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Chronic administration of amphetamines disturbs development of neural progenitor cells in young adult nonhuman primates

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Abstract

The detrimental effects of amphetamines on developmental stages of NPCs are limited to rodent brain and it is not known if these effects occur in nonhuman primates which are the focus of the current investigation. Young adult rhesus macaques either experienced MDMA only, a combination of amphetamines (MDMA, MDA and methamphetamine) or no amphetamines (controls) and hippocampal tissue was processed for immunohistochemical analysis. Quantitative stereological analysis showed that intermittent exposure to MDMA or the three amphetamines over 9.6 months causes > 80% decrease in the number of Ki-67 cells (actively dividing NPCs) and > 50% decrease in the number of NeuroD1 cells (NPCs that have attained a neuronal phenotype). Co-labeling analysis revealed distinct, actively dividing hippocampal NPCs in the subgranular zone of the dentate gyrus that were in transition from stem-like radial glia-like cells (type-1) to immature transiently amplifying neuroblasts (type-2a, type-2b, and type-3). MDMA-alone and the combination reduced the number of dividing type-1 and type-3 NPCs and cells that were not NPCs. These data indicate that amphetamines interfere with the division and migration of NPCs. Notably, the reduction in the number of NPCs and immature neurons were not associated with changes in cell death (via apoptosis) or granule cell neuron numbers, indicating that amphetamines selectively affected the generation and maturation of newly born granule cell neurons. In sum, our

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CDM, RRD and MAT designed the research and RRD and CDM wrote the manuscript. CDM and RRD performed the research. CDM and MAT contributed new reagents and analytic tools. CDM and RRD analyzed the data.

Ethical statement:
All animal experiments comply with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and is indicated in the manuscript that such guidelines have been followed.
The authors have no financial and personal relationships with other people or organizations that could inappropriately influence (bias) the current work.
The current work described here has not been published previously (except in the form of an academic thesis), and is not under consideration for publication elsewhere, and is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out.
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findings suggest that alterations in the cellular composition in the dentate gyrus during chronic exposure to amphetamines can effect neuroplasticity in the hippocampus and influence functional properties of hippocampal neurons.

Keywords

Granule Cell Neurons; Hippocampus; Ki-67; NeuroD1; MDMA; Methamphetamine

Introduction

Illicit drugs have posed serious political, social, and economic problems to countries around the world. Of these drugs, (±) 3, 4- methylenedioxymethamphetamine (MDMA), (±) 3,4-methylenedioxyamphetamine (MDA) and (+) methamphetamine (METH) all belong to a class of illicit substances known as amphetamine type psychostimulants. Although some amphetamines (e.g., d-amphetamine, d-methamphetamine), can be used therapeutically by clinicians to help treat disorders such as attention deficit hyperactive disorder and narcolepsy, most other amphetamines are only used recreationally (Teixeira-Gomes *et al.*, 2015). MDMA, MDA and METH all are psychoactive substances that can act as stimulants, euphorics, anorectics, entatogenics, and/or hallucinogenic agents (Carvalho *et al.*, 2012).

In the brain, a shared feature of MDMA, MDA, and METH is that they all act to release monoamines (dopamine, norepinephrine, serotonin) into the synaptic cleft through a multitude of pharmacological mechanisms (Lewin *et al.*, 2011; Miller, 2011). In addition these drugs can act as direct agonists to the alpha adrenergic and several serotoninergic receptors, interactions that are part of the basis underlying their physiological and psychological effects (Ray, 2010). Repeated use of amphetamines can have lasting detrimental consequences. For example, heavy MDMA use has been associated with changes in mental processing speed, impulsivity, mood and working memory (Wareing *et al.*, 2000; Parrott, 2012). METH is also known to impair both spatial and non-spatial working memory in humans and nonhuman primates, which suggests potential hippocampal neurotoxicity (Camarasa *et al.*, 2012; Groman *et al.*, 2012; Sofuoglu *et al.*, 2016; Zhong *et al.*, 2016).

Neurotoxicity by amphetamines as a function of decreased adult neurogenesis (a phenomenon that continuously generates newly born neurons in the subgranular zone of the dentate gyrus of the hippocampus (Altman, 1969)) has been reported in rodent studies. However, only a small body of research has been devoted to understanding the effect of these amphetamines on the development of neural progenitor cells (NPCs) that contribute to adult neurogenesis. Although it is poorly understood at present, cognitive changes associated with amphetamine use could be due in part to disruption of adult neurogenesis. New neurons come about as a result of either the division of neural stem cells or the division of early neural progenitor cells (NPCs; (Enikolopov *et al.*, 2015; Goncalves *et al.*, 2016)). Not all NPCs survive and mature into new neurons (Dayer *et al.*, 2003), but the ones that survive and mature form functional connections with pre-existing neural circuits (Toni *et al.*, 2008). Even though no functional significance can be attributed to individual neurons as they are

newly incorporated into circuits of the dentate gyrus, much research has gone into establishing correlations between the role of new neurons, such as increased neuronal survival in the dentate gyrus and learning and memory behaviors dependent on the hippocampus (Sahay *et al.*, 2011; Galinato *et al.*, 2017). Understanding how prevalently used amphetamines like MDMA, MDA and METH impact NPCs and their development could help further understanding of the neural basis of the cognitive deficits associated with heavy use.

