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A Single High Dose of Methamphetamine Induces Apoptotic and Necrotic Striatal Cell Loss Lasting Up to Three Months in Mice

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Abstract

Methamphetamine (METH) is an addictive agent that poses a public health problem due to its toxic effects on neural tissue. We have shown that METH induces striatal lesions (cell loss) within 24 hours of administration. Because cell proliferation has been found to follow excitotoxic and other types of lesions in adult brain, we tested the hypothesis that cell proliferation would follow METH-induced striatal cell death. To that end, METH (30 mg/kg ip) was injected into adult male mice followed by a single injection of the proliferation marker 5-bromo-2'-deoxyuridine (BrdU, 100mg/kg ip) at various times post-METH up to 12 weeks. Immunohistochemical analysis of striatal tissue showed that METH-treated animals incorporated BrdU between 24–48 hours post-METH. To determine the survival of the newly generated cells, a sub-group of animals received BrdU 36 hours after METH and were sacrificed at various times up to 12 weeks post-METH. Morphological analysis of striatal tissue from these animals showed that by 12 weeks post-METH, approximately 42% and 30% of the newly generated cells showed pyknotic or necrotic morphology, respectively. Thus, approximately 30% of the newly generated cells survive up to 12 weeks post-METH. Striatal volume was increased by METH and normalized to control levels by 12 weeks after METH. The data demonstrate that a single bolus injection of METH induces cellular changes and responses that persist for months after exposure to METH.

Keywords

methamphetamine; striatum; cytogenesis; proliferation; apoptosis; necrosis

1. INTRODUCTION

Methamphetamine (METH) is a club drug that has been increasing in popularity as a recreational drug and according to recent estimates has become the second most abused drug after cannabis (United Nations Office on Drugs and Crime, 2007). The illicit use of METH is a burden on society because METH use is associated with aggressive behavior that is a major problem for law enforcement (Ellinwood, 1971; Szuster, 1990). Recent studies demonstrate that acute and chronic METH use induces neurotoxic responses in the human brain that can further deteriorate into neural damage including structural abnormalities. For

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example, studies utilizing positron emission tomography showed decreased levels of dopamine transporter (McCann et al., 1998; Sekine et al., 2001) and serotonin transporter sites (Sekine et al., 2006) in various brain regions including the caudate and putamen. Detoxified METH users showed increased glucose metabolism (an index of brain function) in parietal brain regions but decreased glucose utilization in the striatum and thalamus (Volkow et al., 2001). Magnetic resonance imaging studies demonstrated that the brains of METH users display significant deficits in gray-matter in the cingulate, limbic and paralimbic cortices suggesting the loss of neurons (Thompson et al., 2004). A separate study demonstrated the enlargement of the caudate nucleus, globus pallidus and putamen, nucleus accumbens and the parietal cortex of adult METH users (Chang et al., 2005; Jernigan et al., 2005). Studies involving rodents have shown that METH induces death of basal ganglia and cortical neurons (Pu et al., 1996; Eisch and Marshall, 1998; Deng et al., 2001; Zhu et al., 2005, 2006).

In the light of the above it is clear that METH induces neurotoxic injury and cell death in the brain. However, there may be other events contributing to the overall number of cells undergoing protracted cell death in the aftermath of METH, particularly in the striatum. For example, if METH also induces a proliferative response, the fate of these newly generated cells would affect the number of apoptotic cells at later post-METH time points. While studies on METH-induced striatal proliferation have been sparse, there is evidence of striatal cell proliferation in response to ischemic and excitotoxic injury (Kay and Blum, 2000; Dihné et al., 2001; Mao et al., 2001; Parent et al., 2002; Van Kampen et al., 2004; Collin et al., 2005; Tandé et al., 2006). Some proliferating cells differentiate to express markers for glia (Mao and Wang, 2001) and neurons in the striatum (Collin et al., 2005), as well as phenotypic shift to an increase in the number of specific subpopulations of neurons (Tandé et al., 2006). Dihné et al. (2001) found apoptosis as well as a proliferative response to the neurotoxic effects of quinolinic acid injected into the striatum. However, the volume of the striatum was not quantified. Exposure to a toxic dose of-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) increased proliferation in the striatum and the new cells differentiated to express markers for astrocytes but not neurons (Mao et al., 2001). Precursor cells in the SVZ can migrate to the damaged striatum where they differentiate into neuronal or glial phenotypes (Collin et al., 2005; Parent et al., 2002; Jin et al., 2003), suggesting that toxicity-induced damage from METH may be followed by the proliferation of dormant progenitor cells within the striatum or from those that migrate from the SVZ. However, some studies demonstrate that proliferating cells in response to injury are not likely to survive and differentiate into neurons without treatment with trophic factors or other neurogenesis inducing agents (Wagner et al., 1999; Yoshimura et al., 2003; Van Kampen et al., 2004, 2005; Van Kampen and Eckman, 2006).

