MDMA Decreases Glutamic Acid Decarboxylase (GAD) 67-Immunoreactive Neurons in the Hippocampus and Increases Seizure Susceptibility: Role for Glutamate

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Abstract

3,4-Methylenedioxy-methamphetamine (MDMA) is a unique psychostimulant that continues to be a popular drug of abuse. It has been well documented that MDMA reduces markers of 5-HT axon terminals in rodents, as well as humans. A loss of parvalbumin-immunoreactive (IR) interneurons in the hippocampus following MDMA treatment has only been documented recently. In the present study, we tested the hypothesis that MDMA reduces glutamic acid decarboxylase (GAD) 67-IR, another biochemical marker of GABA neurons, in the hippocampus and that this reduction in GAD67-IR neurons and an accompanying increase in seizure susceptibility involve glutamate receptor activation. Repeated exposure to MDMA (3×10mg/kg, ip) resulted in a reduction of 37–58% of GAD67-IR cells in the dentate gyrus (DG), CA1, and CA3 regions, as well as an increased susceptibility to kainic acid-induced seizures, both of which persisted for at least 30 days following MDMA treatment. Administration of the NMDA antagonist MK-801 or the glutamate transporter type 1 (GLT-1) inducer ceftriaxone prevented both the MDMA-induced loss of GAD67-IR neurons and the increased vulnerability to kainic acid-induced seizures. The MDMA-induced increase in the extracellular concentration of glutamate in the hippocampus was significantly diminished in rats treated with ceftriaxone, thereby implicating a glutamatergic mechanism in the neuroprotective effects of ceftriaxone. In summary, the present findings support a role for increased extracellular glutamate and NMDA receptor activation in the MDMA-induced loss of hippocampal GAD67-IR neurons and the subsequent increased susceptibility to evoked seizures.
Keywords
MDMA; Glutamate; GABA; Excitotoxicity

1 Introduction
MDMA, popularly known as ecstasy or, more recently, as Molly, is a synthetic, psychostimulant and a popular drug of abuse. It is well documented that the repeated administration of MDMA results in a persistent deficit in biochemical markers of 5-HT axon terminals in both laboratory animals (Gudelsky & Yamamoto, 2003) and humans (Kish et al., 2010) that has been viewed as evidence of 5-HT neurotoxicity (Capela et al., 2009).

Results from several studies suggest that MDMA-induced neurotoxicity extends beyond 5-HT terminals to include cell bodies in brain regions such as the hippocampus. These studies adduce the MDMA-induced increases in activated calpain-1 and caspase-3, cytochrome C, BAX expression, TUNEL staining, and DNA fragmentation as evidence that MDMA promotes apoptotic cell death (Frenzilli et al., 2007; Soleimani Asl et al., 2012; Tamburini et al., 2006; Wang et al., 2009).

Additional evidence to suggest that MDMA produces hippocampal cell loss includes the findings that the number of parvalbumin-IR interneurons in the DG is reduced following MDMA administration. The MDMA-induced reduction in parvalbumin-IR GABA neurons appears to involve 5-HT2A receptor-dependant and cyclooxygenase-dependent mechanisms (Anneken et al., 2013; Collins et al., 2015a). The involvement of glutamate in this apparent GABAergic neurotoxicity is supported by the finding that MDMA increases the extracellular concentration of glutamate in the hippocampus (Anneken & Gudelsky, 2012) and that MK-801 suppresses the MDMA-induced reduction in parvalbumin-IR GABA neurons (Collins et al., 2015a).

Parvalbumin interneurons in the hippocampus function to provide strong inhibitory control of granule cell neuronal firing (Freund & Buzsáki, 1996). Loss of parvalbumin-IR cells has been reported in patients with epilepsy, underlining the importance of these neurons in maintaining balance between excitation and inhibition (Arellano et al., 2004; DeFelipe et al., 1993). In preclinical studies, Giorgi et al. (2005) and Abad et al (2014) have reported that mice treated with MDMA exhibit an increased sensitivity to kainic acid induced seizures. However, the mechanism by which MDMA treatment results in an increase in seizure susceptibility has not been investigated.

In the present study, we demonstrate that MDMA reduces the number of GAD67-IR neurons in the hippocampus and further investigate the role of glutamate in the reduction of this biomarker of GABA neurons, as well as in the concomitant increase in seizure susceptibility.
2 Materials and Methods

2.1 Animals

Adult, male Sprague-Dawley rats (275–325g) (Harlan Laboratories, Indianapolis, IN) were used in the studies. Animals were housed two per cage in a temperature and humidity controlled room with a 12-hr light/dark cycle and allowed food and water ad libitum. All procedures were in strict adherence to the National Institutes of Health guidelines and approved by the institutional animal care and use committee. Animals were acclimated for at least one week to the housing facilities and diet before being used in the study.