In this context, METH exposure in rodents produces a hostile cellular environment in the progenitor pool (Yuan *et al.*, 2011). For example, METH reduces net proliferation of NPCs and number of immature neurons by reducing the number of proliferating preneuronal neuroblasts (type-2a cells; (Kronenberg *et al.*, 2003)) and increasing the number of proliferating preneuronal progenitor cells (type-3 cells; (Kronenberg *et al.*, 2003; Yuan *et al.*, 2011)), suggesting that a decrease in the number of progenitors and immature neurons, to a large degree, is attributable to the decrease in the ability of neuroblasts to divide and produce stable NPCs that survive as immature neurons (Teuchert-Noodt *et al.*, 2000; Mandyam *et al.*, 2008; Schaefers *et al.*, 2009; Venkatesan *et al.*, 2011; Yuan *et al.*, 2011; Baptista *et al.*, 2014). Parallel work in rodents demonstrates that MDMA affects net proliferation and survival of NPCs, suggesting that similar hostile cellular environment is observed in rodents after either METH or MDMA exposure (Hernandez-Rabaza *et al.*, 2006; Cho *et al.*, 2008; Garcia-Cabrerizo & Garcia-Fuster, 2016). However, these studies on toxic effects of amphetamines on NPCs are limited to rodent models. Therefore, the current study tested the hypothesis that MDMA, MDA and METH reduce net proliferation and survival of NPCs in the dentate gyrus of nonhuman primates, and these effects are attributed to altered division and migration of NPCs.

Materials and Methods

Animals

Fourteen male rhesus monkeys (Macaca mulatta; Chinese origin) participated in this study. Animals were ages 7–10 years old when euthanized. Daily chow (Lab Diet 5038, PMI Nutrition International; 3.22 kcal of metabolizable energy (ME) per gram) was modified individually by the veterinary weight management plan and ranged from 160 to 230 g per day for the animals in this study. Animals were individually housed in a controlled temperature environment (23.5°C), on a 12 h: 12 h light:dark cycle and fed in the home cage. The animals' normal diet was supplemented with fruit or vegetables seven days per week and water was available ad libitum in the home cage at all times. Animals on this study had previously been immobilized with ketamine (5–20mg/kg) no less than semiannually for purposes of routine care and some experimental procedures. Eleven animals also had various acute exposures to scopolamine, raclopride, methylphenidate, SCH23390, Δ9-THC, tetrahydrocannabinol nicotine, and mecamylamine in behavioral pharmacological studies similar to those described in (Taffe *et al.*, 1999; Taffe *et al.*, 2002; Katner *et al.*, 2004; Von Huben *et al.*, 2006; Taffe, 2012). These experimental drug treatments had been administered a minimum of one year prior to the start of MDMA, MDA and METH investigations, involved doses, substances and regimens not reported to have lasting effects on the measures

used here, and thus were not anticipated to have any bearing on the results of the current study. All protocols were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute (La Jolla). The United States National Institutes of Health guidelines for laboratory animal care were followed (Clark *et al.*, 1997).

Experimental Design

Drug naïve group—Hippocampal tissue from three animals that did not experience any amphetamine or drug treatment were used for analysis. Two left hemispheres and one right hemisphere were used for the current study. Brain tissue from the other hemispheres were used for another study published elsewhere (Taffe *et al.*, 2010).

Drug challenge group—For these studies, doses of MDMA (0.0, 0.56, 1.0, 1.78, or 2.4 mg/kg), MDA (0.56, 1.0, 1.78, 2.4 mg/kg) or METH (0.1, 0.32, 0.56, 1.0 mg/kg) were administered intramuscularly in a volume of 0.1 ml/kg saline. MDMA, MDA and METH were provided by the National Institute on Drug Abuse. Treatment was pseudorandomized within compound to the extent possible with the small sample size to minimize the impact of any potential order effects. Generally, the MDMA studies were conducted first, MDA second, and METH last; however, there was some degree of overlap of the schedule across compounds. Animals were injected via brief physical restraint using the moveable back of the home cage, a procedure to which they are well accustomed. All animals remained in home cage for the duration of the study. The dose range was based on pill content analyses suggesting ~75–125 mg MDMA per “Ecstasy” pill thus 1–1.78 mg/kg MDMA for a single pill taken by the standard 70kg person but as much as 2.5 mg/kg in a 50 kg woman or as little as 0.83 mg/kg in a 90 kg man. Relevant dose ranges for MDA and meth were determined initially by reference to MDMA:MDA and MDMA:meth ratios in pills analyzed by ecstasydata.org. These ranges were further refined based on pilot studies conducted for this and other projects and taking into consideration the minimum dose threshold for lasting or neurotoxic effects. All challenges were administered in the middle of the light cycle, with active doses separated by 1–2 weeks. Animals were visually observed for a period of two hours following injections and efforts were made to minimize noise and excitement in rooms during these intervals. Normal daily activity such as afternoon feedings and interactions with other animals not on the study resumed after the two hour interval. Details on the amphetamine regimen and behavioral studies conducted on the animals used in the current study are reported elsewhere (Crean *et al.*, 2006; Taffe *et al.*, 2006; Crean *et al.*, 2007; Von Huben *et al.*, 2007).