In the present study we investigated the generation of new cells in the aftermath of the METH-induced striatal cell loss because of the striatum's vulnerability to METH and as a continuation of our previous studies in this brain region. We were interested in knowing whether significant volume changes accompany the generation of new cells. We have found that METH induces increased cytogenesis compared to saline treated mice for a short time immediately following the cell loss and that the bulk of the newly generated cells die off over a period of 12 weeks. Moreover, the METH-induced cytogenesis is accompanied by enlargement of the striatal volume that normalizes over 12 weeks while many of the new cells die by apoptosis and necrosis.

2. MATERIALS AND METHODS

2.1. Animal Care and Use

All procedures regarding animal use were performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of Hunter College of the City University of New York. The Hunter College animal facility is certified by the American Association for Accreditation of Laboratory Animal Care (AAALAC). Male ICR mice (Taconic, Germantown, NY) between 11 to 12 weeks of age were housed individually on a 12-h light/dark cycle with food and water available *ad libitum*. Mice were habituated to the housing environment and the experimenters for two weeks prior to commencement of intraperitoneal (ip) drug administration.

2.2. Drug preparation and treatment

(+)-Methamphetamine hydrochloride (Sigma, St. Louis, MO) was dissolved in 10 mM PBS and groups of animals received injections (ip) with either saline or one bolus of METH (30 mg/kg of body weight) in a volume of 200 μ L. In addition to drug treatment, 100 mg/kg of the mitotic marker BrdU (Sigma, St. Louis, MO) was injected at one of the following post-METH or post-saline injection times: 2, 24, 36 hours and 2, 3, 4, 5, 6, 7, 14 or 21 days for proliferation experiment. Sacrifice and tissue collection was done within 5 hours of BrdU injections. Five hours was chosen to allow for cells currently undergoing mitosis to incorporate BrdU into the DNA. For survival studies, the animals received 30mg/kg of METH ip and BrdU injected ip 36 hours after METH. Subgroups of animals survived to post-METH times ranging from 2 days to 12 weeks at which point striatal tissue was collected and prepared for BrdU immunohistochemistry. The earliest time after BrdU injection in which an animal could be sacrificed in the survival experiment was 24 hours (2.5 days after METH). This time after BrdU was chosen to allow newly generated cells to complete the cell cycle (cytogenesis). In all of the experiments, sacrifice and striatal tissue collection was done by anesthetizing each animal with a 1:3 mixture of ketamine/acepromazine (100mg/kg of body weight) then transcardially perfusing with 25ml of PBS followed by 25ml of 4% paraformaldehyde in PBS. Brains were dissected out and immediately post-fixed for 12 hours in 4% paraformaldehyde at 4 °C, followed by cryoprotection in 30% sucrose in PBS solution at 4 °C for 48 hrs. Tissue sections were then stored at -80 °C until used for immunohistochemical assays.

2.3. Immunohistochemistry for BrdU-positive nuclei

Coronal sections from each striatal hemisphere were cut at 40 μ m thickness and serially collected from the striatum between bregma 0.02 and 1.4 mm with every sixth sample per striata collected into one of six adjacent sample wells per animal. We collected approximately 36 striatal samples in six wells per hemisphere. For each immunohistochemical assay, we used 2 adjacent wells of samples per animal from the ipsilateral hemisphere. One well was used for immunofluorescence assays and the other for peroxidase DAB assays for light microscopy.