2.2 Drugs and Treatment

MDMA was provided by the National Institute on Drug Abuse (Bethesda, MA), was dissolved in 0.15 M NaCl, and administered ip. Animals were treated with either a single injection of MDMA (1×10 mg/kg, ip), a binge regimen of MDMA, (10 mg/kg, ip, every 2 hr for a total of 3 injections), or vehicle and euthanized either 7 or 30 days after treatment. This binge regimen of MDMA has been well-documented to produce 5-HT neurotoxicity (Puerta et al., 2009; Shankaran et al., 2001), as well as reductions in parvalbumin-IR GABA neurons (Anneken et al., 2013). Ceftriaxone was purchased from Besse Medical (West Chester, OH), was dissolved in 0.15 M NaCl, and administered as a single daily injection of 200mg/kg, ip for 7 days, a dosage regimen similar to that used by others (Rasmussen et al., 2011; Verma et al., 2010). MDMA (3 × 10 mg/kg, ip) or vehicle was given 24 hr following the last injection of ceftriaxone. MK-801 was purchased from Sigma-Aldrich (St. Louis, MO), was dissolved in 0.15 M NaCl, and administered at 0.3mg/kg, sc 30 min prior to each injection of MDMA or vehicle. MK-801 + MDMA treated rats were maintained an elevated ambient temperature (approximately 27° C) in order to maintain MDMA-induced hyperthermia (see results).

To determine the effect of MDMA on seizure susceptibility, animals were treated with a binge regimen of MDMA, (10 mg/kg, ip, every two hr for a total of 3 injections) or vehicle. Seizures were induced by kainic acid (Sigma-Aldrich, St. Louis, MO), 8mg/kg, sc, 7 or 30 days following MDMA treatment (Golden et al., 1995).

2.3 Tissue Preparation

Rats were deeply anesthetized with Euthasol (100–150 mg/kg, ip) and transcardially perfused with 500 ml of physiological saline followed by 500 ml of 4% paraformaldehyde in 0.1M sodium phosphate-buffered saline (NaPBS) (pH=7.4). After perfusion, brains were removed and postfixed in the same fixative at 4° C overnight. Brains were then cryoprotected in 30% sucrose in 0.1 M NaPBS for at least 48 hr. Brains were frozen in the embedding medium and then transferred to the cryostat. Coronal sections (30 μm) of the hippocampus and the nucleus accumbens were cut and kept in the cryoprotective buffer at 4 °C. In the present study, analysis of GAD67-IR was restricted to the dorsal hippocampus (Bregma −3.0 to −3.7mm) and the core of the nucleus accumbens (Bregma +1.3 to +1.7mm).
2.4 Immunohistochemistry

For GAD67-IR interneurons labeled with diaminobenzidine (DAB) chromogen, free-floating sections were stained with antibodies as follows: sections were rinsed in 50 mM potassium phosphate-buffered saline (KPBS), incubated for 10 min in 1% H2O2 in PBS, washed 5 × 5 min in KPBS, incubated for 60 min at room temperature in blocking solution (50mM KPBS with 0.2% Triton X-100, and 0.1% bovine serum albumin (BSA)), and incubated overnight at 4 °C in blocking solution containing monoclonal mouse anti-GAD67 antiserum (1:1000; MAB5406, Millipore, Temecula, CA). Sections were then rinsed 5 × 5 min in KPBS and incubated for 60 min with biotinylated secondary antibody (1:500, Vector) with 0.1% BSA in KPBS. The sections were again rinsed 5 × 5 min and incubated in ABC Elite kit (Vector) for 1 hr. After further washes, the sections were exposed to DAB (Sigma-Aldrich, St. Louis, MO; D5905) in 30% hydrogen peroxide for 3 min. The sections were transferred into KPBS before being mounted onto glass microscope slides in 0.5% gelatin. Slides were allowed to air dry overnight and then dehydrated in increasing concentrations of alcohol, cleared with xylene, and coverslipped with DPX (Sigma-Aldrich, St. Louis, MO).

For GAD67-IR interneurons labeled with the Cy3 fluorophore, free-floating sections were stained with antibodies as follows: sections were rinsed in 50 mM KPBS, washed 5 × 5 min in KPBS, incubated for 60 min at room temperature in blocking solution (50mM KPBS with 0.2% Triton X-100, and 0.1% BSA), and incubated overnight at 4 °C in blocking solution containing monoclonal mouse anti-GAD67 antiserum (1:1000; MAB5406, Millipore, Temecula, CA). Sections were rinsed 5 × 5 min in KPBS and then incubated for 60 min in blocking solution containing Cy3-conjugated AffiniPure Donkey Anti-Mouse IgG (H+L) secondary antibody (1:500; The Jackson Laboratory). The sections were again washed 5 × 5 min with KPBS and 1 X 5 min in potassium phosphate buffer (KPB). The sections were transferred into KPB before being mounted onto glass microscope slides in 0.5% gelatin. Slides were allowed to air dry overnight and coverslipped with gelvatol (Sigma-Aldrich).