Tissue Preparation

After completion of the studies, monkeys were immobilized with 10 mg/kg ketamine/5 mg/kg xylazine and euthanized by 10 mg/kg pentobarbital followed by transcardial perfusion with ice-cold PBS. Brains were hemisectioned, transported on dry ice, and frozen at −80°C. Left or right hemispheres were used for histological analysis (control group: two left hemispheres and one right hemisphere; amphetamine groups: all left hemispheres). The frozen brain hemisphere was thawed on ice to −20°C, and blocks of brain tissue containing the hippocampal formation were dissected and processed for immunohistochemistry (Taffe *et al.*, 2010). Hippocampal-enriched blocks were immediately immersed in cold, freshly

prepared paraformaldehyde solution (pH 7.4) and left to post-fix for 5–7 days at 4°C. Blocks of brain tissue were then cryoprotected in 30% sucrose, after which they were sectioned coronally on a freezing microtome into 40 μm sections. Hippocampal sections (about 250 sections in total for each monkey) were serially collected in six wells and stored in PBS containing sodium azide (0.1%). Nine sections per animal (approximately every 30^{th} section through the hippocampus), each containing different depths of the hippocampus were mounted on slides and were air dried and processed for immunohistochemistry (Perera *et al.*, 2007; Taffe *et al.*, 2010). Slide mounted sections were processed for each of 3 biomarkers, Ki-67, NeuroD, and activated caspase 3 (AC-3) and for the two sets of co-labeling analysis. All sections were mounted and stained by an observer blinded to the study.

Antibodies and Immunohistochemistry

The following primary antibodies were used for immunohistochemistry (IHC): rabbit anti-Ki-67 (1:500, Neo Markers), goat anti-NeuroD1 (1:500, SantaCruz Biotechnology), rabbit anti-AC-3 (1:500, Cell Signaling), goat anti-Sox-2 (SRY (sex determining region Y)-box 2, 1:50; Santa Cruz Biotechnology), chicken polyclonal anti-GFAP (glial fibrillary acidic protein, 1:500; Abcam). The sections used for IHC were pretreated, blocked and incubated with the aforementioned antibodies followed by biotin-tagged secondary antibodies (Taffe *et al.*, 2010).

Microscopic Analysis

Immunoreactive cells in the SGZ (i.e., cells that touched and were within three cell widths inside and outside the hippocampal granule cell- hilus border) were quantified by an observer blinded to the animal groups with a Zeiss Axiophot photomicroscope (x60 magnification) using manual counting for positive cells. Area measurements were done via contouring in the optical fractionator setting of SteroInvestigator. These techniques were both used to quantify the amount of cells per mm2 for Ki-67, Neuro D1, and AC-3.

For type-1, type-2a, type-2b, and type-3 phenotype analysis, five sections through the hippocampus were triple-labeled with one of the combinations GFAP/Sox2/Ki-67 or Sox2/NeuroD1/Ki-67. All Ki-67-IR cells in the SGZ, approximately 15 Ki-67-IR cells from each monkey, were scanned and analyzed for $Ki\text{-}67^{+}/Sox2^{+}/GFAP^{+}$ (type-1), $Ki\text{-}67^{+}/Sox2^{+}/NeuroD1^{-}$ (type-2a), $Ki\text{-}67^{+}/Sox2^{+}/NeuroD1^{+}$ (type-2b), $Ki\text{-}67^{+}/Sox2^{-}/NeuroD1^{+}$ (type-3), or Ki-67-alone labeling (Taffe *et al.*, 2010). All labeling was visualized and analyzed using a confocal microscope (LaserSharp 2000, version 5.2, emission wavelengths 488, 568, and 647 nm; Bio-Rad Laboratories).

Measurement of hippocampal granule cell number was performed as previously described (Taffe *et al.*, 2010). Quantitative analysis to obtain unbiased estimates of the total number of hippocampal granule layer cell bodies was performed on a Zeiss AxioImagerA2 Microscope equipped with MicroBrightField (Colchester, VT) Stereo Investigator software, a three-axis Mac 5000 motorized stage (Ludl Electronics Products, Hawthorne, NY), a digital MRc video camera (Zeiss, Thornwood, NY), PCI color frame grabber, and personal computer workstation. All 40 μm sections from the hippocampus were saved in strict anatomical order. Systematic random sampling of the hippocampus consisted of a one-in-thirty section

analysis, and nine sections were analyzed per monkey. All sections for quantitative analysis were counterstained with Nuclear Fast Red, and all portions of the granule cell layer were examined. Live video images were used to draw contours delineating the dentate gyrus granule cell layer. All contours were drawn at low magnification using a Zeiss Neoflaur 5x objective, numerical aperture 0.15. Granule cell layer area was measured. After determination of mounted section thickness, *z*-plane values, and selection of contours, an optical fractionator analysis was used to determine hippocampal granule cell neuron number. A counting frame of appropriate dimensions denoting forbidden and nonforbidden zones (10 μm × 10 μm × 2 μm counting grid, and a 2 μm top and bottom guard zone) was superimposed on the video monitor, and the optical fractionator analysis was performed using a Zeiss Plan Apochromat 63x oil objective, numerical aperture 1.4 and a 1.4 auxillary condenser lens. Cells were identified as neurons based on standard morphology, and only neurons with a focused nucleus within the nonforbidden regions of the counting frame were counted. Over 500 cells per animal were counted. The total number of granule cells is presented as the average density of cells (cells/μm^3). Granule cell number estimates were made by an observer blind to the study.

