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Chronic administration of psychostimulants reduces hippocampal neurogenesis in young adult non human primates

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by

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EPIGRAPH

Think of scientific knowledge and the progress it begets as the radius of a circle.

The more that you learn about science, the longer your radius becomes.

However, as your radius gets longer, so does the area of the circle,

and that area is everything that science doesn't know and hopes to prove.

Never stop asking questions and embrace the area of the unknown.

Dr. Stefano Gentile PhD

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ABSTRACT OF THE THESIS

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Psychostimulants such as methamphetamine, MDMA, MDA have been used as treatment options for attention deficit disorders and narcolepsy; however, the vast majority of users take such amphetamines recreationally. Pharmacodynamic studies

show that chronic exposure to these drugs produces neurotoxicity which is hypothesized to promote and perpetuate addiction to the drugs. Though monoamine neurotoxicity as a function of cell death is valid for psychostimulant addiction, neurotoxicity by these drugs as a function of decreased neurogenesis in the dentate gyrus of hippocampus can be valid for relapse to psychostimulant addiction. Recent studies have found that both MDMA and methamphetamine significantly reduce levels of neurogenesis in the hippocampus of adult rodents. However, whether the detrimental effects of psychostimulants on developmental stages of neurogenesis are limited to rodent brain or occur in non human primates is unknown and was the focus of the current investigation. Our study investigated the levels of developmental stages of neurogenesis, cell death and the cell density of granule cell neurons after MDMA or MDMA in combination with MDA and methamphetamine exposure in young adult macaque monkeys in hopes to model the effects of these drugs on the young adult human hippocampus. Results from quantitative immunohistochemical analysis show that the two treatment conditions over 9.6 months causes > 80% decrease in the number of Ki-67 (neural progenitor) cells, and > 50% decrease in the number of Neuro D (immature neuron) cells, indicating a neurotoxic environment in the neurogenic niche in the hippocampus by MDMA alone or in combination with other amphetamines.. In sum, our findings suggest that alterations in the cellular composition in the hippocampus during exposure to illicit drugs can promote maladaptive plasticity of hippocampal neurons during withdrawal, which may enhance relapse to drug seeking behaviors.

INTRODUCTION

Drugs of abuse have posed serious political, social, and economic problems to countries around the world. Of these drugs, 3,4methylenedioxymethamphetamine (MDMA), 3,4 methylenedioxyamphetamine (MDA) and methamphetamine (METH) all belong to a class of illicit substances known as amphetamine type psychostimulants. Although amphetamines, such as d-amphetamine, can be used therapeutically by clinicians to help treat disorders such as attention deficit hyperactive disorder and narcolepsy, most other amphetamines are used recreationally (Teixeira-Gomes et al 2014). MDMA, MDA and meth all are psychoactive substances that can act as stimulants, euphorics, anorectics, entatogenics, and/or hallucinogenic agents (Carvalho et al 2012).

According to the World Drug Report 2014 spearheaded by the United Nations Office of Drugs and Crime (UNODC), amphetamine type psychostimulants (ATS) are the second most commonly used illicit substance in the world with an estimated 13.94 million to 54.81 million users (UNDOC World Drug Report 2014 pg 2). Due to the ease of meth synthesis and its high profit margins (Kunalan et al 2009) meth manufacturing has hit an all time high. UNDOC reports that the amount of meth seized in Mexico increased from 341 kg in 2008 to 44,000 kg in 2012. In the United States 29,000 kg were seized in 2012 compared to 9,500 kg in 2008 (UNDOC WR 2014 pg 47). Meth is mainly used by impoverished populations while MDMA is most commonly used as a club drug. Although MDMA isn't as addictive as meth, increases in the number of users seeking treatment in the United Kingdom have been observed (UNDOC WR 2014 pg 67).

A shared feature of MDMA, MDA, and meth is that they all act to release large amounts of neurotransmitter into the synaptic cleft through a multitude of pharmacological mechanisms. First, MDA, MDMA, and meth can act as agonists to the TAAR1 receptor and cause an increase of dopamine, serotonin and norepinephrine release into the synaptic cleft. (Miller et al 2011;Lewin et al 2011). For example, when presynaptic neurons express the TAAR1 receptor, activation of these receptors causes the high concentrations of monoamine neurotransmitter in the synaptic cleft which induces activation of expressing receptors for dopamine, serotonin, and norepinephrine (Miller et al 2011). Secondly, these amphetamine type psychostimulants can also act to alter vesicular monoamine transporter function (VMATs) and disallow neurotransmitter uptake into the vesicles and therefore increase intracellular concentration of these monoamines (Eiden et al 2011). This effect can lead to monoamine induced neurotoxicity, an effect that has been correlated to addiction profile of the psychostimulants (Little et al 2003). Third, MDA, MDMA, and meth act as monoamine oxidase inhibitors as well as monoamine reuptake inhibitors, which again lead to a high concentration of neurotransmitter in the synaptic cleft (Miller et al 2011). Lastly, these drugs can act as direct agonists to the alpha adrenergic and various serotoniergic receptors in many different areas of the brain, interactions that are part of the basis behind their physiological and psychological effects (Manzoni et al 2010).

Pharmacokinetics studies done in MDMA pharmacokinetics have revealed that MDMA reaches peak blood concentration 1-3 hours after oral ingestion (De La Torre 2000). Breakdown of MDMA is catalyzed by hepatic enzymes CYP2D6 and CYP3A4 as well as COMT, and after 24 hours 65% is still excreted as MDMA while about 7% is

excreted as a metabolite, MDA (Verebey 1988). On the other hand, when meth is orally ingested, it reaches its peak blood concentration 3-6 hours post ingestion. Additionally, the chemical structure of meth is lipophillic, so it readily passes thru the blood brain barrier and reaches the brain faster than other amphetamines. Therefore, the combination of these pharmacokinetic characteristics of meth leads to toxic outcomes of the drug on the brain. Meth is also broken down by the same CYP2D6 and CYP3A4 enzymes which aid in the renal excretion of METH. After 24 hours, 41% of METH is excreted in urine while 17% is broken down by the CYP enzymes into amphetamines (METH metabolites) (Schep 2010). Therefore, it is tempting to speculate lower amounts of METH in circulation coupled with higher amounts of neurotoxic metabolite may mean more neurotoxic effects after exposure to METH. Furthermore studies have shown that either administering MDMA or meth in higher doses or administering both meth and MDMA at the same time increases blood concentration of the either drug disproportionately by slowing down elimination of both drugs (Fuchigami et al 2013). Taken together, these pharmacokinetic studies suggest that oral ingestion of psychostimulants such as MDMA and METH are harmful to the body and the brain and support a neurotoxic environment which could assist with the process of addiction to the drug.

Pharmacodynamic studies have demonstrated that neurotoxicity is a broad term that can be categorized as either the result of neuroanatomical changes to the CNS, or functional maladaptive changes in the CNS that cause behavioral defects. In rats, studies have found evidence that suggests that MDMA is neurotoxic and induces neuronal cell death through the release of cytchrome C which activates the caspase signal cascade (Jiménez et al., 2004). Subsequent data has shown that imbalance between reactive

oxygen species (ROS) and internal cellular antioxidants illustrate a potential mechanism as to why MDMA-induced cytochrome C is being released and triggering apoptotic pathways, resulting in neurotoxicity (Franco and Cidlowski 2012). In experiments done in rats, daily doses of MDMA were shown to cause reductions in levels of 5-HT (serotonin), 5-HIAA (serotonin's metabolite), serotonin receptors, and serotonin rate limiting enzyme (tryptophan hydroxylase) (Capela et al., 2009; Lyles and Cadet, 2003; Ricaurte et al., 2000). MDMA is also responsible for structural damage to serotonergic axon terminals in the forebrain after only two injections per day for four days (O'Hearn et al 1988). These results demonstrate a positive link between neurotoxicity and serotoninergic neurons, whereby MDMA exposure leads to toxicity and eventual death of serotonergic neurons.

Pharmacodynamic studies have indicated that METH also induces structural damage to dopaminergic axon terminals in the striatum and nucleus accumbens just 3 weeks after administration of the drug (Ricaurte et al., 1982). Repeated meth administration to rats caused increases in dopamine levels in the synaptic cleft and in the presynaptic neurons as well as dopamine uptake levels in the striatum compared to controls (Wagner et al., 1980). Excess dopamine in dopaminergic neurons is known to cause oxidative stress and cell death (Miyazaki 2008). Meth was found to also activate the caspase signaling cascade via enhanced release of cytochrome C and enhanced ROS resulting in apoptosis (Huang et al., 2015). Taken together, both MDMA and meth have been shown through many modes of experimentation to have detrimental effects on axon terminals, deplete neurotransmitters, activate apoptotic signaling pathways, and exhibit reduced memory performance in intoxicated subjects.