2.5 Image Analysis

Quantitative analysis of the number of DAB-labeled GAD67-IR neurons within the brain regions specified (CA1, CA3, DG, and nucleus accumbens) was performed with Scion Image analysis software. Digital images of each side of the regions specified, as defined by the rat stereotaxic brain atlas of Paxinos and Watson, were captured at 5× magnification with a Carl Zeiss Imager Z.1 (Carl Zeiss Microimaging, Thornwood, New York). At least four images were obtained per animal (one left and one right side from each of two different sections). Using Scion Image analysis, the region of interest in each image was outlined, and the area of the region was measured. The software analyzed the number of particles detected within the outlined region. Particles were considered to be individual cells, and the cell count was divided by the area of the region for statistical analysis.

Stereological analysis of the number of Cy3-labeled GAD67-IR neurons within the brain regions specified (CA1, CA3, and DG) was performed with NIS Elements imaging software. All images were collected with a Nikon A1R confocal on a Nikon Ti Eclipse inverted microscope controlled by NIS Elements interface. Images were captured with a 20x Plan Apo VC (NA 0.75) objective lens. During quantification, every sixth section for a total of six
sections through the dorsal hippocampus were systematically sampled. Confocal images were acquired as Z-stacks (0.95 μm thickness) and the representative image is a maximum intensity projection image from the Z-stack. The numerical densities (ND) of GAD67-IR cells were determined using a modified optical fractionator technique (Gundersen et al., 1999; West et al., 1991) and calculated by the following equation:

$$ND = \frac{\sum \text{Counts}}{h(\text{area})/SV},$$

where \(\sum \text{Counts}\) is the number of GAD67-IR cells per counting frame, \(h\) is the height of the optical dissector, and \(SV\) is the volumetric shrinkage factor (Jinno et al., 1998). Means were derived by averaging the ND from two sections from each animal in the CA1, CA3, and DG. In the present study, stereological analyses were restricted to the dorsal hippocampus (Bregma −3.0 to −3.7mm) and only GAD67-IR cells within the stratum pyramidale of CA1, the stratum radiatum of CA3, and the granular cell layer of the DG were quantified. Due to the lack of homogeneity in cell distribution within the hippocampus and the relative ease which an absolute count of GAD67-IR neurons can be performed (Noori & Fornal, 2011), a single large counting frame was applied to each region of interest and an absolute cell count was performed within the frame. The optical dissector height (\(h\)) was 9.5μm (0.95μm interval x 10 slices) set ~2μm below the lookup section.

2.6 Analysis of Seizure Susceptibility

Rats were transferred from the animal housing facility to the experimental procedure room on the evening prior to injection of kainic acid and allowed to acclimate overnight. Behaviors were videotaped for 3 hr beginning immediately after injection of kainic acid. Seizures were assessed from recorded behaviors using a modified Racine scale: no response (0), frozen posture, staring, and/or facial clonus, (1); myoclonic twitching and tremor (2), forelimb clonus with lordotic posture (3), forelimb clonus with rearing (4), forelimb clonus with rearing, jumping, and falling (5) (Hellier et al., 1998). Only animals which exhibited behaviors consistent with a stage three seizure or above were marked as having seized. Latency to seizure was recorded as the time (min) at which an animal first exhibited a stage 3–5 behavior.

2.7 Microdialysis and Glutamate Analysis

Rats were implanted with a stainless steel guide cannula under anesthesia (ketamine/ xylazine 70/6 mg/kg, ip) 72 hr prior to the insertion of a dialysis probe. On the evening prior to the experiment, a concentric style dialysis probe was inserted through the guide cannula into the dorsal hippocampus; the coordinates of the tip of the probe were: A/P, −3.6mm., L, 2.0mm, and D/V −4.0mm. The active portion of the membrane for the probes was 2.0mm. The probes were connected to an infusion pump set to deliver modified Dulbecco’s phosphate buffered saline containing 1.2mm CaCl₂ and 5mM glucose at a flow rate of 1 μl/min overnight. On the morning of the experiment, the flow rate was increased to 2 μl/min, and the probes were allowed to equilibrate for 1.5hr. Three collections were then taken at 30
min intervals to establish baseline values; thereafter samples were collected every hr for the duration of the experiment. All experiments were performed at an ambient temperature of 24° C. Data were calculated as a percentage of the baseline value for glutamate which was obtained by averaging the three baseline samples.

Glutamate was derivatized according to the method described by Donzanti and Yamamoto (1988). The HPLC consisted of an OPA-HS column (Part #28064. Grace Discovery Science) connected to an amperometric detector (Bioanalytical Systems, West Lafayette, IN) equipped with a glassy carbon target electrode set at +700 mV. The mobile phase consisted of 0.1M Na$_2$HPO$_4$, 50mg/L EDTA, 20% methanol, pH 6.4. Peak heights were recorded with an integrator, and the concentration of glutamate was calculated on the basis of known standards.