Data Analysis

One way randomized block analysis of variance (ANOVA) was employed to evaluate significance in differential expression of Ki-67, NeuroD1, and AC-3, between controls and either MDMA only exposure group or controls and MDA/MDMA/METH exposure group. Total granule cell neuron differences between controls and drug exposure groups also were analyzed by one way ANOVA. Dunnett's post hoc test was applied after each one way ANOVA analysis. Values of $p < 0.05$ were considered statistically significant. Two way ANOVAs with treatment and cell type as a between-subject factor was performed to determine effects of amphetamine groups on co-labeling analysis. Following significant interaction ($p<0.05$), post hoc comparisons were performed to determine individual differences. All analysis was done on GraphPad Prism (v. 6; GraphPad Software, Inc, San Diego CA).

Results

Doses of amphetamines administered to subjects do not differ significantly

The amount of amphetamines administered to each subject in the study did not significantly differ, and the average amount of MDMA administered to all eleven subjects (in both amphetamine exposure groups) was 17.08 (SEM: 1.75) mg/kg (Table 1). The average amount of MDMA administered to five MDMA only exposed animals was 15.02 (1.72) mg/kg (Table 1). The average amount of MDMA administered to six MDA/MDMA/METH animals was 18.80 (2.81) mg/kg (Table 1). The average amount of MDA administered to six MDA/MDMA/METH animals was 7.54 (1.25) mg/kg (Table 1). The average amount of METH administered to six MDA/MDMA/METH animals was 1.55 (0.37) mg/kg (Table 1).

Amphetamines significantly decrease proliferation of NPCs in the dentate gyrus

Ki-67 cell density (cells/mm2) was calculated by dividing the total number of Ki-67 immunoreactive cells per animal by total granule cell layer area per animal. One way

ANOVA analysis confirmed that animals exposed to MDMA and animals exposed to MDA/MDMA/METH had significantly lower number of Ki-67 cells ($F(2,11)= 15.03$, $p=0.0007$) compared to drug naïve controls. MDMA exposure caused an 82% decrease in the number of Ki-67 cells in comparison with controls while MDA/MDMA/METH exposure caused an 87% decrease in the number of Ki-67 cells (Figure 1). Dunnett's post hoc analysis further confirmed a significant effect of MDMA and MDA/MDMA/METH ($p<0.01$, and $p<0.002$ respectively).

Amphetamines significantly decrease maturation of NPCs in the dentate gyrus

NeuroD1 cell density (cells/mm2) was calculated by dividing the total number of NeuroD1 immunoreactive cells per animal by total granule cell layer area per animal. One way ANOVA analysis confirmed that animals exposed to MDMA and animals exposed to MDA/MDMA/METH had significantly lower number of NeuroD1 cells ($F(2,11)= 5.594$, $p=0.0211$). MDMA exposure caused a 55% decrease in NeuroD1 expression in comparison with controls while MDA/MDMA/METH exposure caused a 63% decrease in NeuroD1 expression (Figure 1). Dunnett's post hoc analysis further confirmed a significant effect of MDMA and MDA/MDMA/METH exposure on the number of NeuroD1 cells when compared to controls ($ps<0.05$).

Amphetamines do not significantly increase apoptosis in the dentate gyrus

Cell density of AC-3 (cells/mm2) was calculated and data from each animal was averaged amongst its treatment group (Figure 1). One way ANOVA analysis confirmed that animals exposed to MDMA and animals exposed to MDA/MDMA/METH did not have significantly different levels of caspase activation ($F(2,11)= 0.3246$, $p=0.7295$).

Amphetamines do not significantly alter area of the granule cell layer and total number of granule cell neurons in the dentate gyrus

Area of the granule cell layer was determined for each section used for quantification of granule cell neurons. One way ANOVA did not detect a significant difference in the area between groups (area in mm^2: Controls, 5.8 ± 0.28; MDMA only, 6.8 ± 0.64; MDMA/MDA/METH 6.2 ± 0.42; ($F(2,11)= 0.84$, $p=0.45$). Total granule cell neurons in the dentate gyrus was calculated and data from each animal was averaged among its treatment group (Figure 1). One way ANOVA analysis did not confirm significantly different numbers of total granule cells in animals exposed to MDMA and animals exposed to MDA/MDMA/METH ($F(2,11)= 0.9815$, $p=0.4053$).