Fluorescent immunostaining was visualized as follows: PBS wash, followed by incubation in 65°C solution of 1:1 formamide in 4X SSC for two hours, then incubation in 2N HCL at 37°C for 30 minutes. After a 10-minute rinse in 0.1M boric acid at pH 8.5, non-specific binding sites were blocked with 5% donkey serum in 0.2% triton X100 in PBS at room temperature for an hour. Sections were then incubated in primary antibody 1:500 sheep anti-BrdU (Novus Biologicals, Littleton, CO) in 1% normal donkey serum overnight at 4°C. After 2 PBS washes, sections were incubated for 1 hour in secondary antibody, 1:500 FITC donkey anti-sheep (Novus Biologicals, Littleton, CO) at room temperature. After two PBS

washes, tissue sections were mounted on superfrost glass slides. They were then sealed and cover slipped with Vectashield hard setTM mounting medium for fluorescence (Vector laboratories, Burlingame CA). Each slide was then coded and images taken via Leica TCS SP2 confocal microscope (Leica Microsystems, Germany) for comparison with light microscopy images.

For light microscopy, immunostained BrdU-positive cells were visualized using the Vectastain[®]ABC peroxidase method with DAB. Sections were first washed in PBS and antigen unmasking was accomplished by incubation in a 1:1 solution of formamide and 4X SSC for 2 hours at 65°C, followed by 30 minutes in 2N hydrochloric acid at 37°C. Samples were then rinsed with 0.1 M boric acid, pH 8.5, at room temperature for 10 minutes. Endogenous peroxidases were quenched in 0.3% H₂O₂ for 5 minutes. Non-specific binding was blocked according to instructions from the Vectastain[®] ABC elite sheep kit (Vector Labs, Burlingame CA), followed by overnight incubation in primary antibody, 1:500 sheep-anti-BrdU (Novus Biologicals, Littleton, CO) at 4°C and staining visualized with Impact DAB[®] substrate reaction (Vector Labs, Burlingame, CA). Sections were then washed and mounted on coded superfrost glass slides. Dehydration and clearing was done with 2-minute incubations in a 50% solution of xylene in ethanol followed by 100% xylene. Slides were cover slipped with VectorMount permanent mounting medium (Vector laboratories, Burlingame CA) and stored for unbiased computerized stereological analysis.

An extra level of confirmation for intact versus necrotic morphology was done in a subset of striatal tissue. The assay employed double label immunohistochemistry for BrdU nuclei co-localized with the neural progenitor cytoskeletal protein β -III-tubulin. Histochemical staining was done with an amplification protocol according to the TSA-CY3 kit N5000[®] (Perkin Elmer, Waltham, MA) as follows: Before BrdU immunolabeling two quick PBS rinses followed antigen retrieval with 1M citric acid in dH₂O at 100°C for 8 minutes. Two quick rinses then preceded a one-hour blocking step followed by overnight incubation in primary antibody solution. Blocking was done with IgG from Vector Laboratory basic MOM kit BMK2200[®] (Burlingame, CA). Overnight primary antibody incubation was done in a solution of the diluent from basic MOM kit (Vector Labs) with a 1:2500 dilution of mouse anti- β -III-Tubulin from Millipore (Billerica, MA). On the second day, three quick PBS washes were followed by a one-hour incubation in a secondary antibody solution. Secondary solution consisted of 4 μ l/ml biotin anti-mouse IgG from the basic MOM kit conjugated with 1:1000 Cy3 from TSA-CY3 kit N5000 (Perkin Elmer, Waltham, MA). Secondary antibody incubation was followed by a 10-minute incubation in a solution of 0.1% trypsin in 0.01M Tris buffer and 0.1M CaCl₂ at room temperature. After the trypsin step, two quick washes were followed by 2N HCL incubation for thirty minutes at room temperature and three PBS washes before BrdU staining. BrdU staining commenced with a one-hour blocking step in normal donkey serum followed by overnight incubation in 1:500 sheep anti-BrdU in donkey serum at 4°C. On the third day of the assay, BrdU visualization commenced with FITC secondary antibody as previously described in single label immunofluorescence assay.