Heavy MDMA use has been associated with numerous long term consequences. Of these long term consequences changes in mental processing speed, impulsivity, mood, working memory are most significant. Studies have shown that moderate users show no statistically significant performance differences in neuropsychological tests when compared to controls (abstain from MDMA) while heavy users perform worse in test regarding mental processing speed and impulsivity (Parrot et al 2012). Working memory is among the neurological measures that are affected detrimentally in MDMA users (Wareing et al 2000). Meth is also known to affect memory by impairing both spatial and non-spatial working memory in rats, which suggests potential hippocampal neurotoxicity (Nagai et al., 2007).The relationship between working memory and the hippocampus has been well established as the hippocampus is the location of long term potentiation, the mechanism behind learning. Damage to either this subcortical structure as a whole, or damage to its components- neurons could explain the deficits in working memory produced by long-term exposure to MDMA and METH.

An important aspect of the hippocampus, that could be relevant to the neurotoxicity associated with MDMA and METH use could be the effects of these drugs on adult neurogenesis; a phenomena understood as continuously generated newly born neurons in the subgranular zone of the dentate gyrus of the hippocampus (Bergman et al 2012; Dayer et al 2003; Roberts et al 2012). New neurons come about as a result of either the division of neural stem cells or the division of early neural progenitor cells. Sometimes these newborn cells die before they have a chance to mature (Dayer et al 2003) but more often than not, they will have an opportunity to incorporate in pre-existing neural circuits (Toni 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 (Kempermann 2004; Shors 2002; Gould 1999). Understanding how prevalently used psychostimulants like MDMA and meth impact adult neurogenesis could help reveal the neural basis of the cognitive deficits associated with heavy use.

Change in the cytoarchitecture of the dentate gyrus of the hippocampus due to the neurotoxic effect of drugs is an ongoing topic of investigation. One such study found that giving rats daily/extended access to methamphetamine reduced hippocampal granule neurons and volume of the subgranular zone, changes that were believed to be mediated by the decreased proliferative capacity of the subgranular zone (Mandyam et al 2008). Similarly, studies with MDMA from Hernandez et al found a decrease subgranular zone volume when rats were exposed to MDMA in binge amounts. However, it was found that there was not a significant decrease in the proliferation of neurons, but there was a significant decrease in the survival rate of cells that were incorporated into the subgranular layer. Interestingly they concluded that MDMA affected overall neurogenesis by decreasing the life span of neural precursors, not by altering overall proliferation rates (Hernandez et al., 2006). This result is reinforced by Mandyam et al, as they saw a similar effect in neurogenesis after extended access to methamphetamine. It was observed that rats with long term access to meth showed a decrease in the number of immature neurons than both rats with access to short term meth and control rats (Yuan et al 2011).

In most research thus far, neuronal effects of amphetamine type psychostimulant administration have been studied to a greater degree in rodents as opposed to primates. In rodents it has been shown that amphetamine type psychostimulant exposure decreases cell proliferation in the rodent hippocampus (Hildebrandt et al., 1999). However, results of many studies have shown that fluoxetine, a drug that has a similar mechanism of action as amphetamines, serves to increase neurogenesis in the rodent hippocampus (Malberg et al 2000). These conflicting results provided the motivation to investigate whether amphetamine exposure in a higher order mammal such as the macaque would increase or decrease neurogenesis. Furthermore, the majority of MDMA research in nonhuman primates has been limited to serotonin studies to evaluate neurotoxicity and ambient temperature studies. For example, in a review written by Zhao and Gage (2008) they suggests that levels of serotonin in the brain are positively correlated with neurogenesis in the SGZ. This is evidenced by lesion/grafting of serotonergic neurons in the ralphae nuclei or by pharmacological manipulation of serotonin receptors and measuring the rates of neurogenesis afterwards (Zhao 2008). Studies in human MDMA users have produced data that shows global reductions in serotonin when compared to non-users (Shouw 2012). Therefore it is reasonable to hypothesize that since amphetamine use decreases serotonin levels over time, rates of neurogenesis will also decrease. Earlier studies done on the primates in the present study have shown that increasing ambient temperature leads to a higher degree of hyperthermia, which results in a higher degree of neurotoxicity (Taffe 2007). This evidence from Taffe's lab shows an amphetamine induced hyperthermic reaction to either increases or decreases in ambient temperature that macaque monkeys feel is more similar to the reaction humans have so

they are a better model organism to study stimulant effects than rodents (vonHuben 2007). Additionally, since macaques are closer genetically and behaviorally to humans, it is tempting to ask the question: will exposing nonhuman primates to MDMA or MDMA, MDA and meth adequately provide a closer model to how the human brain is affected by these amphetamine type psychostimulants?

The present study addresses the questions posed by analyzing the subgranular zone of the hippocampus in three groups of young adult macaques: control, MDMA exposed, and MDMA, MDA, meth exposed. These primates were orally and intramuscularly exposed to the respective stimulant drugs. Both routes of administration were used to ensure intoxication. After treatments, animals were euthanized and hippocampal sections were processed for immunohistochemistry; Ki67 was used to determine the levels of cell proliferation of neuronal stem cells, NeuroD1 was used to determine the levels of differentiating and maturing immature neurons, and activated caspase 3 was used to determine the levels of apoptosis. In addition to the investigation of newly born cells, the total number of preexisting granule cell neurons was extrapolated by using StereoInvestigator to count granule cell neurons from 4 samples sites after attaining the total area of the subgranular zone of the dentate gyrus.

MATERIALS AND METHODS

Animals

Fourteen male rhesus monkeys (Macaca mulatta; Chinese origin) participated in this study. Animals were male and ages 7-10 years old when sacrificed. “Daily chow (Lab Diet 5038, PMI Nutrition International; 3.22 kcal of metabolizable energy (ME) per gram) and modified individually by the veterinary weight management plan. Daily chow ranged from 160 to 230 g per day for the animals in this study. 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. Animals also had various acute exposures to scopolamine, racloprie, methylphenidate, SCH23390, THC, nicotine, and mecamylamine in behavioral pharmacological studies. These experimental drug treatments had been administered for a minimum of one year prior to the start of MDMA, MDA, and METH investigations 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. (Von Huben 2007).

Table1: Experimental Design

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.

Experimental Design			
Number of Animals			
Study**:**	**Control**	**MDMA**	**MDA, MDMA, METH**
Ki-67 Cell Count	**3**	**5**	**6**
NeuroD Cell Count	**3**	**5**	**6**
Caspase Cell Count	**3**	**5**	**6**
Total Granule Cell Neurons	**3**	**5**	**6**

Drug Challenge Studies

"For these studies doses of (±) 3, 4- methylenedioxymethamphetamine HCl (0.0, 0.56, 1.0, 1.78, or 2.4 mg/kg), (±) 3,4- methylenedioxyamphetamine HCl (0.56, 1.0, 1.78, 2.4 mg/kg) and (+) methamphetamine HCl (0.1, 0.32, 0.56, 1.0 mg/kg) were administered intramuscularly in a volume of 0.1 ml/kg saline. (±) MDMA was 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 homecage for the duration of the study. All animals were euthanized 2 hours following the final MDMA dose of 5mg/kg. 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” (Crean et al 2006). Animal maintenance/handling as well as drug administration was carried out by another research group.

Tissue Preparation

Blocks of fixed brain tissue were cyroprotected in 30% sucrose solution, after which they were sectioned coronally on a freezing microtome into 40-um sections. Hippocampal sections were serially collected in nine wells and stored in PBS containing sodium azide (0.1%) for subsequent use (Taffe et al 2010). Nine sections each containing different depths of the macaque hippocampus were mounted on two slides for each animal regardless of exposure group. Two slides per animal were used to represent sections 1-250 of the adult macaque dentate gyrus. This procedure was replicated for each of 3 biomarkers, Ki-67, NeuroD, and caspase.

Antibodies and Immunohistochemistry

The following primary antibodies were used for immunohistochemistry (IHC): Ki-67(1:500, Neo Markers), NeuroD (1:500, SantaCruz Biotechnology), AC-3 (activated caspase 3; 1:500 Cell Signaling). The sections used for IHC were pretreated, blocked and incubated with the aforementioned antibodies followed by biotin-tagged secondary antibodies. Avidin was used as a substrate to stain positive cells (Mandyam et al 2004).

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 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 D, and caspase markers. In order to quantify total granule cell neurons in the SGZ of the dentate gyrus, StereoInvestigator used sample counts from 4 sites as well as area size of section and section thickness to extrapolate total granule cell neuron count. This entity was named “estimated variance of estimated cell population” and represented the estimated number of cells found in 62 sections of 40um thick young adult macaque dentate gyrus. Five “estimated variance of estimated cell population” values were generated for five representative sections leading to a total sum of granule cell neurons which represented ~250 sections of the dentate gyrus.