Decreased number of NPCs is attributable to distinct effects on types of actively dividing hippocampal NPCs during their later phases of neuronal development

Using endogenous markers of radial glia-like precursors, proliferating progenitors, and differentiating progenitors, the types of actively dividing NPCs through the phases of neuronal development were identified and quantified (Taffe *et al.*, 2010). For example, Ki-67 was used to mark actively dividing type-1, type-2a, type-2b, and type-3 NPCs. GFAP was used as a stem-like marker to distinguish between type-1 and type-2a NPCs (Steiner *et al.*, 2006; Taffe *et al.*, 2010; Yuan *et al.*, 2011), in which type-1 cells express GFAP and type-2

do not. Sox2 was used as a marker to distinguish between preneuronal type-2 and neuronal type-3 NPCs (Steiner *et al.*, 2006; Taffe *et al.*, 2010), in which type-2 cells express Sox2 and type-3 cells do not. NeuroD1 was used to distinguish type-2a and type-2b NPCs (Steiner *et al.*, 2006; Taffe *et al.*, 2010), in which type-2b express NeuroD1 and type-2a do not. Therefore, using a novel combination of endogenous markers—GFAP, Sox2, Ki-67, and NeuroD1—and the current neurogenesis model for the initial phases of neuronal development of dividing NPCs (Steiner *et al.*, 2006; Taffe *et al.*, 2010), progenitors were distinctly labeled as type-1, type-2a, type-2b, and type-3. Specifically, actively dividing type-1 progenitors were identified as $GFAP^+/Sox2^+/Ki\text{-}67^+$ cells. Actively dividing type-2a progenitors were identified as $Sox2^+/Ki\text{-}67^+/NeuroD1^-$ cells. Actively dividing type-2b progenitors were identified as $Sox2^+/Ki\text{-}67^+/NeuroD1^+$ cells. Actively dividing type-3 progenitors were identified as $Sox2^-/Ki\text{-}67^+/NeuroD1^+$ cells (Figure 2). A total of 62 ± 7 Ki-67-IR cells from controls, 18 ± 2 cells from MDMA group and 19 ± 3 cells from MDA/MDMA/METH group were analyzed for triple labeling analysis (Figure 2). Two-way ANOVA indicated a significant amphetamine treatment x cell type interaction $F(8, 55) = 3.382$, $p = 0.0032$; main effect of amphetamines $F(2, 55) = 23.45$, $p < 0.001$; and main effect of cell types $F(4, 55) = 25.22$, $p < 0.001$. Post hoc analysis revealed significant reduction in the number of Ki-67 cells that were type-1 and type-3 NPCs and non NPCs in the amphetamine groups compared with controls (Figure 2). Analysis of the proportion of Ki-67-IR cells within each experimental groups indicated that a significant number of Ki-67-IR cells were type-1, -2, and -3 NPCs (56.4% in controls *vs.* 47.5% in the MDMA group and 43.6% in the MDA/MDMA/METH group; $p > 0.05$, one way ANOVA; Figure 2).

Discussion

The goal of the present study was to confirm the findings of decreased NPCs and neurogenesis in the hippocampus resulting from amphetamine insult reported in rodents in a higher order organism (Teuchert-Noodt *et al.*, 2000; Hernandez-Rabaza *et al.*, 2006; Cho *et al.*, 2008; Mandyam *et al.*, 2008; Schaefers *et al.*, 2009; Venkatesan *et al.*, 2011; Yuan *et al.*, 2011; Baptista *et al.*, 2014; Garcia-Cabrerizo & Garcia-Fuster, 2016). Many studies in the field have focused on understanding the effects of amphetamines on neurotransmitters and neurotransmitter induced neurotoxicity, but very few have sought to understand neurotoxicity as a function of altered neurogenesis. Furthermore, neurogenesis in the adult mammalian hippocampus is an important mediator of relapse to methamphetamine seeking behavior (Galinato *et al.*, 2017; Galinato *et al.*, 2018). Therefore, the purpose of the current investigation of hippocampal NPCs was to extend earlier research to organisms that are closer evolutionarily to human as well as to further explicate the factors involved in hippocampal plasticity subsequent to stimulant exposure.

By using model organisms in the adolescent and young adult developmental stages (i.e., age matched with humans who typically take MDA/MDMA/METH), a close model for understanding the effects of psychostimulants on the human hippocampus can be established (Crean *et al.*, 2006; Crean *et al.*, 2007). Furthermore, the doses of amphetamines administered to the macaques in each of their respective experimental groups mimicked some aspects of human consumption. For example, the average MDMA dose administered to all animals in both of the experimental groups over a period of 9 to 10 months was 17.08

+ 1.75 mg/kg. Ecstasy pills seized and analyzed by ecstasydata.org showed that ~75 to 125mg of MDMA existed in an individual pill. This corresponds to 1–1.78 mg/kg for the average 70kg human user (Tanner-Smith, 2006; Von Huben *et al.*, 2007). The amount of MDMA administered to subjects in the present study ranged from 1.7–5mg/kg, with changes in dose after each couple administrations. This range of MDMA doses is comparable to those generated in humans after consuming ecstasy pills and, combined with the fact that they were given to animals over a 9.6 month period, this represents a model for club-going recreational use rather than addiction (Rothe et al., 1997; Huang et al., 2003; Parrott, 2004). The experimental group exposed to MDA, MDMA, and METH was critical to include since human club drug users often (knowingly or unknowingly) use different amphetamines either in conjunction (see ecstasydata.org) or separately over a period of time (Fendrich *et al.*, 2008; Halkitis *et al.*, 2008; Daskalopoulou *et al.*, 2014; Hunter *et al.*, 2014). The inclusion of both types of exposure groups, MDMA only and MDA/MDMA/METH, permits a broader inference about the effects of repeated amphetamine use and subsequent effects on NPCs in the hippocampus to help us better understand effects on the human hippocampus.