### 2.8 Core Body Temperature Measurement

A BioMedic Data Systems DAS-7007s Reader System, including the Smart Probe Wand and Implantable Programmable Temperature Transponders 300 (IPTT-300), was utilized to monitor core body temperature in rats which were later assessed for GAD67-IR. Transponders were implanted sc in the shoulder region under isoflurane anesthesia 3–4 days prior to the experiment. Core body temperatures were recorded every 30 min beginning 1 hr prior to and ending 7 hr after the first injection of MDMA. All recordings were carried out while the animals were freely roaming around their cage with the lid removed. The transponder temperature was measured in triplicate (performed in rapid succession) in each rat at every time point, and the mean value recorded. The rats that received MK-801 and MDMA were maintained at an elevated ambient temperature of approximately 27° C in order to maintain MDMA-induced hyperthermia.

### 2.9 Statistical Analysis

The effect of MDMA on GAD67-IR neurons was analyzed in each brain region of interest using either a one-way analysis of variance (ANOVA) or a two-way ANOVA. Multiple pairwise comparisons were performed using Student-Newman-Keul’s test. All seizure data were analyzed using chi-square analysis followed by the fisher’s exact test. Latency to seizure data was analyzed using a t-test. All microdialysis and body temperature data were analyzed using two-way repeated measures ANOVA, and multiple pairwise comparisons were performed using post hoc analysis with a Student-Newman-Keuls test. Treatment differences for all data were considered statistically significant at p < 0.05.

### 3 Results

#### 3.1 MDMA reduces DAB-labeled GAD67-IR in the hippocampus

The binge regimen of MDMA (3×10 mg/kg, ip) significantly (p<0.05) reduced GAD67-IR in subregions of the hippocampus at both 7 and 30 days after treatment (Figure 1A). GAD67-IR in the CA1, CA3, and DG was decreased by 43%, 58%, and 37%, respectively, 7 days after the binge regimen of MDMA when compared to values for vehicle-treated controls. Significant (p<0.05) reductions of ~40 to 50% in GAD67-IR were still evident in the CA1, CA3, and DG 30 days after the binge regimen of MDMA. A single injection of

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MDMA (10 mg/kg, ip) was not sufficient to produce a reduction in GAD67-IR at either 7 or 30 days in any region of the hippocampus examined. GAD67-IR was also assessed in the nucleus accumbens; there was no reduction in GAD67-IR in this brain region following the binge regimen of MDMA (data not shown). A one-way ANOVA indicated a significant main effect of treatment for each region of the hippocampus: CA1 ($F_{(3,17)} = 11.646, p<0.001$), CA3 ($F_{(3,17)} = 10.987, p<0.001$), and DG ($F_{(3,15)} = 23.635, p<0.001$). There was no significant effect of treatment on number of GAD67 cells in the nucleus accumbens ($F_{(1,9)} = 0.211, p=0.657$). Representative images of the hippocampus are presented in Figure 1B.

### 3.2 Involvement of glutamate in the MDMA-induced loss of hippocampal GAD67-IR neurons

The role of glutamate in the MDMA-induced loss of GAD67-IR in the hippocampus was evaluated by treatment of rats with the NMDA glutamate antagonist MK-801 or ceftriaxone, which has been shown to up-regulate the glutamate transporter GLT-1. In this experiment, the number of GABA neurons was quantified by stereological counting of GAD67-IR neurons in the CA1, CA3 and DG.

MDMA treatment significantly ($p<0.05$) reduced the densities of GAD67-IR neurons in the pyramidal layer of CA1, the molecular layer of CA3, and the granular layer of the DG by 33%, 30% and 34%, respectively, when compared to the values for vehicle-treated rats (Figure 2). MDMA did not significantly reduce the number of GAD67-IR neurons in any hippocampal region of rats that had received MK-801 or ceftriaxone (Figure 2). MK-801 or ceftriaxone treatment alone did not affect the number of GAD67-IR neurons. A two-way ANOVA indicated a significant main effect of treatment on the densities of GAD67-IR neurons in each brain region: CA1 ($F_{(1,29)} = 7.17, P=0.012$); CA3 ($F_{(1,28)} = 4.54, P=0.042$); DG ($F_{(1,27)} = 7.16, P=0.012$).

### 3.3 MDMA treatment increases the susceptibility to kainic acid-induced seizures

Kainic acid-induced seizures were evaluated 7 and 30 days following treatment with the binge regimen of MDMA or vehicle. There was no statistical difference in seizure incidence of animals treated with vehicle 7 or 30 days prior to kainic acid administration; therefore, these data for control animals were pooled for the purposes of statistical comparison. Chi square analysis of the data indicated that there was a significant difference in the incidence of seizures across treatment groups ($X^2 = 19.48, p=0.001$) (Figure 3A). Seizures were observed in 29% of rats treated with vehicle and in 89% and 100% of the rats treated with MDMA 7 days or 30 days earlier. Post hoc analysis indicated that seizure incidence was significantly increased in both groups of MDMA treated animals (7 days, $p<0.001$; 30 days, $p<0.002$).