Data Analysis

One way randomized block analysis of variance (ANOVA) was employed to evaluate significance in differential expression of Ki-67, NeuroD, and activated caspase 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. Bonferroni's post hoc test was applied after each one way ANOVA analysis. Values of $p< 0.05$ were considered statistically significant. Correlation analysis between drug dose administered (MDA, MDMA, or meth) and effect on Ki-67, Neuro D, activated caspase 3, or total granular cell neurons was performed. All analysis was done on GraphPad Prism version 5 or Microsoft Excel 2007.

RESULTS

Doses of amphetamines administered to subjects do not differ significantly

Although the amount of drug administered to each animals in the study differed, the average amount of MDMA administer to all eleven subjects (in both drug exposure groups) was 17.08 ± 1.75 mg/kg. The average amount of MDMA administered to five MDMA only exposed animals was 15.02± 1.72 mg/kg (**Table 2)**. The average amount of MDMA administered to six MDA/MDMA/meth animals was 18.80 ± 2.81 mg/kg (**Table 2)**. The average amount of MDA administered to six MDA/MDMA/meth animals was 7.54 ± 1.25 mg/kg (**Table 2)**. The average amount of meth administered to six MDA/MDMA/meth animals was 1.55 ± 0.37mg/kg (**Table 2)**.

Amphetamines significantly decrease proliferation of neural progenitors in SGZ of dentate gyrus

Ki-67 cell density (cells/mm2) was calculated by dividing the total number of Ki-67 immunreactive cells per animal by total SGZ area per animal. Data from each animal was averaged amongst its treatment group. One way ANOVA analysis showed that animals exposed to MDMA and animals exposed to MDA, MDMA, and meth had significantly decreased Ki-67 expression ($F(2,11)= 15.03$, $p=0.0007$). MDMA exposure caused an 82% decrease in Ki-67expression in comparison with controls while MDA, MDMA, meth exposure caused an 87% decrease in Ki 67 expression (**Figure 1)**. Dunnett's post hoc analysis showed a significant effect of MDMA exposure on Ki 67 expression when compared to controls as well as a significant effect of MDA, MDMA, and meth exposure on Ki 67 expression when compared to controls ($p<0.01$, and $p<0.002$ respectively).

Amphetamines significantly decrease survival of immature neurons in SGZ of dentate gyrus

NeuroD cell density (cells/mm2) was calculated by dividing the total number of NeuroD immunreactive cells per animal by total SGZ area per animal. Data from each animal was averaged amongst its treatment group. One way ANOVA analysis showed that animals exposed to MDMA and animals exposed to MDA, MDMA, and meth had significantly decreased NeuroD expression ($F(2,11)= 5.594$, $p=0.0211$). MDMA exposure caused a 55% decrease in NeuroD expression in comparison with controls while MDA, MDMA, meth exposure caused a 63% decrease in NeuroD expression (**Figure 2)**. Dunnett's post hoc analysis showed a significant effect of MDMA exposure on NeuroD expression when compared to controls as well as a significant effect of MDA, MDMA, and meth exposure on Neuro D expression when compared to controls ($p<0.05$).

Amphetamines do not significantly increase apoptosis in SGZ of dentate gyrus

Caspase cell density (cells/mm2) was calculated by dividing the total number of Caspase immunreactive cells per animal by total SGZ area per animal. Data from each animal was averaged amongst its treatment group. One way ANOVA analysis showed that animals exposed to MDMA and animals exposed to MDA, MDMA, and meth did not have significantly different levels of caspase activation ($F(2,11)= 0.3246$, $p=0.7295$). MDMA exposure caused a 17% increase in caspase activation in comparison with controls while MDA, MDMA, meth exposure caused a 29% increase in caspase activation (**Figure 3)**. Dunnett's post hoc analysis showed no significant effect of MDMA exposure on caspase activation when compared to controls as well as no

significant effect of MDA, MDMA, and meth exposure on caspase activation when compared to controls.

Amphetamines do not significantly alter total number of granule cells in SGZ of dentate gyrus

Total granule cell neurons in the SGZ of the dentate gyrus was calculated by extrapolating total granule cell counts in 4 30 um by 30 um sampling frames to fit the total area and volume of a 40 um thick coronal section. Data from each animal was averaged among its treatment group. One way ANOVA analysis showed that animals exposed to MDMA and animals exposed to MDA, MDMA, and meth did not have significantly different numbers of total granule cells ($F(2,11)= 0.9815$, $p=0.4053$). MDMA exposure caused a 72% increase in total granule cell neurons in comparison with controls while MDA, MDMA, and meth exposure cause a 33% increase in total granule cell neurons (**Figure 4)**. Dunnett's post hoc analysis showed no significant effect of MDMA exposure on the number of total granule cell neurons in the SGZ when compared to controls as well as no significant effect of MDA, MDMA, and meth exposure on total granule cell neurons in the SGZ when compared to controls.

Differential drug doses administered to each subject show no correlation with Ki-67, Neuro D, or caspase expression as well as no correlation with total granular cell neurons

No correlation between varied drug doses administered and effect on Ki-67, Neuro D, and activated caspase 3 expression was found. No correlation between varied drug doses administered and total granule cell neurons was found.

DISCUSSION

The goal of the present study was to substantiate the rodent result of decreased neurogenesis in the hippocampus resulting from psychostimulant insult, by replicating the results in a higher order organism (Hildebrandt et al 1999; Hernandez 2006). Many studies in the field have focused on understanding the effects of psychostimulants on neurotransmitters and neurotransmitter induced neurotoxicity, but very few have sought to understand neurotoxicity as a function of altered neurogenesis. Furthermore, psychostimulant research conducted by addiction neuroscientists has found the role of neurogenesis in the adult mammalian hippocampus as an important player in relapse to drug seeking behavior (Koob and Volkow 2010). Therefore, the rationale behind the current investigation of hippocampal neurogenesis exists to replicate earlier research in the field in organisms closer evolutionarily to human as well as to implicate factors involved in hippocampal related behaviors.

By using model organisms 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 2007). Doses of psychostimulant administered to each of the macaques in each of their respective experimental groups differed, but did not differ significantly. The average MDMA dose administered to all animals in both of the experimental groups was 17.08 $\pm$ 1.75 mg/kg. Ecstasy pills seized and analyzed by ecstasypill.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 (von Huben et al 2007). However, the amount of MDMA administered

to subjects in the present study ranged from 1.7-5mg/kg, with escalation in dose after each couple administrations. This range of MDMA doses is much higher than the doses found in average ecstasy pills and combined with the fact that they were given to animals over a 9.6 month period, favors a model for chronic use/ addiction over a model for recreation use (quote from Mandyam 2015?). As for the significance of the experimental group MDA, MDMA, and METH, human poly drug users are known to use different combinations of these psychostimulants either in conjunction (ecstasy) or separately over a period of time, the latter as the model used in the current study. Nevertheless, both types of exposure groups, MDMA only and MDA/MDMA/METH serve as models for chronic amphetamine use and subsequent effects on neurogenesis in the hippocampus found in closer related organisms (macaques) could help us better understand effects on the human hippocampus.

The majority of former psychostimulant studies have focused on neurotransmitter activity causing neurotoxicity and have found that both MDMA and METH are neurotoxic to serotonergic and dopaminergic nerve terminals (Capela et al., 2009; Lyles and Cadet, 2003; Ricaurte et al., 2000; O'Hearn et al 1988; Ricuarte et al., 1982; Wagner et al., 1980). Although few studies, including the present study have used altered neurogenesis in the hippocampus of adult animals as a measure for neurotoxicity, results thus far have been promising (Yuan et al., 2011; Mandyam et al., 2008; Taffe et al., 2010). In the present study it was calculated that chronic MDMA exposure causes an 82% decrease in Ki-67 expression when compared with control animal Ki-67 expression. Ki-67 is a protein expressed during phases of the cell cycle, but is absent from resting cells (Scholzen et al., 2000). Many earlier studies have characterized adult neural stem

cells as newly born cells in the subgranular zone of the hippocampus that have not finished the cell cycle (Ma et al., 2008). Thus changes in Ki-67 expression are an adequate measure of adult neural stem cell proliferation in the hippocampus. Chronic MDA, MDMA, and METH exposure causes an 87% decrease in Ki-67 expression in comparison to control animal Ki-67 expression. After one way ANOVA and Dunnett's post hoc analysis, it was shown that both exposure to MDMA and exposure to MDA, MDMA, and METH caused significant decreases in Ki-67 expression ($F(2,11)= 15.03$, $p=0.0007$). This data coincides with results found in the rat hippocampus after MDMA exposure (Hernandez et al., 2006).