2.4. Quantification

All slides were coded and two different individuals blind to the treatment conditions performed counts of BrdU nuclei and striatal volume estimates using Stereologer (Stereology Research Center; Chester, MD) unbiased stereology software for MAC. Hardware component of the stereology system included a Leica DM 2500 microscope (Leica Microsystems, Germany) attached to a Prior optiscan II motorized XYZ stage system (Prior Scientific, MA), a Sony CCD video camera and a MAC pro computer with the settings described below. A cross section of the striatum from one hemisphere was outlined in 5X magnification for each tissue sample to get an estimate of the structure, but nuclei

counts were done at 100 X magnification. The dissector probe in Stereologer was used with a Cavalieri estimator for separate counts of the mean number of BrdU-positive nuclei that had morphological features of proliferating and surviving intact new cells (Mandyam et al., 2008) as well as pyknotic and necrotic cells (Zeigler and Groscurth, 2004). Intact but visibly mitotic nuclei were selected for proliferation analysis. Mitotic nuclei are usually seen in groups of two nuclei with irregular shapes and high BrdU stain (see Figure 1C). For the survival analysis we selected intact nuclei. Intact nuclei were larger and more regularly shaped than mitotic nuclei. Although contrasting brightness in BrdU-stained chromatin can be seen inside the nucleus of the intact cell, nuclear structure is whole and not fragmented or shrunken as occurs in pyknosis (see Figure 4). Pyknotic nuclei appear in shrunken, round or crescent shaped fragments with intense BrdU staining (see Figure 4). Necrotic nuclei appear with swollen cytoplasm some vacuoles and less intense BrdU staining. When counterstained with a membrane or cytoskeletal marker, fragmented membrane structure is visible (see Figure 5C). All positive nuclei that had the defined morphology and fell within the inclusion lines of the sampling frame without touching the exclusion lines were counted. Sampling frame area was 50 μm^2 and the frame moved in steps automatically set at (x-step $\cdot$ y-step = 100 μm^2) in a raster pattern for approximately 200 frames of striatal tissue per animal. For all groups, Stereologer estimated sampling error (CE) was well within acceptable range and less than 0.1.

For the proliferation study, mean number of mitotic BrdU-positive nuclei were quantified for approximately 8 METH and 6 saline treated mice at each of the following post-drug times; 2, 24 and 36 hours; 1, 2, 3, 4, 5, 6, 7, 14, and 21 days. For the survival study, intact, pyknotic, necrotic nuclei, as well as striatal volume were quantified in 5–7 mice for each of the following post-METH time points: 2.5 days, 1, 2, 4, 8, and 12 weeks. The estimated striatal volume was automatically calculated using the region point probe of Stereologer. After stereological quantification was complete, the group code was broken and statistical comparisons were done using Prism software (GraphPad Inc., La Jolla, CA).

2.5. Statistical analysis

For the proliferation study, a two-way ANOVA compared the difference between the mean number of BrdU-positive nuclei in METH and saline treated mice at each post-treatment time. Post-hoc analysis was done using Bonferroni multiple comparisons test of significant differences between groups at each time point. BrdU-positive nuclei with intact, pyknotic (apoptotic) and necrotic morphologies were quantified by Stereologer. The group mean from the peak proliferation time point in METH-treated mice at 36 hours was the 100% normalizing value for surviving intact nuclei. The group mean for METH-treated mice from the 36-hour time point in the proliferation study was also used to calculate the percent new cells that were pyknotic or necrotic. These percent values were compared to 0% necrotic or pyknotic nuclei in the statistical analysis. Post-METH striatal volume was automatically quantified by stereologer. The group mean volume from the saline-treated mice in the proliferation study was used as the 0% value for normalizing values in METH-treated mice over the study time course. These values were compared to baseline 0% change in volume. Using a one-way ANOVA the percentage of striatal volume change, and the percentage of BrdU-positive nuclei generated at 36 hours that were intact, pyknotic or necrotic over the 12-week time course were compared to their respective baseline for significant differences. Significant ANOVAs were followed by Bonferroni multiple comparisons test for significant differences between a given survival time point and baseline value, as well as differences between the various post-treatment survival groups.