Animals treated with the binge regimen of MDMA, either 7 or 30 days prior to kainic acid, also exhibited a significant ($p<0.05$) reduction in seizure latency compared to animals treated with vehicle (Figure 3B). A one-way ANOVA indicated a significant main effect of treatment on seizure latency amongst groups ($F_{(2,27)} = 3.58, P=0.042$). Post hoc analysis indicated that seizure latency was significantly reduced in both groups of MDMA treated animals (7 days, $p<0.023$; 30 days, $p<0.048$).

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3.4 Involvement of glutamate in the MDMA-induced increase in seizure susceptibility

Kainic acid-induced seizures were recorded 7 days following the administration of vehicle or MDMA in groups of rats that had also received prior treatment with vehicle or MK-801. Chi square analysis revealed significant differences in seizure incidence across treatment groups ($X^2 = 12.464, p=0.006$). Vehicle+MDMA treated rats were significantly ($p<0.05$) more likely to exhibit kainic acid seizures than their appropriate control animals (vehicle +vehicle). However, treatment of rats with MK-801 abolished the increase in seizure susceptibility produced by MDMA. Seizure incidence was significantly ($p<0.05$) less in the MK-801+MDMA treated rats than in the vehicle+MDMA treated animals. Moreover, seizure incidence in MK-801+MDMA treated rats did not differ compared to that in the control group (MK-801+vehicle, $p=1$) (Figure 4A).

In a separate experiment, the effect of ceftriaxone treatment also was determined on the MDMA-induced increase in seizure susceptibility. Seizure incidence was determined to be significant across treatment groups, as determined by Chi square analysis ($X^2 = 15.122, p=0.002$). Again, seizure incidence was significantly ($p<0.05$) increased in the vehicle +MDMA group when compared to the vehicle+vehicle group. Importantly, treatment of rats with ceftriaxone prevented the increase in seizure susceptibility produced by MDMA. Seizure incidence in the ceftriaxone+MDMA treated animals was significantly less than that in the vehicle+MDMA treated rats ($p<0.05$). Furthermore, seizure incidence in the ceftriaxone+MDMA treated group was not significantly different than that in the ceftriaxone +vehicle treated control group (Figure 4B).

3.5 Neither MK-801 nor ceftriaxone alters MDMA-induced hyperthermia

2MK-801 has been shown to attenuate the hyperthermic response to MDMA, and this may confound the interpretation of its potential neuroprotective effects (Farfel & Seiden, 1995). For this reason, all rats treated with MK-801+MDMA were kept at an elevated ambient temperature of approximately 27°C during the MDMA treatment regimen in an effort to maintain the hyperthermic response to MDMA.

Body temperatures in rats treated with the binge regimen of MDMA increased approximately 2–2.5°C during the course of treatment (Figure 5A, B). The magnitude of the MDMA-induced hyperthermia was not significantly ($p>0.05$) different in ceftriaxone-treated (Figure 5A) or MK-801-treated (Figure 5B) rats when compared to rats given MDMA alone.

3.6 Ceftriaxone suppresses the MDMA-induced increase in extracellular glutamate

The ability of ceftriaxone to attenuate the MDMA-induced increase in extracellular glutamate in the hippocampus was assessed by in vivo microdialysis. Extracellular glutamate in the dorsal hippocampus was significantly ($p<0.001$) elevated during the treatment of rats with the binge regimen of MDMA. Extracellular glutamate was elevated compared to baseline values beginning 2 hr following the first injection of MDMA and remained elevated for the next 4 hr (Figure 6). In contrast, the administration of MDMA to rats treated for 7 days with ceftriaxone did not result in an increase in extracellular glutamate ($p=0.136$). A repeated measures ANOVA revealed a significant effect of treatment on extracellular glutamate ($F_{(3,30)}=15.80, p<0.001$).
4 Discussion

The key findings of the present study include the following: 1) exposure to MDMA results in a dose-dependent and persistent reduction in GAD67-IR neurons in multiple regions of the hippocampus, 2) treatment with MDMA results in an increase in susceptibility to kainic acid-induced seizures, 3) blockade of NMDA receptors or enhancement of glutamate re-uptake prevents the MDMA-induced loss of hippocampal GAD67-positive GABA neurons, as well as the increase in seizure susceptibility. Overall, the present findings substantiate a role for glutamate in the MDMA-induced loss of GAD67-IR neurons within the hippocampus and the subsequent increase in seizure susceptibility.