Another viable measure of neurotoxicity as a function of neurogenesis is to look at changes in levels of immature neurons in the adult hippocampus. By using markers like NeuroD, a neuronal differentiation transcription factor that express in immature neurons, we can hypothesize the effect psychostimulant exposure will have on the maturation of developing neurons in the hippocampus. Present findings indicate that chronic MDMA exposure causes a 55% decrease in NeuroD expression, while chronic MDA, MDMA, and METH exposure causes a 63% decrease in NeuroD expression. After one way ANOVA and Dunnett's post hoc analysis both decreases were deemed significant ($F(2,11)= 5.594$, $p=0.0211$). Since NeuroD expression is involved in the terminal differentiation of progenitors into mature neurons, the integrity of the hippocampus as a structure is affected when numbers of immature neurons are decreased (Lee et al., 1995). Although adult neurogenesis still occurs in these animals, decreased rates of proliferation of neural progenitors as well as decrease rates of neurons maturing definitely show a new model of neurotoxicity. Future studies that explore how a decrease

in immature neurons could correlate with changes in hippocampal circuitry because of psychostimulant insult may lead to important insights into how neurotoxicity affects function.

Although the current study uses decreased neurogenesis as a measure of neurotoxicity instead of the more traditional measure of neurotransmitter induced neurotoxicity, neurotransmitters can still be involved in decreasing neurogenesis. Results from studies done on the effect of MDMA and methamphetamine on dopamine and serotonin in the rat striatum have shown that taking both drugs concurrently affects release of neurotransmitters differently (Ikeda et al., 2011). Specifically, rats were exposed to both MDMA and methamphetamine showed a higher striatal concentrations of dopamine than rats that were exposed to either MDMA alone or methamphetamine alone. Interestingly, rats that were exposed to both MDMA and methamphetamine showed about the same (ceiling effect) striatal concentrations of serotonin as rats that were exposed to either MDMA alone or methamphetamine alone (Ikeda et al., 2011). In accordance with the present study, the differences between Ki-67 and NeuroD expression among the MDMA and MDA, MDMA, and METH animals more closely resemble Ikeda's serotonin result, suggesting that serotonin may be a major player associated in the decreasing trend of neurogenesis between these two groups. However, the decreases in neurogenesis seen in the current study could be due to a floor effect in which psychostimulants are so neurotoxic that it won't matter if you have just MDMA or MDA, MDMA and METH; rates of neurogenesis cannot be lowered further. Furthermore, evidence from serotonin transporter (5HTT) knockout studies have shown that MDMA treated wild type (presence of 5HTT) rats show a decrease in hippocampal cell

proliferation while MDMA treated knock out (absence of 5HTT) rats show no change in cell proliferation (Renoir et al., 2008). This finding, the Ikeda result, and the findings of the current study serve to reinforce the role of serotonin in MDMA induced regulation of neurogenesis in the hippocampus.

Another traditional measure of neurotoxicity is evaluating the expression levels of activated caspase 3. It is well established that caspase signaling is involved in apoptosis, and MDMA exposure enhances caspase expression (Capela 2013). However, past studies such as the aforementioned Capela paper establish significant increases in caspase expression in rodents, while the current study shows an increasing trend in caspase expression from control animals to MDMA expose to MDA, MDMA, and METH exposed animals ($F(2,11)= 0.3246$, $p=0.7295$). Interestingly, the hallmark of this experiment was the fact that there was a significant decrease in proliferating newly born cells and immature neurons, along with no significant increase in caspase expression. This means that the death of proliferating and immature neurons was due to something else that was not apoptotic signaling.

If apoptotic signaling serves to understand what happens to cells on an individual basis, measuring total granular cell neurons would help deduce what happens to hippocampal cytoarchitecture as a result of psychostimulant ingestions. Unfortunately, little data can be found regarding the effect of MDMA or METH on total granular cell neurons in the GCL of the hippocampus. The utility of this analysis was to check if the decreases in proliferation and survival found earlier in the study were due to changes in the sum of granular cell neurons in the hippocampus.

Along with the results of the current study, future investigation should be directed towards understanding what genotype of neurons is specifically affected by psychostimulant exposure. First, confocal microscopic analysis can be used to co-label combinations of Ki-67, NeuroD, GFAP, and/or caspase in order to elucidate the exact phase in neuronal development that psychostimulants damage neurons. Second, immunohistochemistry for tyrosine and tryptophan hydroxylase, rate limiting enzymes in dopamine and serotonin synthesis respectively can help us understand the effect that psychostimulants have on neurotransmitter synthesis in the hippocampus. Third, immunohistochemistry for SERT (serotonin transporter) and DAT (dopamine transporter) can help us understand the specific effect that psychostimulants have on transporter expression in the hippocampus, and if an association between transporter expression and neurogenesis exists. Fourth, immunohistochemistry for serotonin receptors (5HT2a and 5HT1a) could help us understand if receptor up-regulation is involved in causing decreased neurogenesis. DiI injections to track neural inputs and outputs to the hippocampus would be useful in understanding the effect psychostimulants have on neural circuitry.

Collectively, this study proposes that administering large escalating doses of psychostimulants to young adult nonhuman primates yields significant reductions in both proliferating and immature neurons, to an extent that the integrity of the hippocampus may be compromise. However, it was shown that decreases in neurogenesis were not due to increased apoptosis; so it is suggested that serotonin may play a role in regulating psychostimulant induced neurogenesis- a phenotype that may induce maladaptive hippocampal related behaviors.

FIGURES AND TABLES

Table 2: **Average Dose Administration per Experimental Group**

Over the span of 9.6 months, 14 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 1.30 mg/kg respectively in a 9.6 month period.

Total Drug Doses over 9.6 months (0.8 years)				
Animal #	Treatment	**MDA**	**MDMA**	**METH**
389	**MDMA ONLY**	-	15.6 mg/kg	-
405	**MDMA ONLY**	-	15.6 mg/kg	-
413	**MDMA ONLY**	-	20.6 mg/kg	-
415	**MDMA ONLY**	-	10.1 mg/kg	-
416	**MDMA ONLY**	-	13.2 mg/kg	-
	AVERAGE:	N/A	15.02 mg/kg	N/A
302	**MDA, MDMA, METH**	6.78 mg/kg	12.12 mg/kg	2.84 mg/kg
320	**MDA, MDMA, METH**	5 mg/kg	11.78 mg/kg	0.32 mg/kg
329	**MDA, MDMA, METH**	5 mg/kg	15.27 mg/kg	1.94 mg/kg
333	**MDA, MDMA, METH**	10 mg/kg	21.55 mg/kg	1.30 mg/kg
410	**MDA, MDMA, METH**	12.52 mg/kg	29.06 mg/kg	2.04 mg/kg
412	**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

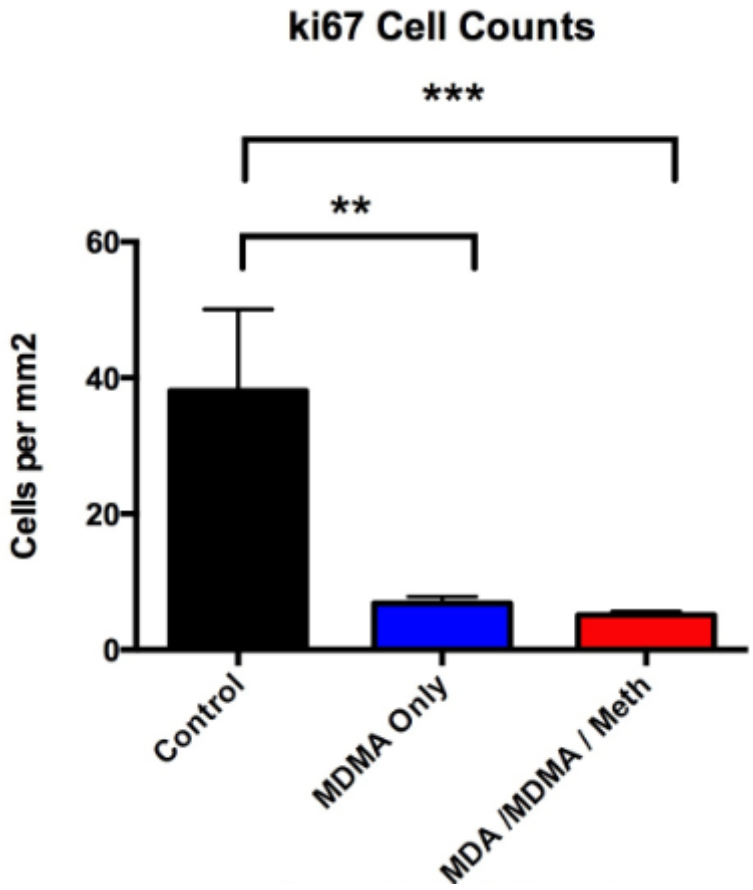


Figure 1: Quantitative analysis of immunoreactive cells stained for ki 67. Data is expressed as mean cells/mm2 ± SEM (control n=3, MDMA only n=5, MDA/MDMA/Meth n=6) ***p<0.002 and **p<0.01 compared with controls.