The majority of prior related studies in nonhuman primates have focused on neurotransmitter-related neurotoxicity and have found that both MDMA and METH are neurotoxic to serotonergic and dopaminergic nerve terminals (Ricaurte *et al.*, 2002; Madden *et al.*, 2005; Ricaurte *et al.*, 2005; Castner & Williams, 2007; Fantegrossi, 2007). The present study provides the first evidence of neurotoxicity by amphetamines in the hippocampus in nonhuman primates in confirmation of recent rodent studies that have found altered neurogenesis in the hippocampus of adult animals (Teuchert-Noodt *et al.*, 2000; Hernandez-Rabaza *et al.*, 2006; Cho *et al.*, 2008; Mandyam *et al.*, 2008; Schaefers *et al.*, 2009; Venkatesan *et al.*, 2011; Yuan *et al.*, 2011; Baptista *et al.*, 2014; Garcia-Cabrerizo & Garcia-Fuster, 2016). For example, in the present study it was found that amphetamines reduce NPCs expressing Ki-67 and NeuroD1 by greater than 80 and 50%. Ki-67 is a transcription factor expressed during multiple phases of the cell cycle, and is absent in quiescent cells (Scholzen & Gerdes, 2000). Therefore, reduction in the number of Ki-67 labeled NPCs suggests depletion of the pool of progenitors available for cell division. Mechanistically, several pathways may be involved in amphetamine's effect on Ki-67 labeled NPCs. For example, amphetamines could alter how proliferating progenitors cycle through the cell cycle (e.g. kill NPCs by producing cell cycle arrest). Cell cycle kinetic studies in adult rats that experienced chronic METH does not support this hypothesis as METH did not alter the synthesis phase of the cell cycle (Yuan *et al.*, 2011). While the limitation of the current study is that this was not accessible, the current study determined whether a particular stage of NPC development was altered by amphetamines. For instance, Ki-67 labeled NPCs in the postnatal hippocampus are not homogeneous, and represent cells in developmental stages that progress in parallel, including cycling cells that are radial glia-like (type 1), preneuronal (type-2a), intermediate (type-2b), and early neuronal (type-3) (Kronenberg *et al.*, 2003; Steiner *et al.*, 2006; Taffe *et al.*, 2010; Yuan *et al.*, 2011). Therefore, amphetamines could be altering a distinct type of NPC. Study conducted in rats revealed that METH reduced the percent of NPCs that were type-2a and enhanced the percent of NPCs that were type-3, indicating an alteration in the population of NPCs in the dentate gyrus (Yuan *et al.*, 2011). Our findings in the nonhuman primate hippocampus demonstrates that MDMA and

MDMA/MDA/METH reduced the number of Ki-67 cells that were type-1 and type-3 NPCs (almost wiped out these cells) and non NPCs. Our findings also indicate that amphetamines did not alter the number of Ki-67 cells that were type-2 NPCs in the dentate gyrus. A potential limitation in the interpretation of these results is that triple labeling studies were conducted with markers expressed in distinct stages of development for examining type-2 cells. While we are confident with the proportion of proliferating cells in each stage of development examined, quadruple labeling of these markers may have been optimal for examining type-2a and type-2b progenitors. Nevertheless, these findings suggest that a combination of amphetamines produced a higher neurotoxic environment in the dentate gyrus by depleting radial glia-like stem cells and early neuronal cells. Overall the reduction in the number of NPCs, in certain cell types contributed to the reduced number of NeuroD1 cells in the dentate gyrus. Future studies would be required to determine any detrimental effects of the depletion of early stem-like precursor cells and immature neurons in the hippocampus on hippocampus dependent behaviors. Both MDMA and MDMA/MDA/METH significantly reduced the number of Ki-67 cells that were non NPC (i.e., neither of the cell types examined). The phenotype of these cells could be endothelial, oligodendroglial and astrocytic cell types (Perera *et al.*, 2007), and a reduction in these cells could alter the cellular composition of the dentate gyrus and perturb the neurogenic niche (Morrens *et al.*, 2012; Ehret *et al.*, 2015). Altogether, the present data indicate that chronic amphetamine exposure decreases hippocampal neurogenesis by initially altering the precursor cell pool, followed by altering the transition of developmental stages of proliferating progenitors from NPCs to immature neurons and altering the neurogenic niche. Whether the altered type-1 and -3 cells of proliferating progenitors harbor specific receptors or neurochemicals that are exclusive to amphetamine's neuropharmacology is yet to be determined and will be a highly productive pursuit for future studies.