It is well documented that the repeated administration of MDMA results in persistent deficits in markers of 5-HT axon terminals that has traditionally been viewed as 5-HT neurotoxicity (Gudelsky & Yamamoto, 2003; McCann & Ricaurte, 2004). The present findings provide evidence that MDMA neurotoxicity extends beyond 5-HT axon terminals. Herein we document that both two-dimensional and three-dimensional (stereological) analyses reveal a dose-dependent and persistent reduction in GAD67-IR in the CA1, CA3, and DG of the hippocampus following treatment with MDMA. To our knowledge, this is the first demonstration that MDMA reduces GAD67-IR in any brain region. These data are consistent with earlier reports that MDMA decreases the concentration of GABA in the hippocampus (Perrine et al., 2010) and reduces the number parvalbumin-IR GABAergic neurons in the hippocampus of rodents (Abad et al., 2014; Anneken et al., 2013; Collins et al., 2015a).

The MDMA-induced reduction in parvalbumin-positive neurons in the rat was shown previously to be evident only within the DG of the hippocampus (Anneken et al., 2013; Collins et al., 2015a). In the present study, MDMA treatment resulted in a decrease in the number of GAD67-IR neurons throughout subregions of the hippocampus, including CA1, CA3, as well as DG. This finding suggests that the loss of biochemical markers of GABA neurons produced by MDMA extends not only beyond parvalbumin-IR neurons but also beyond the DG.

The long-lasting reduction in GAD67-IR could be representative of either GABAergic neuron cell death or a persistent downregulation in GAD67 expression and change in cell phenotype. However, MDMA has been shown to increase biomarkers, e.g., TUNEL staining, caspase-3, and cytochrome C, of cell death in the hippocampus (Frenzilli et al., 2007; Soleimani Asl et al., 2012; Tamburini et al., 2006; Wang et al., 2009) and reduce GABA concentrations (Perrine et al., 2010). Therefore, it is tempting to speculate that the increase in markers of cell death following MDMA-treatment is due to the loss of GABAergic neurons. If, however, the MDMA-induced reduction of GAD67-IR is the result of down-regulation, the long-lasting deficits in GAD67 expression might still result in dysfunctional GABAergic systems in the hippocampus (i.e., functional neurotoxicity).

Several studies have reported that hippocampal GABA neurons are vulnerable to glutamate-mediated excitotoxicity (Kerner et al., 1997; Moga et al., 2003; Nyiri et al., 2003), and MDMA has been shown to selectively increase extracellular glutamate in the hippocampus.
(Anneken & Gudelsky, 2012). Therefore, the MDMA-induced increase in glutamate has been suggested to contribute to the damage to hippocampal GABAergic neurons produced by MDMA. Supporting this view, Collins et al. (2015a) reported that the MDMA-induced reduction in parvalbumin-IR in the DG is dependent upon the activation of NMDA receptors.

In the present study, treatment with either MK-801, an antagonist of NMDA receptors, or ceftriaxone, an inducer of GLT-1 expression (Rothstein et al., 2005), diminished the MDMA-induced reduction in GAD67-IR. Ceftriaxone is a beta-lactam antibiotic and has been shown to provide neuroprotection against glutamate-mediated excitotoxicity (Beller et al., 2011; Hota et al., 2008; Jagadapillai et al., 2014; Liu et al., 2013). Ceftriaxone presumably maintains glutamate homeostasis through an enhancement of glutamate re-uptake into astrocytes, thereby limiting excessive increases in extracellular glutamate. In the present study, ceftriaxone prevented the MDMA-induced increase in extracellular glutamate in the hippocampus. Thus, blockade of glutamate receptors or suppression of the MDMA-induced increase in extracellular glutamate prevented the MDMA-induced loss of GAD67-IR neurons. These findings further support a role for glutamate excitotoxicity in the MDMA-induced loss of GAD67-positive hippocampal neurons.

Hyperthermia is a critical component of the neurotoxicity of amphetamine analogs, including MDMA (Broening et al., 1995; Malberg & Seiden, 1998). In the present study, rats were administered MK-801 concurrently with MDMA at an elevated ambient temperature to maintain MDMA-induced hyperthermia. The core body temperatures of rats treated with the combination of MK-801 and MDMA, as well as ceftriaxone and MDMA, were not significantly different from rats treated with MDMA alone at any time point. This finding excludes the possibility that the protection afforded by MK-801 and ceftriaxone against the MDMA-induced loss of GAD67-IR neurons is due to alterations in MDMA-induced hyperthermia and further supports glutamatergic mechanisms in the role of these drugs.

The systemic administration of kainic acid induces status epilepticus in rats and generates a syndrome of seizures and brain damage that mimics human temporal lobe epilepsy (Goodman, 1998). Presently, we have demonstrated that susceptibility to kainic acid-induced seizure in rats is increased one week after MDMA treatment and persists up to 30 days. Additionally, rats exposed to repeated MDMA treatment exhibited reduced latency to seizure. These results are consistent with previous studies by Giorgi et al. (2005) and Abad et al. (2014) who demonstrated that MDMA increases seizure susceptibility in mice. However, these earlier studies did not address potential mechanisms underlying the MDMA-induced increase in seizure susceptibility. The increased sensitivity to kainic acid seizures reported here following exposure to MDMA is prevented by treatment with MK-801 or ceftriaxone. Thus, glutamate receptor mechanisms, presumably initiating excitotoxicity, appear to contribute to the increase in seizure susceptibility following MDMA administration, as well as the MDMA-induced loss of hippocampal GAD67-IR neurons.