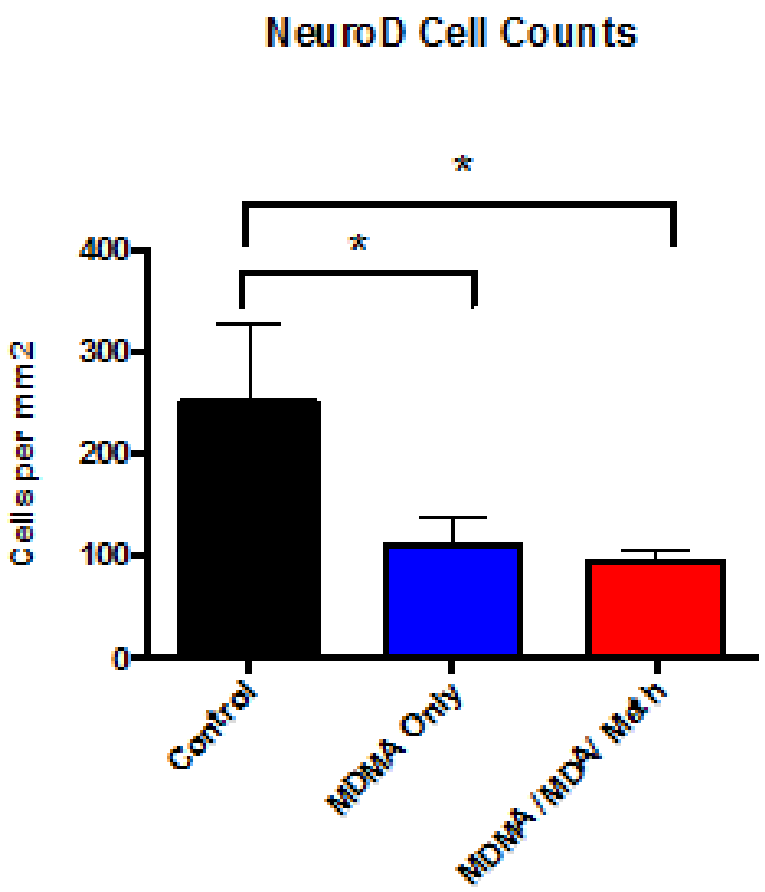


Figure 2: Quantitative analysis of immunoreactive cells stained for NeuroD. Data is expressed as mean cells/mm2 ± SEM (control n=3, MDMA only n=5, MDA/MDMA/Meth n=6) *p<0.05compared with controls.

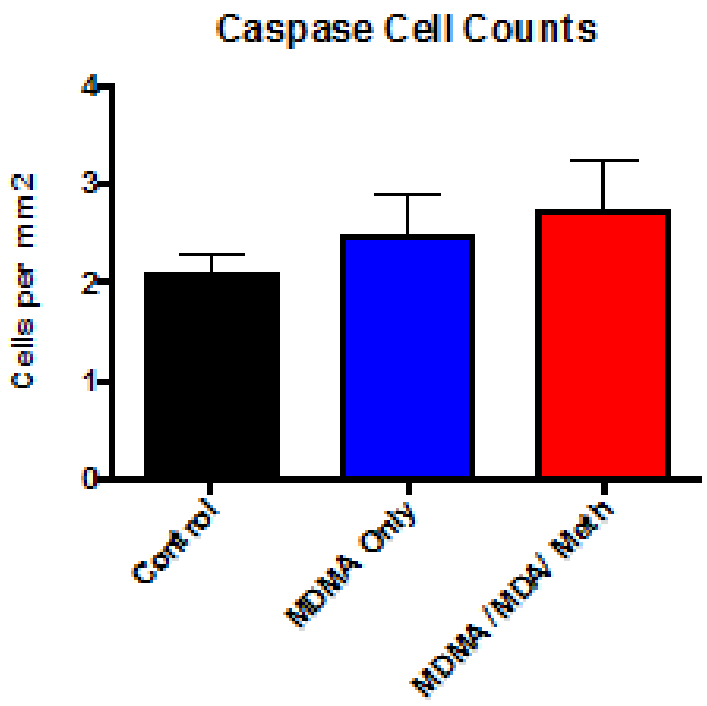


Figure 3: Quantitative analysis of immunoreactive cells stained for Caspase. Data is expressed as mean cells/mm2 ± SEM (control n=3, MDMA only n=5, MDA/MDMA/Meth n=6)

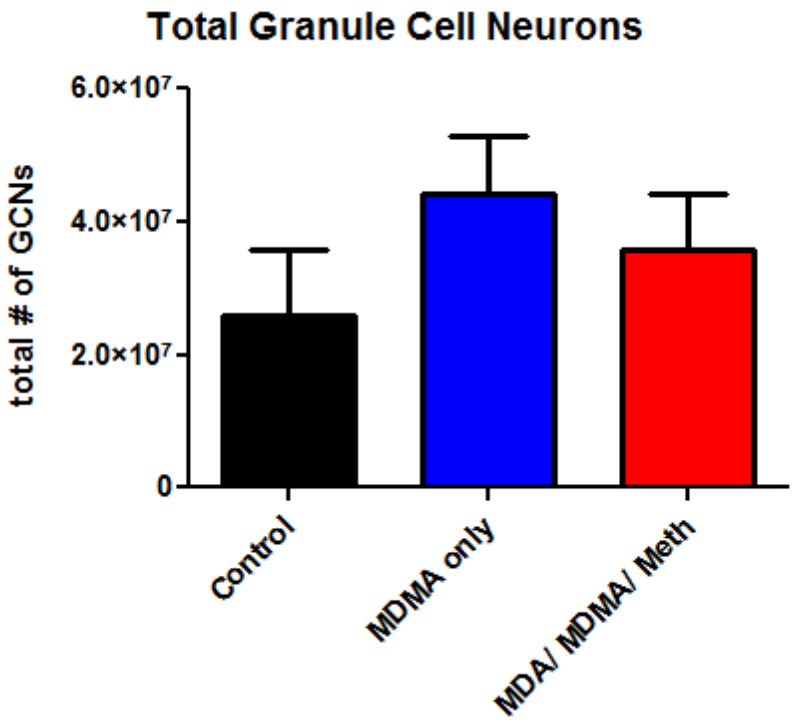
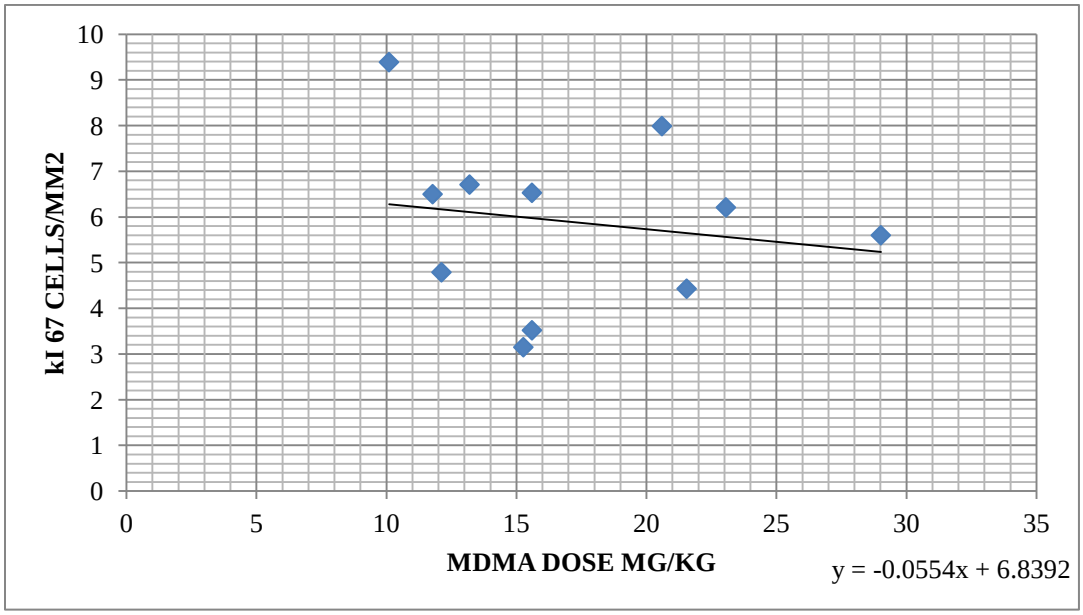
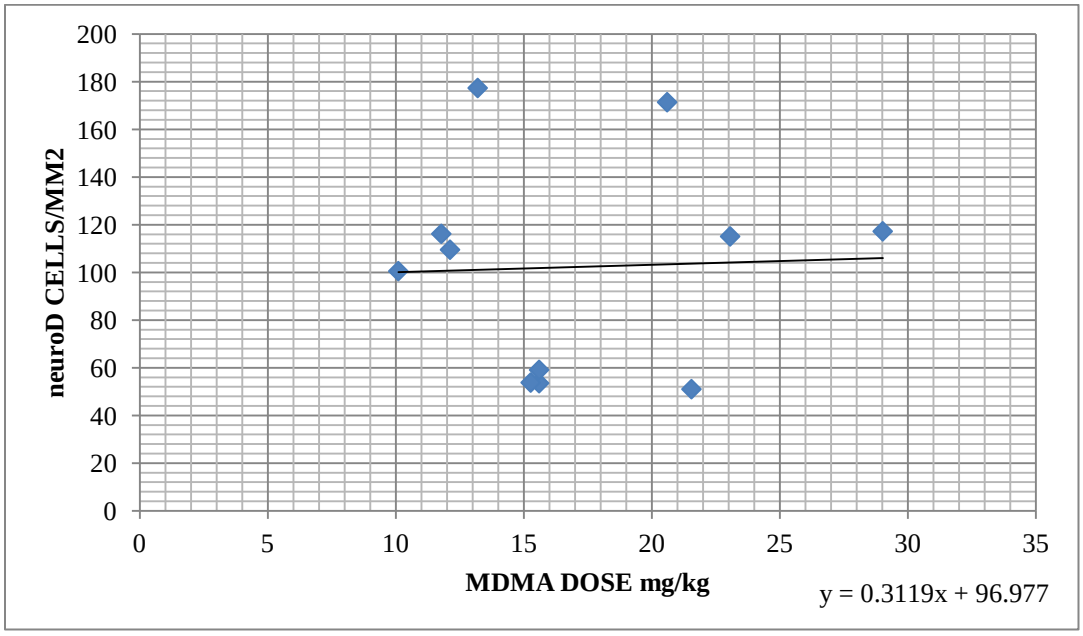