3. RESULTS

3.1. Proliferation and Survival

We have shown that a single high dose of METH (30 mg/kg) induces striatal apoptosis at 24 hours post-METH with just a few and scattered apoptotic cells at 48 hours (Zhu et al., 2006). Paradoxically, we observed an increase of Nissl staining in the striatum 48 hours after METH (data not shown). In order to reconcile this puzzling observation, we hypothesized that cell proliferation followed the METH-induced loss of striatal cells. To test this hypothesis, we gave animals an injection of BrdU, an analog of thymidine that becomes stably incorporated into nascent DNA, at different times after METH and visualized the newly generated cells by immunohistofluorescence. Fluorescent green nuclei can be seen 36 hours post-METH throughout the striatum of a METH-treated animal (Figure 1B) and in the subventricular zone of controls (Figure 1A). The latter structure supports neurogenesis in the adult brain. Many BrdU-positive cells that appear to have divided are observable throughout the striatum (Figure 1C). A two-way ANOVA showed significant interaction effects between the type of treatment and the time after treatment [$F(10,110)=49.2$, $p<.001$], significant drug treatment effect [$F(1,110)=100.2$, $p<.001$] and significant time after treatment effect [$F(10,110)=49.2$, $p<.001$]. Post-hoc analysis showed significantly more BrdU-positive nuclei in METH compared to saline at 24, 36 and 48 hours (Figure 2). This is evidence that the proliferation occurs within a narrow timeframe of 24–48 hours after METH (Figure 2).

A significant number of the BrdU-positive nuclei remaining intact compared to those generated at 36 hours post-METH failed to survive within the 12 weeks measured (Figure 3). Post-hoc analysis showed that at 2.5 days no significant differences in the number of striatal BrdU-positive nuclei compared to those generated at 36 hours post-METH. Additionally, Bonferroni corrected post-hoc test for multiple pair wise comparisons was done with the data. At 1, 2, 4, 8 and 12 weeks after METH the surviving cells were significantly less compared to what was present at 36 hours (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$, respectively; Figure 3). A difference also existed between 2.5 days and 12 weeks (#, $p<0.05$) but no differences were observed between other post-treatment time points.

3.2. Pyknotic and Necrotic Cell Loss

As shown by morphology (Figure 4A–D), a significant percentage of the cells generated at 36 hours underwent apoptosis at the post-METH time points measured [$F(6, 32)=7.7$, $p<0.001$]. However, cell loss was a protracted process. Post-hoc analysis revealed that at 2.5 days, 1 and 12 weeks the percentage of new cells that underwent apoptosis was not significantly different from zero. Apoptotic cells at these same post-treatment times were significantly different compared to the 4-week time point when most of the cells were lost (##, $p<0.01$). In contrast, at 2, 4 and 8 weeks after METH the differences were significant compared to baseline but not different from each other (Figure 4E).

A population of the BrdU-positive cells displayed necrotic morphology including swelling and fragmentation of the membrane and vacuolization (Figure 5), common features of necrosis. The double labeling of BrdU-positive nuclei with the neural progenitor protein β -III-tubulin was used as an extra measure of necrosis in new cells destined for neuronal phenotype. β -III-tubulin has previously been used to demonstrate lipid peroxidation and necrotic cytoskeletal degeneration of neuronal cells in brain tissue undergoing neurodegeneration (Boutte et al., 2006; Montine et al., 2002). Therefore, the double label immunohistofluorescence demonstrated that newly generated progenitors also undergo necrotic cell death after METH (Figure 5A–C). At every time point after METH a significant percentage of the cells generated at 36 hours displayed the necrotic morphology

compared to the baseline value of zero, one-way ANOVA [$F(6, 32) = 9.5, p < 0.0001$]. However, there were no significant differences between any of the other post-METH times when compared to each other (Figure 5D).

Because we observed the most proliferation between 24–48 hours post-METH, we decided to measure the estimated striatal volume at various times after METH using computerized stereology (Figure 6). The striatal volume of the hemisphere measured after METH was significantly larger than the group mean in saline treated mice from the proliferation study [$F(6, 32) = 2.7, p < 0.05$]. Note the large variability within each group after METH. One mouse had greater than 50 percent enlargement of the striatum at 2.5 days post-METH and the mean percent increase over baseline volume in this group was significant ($p < 0.01$). Even though one mouse actually had a small decrease in volume at 12 weeks, the difference from baseline at this or the other post-METH times measured was not significant. Additionally, comparisons between the other post-treatment groups revealed no significant differences (Figure 6).