Cortical excitability is regulated by glutamate and GABA (Petroff, 2002); therefore, imbalances of these neurotransmitters can alter excitability and potentially increase seizure
activity (Schousboe & White, 2009). Moreover, the hippocampus is sensitive to shifts in cortical excitability, and thus is especially prone to generating seizures (Stafstrom, 2010). Given that GABA interneurons provide strong inhibitory control of neuronal excitability, it seems reasonable to speculate that a causal relationship exists between the MDMA-induced loss of GAD67-IR neurons in the hippocampus and the associated pro-convulsant state. Indeed, loss of GABA interneuron activity in the hippocampus has been suggested to be associated with increased vulnerability to epileptogenic processes (Sloviter et al., 2001).

The recent findings of Collins et al. (2015b) also support an association between a purported MDMA-induced loss of GABA interneurons in the DG and increased hippocampal neuronal excitability. These investigators reported that the threshold intensity to drive after-discharges in the DG is reduced in MDMA-treated rats in which there is a reduction in hippocampal parvalbumin-IR neurons. Collins and coworkers (2015b) also demonstrated that paired pulse inhibition (which has been attributed to GABAergic inhibition in the DG) is reduced following MDMA administration.

An alternative possibility is that the MDMA-induced depletion of brain 5-HT underlies the increased seizure susceptibility, inasmuch as 5-HT modulates both glutamatergic and GABAergic neurotransmission (Ciranna, 2006). Indeed, it has been shown that a genetic manipulation of zebrafish can result in reduced 5-HT content and increased seizure activity (Sourbron et al., 2016). However, Giorgi et al. (2005) have reported that dosage regimens of MDMA that do not result in 5-HT depletion still result in increased seizure susceptibility. Moreover, the differential effects of MK-801 on the MDMA-induced depletion of brain 5-HT and increased seizure susceptibility also appear to preclude the involvement of 5-HT neurotoxicity in the alteration in seizure threshold. Although MK-801 was initially shown to prevent MDMA-induced 5-HT neurotoxicity (Farfel et al., 1992), it subsequently was shown not to afford neuroprotection when MDMA hyperthermia is maintained (Farfel & Seiden, 1995), as was done in the present study. Thus, MK-801 prevented the increase in seizure susceptibility, as well as the loss of GAD67-IR neurons, despite the likelihood that depletion of brain 5-HT was still evident. Although the data are only correlational in nature, they support the view that glutamate signaling, rather than 5-HT neurotoxicity, underlies the increase in seizure susceptibility that accompanies the MDMA-induced loss of GAD67-IR neurons.

Nevertheless, other mechanisms also may contribute to the reduced seizure threshold produced by MDMA. For example, hippocampal gene expression may be altered (Weber et al., 2014) during or following the binge regimen of MDMA that would result in changes in cellular excitability. These changes may involve pro-inflammatory cytokines and the immune system (Scharfman, 2007).

Several investigators have examined the relevance of the neurotoxic regimen of MDMA in rats to doses of the drug typically abused by humans (Baumann et al., 2007; Green et al., 2003; Green et al., 2009; McCann & Ricaurte, 2001). The doses of MDMA necessary to produce various physiological, neurochemical and/or behavioral effects in rats appears to be approximately 4 times the dose necessary in humans (Baumann et al., 2007; Green et al., 2009). On the basis of this “effect scaling”, the binge regimen of MDMA employed in the
current study may be 4–5 times a single dose acutely administered recreationally by humans (100 mg or 1.5 mg/kg). However, MDMA pharmacokinetics in humans is non-linear (de la Torre et al., 2000). Consequently, humans acutely administering more than 1 Ecstasy tablet (more than 1.5 mg/kg) may exhibit plasma concentrations of MDMA that approach those of rats given 20–30 mg/kg (Green et al., 2009) which approximates the dosage regimen given rats in the present study.

Human abusers of MDMA have been shown to exhibit long-lasting changes in EEG. Dafters et al. (1999) has reported that previous MDMA abuse correlates with a reduction in EEG coherence (synchrony) and an increase in EEG power in the alpha and beta frequency bands. These results are consistent with those of Gamma et al. (2000), who also reported global increases in alpha 1 and beta 2/3 power, as well as an increase in theta power in human users of MDMA. Such EEG changes have often been related to drug-induced cognitive deficits (Dafters et al., 1999; Gamma et al., 2000). However, EEG alterations also may be indicative of alterations in brain excitability (Dinner et al., 2002; Giorgi et al., 2005). Further clinical studies in human abusers of MDMA may be warranted to address whether seizure threshold is reduced in these individuals.