Figure 4: Quantitative analysis of total granule cell neurons approximated in the dentate gyrus. Data is expressed as total number of granule cell neurons counted ±SEM (control n=3, MDMA only n=5, MDA/MDMA/Meth n=6)

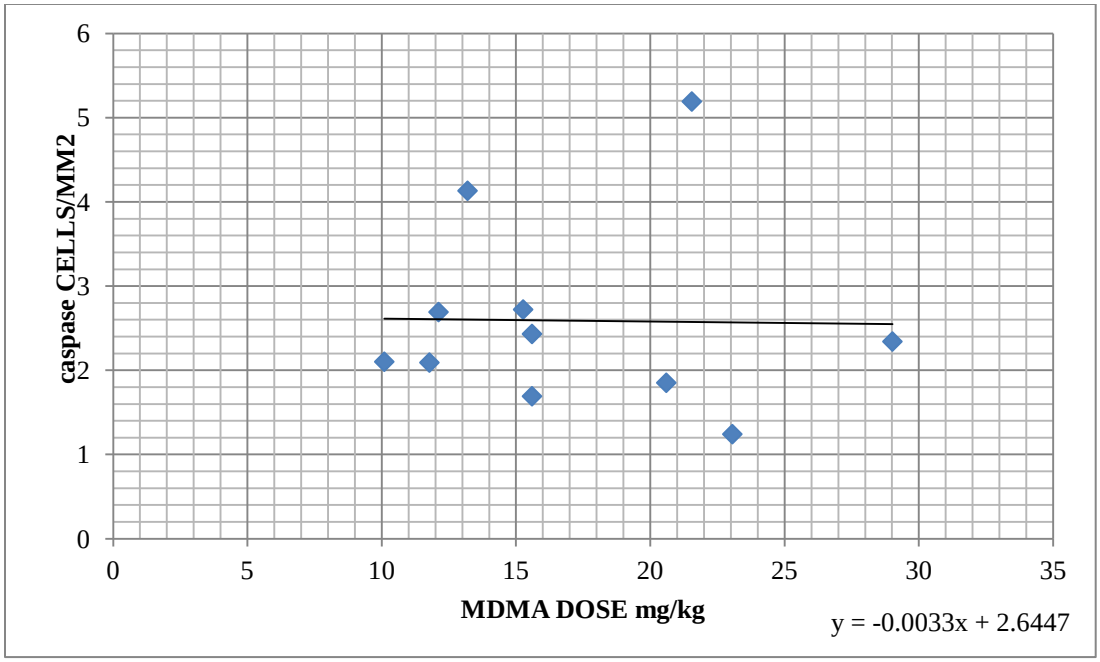
A)


kI 67 CELLS/MM2
MDMA DOSE MG/KG
y = -0.0554x + 6.8392

B)


neuroD CELLS/MM2
MDMA DOSE mg/kg
y = 0.3119x + 96.977

C)



D)

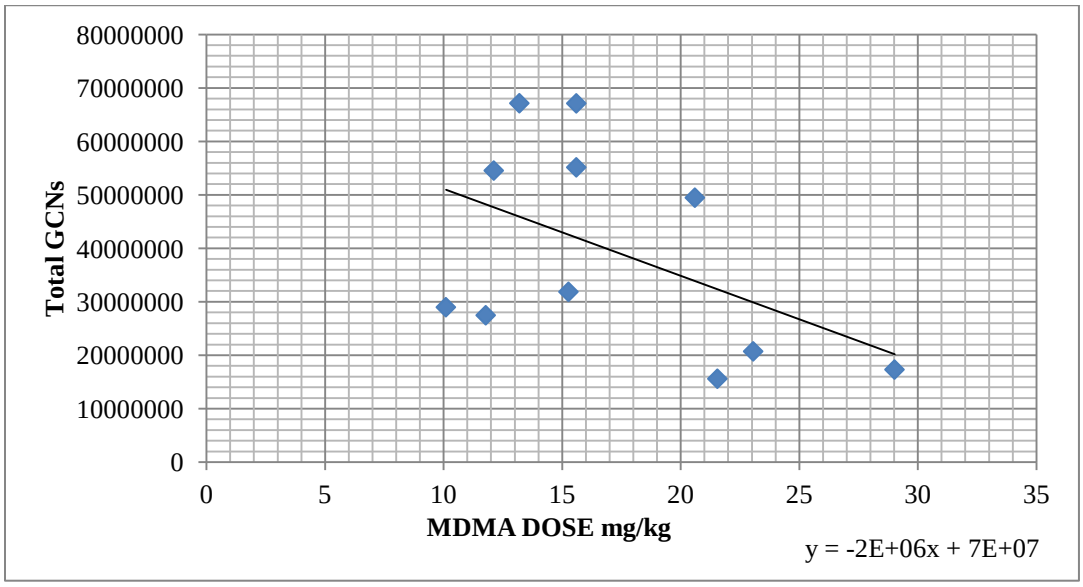
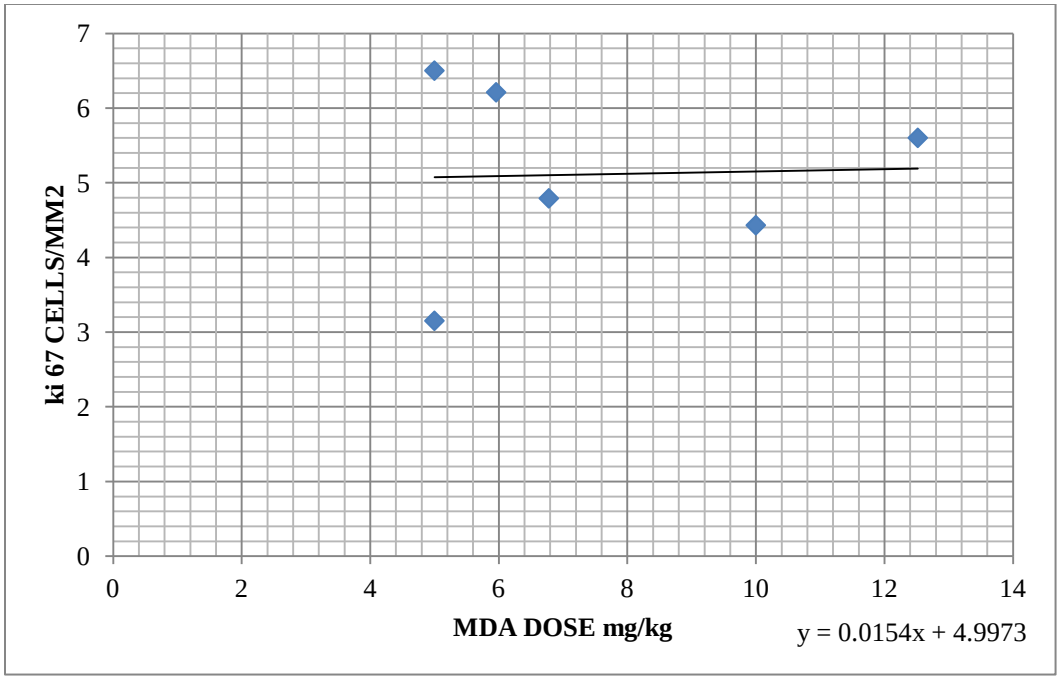
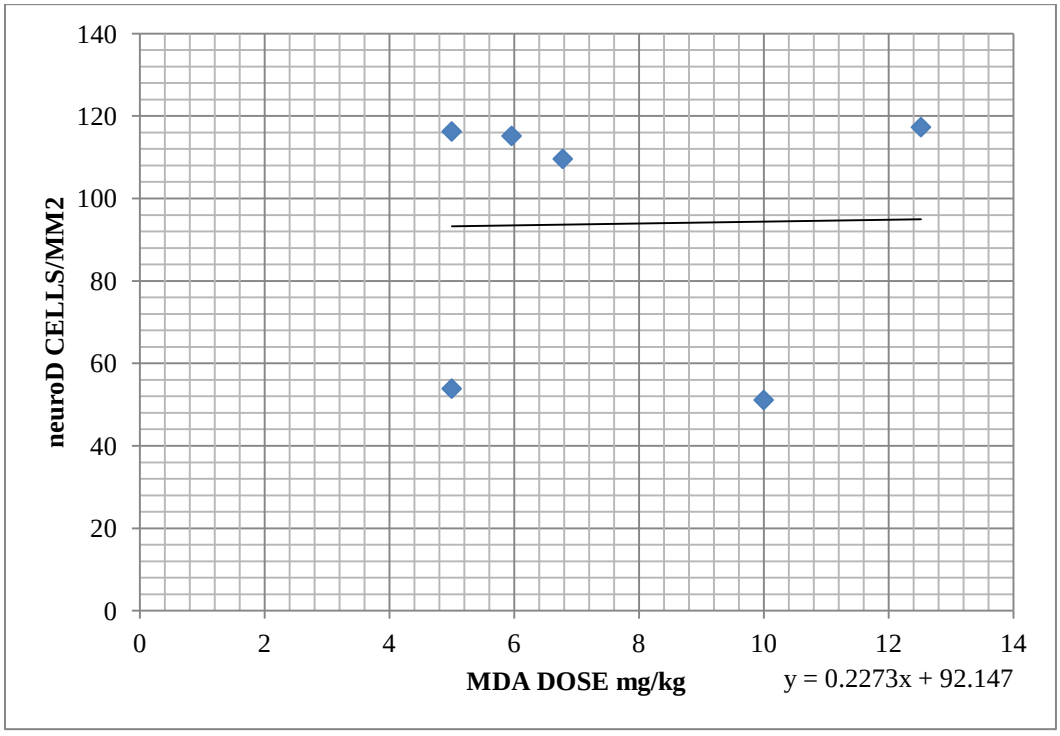