Studies in rodent models have indicated that METH reduces proliferation and neurogenesis in the dentate gyrus through increased cell death (Yuan *et al.*, 2011). Active programmed cell death (apoptosis) was analyzed in the present study. There was no increase in apoptosis found in either amphetamine-exposed group in this study at the time point examined. These data indicate that changes in apoptosis may have occurred at an earlier time point during amphetamine experience, or are short-lived, and NPCs may die through other cell death pathways. We also investigated whether the alteration in the pool of NPCs produces changes in the number of granule cell neurons mostly generated during development in the dentate gyrus. Our results do not show a significant change in the number of preexisting granule cell neurons in the amphetamine groups, suggesting that the effect of amphetamines were more potent on newly born granule cell neurons. Few reasons could contribute to a loss in newly born granule cells without significantly altering the preexisting population of granule cells. First, studies from rodent models show that the number of newly born granule cells is about 6% of the total population of granule cells in the dentate gyrus (Cameron & McKay, 2001). This relationship may be true in nonhuman primates (Gould *et al.*, 2001), and therefore a loss in cell numbers in newly born neurons may not entirely translate to loss in cell numbers of preexisting granule cell neurons. Second, the hippocampus is sensitive to changes in levels of glucocorticoids, and increased levels of glucocorticoids has been associated with reduction in newly born and preexisting granule cell neurons in rodents and nonhuman

primates (Sapolsky *et al.*, 1985; Gould *et al.*, 1991; Coe *et al.*, 2003; Cinini *et al.*, 2014). These studies suggest that factors other than the drug itself could produce profound effects on newly born and preexisting granule cell neurons in the dentate gyrus. Nevertheless, given that emerging studies are indicating functional roles for newly born neurons in behaviors dependent on the hippocampus (McAvoy *et al.*, 2015; Galinato *et al.*, 2017; Galinato *et al.*, 2018), it will be critical to determine if the reduction in NPCs and neurogenesis in nonhuman primates will correlate with hippocampus dependent memory deficits in the context of chronic amphetamine experience.

Collectively, this study proposes that administering repeated intermittent doses of amphetamines to young adult nonhuman primates results in significant reductions in both proliferating NPCs and immature neurons, to an extent that the integrity of the hippocampus may be compromised. It was also shown that decreases in neurogenesis were not directly associated with increased apoptosis at the time point examined; so it is most likely that other forms of cell death play a role in regulating amphetamine-induced depletion of progenitor pool.

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Highlights

- Amphetamines reduce proliferation and immature neurons in the nonhuman primate hippocampus
- Reduced number of progenitors is due to alterations in the proportion of neural progenitor cells undergoing cell division
- Reduced number of progenitors is not related to enhanced apoptosis or loss of granule cell neurons in the hippocampus

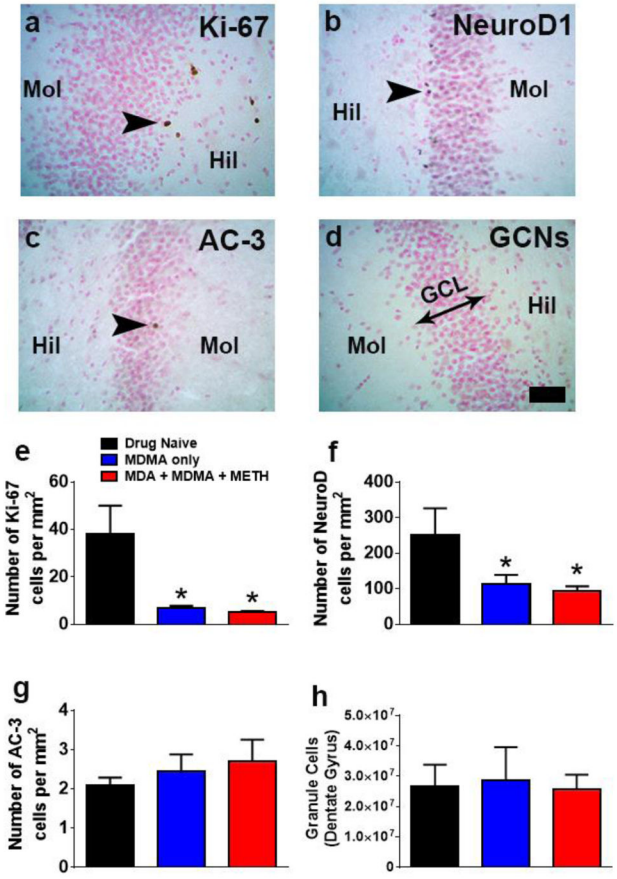

Figure 1.

(a–d) Photomicrographs of immunoreactive cells labeled with Ki-67 (a), NeuroD1 (b), AC-3 (c), and nuclear staining FastRed (d). Scale bar in d is 20μm, applies a–d. Hil, hilus; Mol, molecular layer; GCL, granule cell layer; GCNs, granule cell neurons. (e–h) Quantitative analysis of immunoreactive cells stained for Ki-67 (e), NeuroD1 (f), AC-3 (g) and granule cell neurons (h). Data is expressed as mean cells/mm2 ± SEM (control n=3, MDMA only n=5, MDA/MDMA/METH n=6). *$p<0.05$ compared with drug naïve controls.