4. DISCUSSION

Our results demonstrate that exposure to a neurotoxic dose of METH induces two phases of cell death in the striatum. First, as we previously reported (Zhu et al., 2005, 2009), there is an acute phase of resident cell death that occurs 24 hours post-METH in which approximately 25% of striatal neurons undergo apoptosis. Secondly, as reported here, cell proliferation occurs between 24–48 hours post-METH (12 hours after the peak of acute resident cell death), followed by a protracted phase of apoptosis and necrosis of the newly generated cells extending over 12 weeks. The proliferative response is in agreement with multiple studies demonstrating cell proliferation in response to excitotoxic lesion in the adult brain (Gould and Tanapat, 1997; Dihne et al., 2001). However, a protracted phase of cell death consisting of the newly generated striatal cells in the aftermath of METH is novel and provides opportunities to study the long-term effects of METH in this structure. We have used a single high dose of METH because the single dose is more suited for mechanistic studies. We have shown that a binge of METH consisting of four injections (10 mg/kg ip) at two-hour intervals also induced striatal cytogenesis (Tulloch et al., 2011a).

Of particular relevance to the results reported here, are published results demonstrating that d-amphetamine and MPTP affect striatal cytogenesis. In contrast to our results, acute exposure to d-amphetamine (10 mg/kg) suppressed spontaneous BrdU incorporation (cytogenesis) in the striatum of rats (Mao and Wang, 2001). Unlike the present study, BrdU was given 1, 24 and 48 hours after the single injection of d-amphetamine and cells in one plane of the striatum were counted (Mao and Wang, 2001). We gave the mice an injection of METH (30 mg/kg) followed by BrdU at various times after METH. We observed some BrdU incorporation at 2, 24 and 48 hours, but the peak of BrdU incorporation occurred at 36 hours post-METH. We observed low levels of spontaneous BrdU incorporation in the mouse striatum. In the present study we counted BrdU incorporation using automated unbiased computerized stereology in all rostral-caudal planes of the caudate-putamen. In contrast to d-amphetamine, exposure to systemic MPTP (25 mg/kg, once daily for five days) augmented BrdU incorporation into striatal cells expressing glial phenotypes and the newly generated cells survived for up to 60 days (Mao et al., 2001). It was not determined in the above studies if d-amphetamine and MPTP exposure resulted in loss of striatal neurons. In our study the incorporation of BrdU occurs 12 hours after the peak of METH-induced apoptosis (Zhu et al., 2005) and less than 50% of the newly generated cells survive up to 12 weeks post-METH. β -tubulin, a marker of neuronal progenitor cells is expressed in a population of the newly generated cells (data not shown). Ongoing experiments in our laboratory are attempting to characterize the phenotype of the new cells. The new cells originate

throughout the striatum and not in the SVZ because mitotic cells are evident throughout the striatum of METH-treated mice (Tulloch et al., 2011b).

The acute effect of METH includes dopamine overflow in the striatum followed by dopamine depletion and toxicity (for review see Yamamoto and Bankson, 2005; Krasnova and Cadet, 2009). Some studies have shown that dopamine depletion persists up to 6 months (Seiden et al., 1975) and 4 years (Woolverton et al., 1989) after METH treatment in non-human primates. However, Cass and Manning (1999) showed partial dopamine recovery at 6 months post-METH in rats. They also found full recovery measured as evoked dopamine overflow and striatal tissue levels of dopamine by 12 months post-METH in rats. Since we measured survival of the new cells up to 3 months, it is to be determined if the newly generated cells contribute to the kind of recovery noted in the Cass and Manning study.