Figure 5: Correlation between total MDMA dose given to animal over 9.6 months and number of positive immunoreactive cells counted as well as total GCNs approximated. No significant correlation found.

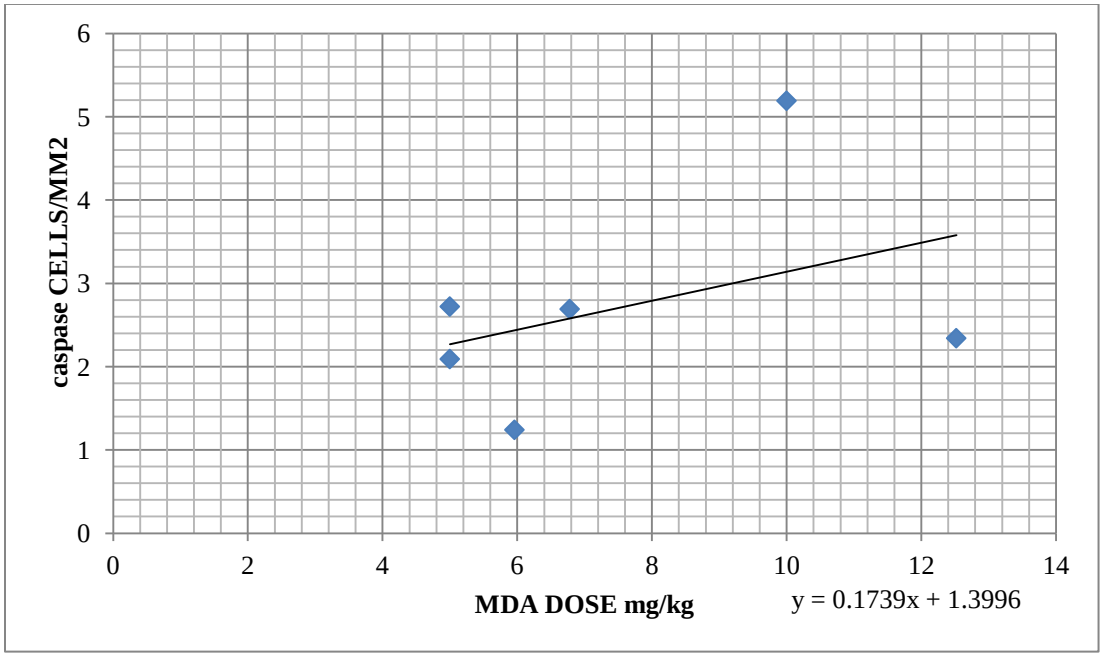
A)


ki 67 CELLS/MM2
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
MDA DOSE mg/kg
y = 0.0154x + 4.9973

B)


neuroD CELLS/MM2
0
20
40
60
80
100
120
140
0
2
4
6
8
10
12
14
MDA DOSE mg/kg
y = 0.2273x + 92.147

C)



D)

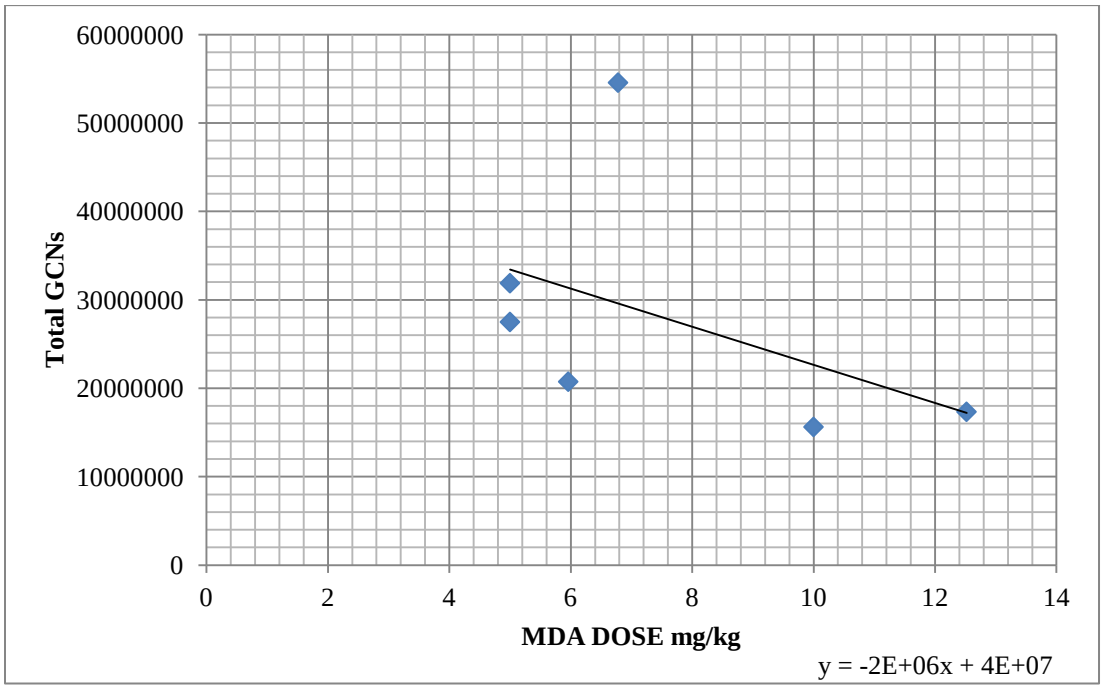
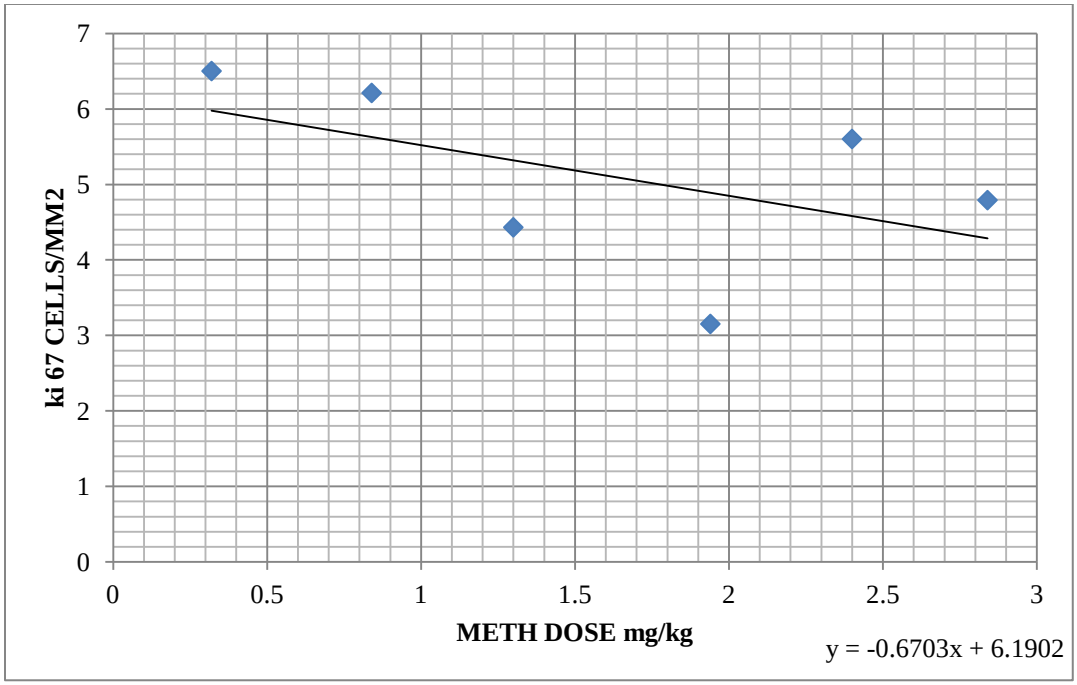
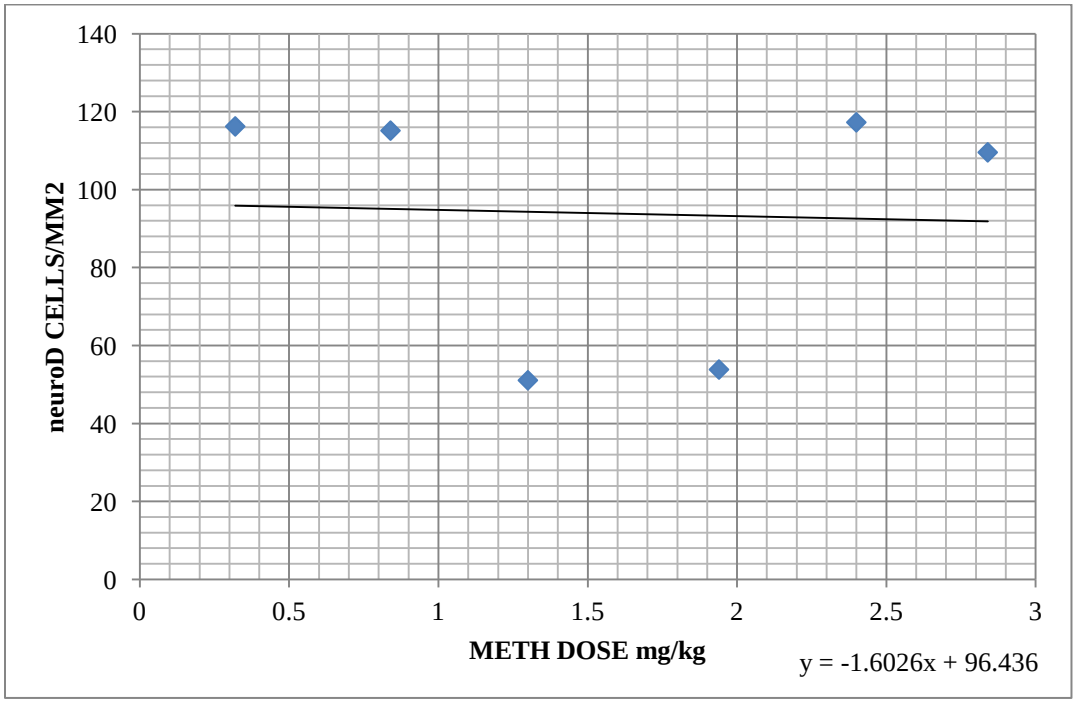