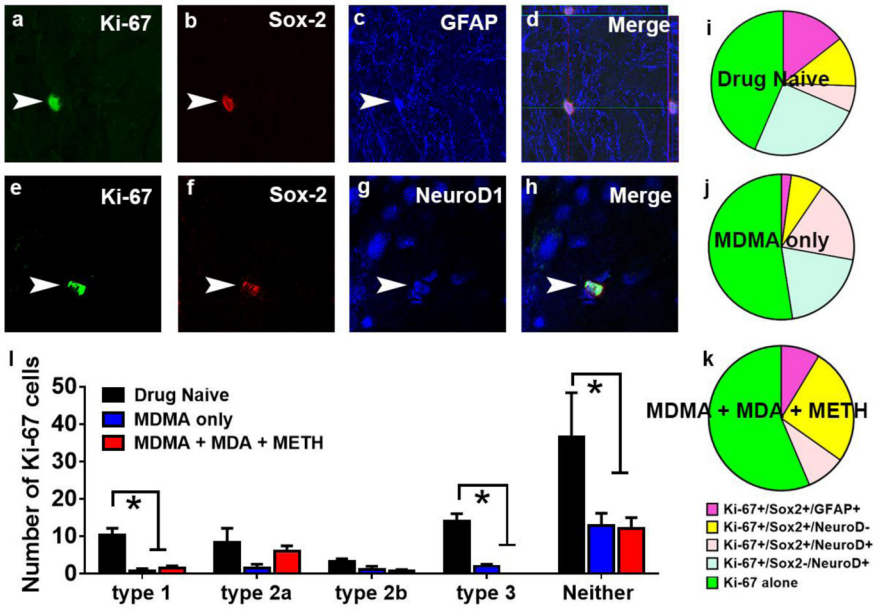

Figure 2.

(a–h) Photomicrographs of confocal images of two sets of triple labeling analysis. (a–d) Ki-67/Sox-2/GFAP triple labeling, with Ki-67 in green (a, FITC), Sox-2 in red (b, CY3), GFAP in blue (c, CY5) and merged orthogonal view of the cell (d). (e–h) Ki-67/Sox-2/GFAP triple labeling, with Ki-67 in green (e, FITC), Sox-2 in red (f, CY3), NeuroD1 in blue (g, CY5) and merged view of the cell (h). (i–k) Pie charts of distribution of types of NPCs labeled with Ki-67. (l) Quantitative analysis of the number of Ki-67 cells that were either type-1, type-2a, type-2b, type-3 cells or neither cell type. Data is expressed as mean ± SEM (control n=3, MDMA only n=5, MDA/MDMA/METH n=6). *p<0.05 compared with drug naïve controls.

Table 1

Experimental Design and Average Dose Administration per Experimental Group

Total amphetamine doses over 9.6 months				
Treatment	**MDA**	**MDMA**	**METH**	**Citations**
MDMA ONLY	-	15.6 mg/kg	-	(Taffe *et al.*, 2006; Von Huben *et al.*, 2007)
MDMA ONLY	-	15.6 mg/kg	-	
MDMA ONLY	-	20.6 mg/kg	-	
MDMA ONLY	-	10.1 mg/kg	-	
MDMA ONLY	-	13.2 mg/kg	-	
AVERAGE:	N/A	15.02 mg/kg	N/A	
MDA, MDMA, METH	6.78 mg/kg	12.12 mg/kg	2.84 mg/kg	(Crean *et al.*, 2006; Crean *et al.*, 2007)
MDA, MDMA, METH	5 mg/kg	11.78 mg/kg	0.32 mg/kg	
MDA, MDMA, METH	5 mg/kg	15.27 mg/kg	1.94 mg/kg	
MDA, MDMA, METH	10 mg/kg	21.55 mg/kg	1.30 mg/kg	
MDA, MDMA, METH	12.52 mg/kg	29.06 mg/kg	2.04 mg/kg	
MDA, MDMA, METH	5.96 mg/kg	23.06 mg/kg	0.84 mg/kg	
AVERAGE:	7.54 mg/kg	18.80 mg/kg	1.55 mg/kg	

A total of 14 young adult rhesus macaque monkeys, age 7–10 years, were used in this experiment. Three of the monkeys were used as controls, five were exposed to doses of MDMA, and six were exposed to doses of MDA, MDMA, and METH. Over the span of 9.6 months, 11 monkeys were given doses of either MDMA only or MDA, MDMA, and methamphetamine according to their respective experimental group. Monkeys given only MDMA received an average dose of 15.02 mg/kg over the 9.6 month period while monkeys given MDA, MDMA, and METH were given doses of 7.54 mg/kg, 18.80 mg/kg, and 1.55 mg/kg respectively in a 9.6 month period. MDMA was administered intramuscularly and doses of 0.00, 0.56, 1.0, 1.78, or 2.4mg/kg in an injection volume of 0.1 ml/kg with saline were separated by 1–2 weeks in a pseudorandomized order. MDMA, MDA, METH were administered intramuscularly and doses of MDMA (0.00, 0.56, 1.0, 1.78, or 2.4mg/kg), MDA (0.00, 0.56, 1.0, 1.78, or 2.4mg/kg) and METH (0.1, 0.32, 0.56, 1.0 mg/kg) were given in an injection volume of 0.1 ml/kg with saline. Similar to the MDMA only group, treatment order was pseudorandomized in the MDMA/MDA/METH group and randomized within compound to the extent possible to minimize the impact of any potential order effects. Generally, the MDMA studies were conducted first, MDA second and the METH last; however, there was some degree of overlap of the schedule across compounds.