An interesting feature of the data reported here is that the peak of cytogenesis occurs 36 hours after the striatal METH-induced apoptosis and is triggered within a 12-hour window following the loss of striatal neurons and acute dopamine depletion sets in (Yu et al., 2002; Zhu et al., 2005). Published studies demonstrate that dopamine D2/D3 receptor signaling promotes progenitor cell proliferation and neurogenesis as well as gliogenesis in both developing and adult brain (Diaz et al., 1997; Van Kampen et al., 2004, 2005; Winner et al., 2009) suggesting that the METH-induced overflow of striatal dopamine may play a role in the induction of cytogenesis observed in the present study. Work in progress in our laboratory is investigating the role of dopamine D1 and D2 receptors on the METH-induced striatal cytogenesis. In contrast to our results, METH has been reported to decrease proliferation in the adult hippocampus and sub-ventricular zone (Teuchert-Noodt et al, 2000; Mandyam et al., 2008; Kochman et al., 2009). For example, Mandyam et al. (2008), reported that methamphetamine reduced adult neurogenesis. However, they also revealed that short-term intermittent METH treatment actually increased hippocampal proliferation as measured by BrdU incorporation in ki-67 and DCX immunoreactive cells. Ki-67 and DCX are markers of neuron progenitor cells and these measures decreased with short and long term daily treatment. Kochman et al (2009) used a treatment regimen of 1.5 mg/kg three times over a 12-hour period. A slight decrease in hippocampus cell proliferation was seen in rats after METH. However, this decrease was not statistically significant from controls. Furthermore, Teuchert-Noodt et al. (2000) reported that a single treatment with METH (25 mg/kg, a dose similar to ours) transiently reduced hippocampus neurogenesis in gerbils. Interestingly, when proliferation was measured 36 hours after METH the levels were normal. It is not unreasonable to consider that the rapid normalization might include increased proliferation before 36 hours to compensate for the initial decrease. Our results suggest that the new cells are derived from striatal progenitors as opposed to the SVZ because dividing nuclei can be seen within the striatum.

The protracted cell death induced by METH is different from the initial phase of cell loss occurring within the first 24 hours after METH. This initial phase involves the loss of approximately 25% of striatal neurons: projection neurons and parvalbumin and cholinergic interneurons. The somatostatin/NPY/NOS interneurons are resistant to METH (Zhu et al., 2006). Other laboratories have also reported the METH-induced loss of some striatal neurons using TUNEL (Deng et al., 2001) or Fluoro-Jade C (Bowyer et al. 2008). The protracted phase of striatal cell death induced by METH is restricted to the newly generated cells as shown by BrdU immunoreactivity.

Our results demonstrate that the production of new cells is associated with enlarged striatal morphology. This observation is particularly significant in the light of recent studies showing enlarged striatal volumes in METH users (Jernigan et al., 2005; Chang et al., 2005, 2007). One study reported that subjects with enlarged putamen and globus pallidus had

relatively normal cognitive performance compared to subjects with smaller striatal structures suggesting a compensatory response (Chang et al., 2005). A recent *in vivo* imaging study reported enlargement of the caudate-putamen in rats exposed to METH (Delis et al., 2010). Our data demonstrate that the enlarged striatal volume normalizes over a period of three months in mice, the same amount of time associated with the bulk of the protracted cell death. The human study that found enlarged striatal volumes (Chang et al., 2005) involved METH users. However, a study assessing abstinent METH users drug-free for approximately two years reported normal striatal volumes (Oh et al., 2005). Our results show that the mouse striatum normalizes over a period of three months as most of the newly generated cells die by apoptosis and necrosis, suggesting that the enlarged striatal volumes observed in METH users may be accounted for by inflammation and the production of new cells.

In conclusion, our data demonstrate that a single high dose of METH induces the proliferation of new cells in the striatum during a narrow window occurring between 24–48 hours post-METH. The bulk of the new cells die off gradually over a period of 12 weeks. The METH-induced cytogenesis coincides with the enlargement of the caudate-putamen, which normalizes to control levels by 12 weeks. Work in progress in our laboratory is characterizing the differentiation and phenotype of the cells surviving 12 weeks after METH.

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Abbreviations used

BrdU	5-Bromo-2'-deoxyuridine
DAB	+3,3'-diaminobenzidine
ICR	Institute for Cancer Research
ip	intraperitoneal
METH	(+)-methamphetamine hydrochloride
PBS	phosphate-buffered saline, pH 7.4
SSC	0.15M sodium chloride/0.015M sodium citrate buffer, pH 7.0
SVZ	subventricular zone

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RESEARCH HIGHLIGHTS

1. A neurotoxic dose of methamphetamine induces cell proliferation in the striatum.
2. A significant population of the newly generated cells survive for up to three months.
3. Methamphetamine induces protracted cellular changes in the striatum.