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References


Collins SA, Gudelsky GA, Yamamoto BK. MDMA-induced loss of parvalbumin interneurons within the dentate gyrus is mediated by 5HT2A and NMDA receptors. Eur J Pharmacol. 2015a; 761:95–100. [PubMed: 25936514]


Noori HR, Fornal CA. The appropriateness of unbiased optical fractionators to assess cell proliferation in the adult hippocampus. Front Neurosci. 2011; 5:140. [PubMed: 22207833]


Shankaran M, Yamamoto BK, Gudelsky GA. Ascorbic acid prevents 3,4-methylenedioxymethamphetamine (MDMA)-induced hydroxyl radical formation and the behavioral and neurochemical consequences of the depletion of brain 5-HT. Synapse. 2001; 40:55–64. [PubMed: 11170222]


Highlights

- MDMA treatment results in a loss of GAD67-IR hippocampal neurons
- MDMA treatment reduces the threshold for kainic acid-induced seizures
- The MDMA-induced decrease in GAD67-IR hippocampal neurons and increase in seizure susceptibility appear to involve glutamate receptor mechanisms.
Figure 1.
MDMA selectively decreases DAB-labeled GAD67-IR in the rat hippocampus. A) Rats were treated with MDMA (1x or 3×10 mg/kg, ip) or vehicle 7 or 30 days prior to sacrifice. GAD67-IR neurons were counted in 6 right and left images of each brain region from 6 rats/treatment group. *Indicates p<0.05 compared to vehicle. B) Representative images of DAB-labeled GAD67 cells in the CA1, CA3, and DG in rats treated with vehicle or MDMA (3×10 mg/kg, ip).
Figure 2.
Glutamate mediates MDMA-induced reductions in GAD67-IR.
Animals received ceftriaxone (CEF) (200 mg/kg, ip, daily) for 7 days prior to MDMA,
MK-801 (0.3mg/kg, sc, 30 min prior to each injection of MDMA) or vehicle prior to either
MDMA (3 x 10 mg/kg, ip) or vehicle for a total of six treatment groups. Animals were
sacrificed 7 days after treatment with MDMA, and GAD67-IR was assessed using a
stereological technique in the CA1 (A), CA3 (B), and DG (C). GAD67-IR neurons were
counted in 2 sections in each brain region from 6–8 rats/treatment group. *Indicates p<0.05 compared to the values for vehicle treated rats.
Figure 3.
MDMA increases susceptibility to kainic acid-induced seizures.

Rats were given kainic acid (8mg/kg, sc) 7 or 30 days following the administration of either vehicle or MDMA (3×10mg/kg, ip). A) The percentage of rats exhibiting stage 3–5 seizures is depicted. N=8–19 rats/group. B) Of the animals that seized in panel A (n=5–17 rats/group), latency to seizure was recorded as the time (min) at which the animal first exhibited a stage 3–5 behavior. *Indicates p<0.05 compared to the values for vehicle treated rats.
Figure 4.
MK-801 and ceftriaxone prevent the MDMA-induced increase in seizure susceptibility. Rats were treated with A) MK-801 (0.3mg/kg, sc) or vehicle 30 min prior to each injection of either MDMA (3×10 mg/kg, ip) or vehicle. B) Ceftriaxone (CEF) (200mg/kg, i.p.) or vehicle was given daily for 7 days prior to either MDMA (3 x 10 mg/kg, ip) or vehicle. Kainic acid (8mg/kg, sc) was administered 7 days after MDMA treatment. The percentage of rats exhibiting stage 3–5 seizures is depicted. N=5–10 rats/group. *Indicates (p<0.05) compared to VEH/VEH. #Indicates (p<0.05) compared to VEH/MDMA.

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Figure 5.
MDMA-induced hyperthermia was maintained in MK-801- and Ceftriaxone- treated animals.

Core body temperatures were recorded every 30 min beginning 1 hr prior and ending 7 hr after the first injection of MDMA. A) Rats were treated with either a single daily injection of ceftriaxone (CEF) (200mg/kg, i.p.) or vehicle for 7 days prior to either MDMA (3 × 10 mg/kg, ip) or vehicle. B) Rats were treated with either MK-801 (0.3mg/kg, sc) or vehicle 30 min prior to each injection of either MDMA (3×10 mg/kg, ip) or vehicle. The values for
animals that received MDMA in addition to either CEF or MK-801 were not significantly different from the values for MDMA-treated rats (p>0.05). N=6–8 rats/group.
Figure 6.
Ceftriaxone prevents the MDMA-induced increase in extracellular glutamate.
Extracellular glutamate was determined in the hippocampus in rats treated daily with ceftriaxone (CEF), (200 mg/kg, ip,) or vehicle for 7 days prior to either MDMA (3 × 10 mg/kg, ip, as indicated by the arrows) or vehicle. N=7–11 rats/group. *Indicates values that differ significantly (p<0.05) from the corresponding average baseline values for vehicle treated animals.