Figure 6: Correlation between total MDA dose given to animal over 9.6 months and number of positive immunoreactive cells counted as well as total GCNs approximated. No significant correlation found.

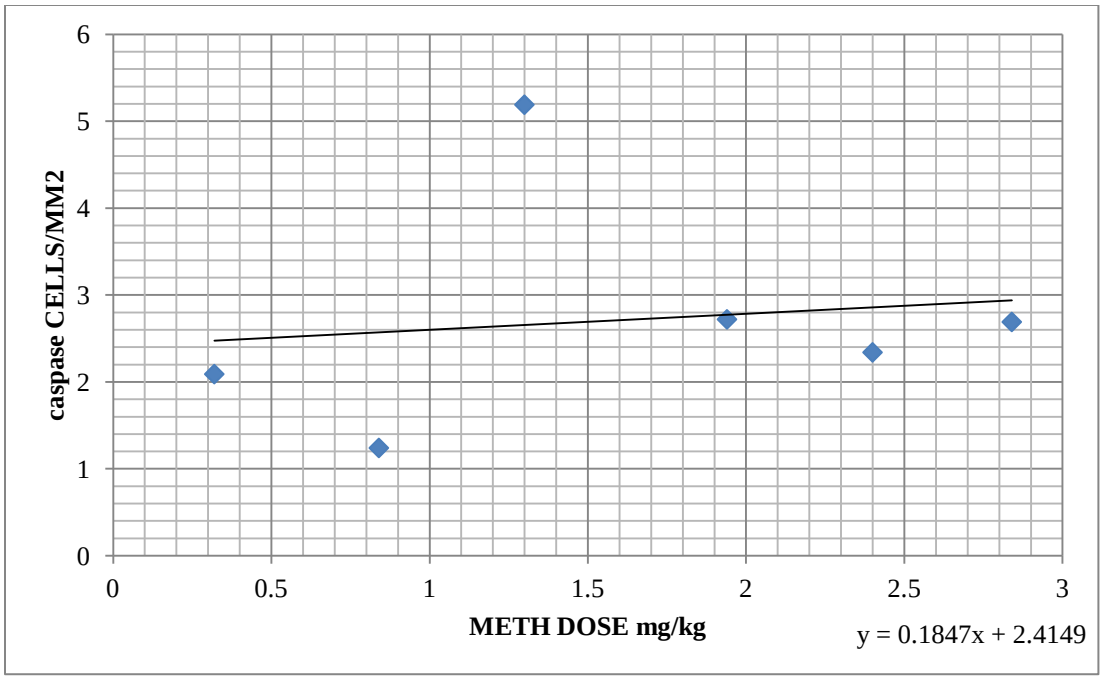
A)


ki 67 CELLS/MM2
METH DOSE mg/kg
y = -0.6703x + 6.1902

B)


neuroD CELLS/MM2
METH DOSE mg/kg
y = -1.6026x + 96.436

C)



D)

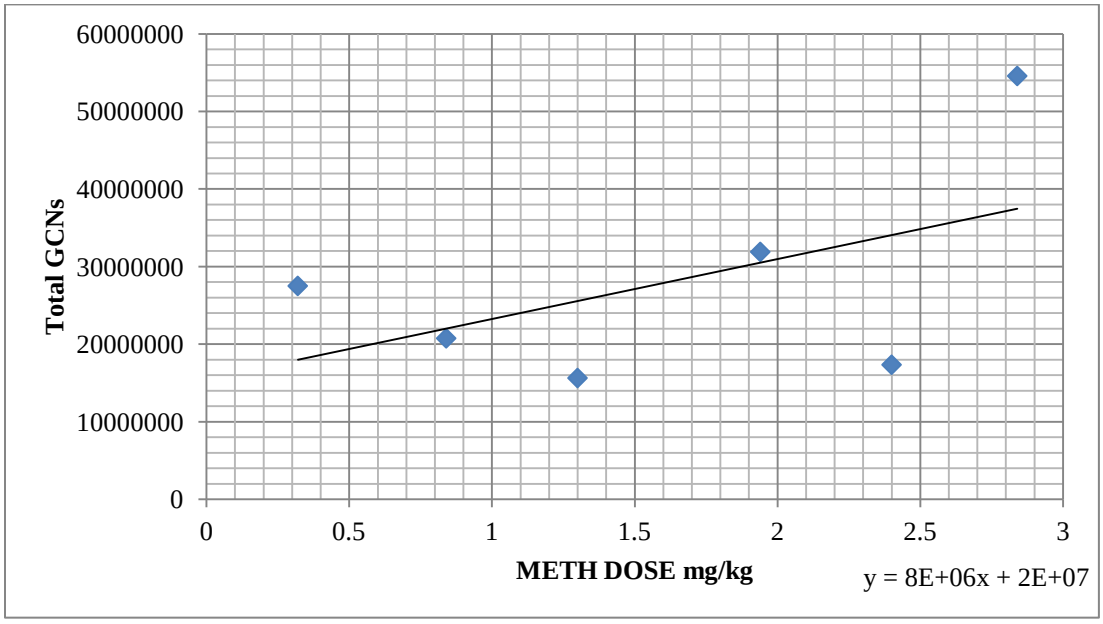


Figure 7: Correlation between total METH dose given to animal over 9.6 months and number of positive immunoreactive cells counted as well as total GCNs approximated. No significant correlation found

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