Figure 1.

METH induces the incorporation of BrdU into newly generated cells in the striatum. Adult mice (n=8) received a single injection of METH (30 mg/kg, ip) or saline (control) followed by BrdU (100 mg/kg, ip) 36 hours after METH. Newly generated cells were visualized by immunohistofluorescence with an antibody against BrdU. Note the lack of BrdU incorporation in control mice (A) and the robust immunostaining 36 hours after METH (B). High magnification photomicrograph shows what appear to be daughter cells (C). BrdU incorporation was observed in the subventricular zone (SVZ) of control and METH-treated adult mice. CC, corpus callosum.

Figure 2.

Time course of incorporation of BrdU as a measure of the METH-induced striatal proliferation. Mean number of BrdU immunoreactive mitotic nuclei. The animals (5–8) were given a single injection of METH (30 mg/kg, ip) or saline followed by an injection of BrdU (100 mg/kg, ip) at the times indicated under the x-axis. Note the narrow window of BrdU incorporation between 24–48 hours post-treatment. *** $p < 0.001$; ** $p < 0.01$.

Figure 3.

The bulk of the newly generated cells disappear by 12 weeks post-METH. Mice (n=42) received METH (30 mg/kg, ip) followed by BrdU (100 mg/kg, ip) 36 hours post-METH. Different groups of animals (n=5–7 per group) were sacrificed at various times after the BrdU injection. In comparison to the new cells generated at 36 hours (100%), note the significant reduction in the percentage of the intact BrdU immunoreactive nuclei. Note that no other significant differences were observed between any of the other pairs of post-METH time points measured by Bonferroni corrected post-hoc for multiple pairwise comparisons. ***p<0.001; **p<0.01; *p<0.05; and #p<0.05 for 12 weeks.

Figure 4.

Protracted death of the striatal newly generated cells by apoptosis (pyknosis). Pyknotic morphology of BrdU-positive nuclei was visualized in the striatum with immunofluorescence (A). Panel B shows a representative pyknotic nucleus at high magnification from the 4-week time point. Panel C shows a 400X magnified image of an intact nucleus from panel A. Panel D shows a 400X magnified image of the newly divided nuclei from Figure 1C (36 hours post-METH). The newly generated cells begin to die at 2.5 days post-METH. A significant percentage of BrdU-positive nuclei generated at 36 hours had pyknotic morphology at later post-METH times up to 12 weeks compared to baseline of zero pyknotic nuclei (C). At 2.5 days, 1 and 12 weeks after METH, significantly less pyknotic nuclei were present compared to peak pyknosis at 4 weeks (## $p < 0.01$). *** $p < 0.001$; ** $p < 0.01$.

Figure 5.

A significant percentage of the new cells undergo necrotic in the striatum. Cells in a cross section of striatal tissue stained for BrdU in green (A) and neuron microtubule protein β -III-tubulin in red (B) that were co-labeled (C). Solid white arrows indicate necrotic morphology of disrupted microtubule and disintegrating nucleus. Hollow arrows denote intact morphology of β -III-tubulin-labeled BrdU-positive cells. Quantification of the percent BrdU positive cells co-labeled with β -III-tubulin showing necrotic morphology of neural progenitors is shown in panel D. The mice ($n=5-7$) per time point were treated as in Figure 4 and necrotic morphology was assessed from 2.5 days to 12 weeks post-METH and compared to baseline necrotic nuclei 1.5 days after METH. Additionally, significantly less necrotic BrdU nuclei were observed at 2.5 days and 4 weeks compared to peak necrosis at 2 weeks (# $p<0.05$). *** $p<0.001$; ** $p<0.01$; * $p<0.05$.

Figure 6.

Exposure to METH induced an increase in striatal volume. Striatal volume was determined using computerized unbiased stereology at various times after METH (30 mg/kg, ip) from 2 days to 12 weeks. Note that striatal volume returns to nearly control values at 12 weeks post-METH. *** $p < 0.0001$ when compared to percent change in baseline (saline group mean percent change). No significant differences between other post-treatment groups were observed.