocaine treatment was decreased after 1 day (22.3%), but increased after 3 days (14.5%) and 5 days (26.1%) labeling, suggesting an initial inhibition of proliferation with a subsequent and rapid rebound increase upon cocaine withdrawal or a selective effect on the survival of immature cells. Similar trends were reported after ethanol, methamphetamine, and cocaine exposure [31,37]. Decreases in the SGZ and aSVZ proliferation were no longer apparent after 4 weeks of abstinence [31]. Our study also provides evidence for a reestablishment of normal BrdU labeling upon cocaine abstinence in the SGZ and suggests that this may occur after only five days of cocaine withdrawal.

Previous studies differ from the current study both in terms of species examined and the cocaine dosing protocols that were used. In this study we chose 10mg/kg b.i.d. cocaine administrations, since this concentration has been shown to produce physiologically relevant brain concentrations of cocaine and its metabolites, similar to those reported after human intranasal use in the absence of anoxia [27]. Cocaine-induced vasoconstriction is dose-dependent and hypoxia is known to increase adult hippocampal (but not aSVZ/RMS) proliferation through erythropoietin and BDNF-dependent mechanisms [40]. A large body of literature implicates BDNF and other growth factors in brain plasticity, the survival and differentiation of newborn adult hippocampal cells, spatial learning, mood regulation as well as cocaine-related reinforcement, locomotor sensitization and withdrawal [19,21,34]. Therefore, it is possible that the selective cocaine-dependent increases in BrdU labeling in the SGZ noted in this study reflect model-dependent indirect alterations in circulating factors (e.g., transmitters or growth factors) that specifically regulate hippocampal proliferation and survival [4]. Our results might also reflect direct, receptor-dependent actions of cocaine on SGZ proliferation and survival: a hypothesis that is supported by the observed spatial and temporal effects noted in the dorsal SGZ at 1 and 3 days post labeling/cocaine abstinence (Figure 2).

The hippocampus has at least two functionally distinct components. The dorsal hippocampus is attributed a role in spatial memory, while the ventral hippocampus seems well suited to emotional processing [15]. Adult neurogenesis also differs along the dorso-ventral axis with ~70% of BrdU labeling occurring in the dorsal hippocampus [34]. Consistent with previous studies and regardless of treatment, we found that the majority of BrdU labeling was located in the dorsal hippocampus (~80%). In addition, we found that the cocaine-dependent increase in BrdU labeling at one and three days represented changes occurring in the dorsal SGZ.

Although approximately 50% of the newborn cells in the SGZ do not survive, many differentiate into neurons, migrate and are functionally incorporated into the hippocampal circuitry, where they are thought to be involved in spatial learning and contextual fear conditioning [4]. Dopamine fibers from the midbrain ventral tegmental area innervate the hippocampus where they regulate the persistence of long-term memory as well as the proliferation and survival of SGZ cells [33]. Dopamine, specifically the D1 receptor pathway in the dorsal hippocampus, has been linked to synaptic plasticity, memory processes and cocaine-dependent cellular and behavioral changes, such as conditioned place preference [33,38,42]. Taken together, these studies suggest that the observed increase in BrdU+ cells in the dorsal SGZ might contribute to a functional effect of creating long-term persistence to spatial and contextual information, such as those formed during drug

addiction. These types of long-term memories contribute to cue-dependent drug cravings and are a major factor in relapse and target for treatment [14].

Adult neurogenesis is a complex process involving proliferation, differentiation, migration, maturation and survival [4]. Progression from a newly formed cell to a mature neuron likely requires a number of intrinsic and extrinsic signals, many of which are mediated by dopamine, perhaps through a common intracellular signaling pathway involving cdk5 [4,23]. We observed a significant increase in cocaine-treated animals in BrdU labeling in the SGZ 1 day and 3 days after cocaine abstinence/BrdU labeling when compared to saline-treated animals with no difference noted at 5 days (Figure 2B). There was a 37% difference in the number of labeled cells at 1 day compared across treatments and a 44% difference at 3 days. However, BrdU labeling in the cocaine treated animals decreased from 1 day to 3 days by 55% with a 66% decrease observed in the saline treated animals. The difference in labeling in cocaine treated and saline treated animals from 3 to 5 days decreased 62% and 32%, respectively. Taken together, these data support a temporal effect of cocaine on the survival of newly born cells.

Although the exact role of the hippocampus in addiction is unclear, it is implicated in regulating emotional memories, conditioned place preference, and self-administration to psychostimulants and other conditioned reinforcers. Since adult neurogenesis is implicated in the function of the hippocampus, alterations in adult SGZ neurogenesis could have a lasting impact on drug-related contextual memories, addictive behaviors, and relapse, regardless of the direction of effect. Recent studies support a role for the hippocampus in drug addiction and include its involvement in general drug-related and specific cocaine-related reinforcement, relapse, plasticity and toxicity [5-7,11,20,38]. Alterations in adult hippocampal neurogenesis may underlie some of the neuroadaptations associated with cocaine dependence, thus providing novel strategies for treating cocaine addiction and associated mood and cognitive disruptions [11]. More studies are needed to elucidate the timing, duration and consequences of cocaine-induced disruption of SGZ neurogenesis.

***Research Highlights**

1. Chronic cocaine exposure did not alter aSVZ BrdU labeling in mice.
2. Chronic cocaine exposure increased SGZ BrdU labeling in mice in a spatio-temporal manner.
3. BrdU labeling increases were restricted to the dorsal and not ventral SGZ and were noted after 1 and 3, but not 5 days of cocaine abstinence/post BrdU labeling.

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Figure 1. The effects of cocaine on BrdU labeling in the aSVZ/RMS

The effects of chronic cocaine exposure on the number of BrdU+ cells in the entire aSVZ/RMS (A) or across sagittal tissue sections (B-D) are reported. The total number of BrdU+ cells for saline and cocaine-treated animals in seventeen tissue sections chosen for their relative position to the center of the aSVZ/RMS (A). The analyses were performed at 1 day (B), 3 days (C) and 5 days (D) after the last BrdU injection. Aside from a main effect of section, no significant differences were noted. Error bars represent SEM.

Figure 2. The effects of cocaine on BrdU labeling in the SGZ

The effects of chronic cocaine exposure on the number of BrdU+ cells in the SGZ (A), at 1, 3 and 5 days post labeling (A), and according to spatial distribution along the dorso-ventral axis (B). Chronic cocaine treatment results in a significantly greater number of BrdU+ cells in the SGZ and the total number of BrdU+ cells significantly decreased as the post-labeling time increased from 1 to 3 and 1 to 5 days post labeling/cocaine abstinence (A). The increases in BrdU+ cells at 1 and 3 days were due to alterations in the dorsal and not ventral SGZ (B). Error bars represent SEM. * $p < .05$